Topographical Rearrangements of Visna Virus Envelope Glycoprotein during Antigenic Drift

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Visna virus undergoes antigenic drift during persistent infection in sheep and thus eludes neutralizing antibodies directed against its major envelope glycoprotein, gp135. Antigenic variants contain point mutations in the 3' end of the genome, presumably within the envelope glycoprotein gene. To localize the changes in the viral proteins of antigenic mutants, we isolated 35 monoclonal antibodies (MAbs) against the envelope glycoprotein gp135 or the major core protein p27 of visna virus. The MAbs defined five partially overlapping epitopes on gp135. We used the MAbs and polyclonal immune sera directed against visna virus, gp135, or p27 in enzyme-linked immunosorbent assays to compare visna virus (strain 1514) with antigenic mutants (LV1-1 to LV1-6) previously isolated from a single sheep persistently infected with plaque-purified strain 1514. Polyclonal immune sera and anti-core p27 MAbs failed to distinguish antigenic differences among the viruses. By contrast, the anti-gp135 MAbs detected changes in all five epitopes of the envelope glycoprotein. Three gp135 epitopes, prominently exposed on strain 1514, were lost or obscured on the mutants; two covert gp135 epitopes, poorly exposed on strain 1514, were reciprocally revealed on the mutants. Even virus LV1-2, which is indistinguishable from parental strain 1514 by serum neutralization tests and which differs from it by only two unique oligonucleotides on RNase-T1 fingerprinting, displayed global changes in gp135. Our data suggest that (i) visna virus variants may emerge more frequently during persistent infection than can be detected by serological tests involving the use of polyclonal immune sera, and (ii) the extent of phenotypic changes in their envelope glycoproteins may be greater than predicted by the small number of genetic changes previously observed. We suggest that topographical rearrangements in the three-dimensional structure of gp135 may magnify the primary amino acid sequence changes caused by point mutations in the *env* gene. This may complicate strategies to construct lentiviral vaccines by using the envelope glycoprotein.

Visna virus is the prototype lentivirus, a nononcogenic retrovirus of sheep and goats which causes slowly progressive encephalitis, pneumonitis, and arthritis (31). By morphology and nucleotide sequence homology, visna virus resembles three other lentiviruses: caprine arthritis encephalitis virus (26, 27, 39), equine infectious anemia virus (34), and human immunodeficiency virus, the putative cause of acquired immune deficiency syndrome (12, 32). The lentiviruses contain a single linear strand of messenger-sense RNA within an enveloped capsid. Three major structural genes, 5'-*gag-pol-env*-3', respectively encode the core proteins, RNA-dependent DNA polymerase (reverse transcriptase), and the major envelope glycoprotein (4, 14, 28). The envelope glycoprotein is the target for neutralizing antibodies (29) and may also mediate virus attachment to cells (18).

The lentiviruses share the remarkable ability to replicate continuously in immunocompetent hosts (6). Visna virus has evolved the following three strategies of replication to elude humoral immune elimination: (i) integration of proviral DNA into the host stem cell genome (3, 11, 15); (ii) restriction of viral gene expression in a narrow range of target cells (promonocytes, monocytes, and specific populations of macrophages) (10, 11, 24, 25); and (iii) escape from neutralizing antibodies directed against the major envelope glycoprotein, gp135, by progressive antigenic drift (5, 8, 13, 22, 29).

We previously studied antigenic variation of visna virus in a single persistently infected animal by inoculating a sheep

with plaque-purified visna virus (strain 1514) and monitoring the infection for 3.5 years (22, 23). Soon after infection, the sheep developed serum antibodies which neutralized the virus. Of six visna virus strains isolated from peripheral blood after 1 year however, five could no longer be neutralized by the sheep's own serum (23). These six antigenically distinct viruses (called leukocyte viruses from sheep 1 [LV1-1 to LV1-6]) contained point mutations in the 3' ends of their genomes, presumably within their envelope glycoprotein genes (5, 8). We postulated that changes in gp135 might confer a selective advantage for visna virus survival and spread by allowing a temporary escape from neutralization. Similar changes in the envelope glycoproteins of equine infectious anemia virus were subsequently linked to the recurrent waves of fever and anemia seen in persistently infected horses (19). Together, these studies suggested that all lentiviruses may undergo antigenic drift.

To determine how visna virus proteins change during antigenic variation of the virus, we isolated 35 anti-visna virus monoclonal antibodies (MAbs) directed against five spatial epitopes on gp135 or against the major core protein, p27. We used the MAbs to probe for antigenic differences among visna virus (strain 1514) and the six LV1 mutant viruses we had previously genetically characterized. We show here that during antigenic drift, the topography of the envelope glycoprotein is globally rearranged but the core protein is not measurably altered. We suggest that topographical rearrangements in the three-dimensional structure of gp135 may magnify the primary amino acid sequence

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changes caused by a small number of point mutations in the *env* gene.

MATERIALS AND METHODS

Mice. We used 6- to 12-week-old BALB/c AnNrIBR female mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.).

Virus. Icelandic visna virus strain 1514 was plaque purified from primary sheep choroid plexus cells (23). Virus was treated with 5.3 M NaCl and purified by equilibrium centrifugation on a 20 to 50% discontinuous sucrose gradient (7). Envelope glycoprotein gp135 was purified by affinity chromatography on a *Lens culinaris* Sepharose 4B column (Pharmacia Fine Chemicals, Piscataway, N.J.) (27).

Antisera. Preparation of hyperimmune sheep antisera to detergent-disrupted, inactivated visna virus or to purified core protein p27 (21) and guinea pig antisera to purified gp135 and the putative gp135-p15(E) complex (27) has been described previously.

Isolation of MABs. (i) Immunization. A female BALB/c mouse was injected with 195 μ g of purified gp135 over 9 months: 20 μ g subcutaneously in complete Freund adjuvant initially; 25 μ g subcutaneously in incomplete Freund adjuvant after 30 days; 50 μ g intraperitoneally in phosphate-buffered saline (PBS) (pH 7.4) after 150 and 180 days; and 50 μ g intravenously in PBS with 0.05% dithiothreitol [to ensure dissociation of any contaminating gp135-p15(E) complexes] 3 days before fusion at 9 months. Mouse anti-gp135 immune serum was obtained from the mouse used for fusion.

(ii) Hybridomas. Our fusion protocol has been detailed previously (33). Briefly, 1.2×10^8 spleen cells were fused to 6.6×10^7 P₃ NS1-Ag-4-1 myeloma cells with 50% polyethylene glycol 1540 (BDH, Poole, England) and were grown in hypoxanthine-aminopterin-thymidine selective medium. Fused cells were mixed with a feeder layer of 5.6×10^6 BALB/c mouse peritoneal cells (>95% esterase positive). Hybridomas were cloned one to three times by limiting dilution in soft agar (9). MAB ascites fluid was then made and filtered as described previously (33).

(iii) ELISA. Immulon-II 96-well plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with either purified visna virus (200 ng of protein per well either in 100 μ l of PBS for enzyme-linked immunosorbent assay (ELISA) titers or in 100 μ l of 0.5 M CO₃-HCO₃ [pH 9.5] for hybridoma screening) or affinity-purified gp135 (25 to 50 ng per well in 100 μ l of PBS). Plates were blocked with PBS containing 2% bovine serum albumin. Hybridoma supernatant fluid, ascites fluid, and immune serum were detected by horseradish peroxidase-conjugated rabbit anti-mouse, anti-goat, and anti-guinea pig immunoglobulin antisera (DAKO Corp., Santa Barbara, Calif.). The ELISA titer was the greatest dilution of ascites or serum yielding an optical density at 492 nm, as measured by a Titertek multiscan, greater than twice that obtained by substituting anti-Sindbis virus MABs or normal serum. Relative MAB-binding avidity was defined as the log₁₀ amount in nanograms of protein of ammonium sulfate-purified ascites required to yield an optical density at 492 nm of 0.4 (1). Relative avidity was defined as high if 10 ng or less of purified MAB ascites protein was required to yield an optical density of 0.4 (relative avidity, ≤ 1.0).

(iv) Isotype. Hybridoma antibody class was determined by ELISA as described previously (33).

(v) Radioimmunoprecipitation. Either L-[³⁵S]methionine (>600 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), L-[4,5-³H]leucine (40 to 60 Ci/mmol; Amersham), or D-[1-

³H]glucosamine hydrochloride (2 to 5 Ci/mmol; Amersham) was added to visna virus-infected sheep choroid plexus cells as described previously (27). Labeled virus purified by equilibrium density centrifugation was lysed in 0.02 M Tris hydrochloride buffer (pH 7.6) containing 0.15 M NaCl, 0.5% sodium deoxycholate, 0.5% Zwittergent 3-14 (Calbiochem-Behring, La Jolla, Calif.), 0.1% sodium dodecyl sulfate (SDS; Bio-Rad Laboratories, Richmond, Calif.), 1 mM disodium EDTA, and 1 mM phenylmethylsulfonyl fluoride. MAB ascites fluid (1 to 25 μ l), hybridoma supernatant fluid (0.5 to 1.0 ml), or immune serum (1 to 25 μ l) was incubated with labeled lysed virus (2×10^5 cpm) for 2 h at 37°C, and the mixture was then added to 35 μ l of a 50% suspension of protein A coupled to Sepharose CL4B (Pharmacia Fine Chemicals) for 12 to 16 h at 4°C. To allow immunoprecipitation by immunoglobulin A (IgA), IgM, and some IgG1 MABs or by sheep or goat serum, protein A-Sepharose beads were first mixed 1:1 (vol/vol) with rabbit anti-mouse immunoglobulin, IgA, or IgM antiserum (DAKO) or rabbit anti-goat immunoglobulin antiserum (DAKO). Precipitated proteins were boiled for 5 min in 30 to 40 μ l of 0.05 M Tris hydrochloride (pH 6.9) with 1% SDS, 2% 2-mercaptoethanol, 10% glycerol, and 0.1% bromophenol blue (polyacrylamide gel electrophoresis [PAGE] buffer), separated by SDS-PAGE in continuous 5 to 20% acrylamide gels (16), and analyzed by fluorography (2).

(vi) Protein immunoblot. Purified visna virus (400 to 500 μ g of protein) or gp135 (25 μ g of protein) was separated by SDS-PAGE on 5 to 20% gels. Proteins were then transferred electrophoretically to nitrocellulose paper (36), where binding of anti-visna virus antibodies was detected by immunoperoxidase staining as described previously (33).

Competition binding assay. Ammonium sulfate-purified MAB ascites (5 to 10 mg/ml) was biotinylated and detected in our ELISA by the method of Waxham and Wolinsky (38). Visna virus- or gp135-coated wells were incubated with duplicate serial dilutions of the competing unbiotinylated purified MAB ascites (50 ng to 10 μ g of protein in 100 μ l of

FIG. 1. *L. culinaris* affinity column purification of [³H]leucine-labeled visna virus gp135 shown by SDS-PAGE on a 5 to 20% acrylamide gel. Fluorograph lanes: 1, purified visna virus before application to affinity column; 2, viral proteins not bound by the column (gp135 has been quantitatively bound by the column); 3, purified gp135 eluted from column; 4, longer fluorographic exposure of the gp135 preparation demonstrating minor contamination (arrows) by 90-, 50-, and 27-kDa proteins. Major visna virus proteins and molecular weight (10^3) protein standards are labeled.

TABLE 1. Biological properties of MAbs and immune sera against visna virus

Hybridoma or control	Isotype ^a	ELISA titer ^b		Antigenic specificity ^c						
		Virus	gp135	Envelope					Core	
				gp150 ^{env}	gp135	gp105	p90	p50	p55	p27
Anti-envelope										
50-1	IgG1	2.0	—			○				
51-5	IgG1	>5.0	—		○					
52-4	IgG1	3.0	2.0		●, ○					
53-1	IgG1	3.0	—		○					
56-3	IgG1	>5.0	3.0		○					
60-3	IgG1	>5.0	2.0	○	●, ○	●, ○	○			
61-6	IgG1	>5.0	4.0	○	●, ○	●, ○	○			
63-2	IgG1	>5.0	3.0		○	○	○			
66-3	IgG1	>5.0	—		●					
74-2	IgG1	—	—		●	●				
80-3	IgG1	>5.0	4.0		●, ○	●				
83-4	IgG1	4.0	2.0		●, ○					
88-3	IgG1	>5.0	>5.0		●, ○	○	○			
114-4	IgG1	—	2.0	○	●	●				
115-2	IgG2B	—	2.0		●					
118-2	IgG1	3.0	2.0	○	●	●, ○		●, ○		
120-3	IgG2B	2.0	4.0		●, ○	●		●		
121-1	IgG1	4.0	—		●	●		○	●, ○	
123-1	IgG1	—	>5.0	●, ○	●, ○	●		●, ○		
128-2	IgG1	>5.0	4.0		●, ○	●, ○	○	●, ○		
130-2	IgG1	2.0	>5.0	○	●, ○	●, ○	○	○		
133-1	IgG1	>5.0	—		●, ○	●, ○				
135-4	IgG1	>5.0	—		●					
141-1	IgG1	>5.0	—		○					
143-3	IgG2A	2.0	4.0	●	●	●		●, ○		
164-3	IgG1	—	—		●, ○	●				
168-6	IgG1	>5.0	4.0	○	●, ○	●, ○	○	●, ○		
170-1	IgG1	2.0	3.0		●, ○					
200-1	IgG2A	>3.0	—	○	○					
Anti-core										
64-1	IgG1	4.0	—					○	○	●, ○
71-5	IgG1	3.0	3.0						●, ○	
75-4	IgM	4.0	2.0						●, ○	
78-2	IgM	—	—						●, ○	
122-3	IgG1	2.0	2.0	○	○		○		●, ○	○
161-6	IgM	3.0	—					○	○	
3076 ^d	IgG2A	3.0	—						●, ○	
Controls ^e										
Anti-SV Mabs	IgGs	—	—							
	IgM	—	—							
Immune sera										
Mouse anti-gp135		3.0	3.0	○	●, ○	●, ○			○	
Sheep anti-visna virus		4.0	5.0	●, ○	●, ○	●, ○	●, ○	●	●, ○	●, ○
Guinea pig anti-gp135		5.0	5.0	●, ○	●, ○	●, ○	○			
Goat anti-p27		5.0	2.0						●, ○	
Normal sera (all species)		—	—							

^a Determined by ELISA with isotype-specific rabbit anti-mouse immunoglobulin sera.

^b ELISA titers are the greatest log₁₀ dilutions of ascites fluid or sera binding purified virus or affinity-purified gp135 to an optical density at 492 nm greater than twice the background level (determined by substituting anti-Sindbis virus MAb ascites fluid or normal serum). Titers less than 2.0 (1:100) were considered negative (—).

^c Determined by immunoprecipitation (●) of radiolabeled visna virus or by protein immunoblot (○) of visna virus or purified gp135. Proteins gp150^{env} and p55 are, respectively, the cellular precursors of gp135 and the three core proteins (p16, p27, and p14). Proteins gp105, p90, and p50 may be degradation products or precursors of gp135.

^d Gift of Neal Nathanson.

^e Anti-Sindbis virus (SV) MAbs matched for isotype and normal sera matched for species served as negative controls.

PBS–2% bovine serum albumin) for 1 h at room temperature. Biotinylated MAb (diluted 1:100 to 1:10,000 to yield an optical density at 492 nm in the linear range of 0.4 to 1.0) was then added for 2 h at room temperature. Bound biotinylated MAb was detected by using 200 µl of streptavidin-horseradish peroxidase (Vector Laboratories, Inc., Burlingame, Calif.) per well diluted 1:1,000 in PBS. Competitive

binding was defined as greater than 50% reduction in biotinylated MAb ELISA binding; most competitive MAbs inhibited binding by more than 90%.

Antigenic variants. We had previously inoculated Hampshire sheep no. 1 with plaque-purified visna virus strain 1514 and over the next 3.5 years had sequentially isolated six stable, virulent visna virus mutants (LV1-1 to LV1-6) from

FIG. 2. Immunoprecipitation of visna virus proteins by immune sera and MAbs. Composite fluorographs from 12 SDS-polyacrylamide (5 to 20%) gels. Visna virus proteins and the molecular weight (10^3) protein standards are marked. (A) Antigenic specificities determined by using [35 S]methionine-labeled virus. Lanes: 1, purified virus; 2, sheep anti-visna virus serum; 3, sheep anti-core (p27) serum; 4, normal sheep serum; 5, guinea pig anti-gp135 and anti-p15(E) serum; 6, guinea pig anti-gp135 serum; 7, normal guinea pig serum; 8, mouse anti-gp135 serum; 9, normal mouse serum; 10, Sindbis virus IgG MAb; 11 to 22, MAbs as marked. Small amounts of p27 are nonspecifically coprecipitated by several anti-gp135 sera and MAbs. (B) Viral glycoproteins identified by immunoprecipitation of [3 H]glucosamine-labeled virus (lanes 2 to 11). Lanes: 1, [35 S]methionine-labeled virus for comparison; 2, [3 H]glucosamine-labeled virus; 3, sheep anti-visna virus serum; 4, normal sheep serum; 5, guinea pig anti-gp135 serum; 6, normal guinea pig serum; 7, Sindbis virus IgG MAb; 8 to 11, visna virus MAbs as marked.

cultures of its peripheral blood (5, 21–23). For these experiments, the six LV1 viruses were plaque purified, purified by equilibrium density centrifugation, and then coated onto Immulon II (Dynatech) plates at 200 ng of protein per well in PBS. We compared the LV1 viruses with strain 1514 by measuring differences in ELISA binding by MAb ascites fluid or polyclonal anti-visna virus, anti-gp135, or anti-p27 immune sera (diluted 1:250 to 1:64,000 in PBS–2% bovine

serum albumin and assayed in triplicate). Background binding by isotype-matched Sindbis virus MAb ascites or by nonimmune sera was subtracted at each dilution.

RESULTS

MAbs against visna virus. Our initial fusion experiments with mice immunized with purified visna virus produced only hybridomas secreting anti-p27 (core protein) MAbs

FIG. 3. Composite immunoblots of visna virus. Lanes: 1, visna virus lysate stained for protein with 1% amido black–1% aniline blue in 45% methanol–10% acetic acid; 2 and 3, two sheep anti-visna virus sera; 4, sheep anti-p27 serum; 5, normal sheep serum; 6, guinea pig anti-gp135 serum; 7, normal guinea pig serum; 8, mouse anti-gp135 serum; 9, normal mouse serum; 10, Sindbis virus IgG MAb; 11 to 14, anti-core MAbs as marked; 15 to 28, anti-envelope MAbs as marked. The anti-gp135 MAbs displayed five patterns (I to V) of immunoblot reactivity. Visna virus proteins are marked.

(data not shown). To obtain MAbs specific for gp135, we purified the envelope glycoprotein from virus by affinity chromatography and used it to more selectively immunize mice (Fig. 1). A total of 170 anti-visna virus hybridomas were isolated by virtue of their binding to either visna virus or purified gp135 in our ELISA; 80 were further biologically characterized, and 35 were used in this study. The isotypes of the MAbs are 80% IgG1, 5.7% IgG2A, 5.7% IgG2B, and 8.6% IgM (Table 1).

The antigen specificity of each MAb was determined by immune precipitation of radiolabeled purified visna virus (Fig. 2; Table 1). Six MAbs (Fig. 2A, lanes 21 and 22), like anti-p27 immune sheep serum (lane 3), precipitated the major core protein, p27. Of the 35 MAbs, 22 precipitated gp135: 7 precipitated gp135 alone; 10 precipitated both gp135 and a prominent 105-kilodalton (kDa) protein not seen in lysates of purified virus (Fig. 2A, compare lanes 12 and 14 with lane 1); and 5 precipitated gp135, the prominent 105-kDa protein, and a broad 50- to 55-kDa protein band (p50; Fig. 2A, lane 13). Four polyclonal anti-visna virus or anti-gp135 immune sera also precipitated gp135 and the 105-kDa protein (Fig. 2A, lanes 2, 5, 6, and 8). Unlike the immune sera however, many anti-gp135 MAbs precipitated the 105-kDa protein better than gp135 itself (Fig. 2A, lanes 15 to 20). Immune precipitations with [³H]glucosamine-labeled virus showed that the 105-kDa protein was significantly glycosylated (gp105; Fig. 2B). The 50- to 55-kDa protein (p50) was not labeled by [³H]glucosamine.

FIG. 4. ELISA binding. Dilutions of purified MAb ascites fluid were bound in duplicate to visna virus (● and ■) or purified gp135 (○ and □). Background binding by isotype-matched anti-Sindbis virus MAbs was subtracted. (A) Equal virus and gp135 binding (MAb 63-2); (B) virus-predominant binding (MAbs 60-3 and 61-6); (C) glycoprotein-predominant binding (MAbs 143-3 and 123-1).

FIG. 5. Epitope mapping of gp135 by competitive-inhibition ELISA. Visna virus-coated wells were preincubated with unlabeled MAb (50 ng to 10 µg) before biotinylated MAb 128-2 was added; bound biotinylated MAb 128-2 was detected by using peroxidase-conjugated streptavidin. Unlabeled MAbs 128-2, 60-3, 88-3, and 61-6 completely inhibited ELISA binding by biotinylated MAb 128-2; Sindbis virus (SV) MAb and 12 other visna virus MAbs (e.g., 143-3 and 50-1) did not.

To exclude the possibilities that gp105 and p50 had been either degraded from gp135 after immunoprecipitation or nonspecifically precipitated while complexed to gp135, we reacted immune sera and MAbs with purified visna virus proteins on immunoblots (36). Composite immunoblots, which are shown in Fig. 3 and summarized in Table 1, confirmed the immunoprecipitation results. Sheep anti-visna virus serum (lane 3) and guinea pig anti-gp135 serum (lane 6) again bound gp135, gp105, and p50. Nearly half of the 20 MAbs which bound gp135 on immunoblot also bound gp105 or p50 (lanes 18 to 27). Seven MAbs also bound a 90-kDa polypeptide (p90) which was neither prominent in purified virus nor labeled by [³H]glucosamine (lanes 18 to 22; compare Fig. 2B).

From the combined immunoprecipitation and immunoblot results summarized in Table 1, we determined that 29 MAbs were directed against gp135 and 6 were directed against p27. Of 29 anti-gp135 MAbs, 18 (62%) also recognized one or more of three proteins nearly absent from lysates of purified visna virus: a smaller glycoprotein, gp105, and two proteins, p90 and p50, which could not be labeled by [³H]glucosamine. These three antigenically related proteins may be (i) gp135 degradation products; (ii) gp135 precursors (37); or (iii) distinct proteins which share sequence homology with the major envelope glycoprotein [e.g., the outer membrane and transmembrane proteins predicted by Sonigo et al. (32)].

Although we used purified gp135 to induce the MAbs, we observed that 31% of the anti-gp135 MAbs preferentially bound virus greater than purified gp135 in our ELISA (Fig. 4B); 10% of the anti-gp135 MAbs preferentially bound purified gp135 greater than virus (Fig. 4C); and 59% of the anti-gp135 MAbs bound virus and purified glycoprotein equally well (Fig. 4A). Unequal binding implied that (i) gp135 may have been altered by purification or by its release from the viral envelope and (ii) a gp135 domain exposed on the native virion might be covert on the purified glycoprotein; conversely, a gp135 domain exposed on the isolated glyco-

TABLE 2. Topography of visna virus gp135 mapped by reciprocal competitive-inhibition ELISA^a

Unbiotinylated competing MAb	Relative avidity ^b		Inhibition of biotinylated MAb:									
	Virus	gp135	83-4	61-6	88-3	128-2	63-2	66-3	121-1	123-1	130-2	143-3
83-4	1.7	3.7	+									
120-3	2.7	2.7	+									
60-3	0.7	3.7		+	+	+						
61-6	0.7	>4.0		+	+	+						
88-3	<0.0	3.7		+	+	+						
128-2	0.7	3.7		+	+	+	+					
63-2	<0.0	<0.0					+					
66-3	2.7	>4.0					+	+	+			
121-1	2.7	>4.0						+	+			
50-1	2.7	>4.0						+				
135-4	1.7	>4.0						+	+			
123-1	>4.0	1.7								+	+	+
130-2	>4.0	0.7								+	+	+
115-2	ND	ND								+	+	+
143-3	4.0	3.7										+

^a Duplicate wells were coated with visna virus or gp135, preincubated with serial dilutions of unbiotinylated competing MAb (10 µg to 50 ng) for 1 h, and then incubated with 1 of 10 biotinylated MABs. Bound biotinylated MABs were measured by using peroxidase-conjugated streptavidin. A >50% reduction in biotinylated MAB binding was defined as inhibition (+). Five partially overlapping epitopes are represented by boxes in the field of the table (epitopes gp135^a through gp135^e are shown from left to right).

^b Relative avidity is defined as the log₁₀ amount of purified MAB in nanograms of protein required to yield an optical density at 492 nm of ≥ 0.4 in our ELISA (1). Relative avidities 1.0 ≤ (10 ng of MAB) were considered high.

^c ND, Not done.

protein might be concealed within the envelope of the native virion.

Topographical changes in the visna virus envelope during natural antigenic drift. We used 15 anti-gp135 MABs to map the spatial arrangement of epitopes on gp135. Results from a typical reciprocal competitive inhibition ELISA are shown in Fig. 5 and summarized in Table 2. Our MABs defined five distinct, partially overlapping epitopes on gp135, designated gp135^a through gp135^e. MABs against each of these epitopes differed in their relative avidities for virus or purified gp135; MABs with the highest avidity for virus were directed against epitopes gp135^b and gp135^c (Table 2). MABs against epitope gp135^d did not bind to immunoblotted viral proteins. This suggested that SDS-PAGE had destroyed a secondary or tertiary conformational structure necessary for antibody recognition.

We next used the MABs to examine how the structure of the visna virus envelope had changed during sequential mutations of the virus in a single, persistently infected sheep. We had previously isolated six stable, virulent mutant viruses (LV1-1 to LV1-6) from a sheep inoculated with plaque-purified visna virus (strain 1514) (23). Strain 1514 and early mutant LV1-2 had both been strongly neutralized by the sheep's own early-postinfectious serum, but the five more genetically distant mutants had escaped neutralization by this serum (Fig. 6, bottom). We purified the six LV1 viruses and then compared them with parental strain 1514 by using polyclonal immune sera or MAB binding in our ELISA. Immune sera directed against strain 1514 (Fig. 6A), core protein p27 (Fig. 6C), or gp135 purified from strain 1514 (Fig. 6B) bound strain 1514 and all the LV1 viruses equally well; no antigenic changes could be detected. The sheep's own early- or late-postinfectious sera, which had distinguished strain 1514 and LV1-2 from the other variants by neutralization tests (Fig. 6, bottom), failed to reveal any antigenic differences among the viruses by ELISA (data not shown). Binding by anti-p27 MABs similarly failed to detect any structural changes in the major core protein during antigenic drift (data not shown). Polyclonal antisera and anti-core MAB-binding results emphasized the close anti-

genic relatedness of these seven visna virus strains and also assured that subsequent differences in MAB binding could not be attributed to different amounts of viral antigens bound to the ELISA plates.

By contrast, MABs directed against each of the five gp135 epitopes clearly distinguished parental strain 1514 from all of the antigenic mutants (Fig. 7). Two variant viral subsets were also distinguished: (i) LV1-1, LV1-2, LV1-3, and LV1-4; and (ii) LV1-5 and LV1-6. Nearly all MABs directed against a particular epitope produced identical ELISA patterns (e.g., four MABs against gp135^d in Fig. 6D). Subtle differences in ELISA patterns allowed epitopes gp135^b and gp135^c to be subdivided (Fig. 7); the ELISA differences suggested that epitope gp135^b may be composed of at least two fine antigenic determinants; decreased antibody avidity alone could account for the two gp135^c patterns.

During natural antigenic drift of visna virus, all five epitopes of gp135 topographically shifted (Fig. 7). Binding of the highest-avidity MABs to epitopes gp135^a, gp135^b, and gp135^c, which are exposed on strain 1514, decreased during antigenic drift; these three epitopes were obscured or lost on the variants (Table 3). LV1 viruses treated with nonionic detergents or reducing agents sometimes retained MAB-binding abilities against these three epitopes (data not shown). This suggests that these surface epitopes were sometimes blocked or buried rather than destroyed during antigenic variation. Binding of lower-avidity MABs to epitopes gp135^d and gp135^e, which are covert or less accessible on strain 1514, increased during antigenic drift; these two epitopes were reciprocally exposed or unblocked on the variants. Treatment of strain 1514 with reducing agents increased MAB binding to covert epitope gp135^e three- to fivefold (data not shown). This suggests that disulfide bonding on gp135 may diminish MAB binding to epitope gp135^e on strain 1514; enhanced MAB binding to this epitope on the LV1 viruses may be due to conformational rearrangements of gp135 which involve altered disulfide linkages. MABs against epitope gp135^{e1} (e.g., 123-1, 143-3, and 130-2) bound the LV1 viruses better than they bound strain 1514, just as they had bound gp135 purified from strain 1514 better than

they bound strain 1514 itself (compare Fig. 7 and 4C). This suggests that during antigenic variation, the covert gp135^{e1} epitope may have been exposed on the LV1 viruses in a conformation resembling that caused by purification of gp135 from the envelope of strain 1514.

By ELISA, MAbs against epitopes gp135^a through gp135^d clearly differentiated strain 1514 from its closest progeny, LV1-2, whereas the antibodies in early- or late-postinfectious immune sheep sera had been unable to distinguish the two viruses in neutralization tests. Paradoxically, by ELISA, the MAbs did not separate LV1-2 from LV1-1, LV1-3, or LV1-4, whereas serum neutralization tests had separated them (Fig. 6, bottom). It is of interest that the anti-gp135 MAbs we used did not neutralize visna virus (data not shown). It is thus possible that the five epitopes on gp135 recognized by our MAbs are distinct from the epitopes responsible for neutralization. This suggests that antigenic drift is associated with complex rearrangements of both neutralizing and nonneutralizing epitopes on the visna virus envelope glycoprotein.

DISCUSSION

We have characterized 35 MAbs directed against visna virus (strain 1514) envelope glycoprotein or core protein p27 and have used them to map the phenotypic changes in six antigenic variant viruses (LV1-1 to LV1-6) isolated from a single sheep previously infected with plaque-purified strain 1514 (22, 23). Prior RNase-T1-resistant oligonucleotide mapping and sequence analysis of strain 1514 and the LV1 mutants had shown that antigenic drift was accompanied by point mutations in the 3' end of the viral genome (5, 8). We had postulated that the mutations lay within the *env* gene. Similar nucleotide changes in antigenic variants isolated from a second sheep inoculated with strain 1514 and from tissue cultures infected with strain 1514 and maintained in neutralizing sheep antisera had been detected (5, 8, 21). This implied that the antigenic variants had been selected by immune sera because they contained a specific set of mutations which diminished antibody binding to strain 1514-specific neutralization epitopes on gp135. Our anti-gp135 MAb-binding studies extend and support our earlier conclusion from viral genetic analyses that changes in the antigenicity of visna virus during drift are preponderantly caused by mutations in the *env* gene. The MAbs define five partially overlapping epitopes on gp135 which comprise two large domains: one overt or exposed on the native virion (antigenic sites a, b1, b2, and c) and one covert on the virus (antigenic sites d, e1, and e2) but exposed on purified glycoprotein (antigenic sites e1 and e2). All five epitopes of visna virus gp135 were altered in the LV1 viruses isolated during persistent infection. No similar antigenic changes in major core protein p27 were detected when anti-p27 MAbs were used. Since early mutant LV1-2 was indistinguishable from strain 1514 by immune serum neutralization tests and yet displayed substantial phenotypical changes in five gp135 epitopes by MAb binding, the selective pressure that promoted the survival of these visna virus antigenic variants may not have been neutralization alone. Indeed, since our MAbs do not neutralize visna virus infectivity, our results suggest that both neutralizing and nonneutralizing epitopes on the envelope glycoprotein are altered during antigenic drift.

Two recent studies involved the examination of visna viruses which had been sequentially isolated from sheep

FIG. 6. Comparison of visna virus with natural antigenic variants by antibody binding in ELISA. Immune sera or MAbs were bound in triplicate to gp135 purified from visna virus strain 1514, visna virus (strain 1514), or one of six visna virus mutants (LV1-1 through LV1-6) (represented along the abscissa). Previously published neutralization and RNase-T1 fingerprint data are included below each virus (5); unique oligonucleotides are labeled A through F (4). (A) Hyperimmune sheep serum to detergent-disrupted strain 1514 virions; (B) guinea pig anti-gp135 serum (●) or mouse anti-gp135 serum (○); (C) sheep anti-p27 core protein serum; (D) ascites from four MAbs (○, 135-4; ●, 50-1; ■, 66-3; □, 121-1) directed against the gp135^d epitope. Average optical densities at 492 nm (O.D. 492nm) are shown with standard deviations (S.D.) except in panel D.

persistently infected with strain 1514 (17, 35). In these studies neutralization tests with polyclonal postinfectious sheep sera classified 16 to 25% of the visna viral isolates as antigenic mutants, and yet the surprising conclusion of the studies was that natural antigenic variation is rare. We interpret these studies and our own to show not only that antigenic drift of visna virus is a frequent event when tested by using postinfectious sera but also that the true frequency of significant phenotypic changes in the virus during long-

FIG. 7. Topographical changes in visna gp135 during antigenic drift, shown by differential MAb binding to purified gp135 (strain 1514), strain 1514, or the six LV1 antigenic variants. The LV1 viruses are ordered along the abscissa from left to right by increasing genetic divergence from strain 1514. The five gp135 epitopes are marked a through e (boxes); MAb against epitopes b and e displayed two distinct ELISA patterns, which suggested that each epitope might be subdivided (subscripts 1 and 2). Abbreviations: O.D. 492nm optical density at 492 nm; S.D., standard deviation.

term infections may be even higher when viruses are compared by using MAb. Our study suggests that polyclonal immune sera may completely fail to distinguish antigenic variants by neutralization test or by ELISA binding. For example, strain 1514 and mutant LV1-2, which are clearly

distinguishable by MAb binding, appear identical in both tests that involve the use of immune sera.

Clear separation of parental strain 1514 from early mutant LV1-2 further suggests that even a few point mutations in the *env* gene may globally rearrange the topography of the

TABLE 3. Summary of topographic changes in gp135 during antigenic variation as detected by MAb binding on ELISA^a

Epitope	Prototype MAb	Relative avidity ^b		MAb binding to viruses		
		Strain 1514	gp135 (strain 1514)	Strain 1514	LV1-1, LV1-2, LV1-3, LV1-4	LV1-5, LV1-6
Overt domain						
a	83-4	Low	Low	++++	—	—
b1	88-3	High	Low	++	—	—
b2	128-2	High	Low	+++	—	+
c	63-2	High	High	+++	—	+
Covert domain						
d	121-1	Low	Low	+	+++	++++
e1	130-2	Low	High	—	+++	+++
e2	115-2	ND ^c	ND	—	—	—

^a MAb-binding results are summarized from Fig. 7; — represents less than twice the background level and ++++ represents greater than four times the background level.

^b Relative avidity (defined in Table 2, footnote b) was considered high if ≤ 10 ng of immunoglobulin was required to yield an optical density at 492 nm of ≥ 0.4 in our ELISA.

^c ND, Not done.

envelope glycoprotein. By RNase-T1 fingerprinting, mutant LV1-2 differs from strain 1514 by only two oligonucleotides (at least two base changes) (5). Since RNase-T1 fingerprinting analyzes only about 10% of viral RNA, the actual number of nucleotide changes may total 20. Assuming that the sequence changes detected between strain 1514 and LV1-2 on the two unique oligonucleotides are representative of changes across the full genome, we estimate that the nucleotide sequence of the *env* gene of LV1-2 had probably diverged from that of strain 1514 by less than 2%. Yet, unexpectedly, this small number of point mutations appears to have transfigured the LV1-2 glycoprotein. All five gp135 epitopes of LV1-2 were altered. We suggest that topographical flip-flop or refolding of the tertiary structure of gp135 may phenotypically amplify genetic changes in variable regions of the *env* gene. Topographical flip-flop of gp135 might further explain the symmetrical losses and gains of MAb-binding sites on the viral glycoprotein during antigenic drift (Table 3). Three epitopes exposed on strain 1514 (gp135^a, gp135^b, gp135^c) were lost or obscured on the LV1 viruses, while two covert epitopes on strain 1514 (gp135^d and gp135^e) were reciprocally exposed or unblocked on the LV1 mutants.

Topographical permutability may dramatically increase the amount of information stored on the envelope glycoprotein and may allow visna virus to sequester biologically active epitopes from the immune system. We had earlier observed that neutralizing antiserum from a sheep infected with visna virus strain 1514 did not neutralize the mutant LV1-1 virus which was isolated later from the same sheep (22, 23). However, when another sheep was hyperimmunized with nonionic detergent-disrupted strain 1514, the animal developed antibodies that neutralized both strain 1514 and the LV1-1 virus. This suggests that strain 1514 contains the neutralization epitope of LV1-1 but that this site on its envelope glycoprotein is conformationally concealed or blocked until it is exposed to the immune system by detergent solubilization. Such exposure of conformationally hidden neutralization epitopes on strain 1514 gp135 strikingly resembles the manner in which covert epitopes gp135^d and gp135^e were topographically exposed or unblocked on the LV1 viruses during antigenic drift in this study.

Whether all lentiviruses, like visna virus and equine infectious anemia virus, undergo significant antigenic variation is not yet clear, but all lentiviruses show extensive envelope gene polymorphism (4, 5, 8, 19, 20, 30, 39). Rabson and Martin (28) compared two human immunodeficiency virus isolates and determined that the primary amino acid sequences of their major envelope glycoproteins differed by 21%. The genetic changes we have shown between visna virus strain 1514 and early mutant LV1-2 are much less substantial (5, 8), yet they are sufficient to globally alter the envelope glycoprotein. Our results suggest that topographical rearrangements in the three-dimensional structure of gp135 may magnify the primary amino acid sequence changes caused by point mutations in the *env* gene. A small number of genetic changes in the envelope gene during antigenic variation of the virus may thus disproportionately alter glycoprotein antigenicity. If, during persistent infection, the neutralization epitopes on the envelope glycoproteins of visna virus and the other lentiviruses undergo the same conformational rearrangements we have demonstrated for five nonneutralizing epitopes on visna virus gp135, the combinatorial complexity of lentiviral glycoprotein structure may make it very difficult to construct a lentiviral glycoprotein vaccine that is more than strain specific.

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