Separate Epitopes in the Envelope of Visna Virus Are Responsible for Fusion and Neutralization: Biological Implications for Anti-Fusion Antibodies in Limiting Virus Replication

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Visna virus is a lentivirus which causes fusion of infected cells in vitro. Two types of fusion occur. Fusion from without requires no viral replication and a relatively high multiplicity of infection; fusion from within results from the replication of virus in cells. By using fusion from without as an assay, the mechanism of fusion by visna virus was investigated. Immune sera which contained both anti-fusion and neutralizing antibodies interacted with the virus with rapid kinetics in blocking fusion but relatively slow kinetics in the virus neutralization assay. By using visna virus and an antigenic variant, the epitopes responsible for fusion and virus neutralization were shown to be different. Antigenic variation of visna virus resulted in alteration of the neutralization epitope and conservation of the fusion epitope. This suggested that there were two populations of antibodies and that the viral epitopes for fusion and neutralization were separate. These data suggest that visna virus is capable of infecting cells via two pathways: one via the fusion site and the other via the viral epitope which mediates neutralization.

Visna is a neurological and wasting disease of sheep characterized by a long incubation period, an insidious onset, and a slowly progressive course that is fatal (18). The disease is caused by an exogenous, replication-competent, nononcogenic retrovirus that is closely related to caprine arthritis-encephalitis virus (CAEV) (5, 15, 18, 23, 24). Both visna virus and CAEV cause persistent infections in their natural hosts. Visna virus induces neutralizing antibodies which provide a selective advantage for the emergence of antigenic variants (4, 19, 20). These variant viruses are virulent both in vivo and in vitro and differ from one another only in their neutralization patterns (19, 20). The biochemical properties of these viruses, their molecular mechanisms of replication, and their biological interactions with their host animals are prototypic of the human immunodeficiency viruses (3, 10, 11, 26, 28; O. Narayan and J. E. Clements, in *R. C. Gallo and F. Wong-Staal, ed., Retrovirus Biology: an Emerging Role in Human Biology*, in press) and have been used as model systems for dissecting the complex interactions between the latter agents and their human hosts.

Both the animal and the human viruses infect monocyte-macrophage cell types in vivo, and in these cells, the virus life cycle is arrested at different stages, depending on the state of maturation of the cell. More mature cells are more permissive for virus replication (8, 9, 21, 22). In contrast, the viruses replicate productively in certain host cells in culture and cause cytopathic effects characterized by cell fusion (12, 17, 27). Unlike the human viruses, which require suspensions of activated T lymphocytes for productive replication, the animal viruses achieve the same results in monolayer cultures of fibroepithelial cells (17, 27). CAEV-infected cultures produce virus continuously for 2 to 3 weeks with development of fusion in about 50% of the cells (17). Visna virus, in contrast, is more virulent, killing the cells between 4 and 7 days after inoculation at a multiplicity of infection (MOI) of 1. Cell fusion progresses to lysis in 1 to 2 days

during the productive phase of replication (12, 27). This is fusion "from within," occurring late in the virus life cycle. Uniquely, visna virus also causes fusion "from without" when inoculated at an MOI of greater than 3. This type of fusion does not require replication; it occurs within a few hours after inoculation and causes cell degeneration by "cytotoxic fusion" before completion of a single cycle of replication (12, 17). This type of fusion can also be caused by UV-inactivated virus and is similar to that caused by paramyxoviruses (2, 14).

The biological importance of the cell fusion property of lentiviruses is not clear. In this report, we have characterized some of the mechanisms of fusion by visna virus, using specific neutralizing antisera and antigenic variant viruses obtained from persistently infected sheep. Our data show that immune sera from sheep hyperimmunized with visna virus neutralized virus infectivity and prevented cell fusion, suggesting a generally accepted linkage between these two phenomena. However, monospecific sheep and guinea pig sera which neutralized parental viruses but not antigenic variant viruses prevented cell fusion by both parental and variant viruses. These data suggested that the viral epitopes responsible for fusion and neutralization were separate. Thus, fusion of the inoculated cells is not a prerequisite for attachment and penetration by the virus. Kinetic studies on fusion and neutralization showed wide differences between these two functions. In combination with selective adsorption of sera containing neutralizing and anti-fusing antibodies, these data suggest that the viral determinants responsible for fusion of cells and those responsible for binding to neutralizing antibodies are separate entities with different biological functions in vivo.

MATERIALS AND METHODS

Viruses. Ovine visna virus strain 1514, its antigenic variant LV1-1, and CAEV strain CO were used in these studies. The origin of these viruses has been described previously (6, 20).

Virus cultivation. Cell lines derived by preparation of

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explants from the choroid plexus of newborn lambs (sheep choroid plexus [SCP] cells) and from the synovial lining of carpal joints of newborn goats (goat synovial membrane [GSM] cells) were used to propagate the viruses (17, 19, 27). Visna viruses replicate productively in both types of cultures but were grown in SCP cells because these cells reach higher saturation than GSM cells and therefore produce more virions. SCP cells were grown to confluence in minimal essential medium (MEM) supplemented with 10% fetal bovine serum. Cultures were inoculated with virus at an MOI of 0.5 and maintained in MEM plus 1% lamb serum, heated at 56°C for 30 min (6, 20). Supernatant fluids were collected twice daily after cells developed cytopathic effects (CPE) and were stored at 4°C. CAEV replicates only in GSM cultures. These cells were propagated similarly to SCP cells and were inoculated at an MOI of 1. Supernatant fluids were collected as described for visna virus (24). The pooled supernatant fluids from each virus suspension were clarified by low-speed centrifugation, and portions were then stored frozen at -70°C for use in neutralization tests. Virus was then purified from the remainder of the fluids as previously described (24). Briefly, particles were concentrated by filtration of the fluid through a Millipore Pellicon membrane system and sedimentation of this concentrate on discontinuous 17 to 60% sucrose gradients. Virus which banded at the 17 to 60% sucrose interphase was removed and pelleted by centrifugation at $20,000 \times g$ for 2 h (24). Virus pellets were suspended in NET buffer (0.15 M sodium chloride, 0.02 M Tris [pH 7.5], 1 mM EDTA), stored at 4°C, and used in assays for fusion and in antibody-mediated inhibition of fusion.

Fusion. GSM cells were used exclusively for these tests. Monolayers of these cultures were inoculated with visna virus 1514 or LV1-1 suspended in fusion medium. This consisted of MEM, 0.5% fetal bovine serum, 25 mM Tris [pH 8], and 10% horse serum. Cultures were inoculated at a multiplicity of approximately 5 PFU per cell and incubated at 37°C. The time course for fusion from without was characterized, and an optimal incubation period of 16 h at 37°C was selected. At this time, the medium was removed and the cells were fixed with methanol and stained with Giemsa by using the Diff-Quik stain set (American Scientific Products). Cells were examined under a phase-contrast microscope, and the fusion index was determined by counting the average number of nuclei per cell. One hundred nuclei in an area of optimal fusion were counted, and the number of cells which contained these nuclei was determined (14). The fusion index was obtained by examination of multiple fields in two wells.

For fusion inhibition assays, a mixture of dilutions of specific antisera and a constant amount of virus were incubated at either 4 or 37°C for various intervals prior to addition to GSM cultures. To determine the dilution of serum that inhibited fusion by visna virus strains LV1-1 and 1514, monolayers of GSM cells were rinsed with fusion medium and inoculated with 1514 and LV1-1 at an MOI of 3. After incubation at 4°C for 1 h, the cultures were rinsed with phosphate-buffered saline (pH 7.5) and dilutions of immune serum were added to replicate cultures. These dishes were then incubated at 37°C for 16 h. For 4°C adsorption, 1514 (2.5×10^6 TCID₅₀, MOI of 2.27) was inoculated onto GSM cells and virus was allowed to adsorb to cells for 1 h at 4°C. At this time, cells were washed with sterile phosphate-buffered saline, and dilutions of serum were added to cells and allowed to incubate at 37°C for 16 h. For the 1-h assay, LV1-1 (8.0×10^5 TCID₅₀, MOI of 0.72) was added directly

onto GSM cells in the presence of sera at various dilutions and incubated at 37°C for 1 h. At this time, the virus and serum were removed, cells were washed with phosphate-buffered saline, and fusion medium was added for a 5-h incubation at 37°C.

Antisera. (i) **Monospecific neutralizing antiserum specific for visna virus 1514.** This immune serum was obtained from a sheep (S1) infected with a plaque-purified stock of visna virus 1514 as described previously (20). This animal developed neutralizing antibodies to the virus 2 months later (early antibodies). Approximately 1.5 years later, viruses were isolated from leukocytes of this animal and plaque purified (19, 20). One of these viruses was indistinguishable from the parental virus in neutralization tests with the early antibodies. However, one of the viruses, LV1-1, was not neutralized by early antibodies. This agent was designated an antigenic variant of 1514, and serum containing the early antibodies was designated monospecific.

(ii) **Antiserum to antigenic variant virus LV1-1.** LV1-1 maintained its unique antigenicity (lack of neutralization by early antibodies to visna virus 1514) when propagated in SCP or GSM cell cultures. A new sheep, no. 11, inoculated with this virus produced neutralizing antibodies to both the homologous virus LV1-1 and the parental virus 1514 (19, 20). This serum was designated S-11 anti-LV1-1.

(iii) **Hyperimmune antibodies to visna viruses.** Concentrated preparations of plaque-purified visna virus 1514 were disrupted with Nonidet P-40 detergent and injected repeatedly into a sheep, NN, at sequential intervals by using Freund complete adjuvant followed by antigen incorporated in incomplete adjuvant and then by several intravenous injections of the antigen (16). This animal produced antibodies that neutralized both visna virus 1514 and LV1-1 at high dilutions.

(iv) **Guinea pig antibodies to purified envelope protein of 1514.** Envelope antigens of visna virus 1514 were purified from a concentrated suspension of virus by lysing the particles with detergent and passage of the lysate through a column of lentil lectin Sepharose 4B which selectively bound the glycoproteins (24). Eluates from this column were characterized electrophoretically and shown to contain only the outer membrane glycoprotein of the virus (gp135 of visna virus) (24). This material was inoculated repeatedly into two guinea pigs. The animals developed antibodies that neutralized visna virus 1514 but not LV1-1. None of the sera described above neutralized CAEV-CO virus.

Adsorption of sera with virus-infected cells. Normal and CAEV- and visna virus 1514-infected GSM cells were used for adsorption of sera. Initial adsorption studies were done on CAEV-infected cells because of the persistent nature of the CAEV infection in contrast to the cytolytic infection of visna virus. Cell cultures in 150-cm² flasks were dispersed with EDTA solution (trypsin-EDTA heated at 60°C for 2 h; GIBCO Laboratories). Cell pellets from five flasks were suspended with 5 ml of specific immune sera diluted 1:10 in MEM and incubated in a shaker water bath at 37°C for 2 h and then overnight at 4°C. The cell pellets were removed by centrifugation, and the serum was subjected to another cycle of adsorption. The sera were then heated at 56°C for 10 min to inactivate any contaminating virus from the cultures and then used in neutralization and fusion inhibition experiments.

Virus neutralization. Neutralization tests were performed by inoculating cultures of GSM cells in microtiter plates with mixtures of twofold dilutions of serum and 100 50% tissue culture infectious doses (TCID₅₀) or PFU of virus. At 2 to 3

FIG. 1. Time course of fusion from without. Visna virus (4×10^6 TCID₅₀/ml) in MEM with 0.5% fetal bovine serum, 10% horse serum, and 25 mM Tris (pH 8.0) (fusion medium) was inoculated onto GSM cells (MOI of 4). The cells were incubated at 37°C with rocking for 4, 6, 8 and 16 h, fixed with methanol, and stained with the Diff-Quik Stain Set (American Scientific Products). Fusion indices of duplicate wells were obtained by the method of Kohn and Fuchs (14).

weeks after incubation at 37°C, the cultures were fixed with methanol and stained with Giemsa. Neutralization endpoints were then determined as indicated by CPE. Kinetic neutralization tests were also performed as described previously (13). Briefly, suspensions of virus containing approximately 10^6 TCID₅₀ were mixed with dilutions of the serum and incubated at various intervals from 5 to 45 min at 37°C prior to assay for residual infectivity.

RESULTS

Assay of fusion from without by visna virus. GSM cell cultures inoculated with visna virus 1514 or LV1-1 at a multiplicity of 3 to 5 PFU per cell developed cell fusion in less than 20 h after incubation at 37°C. The time course of fusion was determined; fusion was detected at 4 h after inoculation and increased with further incubation (Fig. 1). Optimum fusion occurred at 16 h after inoculation. Fusion was dependent on temperature and developed optimally at 37°C. No fusion occurred at $\leq 35^\circ\text{C}$, irrespective of the size of the inoculum or the length of the incubation period (data not shown). A survey of the pH range from 6 to 8.5 showed that optimal fusion occurred at pH 8.0. Routine fusion analyses were performed, therefore, at pH 8.0, with an incubation period of 16 h at 37°C.

Inhibition of fusion by specific antisera. Previous studies have shown that fusion from without by visna virus could be blocked by antiviral antibodies (12). However, the time course for the virus and antibody interaction and the specificity of the antibodies (neutralizing versus nonneutralizing sera) had not been investigated. Initial studies were performed with the hyperimmune NN serum. In fusion inhibition tests, twofold dilutions of this serum, incubated with concentrated visna virus (MOI of 5) for 2 h at 37°C, inhibited fusion of the virus up to a dilution of 1:320 (Fig. 2). These same dilutions neutralized 100 TCID₅₀ of virus up to a dilution of 1:160 (Table 1). These results corroborated the findings reported originally by Harter and Chopin (12).

In a previous report on kinetics of neutralization of visna virus, we had shown that neutralization required preincubation of antibodies with virus for at least 15 min at 37°C (13). Neutralization was first detected at this time and increased

slowly during the following 90 min. To test whether the inhibition of fusion followed similar kinetics, dilutions of the hyperimmune NN serum and virus were mixed and inoculated immediately into GSM cultures. After an incubation period of 1 h at 37°C, the inocula were removed and the cultures were washed with medium and reincubated at 37°C. No fusion occurred during the first 24 h. Further, fusion was inhibited by the dilution of serum that had been obtained when dilutions of the serum were incubated with virus at 37°C for 2 h prior to inoculation of the cells. Thus, binding between the fusion epitope and the fusion-inhibiting antibodies was apparently very rapid.

Despite the inhibition of fusion in this experiment, virus replication proceeded and cells died, with development of CPE by day 3. This experiment showed that the kinetics of antibody-mediated prevention of infection (neutralization) and prevention of fusion were distinct. Binding between antibodies and neutralization epitopes was slow, while binding between the antibodies and the fusion epitopes was very rapid.

In the previous report on kinetics of neutralization of visna virus, we had also shown that when virus-inoculated cultures were maintained at 4°C, the virus went through two phases of attachment to the cells (13). First was a loose attachment which could be disrupted by rinsing the cultures with medium. Loose attachment was followed by a tighter type of binding which could be broken only by enzymatic digestion with trypsin. Neutralizing antibodies added to virus-inoculated cells at the stage of loose attachment failed to prevent infection by the virus when the temperature was raised to 37°C. This was in keeping with the slow kinetics of neutralization that allowed virus to reach the stage of irreversible binding and penetration. This test was repeated in the fusion inhibition assay system. Virus was inoculated onto GSM cultures at 4°C for 1 h prior to inoculation of dilutions of the antiserum. After the addition of the antiserum, the cultures were transferred to 37°C and examined 16 h later. 1514 fusion was inhibited at a dilution of $<1:10$ by normal serum and at a dilution of 1:160 (range, 1:80 to 1:320) by hyperimmune NN serum. 1514 was neutralized at a dilution of $<1:10$ by normal serum and at a dilution of $<1:20$ by hyperimmune NN serum. No fusion had occurred in any of the cultures. However, antibodies failed to neutralize the virus under the same conditions. This experiment added further evidence that the mechanisms of neutralization and

TABLE 1. Neutralizing and anti-fusion activity of immune sera^a

Serum	Dilution			
	Neutralization		Inhibition of fusion	
	1514	LV1-1	1514	LV1-1
Normal lamb	$<1:10$	$<1:10$	$<1:10$	$<1:10$
NN-hyperimmune	1:160	1:160	1:80	1:320
NN-hyperimmune (adsorbed) ^b	$<1:10$	ND	1:80	ND
Guinea pig anti-gp135	1:160	$<1:10$	1:320	1:1,280
Guinea pig anti-gp135 (adsorbed) ^c	$<1:10$	ND	1:160	ND
S1	1:160	$<1:10$	1:320	1:640

^a Highest dilution of serum which exhibited neutralizing or antifusion activity. Equal concentrations of both viruses (4×10^6 TCID₅₀, MOI of 4) were preincubated with various dilutions of serum for 2 h at 37°C prior to inoculation onto GSM cells, which were then incubated for 16 h at 37°C.

^b Serum adsorbed with CAEV as described in Materials and Methods.

^c Serum adsorbed with either CAEV or 1514 as described in Materials and Methods.

FIG. 2. Fusion from without by visna virus. GSM cells were incubated in fusion medium for 16 h at 37°C in the absence of visna virus and fusion-inhibiting serum (a), in the presence of visna virus (4×10^6 TCID₅₀/ml, MOI of 4) in fusion medium (b), and in the presence of a mixture of visna virus (4×10^6 TCID₅₀/ml, MOI of 4) and hyperimmune serum (NN) at dilutions of 1:320 (c) and 1:1,280 (d) which had been preincubated for 2 h at 37°C.

inhibition of fusion by visna virus were distinct and not interdependent.

Differences in antigenicity between the fusion and neutralization epitopes of visna viruses. Having established that the fusion and neutralization epitopes of visna virus had distinct reactivities with antibodies and cells, we next sought to determine whether the two epitopes were antigenically distinct. Parental visna virus 1514 and its antigenic variant, LV1-1, were used in these studies. The two viruses have similar rates of replication in cell culture (20), and studies of fusion by the two agents showed that they were equally efficient at causing fusion from without (data not shown). The monospecific early immune serum which neutralized visna virus 1514 but not LV1-1 was used to determine whether the antibodies would have a similar reactivity in inhibition of fusion as in neutralization. Surprisingly, the antibodies completely inhibited fusion by LV1-1 despite their inability to neutralize this virus (Table 1). The titer of fusion-inhibiting antibodies and the kinetics of the reaction mediated by these antibodies were similar in fusion inhibition tests with both viruses. Sera from guinea pigs immunized with the envelope glycoprotein of visna virus 1514 were used in the fusion inhibition test against both viruses.

These antibodies neutralized 1514 but not LV1-1. However, the antibodies inhibited fusion by both viruses at similar dilutions (Table 1). Use of these two polyclonal sera thus established that the fusion and neutralization epitopes of visna virus were antigenically distinct.

Separation of neutralization and anti-fusion antibodies in hyperimmune serum by adsorption. The previous experiments suggested that the fusion and neutralization epitopes of visna virus were antigenically distinct. This suggestion led us to predict that antibody populations in sera that inhibited both infection and fusion functions of the virus may therefore be specific and possibly separable. We tested this by adsorbing the bifunctional hyperimmune NN serum and the monospecific guinea pig serum with suspensions of cells infected with either CAEV (for NN and guinea pig) or visna virus (for guinea pig). Adsorption of the serum with uninfected cells had no effect. However, adsorption of this serum with either CAEV- or visna virus-infected cells resulted in removal of all of the neutralizing antibodies from the serum (Table 1). The serum, however, retained its antifusion capacity and titer. This showed that the antibody populations to the two epitopes are distinct and clearly separable.

DISCUSSION

We examined the mechanisms and kinetics of induction of fusion of cells by visna virus. Our results suggest that this function of the virus is mediated by a pair of viral-cellular determinants that is distinct from the pair whose binding is prevented by neutralizing antibodies. The results show that the envelope of visna virus contains two biologically important epitopes: one that causes fusion of the cells and another, responsible for penetration and infection, that binds neutralizing antibodies. Previous studies had shown that visna virus caused fusion from without and that this fusion could be prevented by anti-viral antibodies (12). Using visna virus-induced fusion from without as an assay for fusion, we have extended these studies.

Since anti-viral antibodies in hyperimmune sera blocked virus-induced fusion, these antibodies were used to investigate the time course of virus and antibody interaction and the location of the virus fusion epitope and to determine whether the fusion and neutralization epitopes were separate. A series of experiments with the antisera provided evidence that the interaction of antibodies with the viral fusion epitope was rapid and efficient and required no preincubation of the virus with antibodies. Antibodies blocked virus-induced cell fusion even when the virus was allowed to attach to the cells at 4°C for 1 h prior to the addition of antibodies. This same anti-viral serum neutralized the virus, but neutralization required preincubation of

virus and antibody for several minutes at 37°C. These data indicated that the interaction of antibodies with the viral-fusion epitope and viral epitope responsible for neutralization occurred with very different kinetics, suggesting that the two epitopes are different.

The outer membrane glycoprotein of visna virus, gp135, had previously been shown to elicit neutralizing antibodies (24, 25). Guinea pig antisera prepared against purified gp135 completely blocked virus-induced cell fusion. This strongly suggested that both the fusion and neutralization epitopes of visna virus are located on gp135.

Neutralizing antibodies against visna viruses are strain specific (16, 20). Thus, antibodies elicited against the 1514 strain of the virus do not neutralize antigenic variants of that strain. LV1-1, an antigenic variant of visna virus 1514, was used to determine whether the fusion and neutralization epitopes were separate. Both the monospecific sheep and guinea pig sera neutralized visna virus 1514 but not LV1-1. However, both of these sera blocked fusion by both viruses. This suggested that the viral fusion epitope was the same (and conserved) in the two viruses, while the neutralization epitope had been altered by mutation (4, 20). An alternative possibility was that a single epitope functioned for both viral activities but only a portion of the epitope was altered by mutation of 1514 to LV1-1. To distinguish between these two possibilities, the two sera from the sheep and guinea pig that had antibodies against both functions (anti-fusion and anti-neutralization) were adsorbed on infected cells and the resulting sera were tested for both virus neutralization and blocking virus-induced cell fusion. The neutralizing antibodies were removed by adsorption, but both adsorbed sera retained their anti-fusion antibodies. This supported a hypothesis for the existence of two epitopes on the virus, each inducing a specific population of antibody molecules.

Fusion by visna virus is analogous to that caused by paramyxoviruses and may thus be a mechanism for entry of virus into cells (1, 2). Since antibody populations that block cell fusion by visna virus permit execution of the virus life cycle, it is probable that the virus is capable of infecting host cells via two pathways: one via the fusion site and the other via the neutralization site. Further, since fusion is mediated by the viral glycoprotein, it would be possible for an infected cell producing viral glycoproteins to fuse with another cell and transmit the viral nucleic acids without having produced virions. In fact, the transmission of lentivirus infectivity from a macrophage to a fibroblast via fusion has been demonstrated in vitro (22). This may be relevant in vivo because in tissues of sheep infected with visna virus, many macrophages are laden with viral RNA transcripts but few have virus particles. Further, there are fewer infected cells in tissues of animals that develop neutralizing and anti-fusion antibodies than in animals without such antibodies (O. Narayan, unpublished observations). Given the poor kinetics of virus neutralization and the lack of infectivity in tissues, it seems doubtful that neutralizing antibodies have a curative function. In this setting, anti-fusion antibodies may play a more protective biological role because, by their efficient and rapid block of cell fusion, they could limit the spread of virus much more effectively than neutralizing antibodies. This concept is illustrated in Fig. 3. Although speculative, it correlates with the events in vivo. Future studies are directed to this problem. If it is proven true, then the efficacy of vaccines against lentiviruses should be evaluated less on the ability to induce neutralizing antibodies and more on their potential for inducing anti-fusion antibodies.

FIG. 3. Hypothesized role for anti-fusion antibodies in limiting virus replication. Completion of the virus life cycle in vivo depends on maturation of latently infected macrophage precursors in bone marrow to active macrophages in tissues. In most of these cells, the virus life cycle is arrested at a late stage, prior to production of virus particles. As cells become effete, a few infected macrophages produce virus particles. Viral antigens in both mature and effete macrophages induce neutralizing antibodies and anti-fusion antibodies. Experimentally infected animals with these antibodies have only few infected mature macrophages. We speculate that the anti-fusion antibodies may prevent fusion of infected mature macrophages with other cells and thus prevent spread of viral nucleic acids. Neutralizing antibodies react only with virus particles, and some of these probably escape neutralization to initiate a new cycle of infection.

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