Lack of Neutralizing Antibodies to Caprine Arthritis-Encephalitis Lentivirus in Persistently Infected Goats Can Be Overcome by Immunization with Inactivated *Mycobacterium tuberculosis*

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The pathogenesis of the persistent progressive diseases of sheep (visna-maedi) and goats (arthritis-encephalitis) is dependent on continuous replication of the causative lentiviruses. One subgroup of these viruses, Icelandic visna virus, accomplishes this form of replication by undergoing antigenic mutation. Mutant viruses arising late in the infection escape neutralization by antibodies directed to the parental virus. In contrast, we show here that viruses obtained from persistently infected sheep and goats with natural disease in this country do not induce virus-neutralizing antibodies, although antibodies to virus core proteins were produced. The lack of neutralizing antibodies was not overcome by hyperimmunization of animals with concentrated preparations of live or inactivated virus. Thus, failure to produce these specific antibodies was not due to lack of sufficient antigen or interference with the immune response because of the ability of these viruses to infect macrophages. The hyporesponsive state, however, was overcome by immunization of animals with virus and large amounts of inactivated *Mycobacterium tuberculosis*. Induction of agglutinating and neutralizing antibodies by this method was probably due to a unique form of antigen processing by macrophages activated by *M. tuberculosis*. Neutralizing antibodies were produced for the first time against the caprine arthritis-encephalitis virus by this method. These antibodies have similar biological properties to those induced by Icelandic visna virus. They belong to the immunoglobulin G1 subclass, they are effective against a narrow range of caprine arthritis-encephalitis viruses, and they identify (for the first time) antigenic variants among these caprine agents.

Visna-maedi virus of sheep and caprine arthritis-encephalitis virus (CAEV) of goats are serologically related retroviruses which cause persistent chronic-active inflammatory lesions in the brain, lungs, joints, and mammary glands leading to progressive debilitating disease in infected animals (6, 9-11, 22, 30). The perpetuation of this smoldering and frequently cyclical pathological process seems dependent on (i) restricted virus replication in infected cells (15) and (ii) failure to eliminate virus-producing cells despite competent immune defense mechanisms of the host. Restricted replication has been explained in part by integration of viral genomes in the host cell DNA and host cell-controlled regulation of transcription from these templates (4). One such regulatory mechanism is the level of maturation of the infected cell as exemplified by the infection in macrophages. In these cells, infected monocytes (promacrophages) have viral genomes but produce minimal quantities of infectious virus in contrast to the large amounts produced after they differentiate and mature into macrophages (24).

The failure of immune clearance is a complex issue, and the mechanisms may vary among the different groups of lentiviruses. Icelandic visna viruses elicit neutralizing antibodies during infection. Although these antibodies are highly specific, they have a narrow effective range of neutralization, and mutants with minor changes in the envelope glycoprotein escape neutralization (5, 23). Selective replication of mutants in the presence of antibodies which neutralize the parental viruses thus provide an effective mechanism for escape from the immune system in an immunocompetent animal.

Viruses associated with the natural disease complex in the sheep and goats in this country share antigenic determinants with Icelandic visna virus and replicate in similar types of cells, such as macrophages, in the animal (22, 25). However, preliminary studies have shown that animals infected with these field viruses do not produce neutralizing antibodies (13, 18; O. Narayan, Abstr. Annu. Meet. Am. College of Vet. Pathol. 1980, p. 68-69). Although this provides another mechanism for continuous virus replication, the failure of persistently infected animals to produce neutralizing antibodies raises many interesting questions regarding the pathogenesis of these infections. This report explores some of the potential mechanisms related to this lack of neutralizing antibody. Evidence is presented which demonstrates that the lack of neutralizing antibodies in these animals is due to a hyporesponsive state which can be overcome by simultaneous inoculation of virus and heat-inactivated *Mycobacterium tuberculosis* organisms. Characterization of these antibodies showed that they were similar in character to the antibodies induced by Icelandic visna virus. The antibodies belong to the immunoglobulin G1 (IgG1) subclass, are highly strain specific, and fail to neutralize other lentiviruses, including other strains of CAEV. This identifies for the first time distinct antigenic types among these caprine viruses.

MATERIALS AND METHODS

Naturally infected animals. Sheep and goats with clinical disease, reflecting the multisystem disease potential of this group of viruses, were acquired from different farms and studied for variable periods of time under laboratory conditions. Aspects of the infection and the disease in some of

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these animals have been reported previously (25). BL-19 was a dyspneic 5-year-old Border Leicester sheep; COR-102 and COR-104 were 3.5- to 4-year-old Corriedale sheep, the former with dyspnea and ataxia and the latter showing only dyspnea and emaciation; OPP-5 and OPP-9 were an unthrifty sheep and a sheep with ataxia and arthritis, respectively; SHROP-1 was a 4-year-old Shropshire sheep with dyspnea; and G-3 was a congenitally and persistently infected Toggenberg goat maintained for 5 years without any evidence of clinical disease or pathological lesions. G-77 was a thin, 5-year-old goat with severe chronic arthritis and mastitis (24).

Virus was obtained initially from monocyte cultures of peripheral blood from these animals and reisolated from tissues after they were killed. The cultivation of these field viruses and their preferential replication in macrophages have been reported previously (25).

Viruses and cell culture systems. Icelandic visna virus (V1514) is cultured routinely in fibroblasts derived from the choroid plexus of sheep (SCP) as described previously (23). Viruses from naturally infected sheep and goats were obtained initially from cultures of blood monocytes from the respective animals (24, 25). Additional stocks of sheep viruses were prepared in cultures of macrophages derived from a normal sheep.

Viruses from goats (CAEV) (10) or goat leukoencephalitis virus (8) were propagated in macrophage-like cultures derived from the synovial membranes of newborn goats (GSM) as described previously (22). Since this report identifies for the first time antigenic variants among these viruses from goats, we now designate our original virus described previously (6, 9, 22) as strain CAEV/CO.

Although sheep field viruses do not replicate efficiently in SCP cells (25), all of these viruses replicate and cause a syncytial type of cytopathic effect (CPE) in GSM cells similar to that seen with the caprine agents. Neutralization tests (see below) with V1514 and anti-V1514 sera performed in SCP, sheep macrophage cultures, and GSM cells showed identical neutralization end points (unpublished data). In this study, therefore, all assays for infectivity and neutralizing antibody were performed in GSM cells.

Virus neutralization. Virus neutralization was performed as previously described (23). Basically, the tests were performed by incubating mixtures of equal volumes containing 100 50% tissue culture infective doses (TCD_{50}) of virus and doubling dilutions of serum at 37°C for 30 min and then inoculating each mixture into four replicate cultures of GSM cells in microtiter plates or tissue culture dishes. In some experiments (identified in the text) variations of incubation conditions (time and temperature) of virus-serum reactions were altered, and in others, other sera were added to the mixtures in attempts to interfere with or enhance neutralization. Neutralization titers are expressed as the highest dilution of serum which prevented viral-induced fusion in GSM cells. Kinetic neutralization tests were performed by reacting multiple samples of 1-ml suspensions containing 10^6 TCD_{50} of virus with 0.1 ml of serum at 37°C in a shaker water bath. At specific intervals, one reaction mixture was diluted in 100 ml of cold medium, and further dilutions in 0.5 $\log_{10}$ units were inoculated into indicator cells in microtiter plates.

Virus concentration. Supernatant fluids were removed from infected cell cultures when the CPE was advanced and were stored at 4 or -70°C. The fluids were clarified by centrifugation and concentrated 100-fold either by precipitation with ammonium sulfate (14) or by ultrafiltration through a Millipore Pellicon Cassette. Concentrates had infectivity titers between 10^8 and 10^9 TCD_{50} /ml. These virus concen-

trates were used for immunization of animals as well as for serological tests.

Animals. One-year-old crossed Corriedale-Dorset sheep were used for these studies. Similarly aged mixed-breed goats were procured from the Johns Hopkins University Farm where a closed herd has been maintained free of infection with CAEV.

Immunizations. (i) **Live virus.** CAEV concentrate (2 ml) was emulsified in 2 ml of complete Freund adjuvant and injected into four intradermal sites in the axillary and flank regions of two goats. Concentrated virus emulsified in incomplete Freund adjuvant was given at 3-week intervals at other sites either intradermally or by deep intramuscular injection. A series of four or five of these inoculations was followed by about six weekly, intravenous administrations of virus concentrate supplemented with freshly harvested cells (10^8) and supernatant fluids from cultures at the height of virus production.

(ii) **Inactivated virus.** Infectivity in virus concentrates was inactivated either by UV irradiation or by detergent treatment with Zwittergent SB-14 (Calbiochem-Behring, La Jolla, Calif.). Both preparations were then used for immunization with a similar course of inoculations as described above.

(iii) **M. tuberculosis.** Heat-killed *M. tuberculosis* was kindly provided by A. Dannenberg, School of Hygiene and Public Health, Johns Hopkins University. Of these organisms, 10 mg was suspended in 2 ml of virus concentrate and dispersed in 20 ml of mineral oil by brief ultrasonic treatment with a microtip probe. This was administered by slow intravenous injection.

Serological tests. (i) **ELISA.** Enzyme-linked immunosorbent assay (ELISA) was performed by a modification of methods reported previously (1, 17). Virus concentrates were used as a source of antigen. This material was diluted to 10^{-3} in coating buffer (0.5 M CO_3-HCO_3 [pH 9.5]), and 200 μ l was used to coat wells in 96-well microtiter plates (Dynatech Immulon II). After 4 h at room temperature, the wells were washed three times with ELISA washing buffer (phosphate-buffered saline [PBS; pH 7.4] and 0.1% Tween 20), and dilutions of test sera in ELISA buffer (washing buffer plus 4% heated fetal bovine serum) were added. Plates were incubated overnight at 4°C and washed before the application of peroxidase-conjugated rabbit anti-goat (sheep) immunoglobulin (Cappel Laboratories, Cochranville, Pa.). After 4 h at room temperature, plates were washed six times, and freshly prepared substrate, containing citrate phosphate buffer (pH 5), 6.25 mg of *o*-phenylenediamine, and 25 μ l of hydrogen peroxide, was added. Color development was stopped after 30 min with 5 N H_2SO_4 , and plates were read at an absorbance wavelength of 492 nm in an ELISA reader (Flow Multiskan).

(ii) **Immunodiffusion.** Zwittergent-treated virus concentrates were used as antigen for the immunodiffusion test which was performed according to previously reported methods (22).

Serum fractionation. Crude IgG fractions were prepared from sera by precipitation with 33% saturated ammonium sulfate. The precipitate was dissolved in saline and dialyzed extensively against normal saline. IgG1 and IgG2 were separated from serum on columns of staphylococcal protein A Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.). The Sepharose was equilibrated in PBS, and eluates were collected in a UV-monitored LKB fraction collector. IgG1 eluted with PBS and the bound IgG2 and was eluted with 1 M acetic acid. The fractions were dialyzed for 24 h against PBS and diluted 10-fold from the original serum

volume. All immunoglobulin reagents were sterilized by membrane filtration.

RESULTS

Antibody response in naturally and experimentally infected animals. All clinically ill animals were viremic and had characteristic chronic inflammatory lesions in one or more of the usual target tissues affected in this disease complex (brain, lung, mammary gland, and/or joint) (data not shown). Furthermore, virus was obtained from cultures of macrophages aspirated from lungs of sheep with pneumonia and from synovial fluid of joints of goats with arthritis. All the animals also had virus-specific antibodies as determined by ELISA and immunodiffusion techniques (Table 1). Binding antibodies were detected in the ELISA test when CAEV or V1514 was used as antigen and precipitin lines in the immunodiffusion test coincided with those produced by antibodies to the core protein (p30) of V1514. Thus, these animals produced antibodies to the group-specific antigen common to these lentiviruses. However, none had any detectable neutralizing antibodies to the viruses obtained from their tissues.

A similar lack of virus-neutralizing antibodies was found in a series of 15 goats experimentally infected with the CAEV/CO virus (9). These animals were killed sequentially during a pathogenesis study which lasted 18 months. Although all of these animals had viremia and had developed typical inflammatory histological lesions in their brains and joints, none had produced virus-neutralizing antibodies.

Immunization with live virus. Since these lentiviruses do not replicate extensively in host tissues during persistent infection, one of the reasons for the lack of neutralizing antibodies may have been the lack of an adequate mass of viral antigens required to induce such antibodies. Three sheep and three goats were therefore given a course of multiple inoculations of virus extending over a period of 6 months. One of the sheep was given laboratory strain V1514 and two were given OPP-5 and COR-102 viruses, respectively (Table 2). The latter two viruses were obtained from naturally infected sheep identified in Table 1. The three goats were given CAEV/CO. As described above, each animal was given an initial injection of virus emulsified in Freund complete adjuvant, followed by four injections of virus in incomplete adjuvant. This was then followed by a

TABLE 2. Antibody response to goats and sheep hyperimmunized with live viruses

Animals	Immunizing virus strain ^a	Viremia ^b	ELISA Ab		ID	Neut. titer
			CAEV/CO	V1514		
Sh-15	V1514	Transient	1/40	1/40	+	1:1,280
Sh-11	OPP-5	Persistent	1/1,280	1/640	+	<1:10
Sh-771	COR 102	Persistent	1/40	1/80	+	<1:10
G-18	CAEV/CO	Persistent	1/50	1/50	+	<1:10
G-22	CAEV/CO	Persistent	1/640	1/5,120	+	<1:10
G-62	V1514	Transient	1/1,280	1/2,560	+	1:640
	+					
	CAEV/CO	Persistent				<1:10

^a Normal sheep (Sh) and goats (G) were injected sequentially multiple times with various viruses. ELISA antibody (ELISA Ab) titers to CAEV and V1514, and immunodiffusion (ID) and virus neutralization titers (Neut. titer) of serum to homologous immunizing viruses are as stated in Table 1.

^b Viremia was determined by addition of leukocytes from each animal to cultures of SCP and GSM culture.

course of weekly intravenous injections of large volumes of supernatant and cells from infected cultures at the height of virus production. One of these goats, G-62, was given two injections of V1514 several weeks before getting the course of CAEV immunizations.

Viremia in these animals was measured by cultivating 10-fold dilutions of peripheral blood leukocytes with SCP or GSM cells. V1514 causes CPE in both cell types, but the other viruses were cytopathic only in GSM cells. CPE in SCP cells, therefore, was indicative of replication by V1514. This virus caused a transient viremia in Sh-15 and G-62 animals, measuring at a ratio of approximately one cell to 10⁶ mononuclear cells at 1 month after the initial virus inoculation. This level, however, fell to a ratio of less than 1:10⁷ cells at 12 weeks when high levels of virus-neutralizing antibodies were detected in the sera (Table 2). The two other sheep viruses and CAEV caused higher levels of viremia, varying from 1:5,000 to 1:50,000 mononuclear cells. Moreover, the viremia persisted until the animals were killed several months later.

All infected animals developed binding antibodies to the viruses as demonstrated by ELISA and immunodiffusion tests; however, none of the animals produced neutralizing antibodies to the field viruses. G-62, which was immunized with both viruses, had thus produced neutralizing antibodies to V1514 but none to CAEV. In other studies (unpublished data) goats persistently infected with CAEV were immunized secondarily with either V1514, Sindbis virus, or parainfluenza 3 virus. These animals responded similarly to G-62 in that they readily developed neutralizing antibodies to the second virus but none to CAEV. These double immunization studies not only emphasized the antigenic disparity between V1514 and CAEV with regard to sharing of viral epitopes responsible for neutralization, but also showed that the ability of the animal to produce antibodies to heterologous agents was not compromised by persistent infection with CAEV.

Immunization with inactivated virus. The fact that the massive virus inoculation scheme with field viruses resulted only in heightened infection suggested that failure of the animal to develop neutralizing antibodies was not due to the inadequacy of antigenic stimulation. We therefore examined whether the lack of production of these antibodies was related to the known infectivity of these agents for macrophages. Conceptually, such infection could impair the immu-

TABLE 1. Antibodies to maedi-visna and CAEV in naturally infected animals^a

Animal	Disease	Virus isolation	ELISA Ab		ID	Neut. titer
			CAEV/CO	V1514		
Sh BL-19	P	+	1/160	<1/10	+	<1:10
Sh COR-102	PE	+	1/160	1/160	+	<1:10
Sh COR-104	P	+	1/20	1/20	+	<1:10
Sh OPP-5	PAE	+	1/20	<1/10	+	<1:10
Sh OPP-9	P	+	1/20	1/20	+	<1:10
Sh SHROP-1	P	+	1/640	1/320	+	<1:10
G-77	A	+	1/80	1/20	+	<1:10
G-3	—	+	1/10	<1/10	—	<1:10

^a Abbreviations: Sh, Sheep; G, goat; P, pneumonia; E, encephalitis; A, arthritis; ELISA Ab, highest dilution of serum from the particular animal producing a positive response with CAEV/CO and V1514 antigens; ID, immunodiffusion; ID +, coincidence of precipitin line with that caused by anti-P30 of V1514; neut. titer, highest dilution of serum causing inhibition of CPE by homologous virus in GSM cell cultures.

TABLE 3. Responses of animals immunized with inactivated virus antigens and challenged with homologous live viruses

Animals	Immunizing viral antigen ^a	ELISA Ab ^b		Neut. titer	Postchallenge	
		CAEV/CO	V1514		Viremia	Lesions
G-1	V1514	1/320	1/640	1:640	—	None
Sh-38	COR-102	1/640	1/5120	<1:10	+	Not examined
Sh-39	COR-102	1/640	1/2560	<1:10	+	Not examined
G-40	CAEV/CO	1/2560	1/5120	<1:10	+	Arthritis
G-73	CAEV/CO	1/2560	1/1280	<1:10	+	Arthritis
G-74	CAEV/CO	1/5120	1/1280	<1:10	+	Arthritis

^a Normal sheep (Sh) and goats (G) were injected multiple times with inactivated and disrupted immunizing viruses and then challenged with live homologous virus. All three goats immunized with CAEV antigens developed arthritis after challenge.

^b ELISA antibody (ELISA Ab) to CAEV/CO and V1514 antigens, neutralizing antibody titers (Neut. titer) to homologous viruses, and viremia determinations are as described in Table 1.

nological function of these cells. The experimental strategy was, therefore, to administer repeated inoculations of inactivated and disrupted virus preparations to test animals. The results of this experiment are shown in Table 3. Four goats were used in this study: one was given V1514 and three were given CAEV. Two sheep were given COR-102 virus. Two intradermal injections of inactivated and disrupted virus in Freund complete adjuvant at 3-week intervals were followed by five intramuscular injections of antigen in incomplete adjuvant; this was followed by three intravenous inoculations of antigen at weekly intervals. These animals were then bled for antibody determination and challenged by intravenous inoculation of 10^7 TCD₅₀ of live homologous virus to test whether there was any correlation between neutralizing antibodies in cell culture and protective antibodies in vivo. The goat (G-1) receiving inactivated V1514 developed a neutralizing antibody titer of 1:640 to this agent, and this antibody level was boosted fourfold by the virus challenge. Viremia was not detected in this animal. In contrast, none of the other animals receiving the other viruses developed neutralizing antibody. Furthermore, these animals developed viremia within 1 month after inoculation with live virus, and the agents were readily recovered from explants of spleen and lung, in addition to blood, when these animals were killed. These results thus showed that the infection per se of cells of the reticuloendothelial system was not the deciding factor in the failure of induction of neutralizing antibodies. Furthermore, this experiment suggested that the antibodies which neutralized virus in cell culture were probably also important in the protection of animals against infection because of the good correlation between the two phenomena.

Immunization with virus plus *M. tuberculosis*. Having eliminated antigenic mass and infection of immune cells as direct causes for lack of neutralizing antibodies to field viruses, we next attempted to determine whether the hyporesponsiveness of the animals may have been due to the inability of resident macrophages to process viral antigens. We therefore attempted to alter the macrophage population by injecting large amounts of *M. tuberculosis* organisms, which are known to cause macrophage activation (12, 19). Since intravenous administration of heat-killed BCG organisms suspended in mineral oil is known to stimulate large numbers of activated macrophages in the lungs of rabbits (21), we adopted this procedure for immunization of goats. Two goats, G-76 and G-81, which had been previously

infected with CAEV and were typically viremic and lacking neutralizing antibodies, were given two intravenous injections of 10 mg of *M. tuberculosis* in 2 ml of virus concentrate and 20 ml of mineral oil, two weeks apart. This was followed by four weekly intravenous inoculations containing 10^7 virus and 1 mg of *M. tuberculosis*. These animals developed serum neutralizing antibodies with titers of 1:80 to 1:160 at the end of the immunization period.

Characterization of antibodies to CAEV. Preliminary characterization of neutralizing and nonneutralizing goat anti-CAEV sera as exemplified by G-76 (neutralizing) and G-62 (nonneutralizing) sera is shown in Table 4 and Fig. 1. G-76 serum (anti-CO) had a neutralization titer of 1:80 against 100 TCD₅₀ of CAEV/CO. The neutralizing capacity of the serum was not affected by heating at 56°C for 30 min. The antiviral activity was found exclusively in the IgG fraction and was eliminated by prior precipitation of the immunoglobulin with anti-immunoglobulin serum. Further purification of the serum showed that all of the antibodies were in the IgG1 fraction with a corresponding lack of activity in the IgG2 fraction. The kinetic neutralization curve (Fig. 1) showed that the reaction was relatively slow, requiring almost 2 h to achieve 99% reduction of infectivity. Anti-immunoglobulin serum was added to the virus-serum mixtures after 30, 60, and 120 min and overnight incubation. Each of these samples was then incubated for an additional 30 min at 37°C and centrifuged at $10,000 \times g$ for 15 min. Assay of supernatant fluids showed an additional significant reduction of infectivity. Similar treatments of virus preparations with the anti-immunoglobulin alone had no effect. These results suggested that, in addition to neutralizing a portion of the infectivity in the classical manner, immunoglobulin molecules in the immune serum bound to virions which were still infectious. These particles were presumably agglutinated by the anti-immunoglobulin serum causing a further reduction in the infectivity in virus suspensions.

The specificity of the antibodies for the CAEV/CO strain was substantiated by testing the serum with progeny from four plaques of the virus, as well as with a crude virus preparation of the same CO stock which had undergone multiple passages in goats during the previous 6 years. The

TABLE 4. Factors in the neutralization of CAEV by immune goat serum

Reactants (viruses + sera)	Neutralization titer
CAEV/CO + anti-CO	1:80
CAEV/CO + (anti-CO + anti-Ig) ^a	1:20
CAEV/CO + IgG1 of anti-CO	1:80
CAEV/CO + IgG2 of anti-CO	<1:10
CAEV/CO + G-62 serum	<1:10
(CAEV/CO + G-62 serum ^b) + anti-CO	1:80
CAEV/G-77 + anti-CO ^c	<1:10
CAEV/G-3 + anti-CO ^c	<1:10

^a A total of 100 TCD₅₀ of CAEV/CO, the original strain of CAEV used in our laboratory, was neutralized by anti-CO (G-76 serum) at a dilution of 1:80. Preincubation of this serum with anti-immunoglobulin serum (anti-Ig) reduced the neutralizing capacity of the serum, proving that the neutralizing substance was immunoglobulin. All of these antibodies were confined to the IgG1 subclass.

^b Preincubation of virus with nonneutralizing G-62 "immune" serum in an attempt to block or enhance subsequent neutralization by G-76 serum had no effect.

^c Anti-CAEV/CO (G-76) serum did not neutralize other CAEV strains (G-77 and G-3 viruses).

serum had a titer of 1:80 against all five viruses. In contrast to these, the serum did not neutralize any other of the lentiviruses. These included two other strains of CAEV virus, G-77 and G-3 (Table 4).

The non-CAEV-neutralizing serum from hyperimmunized animals showed very little binding capacity for infectious particles. Serum from G-62 which had high levels of IgG1 neutralizing antibodies to V1514 but none to CAEV (Table 2) was typical of this group. Incubation of portions of 5×10^6 TCD₅₀ of CAEV/CO with dilutions of 1:10, 1:100, 1:1,000, and 1:5,000 of this serum for up to 4 h at room temperature, followed by further incubation overnight at 4°C, failed to cause reduction in infectivity (Fig. 1). Furthermore, addition of anti-immunoglobulin to a duplicate of each of the previous samples followed by incubation for 1 h at 37°C also failed to cause reduction in infectivity of the virus suspensions. These tests suggested that only negligible binding of the G-62 antibodies had occurred on the surface of the infectious virions.

Effect of nonneutralizing antibody on neutralization. A further test for potential binding of this G-62 serum to CAEV/CO was an attempt to interfere with the neutralizing capacity of G-76 serum. Since 100 TCD₅₀ of the virus could be neutralized with G-76 serum up to a dilution of 1:80, replicate tubes containing this amount of infectious virus were first incubated with G-62 post- and G-76 preinoculation sera at dilutions of 1:10 and 1:100 for 1 h at 37°C. These "sensitized" virus samples were then used in a standard neutralization test against immune G-76 serum (Table 4). Neutralization by the serum occurred at a dilution of 1:80, suggesting that the antibody molecules in G-62 serum had not bound to the virus particles in any manner which could block or enhance neutralization by the immune serum.

FIG. 1. Portions of CAEV/CO virus containing 5×10^6 TCD₅₀ were mixed with 1:10, 1:100, 1:1,000 and 1:5,000 dilutions of "immune" G-62 serum (see Table 2) and incubated at room temperature (26°C) for 4 h and 4°C overnight before assay for residual infectivity. Anti-immunoglobulin was added to duplicates of each sample and incubated for an additional 1 h at 37°C before assay. Neither "immune" serum (Δ) nor the "immune" serum plus anti- (▲) caused any significant reduction in the virus infectivity titer. In contrast, a 1:10 dilution of G-76 serum caused a steady reduction in virus infectivity (○) which was decreased further by addition of anti-immunoglobulin at specific time periods (●).

DISCUSSION

This report shows that persistent virus replication and lack of neutralizing antibodies occur frequently in lentivirus infections of sheep and goats and are similar in this way to certain other chronic viral diseases such as Aleutian disease of mink (28) and African swine fever (7). Although the precise mechanisms for lack of neutralizing antibodies are not understood, our results suggest that this was not due to insufficiency of viral antigens, infections of macrophages, or viral immunosuppression. Other studies of this phenomenon also rule out the possibility that such antibodies may have been bound in immune complexes (18). Rather, the consistent lack of neutralizing antibodies to field viruses may be due to poor antigenicity of the viral glycoprotein antigen which normally elicits such antibodies (29).

It is clear that *M. tuberculosis* organisms had an important role in the immunopotential process leading to the development of virus-neutralizing antibodies. The immunoregulatory potential of these organisms is well established and is mediated by macrophages which acquire new functional properties. Among these are alterations in receptor function (2), increased efficiency in phagocytosis (12), development of cytolytic properties (26), and development of resistance to growth of obligate intracellular parasites. Affected organisms range from protozoa (16, 27) and bacteria (3) to macrophage tropic viruses (19). Previous studies have shown that the ovine-caprine viruses described in this report replicated preferentially in macrophages (25). In this study, it is of interest that the level of replication of CAEV in *M. tuberculosis*-induced caprine alveolar macrophages was about 100-fold less than in normal alveolar macrophages (unpublished data). Since intravenous administration of virus to *M. tuberculosis*-treated animals would have targeted the agent to these activated macrophages, it is tempting to speculate that the virus-neutralizing antibody production in these animals was the result of a novel form of antigen processing by a population of macrophages which are not normally present in the animal.

Reasons for the difference in neutralization immunogenicity between Icelandic and field viruses are not understood, but the difference cannot be explained solely on the macrophage permissiveness for replication. These agents replicate only in sheep and goats, yet V1514 induces neutralizing antibodies in rabbits (20) and guinea pigs and mice (unpublished data), whereas CAEV does not. Rabbits immunized with the latter virus do, however, develop an apparent complement-dependent virolytic antibody (18), but this is clearly different from the classical neutralizing antibodies described in this report. Since the Icelandic visna virus is a laboratory strain which has been selected for lytic growth in sheep fibroblasts, it is possible that in the selection process the agent may have developed glycoprotein antigens which are highly immunogenic.

Immunization with V1514 showed that there was a good correlation between neutralizing antibodies in tissue culture and protective antibodies in vivo since immunization with inactivated virus protected against intravenous challenge with live virus. The corollary was also true in goats immunized with CAEV, since these animals failed to develop neutralizing antibodies and became infected after challenge with live virus. These results thus emphasize the interdependence between continuous virus replication and lack of neutralizing antibodies because, had the animals developed these antibodies, replication of that particular agent would have been curtailed.

The comparison between goat sera with neutralizing and

nonneutralizing abilities for CAEV provided novel insights into virus-antibody reactivities. For example, binding of immunoglobulins in the G-76 serum to virus was clearly demonstrable by the use of the anti-immunoglobulin serum. In contrast, immunoglobulin in nonneutralizing sera showed little evidence of binding to infectious particles either by use of anti-immunoglobulin in the agglutination test or in the blocking and enhancing neutralization test by proven neutralizing antibodies. Despite this, unpublished immunoprecipitation studies in our laboratory have shown that the immunoglobulin in nonneutralizing sera precipitated viral glycoproteins which make up the virus envelope. The viral glycoprotein is the antigen responsible for inducing neutralizing antibodies (29). One possible explanation for this incongruity is that antibodies in the nonneutralizing sera may have recognized epitopes in the virus envelope which are not on the surface of the particle. This lack of antibody recognition of specific viral epitopes, thus, may be important for the maintenance of persistent virus replication.

An additional point of interest in the neutralizing antibodies to CAEV was the narrow monospecificity of the antibody. This is very similar to the immune responses of sheep to Icelandic visna virus (V1514), both in terms of the antibody class and subclass, as well as the narrow spectrum of virus neutralization. The fact that the antibody to CAEV/CO did not recognize other CAEV strains identifies for the first time that antigenic variants of these viruses exist in nature. The antigenic stability of our CO strain after multiple clonings, as well as after multiple animal passages, is very similar to that of Icelandic visna virus without antibody selection pressures for development of mutants. Since CAEV does not normally induce such antibodies, there is no mechanism for selection of mutants. However, random mutation in the gene coding for the virus glycoprotein could conceivably account for the antigenic disparity in strains of the virus recognized by the antibodies to the CAEV/CO virus strain. From a practical point of view, in addition to the poor immunogenicity of these viruses in terms of elicitation of protective antibodies, the existence of antigenic variants creates additional potential problems for possible curtailment of this virus by any immunization scheme.

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