Distinct Superinfection Interference Properties yet Similar Receptor Utilization by Cytopathic and Noncytopathic Feline Leukemia Viruses

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Cell killing by cytopathic retroviruses is often associated with a delay or failure in the establishment of superinfection interference. Superinfection has been observed during T-cell killing and fatal immunodeficiency disease induction by the feline leukemia virus (FeLV) chimera FeLV-FAIDS-EECC, containing the surface envelope glycoprotein (SU) of FeLV-FAIDS clone 61C. We demonstrate here that 61C SU has a defect that results in a nearly complete failure to establish superinfection interference against homologous virus challenge. This failure was evident only in feline T (FeT) cell clones expressing envelope protein, not in the rare cells that have survived cytopathic infection to become chronically infected. The regions of SU responsible for this defect were the same as those previously identified as responsible for T-cell killing. The superinfection interference properties of a noncytopathic molecular clone, FeLV-FAIDS-61E, were different in that 61E established interference to homologous virus challenge, both in SU-expressing cell clones and in chronically infected cells. Neither 61E nor EECC established interference against heterologous virus challenge. Viruses expressing chimeric SU proteins displayed varied and intermediate interference properties. Purified 61E and 61C SU competed for binding sites on FeT cell surfaces, and purified 61E SU blocked infection of virus bearing 61E or 61C SU. In addition, purified 61E and 61C SU each coprecipitated 70-kDa FeT cell surface proteins. Our data are consistent with the hypothesis that there are multiple cellular components necessary for 61E and 61C attachment to and penetration of FeT cells, a primary receptor that is utilized by both 61E and 61C, and secondary receptors that are likely to be virus specific.

Infection by cytopathic retroviruses is associated with continual reinfection (superinfection). Accumulation of un-integrated viral DNA (UVD), a replicative intermediate, has been observed during cytopathic infection by avian leukosis virus, visna virus, spleen necrosis virus (SNV), human immunodeficiency virus type 1 (HIV-1), avian hemangioma-inducing virus, and feline leukemia virus (FeLV) (12, 18, 23, 35, 40, 43, 47, 54, 55). These observations have led to the hypothesis (reviewed in reference 51) that cell killing involves a delay or failure in the establishment of superinfection interference, a process whereby envelope protein expressed in an infected cell renders the cell resistant to further infection by virus bearing envelope protein of the same receptor specificity. Cell clones expressing SNV or HIV-1 envelope protein establish superinfection interference against homologous virus challenge (9, 49), suggesting that UVD accumulation during SNV and HIV-1 infections in vitro is due to a delay in the establishment of rather than a failure to establish superinfection interference.

Envelope protein and receptor interactions are also associated with disease induction. For example, determinants for disease induction by FeLV-C-Sarna, FeLV-FAIDS-61C, and the murine viruses Cas-Br-E and spleen focus-forming virus have been mapped to the *env* gene (1, 11, 34, 41).

61C and 61E are molecularly cloned proviruses derived

from the FeLV-FAIDS strain (33). 61E is replication competent and minimally pathogenic; 61C is replication defective but, when provided with 61E as helper virus, causes fatal immunodeficiency disease in cats (33). Analysis of tissue DNA samples from cats infected with the FeLV-FAIDS strain, a mixture of 61E and 61C, or EECC (also referred to here as EE[CCCC]C, a chimera made between 61E and 61C that contains the 61C *env* gene and 3' long terminal repeat [LTR]) indicated that high levels of UVD were present immediately preceding the onset and during immunodeficiency disease (11, 29, 33). High levels of UVD were also observed during cytopathic infection of feline T (FeT) cells in vitro (11). Thus, impairment in the establishment of superinfection appears to occur in vivo and in vitro.

The clinical outcome of FeLV infection is related to virus subgroup, a property historically considered to indicate use of different and specific receptors (10, 21, 22, 32, 41, 42). FeLVs have been placed into three subgroups based on superinfection interference properties determined by the surface envelope glycoprotein (SU) (45). An FeT cell line chronically infected with either FeLV-A, -B, or -C maintained cell surface binding sites for purified FeLV-A, -B, and -C SU proteins in homologous and heterologous receptor-binding experiments (8, 16), suggesting that partial downregulation of receptor (16) is sufficient for interference, or that FeLV subgroup-specific superinfection interference might not be due to blockade or downregulation of the primary virus-binding receptor but rather might be due to blockade or downregulation of other cell components required for virus entry.

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The association between superinfection and EECC-mediated T-cell killing in vitro and disease induction in vivo and the suggestion that FeLVs of different subgroups might use common as well as unique cell surface components during virus entry prompted us to characterize the superinfection interference and receptor-binding properties of 61C and 61E SUs. In this report, we demonstrate a nearly complete failure in the ability of 61C SU to establish superinfection interference, map the regions responsible for this failure, show that 61E and 61C SU compete for the same cell surface-binding sites, identify cell surface proteins of the same size coprecipitated by purified 61C SU and 61E SU, and propose models for 61E and 61C SU receptor use and interference.

MATERIALS AND METHODS

Cells and viruses. The FeLV-negative 3201-B FeT lymphoma line (48) was maintained in a 1:1 mixture of RPMI 1640–Leibowitz-15 media (GIBCO) with 15% fetal calf serum and 1% penicillin, streptomycin, and L-glutamine.

The molecular cloning of 61E and 61C and the creation of chimeric clones have been described elsewhere (11, 33). We will refer to full-length molecular clones with four-letter designations that describe the origins of the 5' LTR/*gag*, *pol*, SU, and transmembrane protein (TM)/3' LTR sequences. The letter describing the origin of SU coding sequences is further subdivided into four segments. As an example, EECC, which contains 5' LTR/*gag* and *pol* sequences of 61E origin and SU and TM/3' LTR sequences of 61C origin, is referred to in this report as EE[CCCC]C. Molecular cloning and sequencing of the subgroup A FeLV strain Glasgow-1 (FGA) *env* and 3' LTR sequences have been described elsewhere (50).

FeLV inocula were generated following transfection of cloned DNA and titers were determined as described previously (11).

Envelope protein-expressing single-cell clones were generated by coelectroporation of individual envelope protein expression plasmids and the dominant selectable marker, pSV2his (19), at an 8:1 molar ratio; 24 h after electroporation, cells were incubated in fresh medium containing 3.5 mM L-histidinol (Sigma). Cells were single-cell cloned twice beginning 14 to 21 days after electroporation and were continuously maintained in 3.0 mM L-histidinol.

Flow cytometry. FeLV envelope protein-expressing single-cell clones were stained with fluorescein isothiocyanate-conjugated monoclonal antibody C11D8 (17) and analyzed by using a Coulter EPICS Profile flow cytometer as previously described (38).

Nucleic acid manipulations. Standard recombinant DNA techniques were used for all plasmid manipulations (44). 61E envelope protein expression plasmid p61EΔ was created by restriction digestion of full-length FeLV clone p61E with *Bgl*II (at base 1106 of the 61E [or F6A] sequence [10]) and *Hpa*I (base 5576), filling in of the recessed *Bgl*II overhang with Klenow polymerase, and blunt-end religation (44). This construct retains the *env* splice donor and acceptor sequences but is deleted of most of the *gag* and *pol* sequences. 61C and FGA envelope protein expression plasmids p61C*Δ and pFGAΔ, respectively, were similarly created by restriction digestion of plasmids containing full-length 61C or FGA provirus with *Bgl*II and *Bam*HI (base 5318) and religation of the resulting cohesive ends. Envelope protein expression plasmids p61C*Δr[XXXX], in which [XXXX] corresponds to the [CECE], [CEEC], [CEEE], [EECC], [EECE],

[EECC], or [EEEC] SU sequence, were created by ligation of the 5' portion of p61C*Δ to the 3' half of the full-length virus constructs (12) at the *Xho*I site just 5' of *env*.

Southern transfer and hybridization analyses and genomic DNA preparation procedures were as described previously (11, 28).

CHO cell expression of PI-linked envelope protein. 61E and 61C SUs were purified after expression in Chinese hamster ovary (CHO) cells, using a phosphatidylinositol (PI) glycan linkage expression system previously described for expression and purification of integral membrane proteins (26). Creation of expression vectors was initiated by polymerase chain reaction (PCR) amplification of 61E TM sequences, using primers that amplified the 5' portion of TM up to the transmembrane-spanning domain (76 amino acids from the C-terminal end of TM). The PCR product was digested with *Xho*I, which cleaved in the 5' primer, and *Nar*I, which cleaved in the 3' primer. The digested product was ligated to *Xho*I-*Nar*I-digested pHPAP vector in front of the sequence encoding the C-terminal 38 amino acids of the human placental alkaline phosphatase (HPAP) gene, which serve as a signal sequence for the attachment of otherwise secreted proteins to the cell surface by means of a PI linkage. The TM/HPAP sequences were then removed with *Xho*I and *Not*I and ligated to *Xho*I-*Not*I-digested mammalian expression vector pSR1neo between the SRα promoter (simian virus 40 promoter and enhancer combined with the human T-cell lymphotropic virus type 1 R-U5 region) and simian virus 40 polyadenylation signal. This expression plasmid also contains a neomycin resistance gene. *Xho*I-*Sac*II restriction fragments of 61E and 61C SU were then ligated to the TM/HPAP sequences, generating a plasmid that expressed PI-linked SU and the extracellular domain of TM. Plasmid DNA was introduced into CHO cells by electroporation (25), and envelope protein-expressing cells were sorted by fluorescence-activated cell sorting on a FACSTAR Plus (Beckton Dickinson) for high surface SU expression with use of monoclonal antibody C11D8, single-cell cloned, and cultured in a hollow-fiber bioreactor. Perfusion of phospholipase C (Sigma) into the bioreactor allowed cleavage of the PI-linked envelope protein, and SU was immunoaffinity purified with C11D8 as described previously (16).

SU competition binding assay. Purified 61E and 61C SU (10 μg) were labeled with ¹²⁵I as described previously (16). Exponentially growing FeT cells (3 × 10⁶) were washed twice in binding buffer (phosphate-buffered saline [pH 7.4], 55 mM glucose, 1 mM CaCl₂, 1 mg of bovine serum albumin [BSA] per ml) and then resuspended in 0.5 ml of binding buffer. Cells were preincubated with 20 to 25 μg of unlabeled SU (61E or 61C) or no competitor SU for 1 h at room temperature, and then ¹²⁵I-labeled SU (200 ng, 2 × 10³ cpm/ng) was added and incubated for 2 h at room temperature with rotation. At the end of the incubation, cells were pelleted by centrifugation (400 × g for 10 min) and washed twice with binding buffer. Cells were solubilized in 75 μl of lysis buffer (300 mM NaCl, 50 mM Tris [pH 7.4], 0.5% Nonidet P-40), nuclei were removed by centrifugation (400 × g for 10 min), and the supernatant was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide (7.5%) gel electrophoresis under reducing conditions (46), followed by autoradiography.

Infection blockade assay. FeT cells (2.5 × 10⁵) in 400 μl of complete medium were preincubated with 25 μg of BSA for 15 min at 37°C, and then 0, 0.25, 2.5, or 25 μg of competitor SU was added and incubated at 37°C for 1 h with rotation; 10³ infectious units of either EECC.MUT.NS or EECC.

FIG. 1. Mutagenesis of FeLV SU coding sequences. Shown is a schematic diagram of the FeLV genome and the four segments delineated within the SU coding sequence. PCR primers were used to create tagged mutant viruses as described in Materials and Methods. Primers JM.PCR.1 (1) and TR.PCR.8 (2) were used in PCRs designed to specifically detect tagged provirus in previously infected FeT cells. The hybridization probe is represented by the solid black bar (*). The conserved amino acid sequence and the nucleic acid sequences of the parental and mutant viruses over the 19-bp tagged region are shown at the bottom, with substituted bases underlined.

MUT.NS (see below) was then added to the suspension and incubated at 37°C for 1 h. Cells were washed three times with complete medium and replated in 1 ml (total volume). Total nucleic acid was isolated 2 days after infection, and the presence of challenge virus DNA was assayed by using PCR primers JM.PCR.1 and TR.PCR.8 as described below.

Receptor coprecipitation assay. Coprecipitation of ¹²⁵I-labeled FeT cell surface protein with purified 61E and 61C SU was performed as described previously (16).

PCR mutagenesis. FeLV clones containing a hybridization tag of silent mutations in seven contiguous codons in the SU coding sequence were generated by the method of Higuchi et al. (20). By using plasmid containing the 3' genome half of either 61E or 61C, primer pairs JB.PCR.23/TR.MUT.10 and TR.MUT.11/JM.PCR.4 were used in separate, first-round PCRs to introduce the mutated sequences. Aliquots of product from each of the first-round reactions were mixed together and then amplified with primer pair JB.PCR.23/JM.PCR.4 in second-round reactions to generate full-length, mutated product suitable for cloning, TR.MUT.10 and TR.MUT.11 having specified the altered DNA sequences. Second-round products were digested with *Nco*I and *Sac*II and cloned into a vector containing the 3' half of the 61E genome or were digested with *Sac*II and cloned into a vector containing the 3' half of the 61C genome. In both cases, all of the fourth segment of the SU coding sequence (Fig. 1) was replaced, and in the case of 61C, a portion of the third segment of SU was also replaced. Tagged virus containing the 61E or 61C SU coding sequence was named EE[EEEE]C.MUT.NS or EE[CCCC]C.MUT.NS, respectively, to des-

ignate the presence of mutated sequences in the *Nco*I-*Sac*II portion of the SU coding region. Sequences generated by PCR were confirmed by double-stranded DNA sequencing by the dideoxy method (44).

PCR. The following oligonucleotide primers were synthesized on an Applied Biosystems model 394 DNA/RNA synthesizer (5' to 3'): TR.PCR.4 (bases 1654 to 1630 of the 61E or F6A genome [10]), GCCAACCGGGTACCCTC; JM.PCR.5 (bases 30 to 54), GCTACTGCAGTGGTGCCATTTCACA; JB.PCR.23 (bases 6608 to 6631), CGACTATACCGTACAGGATATGAC; TR.MUT.10 (bases 7215 to 7187 plus a 19-base overhang on the 5' end that specifies the mutated sequences introduced into EE[EEEE]C.MUT.NS and EE[CCCC]C.MUT.NS), CAGTACGCAGAACTCGCTAGTC(A/C)AA TTGAGCACC GCCATGGAAATGCA; TR.MUT.11 (bases 7235 to 7260 plus a 19-base overhang on the 5' end complementary to the 5' overhang on TR.MUT.10), TAGC GACTTCTGCGTACTGATCGAATTATGGCCAGAGT GACTTA; JM.PCR.4 (bases 7401 to 7382), GCTTTAGTCCTGTTCGGAC; JM.PCR.1 (bases 6169 to 6188), AGGAA CCTTAACCGATGTCT; and TR.PCR.8 (bases 7234 to 7216), CAGTACGCAGAAAGGTCGCTA.

The exogenous FeLV LTR-specific primer JM.PCR.5 and the exogenous FeLV *gag*-specific primer TR.PCR.4, which generate 1.6-kbp products, were used in 100-μl reactions for 35 cycles of 94°C denaturation for 30 s, 52°C annealing for 20 s, and 72°C extension for 20 s. The exogenous FeLV SU-specific primer JM.PCR.1 and the FeLV SU tag-specific primer TR.PCR.8, which generate 1.1-kbp products (Fig. 1), were used in 100-μl reactions for 35 cycles of 94°C denaturation for 30 s, 57°C annealing for 20 s, and 72°C extension for 20 s. One microgram of total nucleic acid template was amplified, and 15 μl of product was electrophoresed on a 1% agarose gel and subjected to Southern transfer and hybridization using either the exU3 probe (30) (for products generated with JM.PCR.5/TR.PCR.4) or the 744-bp *Hind*III-*Nco*I SU fragment from p61E (for products generated with JM.PCR.1/TR.PCR.8). Hybridization probes were generated by the random primer method (14). Each filter contained an equivalent aliquot of a stock of PCR product generated from a plasmid template as a hybridization control standard. Hybridized filters were exposed for 3 to 24 h, and bands were excised and counted by liquid scintillation. Results are presented as superinfection indices (SIs), which represent (cpm of hybridizing probe in test sample band/cpm of hybridizing probe in hybridization control band)/(cpm of hybridizing probe in virus-challenged, uninfected FeT cell control band/cpm of hybridizing probe in hybridization control band). Multiple dilution series of infected cell nucleic acid showed linear relationships between input template and hybridization to filter-bound PCR product. The correlation coefficients of linearity were 0.91 and 0.90 for primer pairs JM.PCR.5/TR.PCR.4 and JM.PCR.1/TR.PCR.8, respectively (data not shown).

RESULTS

Superinfection of chronically infected FeT cells. To determine superinfection interference properties of FeLV-infected FeT cells, we created virus clones that could be distinguished from FeLV provirus already present in infected cells. For these experiments, we used both the minimal and acutely cytopathic variants of the FeLV-FAIDS strain, 61E (also referred to as EE[EEEE]C) and EE[CCCC]C, and FGA, a derivative containing the subgroup A FeLV strain Glasgow-1 (50) envelope gene (EE[GGGG]C). The

FIG. 2. Superinfection interference properties of chronically infected FeT cells. FeT cells chronically preinfected with the virus chimeras shown were challenged with equivalent titers of tagged mutant virus and analyzed by PCR for the presence of challenge virus DNA. Results are presented as SI (see Materials and Methods) and are from a minimum of two experiments performed with each challenge virus and cell population. Included is a summary of the T-cell cytopathic properties of the chimeric viruses shown, previously determined by Donahue et al. (11).

FGA derivative was included since whereas both 61E and FGA belong to subgroup A and utilize the same receptor, FGA but not 61E interferes with the cytopathic effects of EE CC (25). As diagrammed in Fig. 1, 10 silent point mutations in seven contiguous codons were introduced into EE [EEEE]C and EE[CCCC]C SU coding sequences by PCR primer-directed mutagenesis (20), resulting in the viruses EE [EEEE]C.MUT.NS and EE[CCCC]C.MUT.NS, respectively. One week after superinfection of previously infected cells, PCR was performed to detect challenge virus DNA, using specific oligonucleotide primers. The products were subjected to Southern transfer and hybridization, followed by excision and quantitation of bands by liquid scintillation counting. Results are presented as SIs (described in Materials and Methods).

EE[EEEE]C-tagged challenge virus (EE[EEEE]C.MUT.NS) infection of FeT cells infected with EE[EEEE]C, EE[EEEC]C, EE[EECE]C, EE[ECCC]C, EE[GGGG]C, and EE[EEEE]C plus EE[CCCC]C resulted in all cases in SIs of <0.05 (Fig. 2), indicating strong interference. EE [EEEE]C.MUT.NS challenge of EE[EECC]C-infected FeT cells resulted in scattered SIs ranging from 0.01 to 0.94. SIs of 0.20 to 0.64 were obtained after challenge of EE[CEEC]C-, EE[CECE]C-, or EE[CCCC]C-infected FeT cells, indicating weak interference. Chimeric viruses that interfered with EE[EEEE]C.MUT.NS superinfection were found previously to be minimally to moderately T-cell cytopathic, whereas chimeric viruses that did not appreciably interfere with EE[EEEE]C.MUT.NS infection were found previously to be moderately to highly T-cell cytopathic (11) (Fig. 2).

EE[CCCC]C-tagged virus (EE[CCCC]C.MUT.NS) challenge of infected FeT cells also resulted in a spectrum of SIs. EE[CCCC]C.MUT.NS superinfection of EE[EEEE]C- or EE[GGGG]C-infected cells resulted in SIs ranging from 0.77 to 1.55, indicating weak or no interference. The degree of interference increased as more 61C SU coding sequence was exchanged into the EE[EEEE]C virus backbone of the preinfecting virus. For example, the SIs obtained after

EE[CCCC]C.MUT.NS challenge were 1.55 and 1.48 for EE[EEEE]C-infected cells, 0.07, 0.10, and 0.10 for EE[EECC]C-infected cells, and <0.01 for EE[ECCC]C-infected cells. Therefore, the more cytopathic a virus was for FeT cells (11) and the more 61C-derived SU sequences it contained, the more strongly it interfered with EE[CCCC]C.MUT.NS superinfection.

Since the chimera EE[ECCC]C established interference against both EE[EEEE]C.MUT.NS and EE[CCCC]C.MUT.NS challenge viruses, although weakly against the latter, we conducted the reciprocal experiment and determined which preinfecting viruses interfered with EE[ECCC]C.MUT.NS. EE[EEEE]C and EE[CCCC]C interfered reciprocally with virus bearing [ECCC] SU protein. Strong interference was obtained after homologous virus challenge of EE[ECCC]C-infected cells (SI of <0.01), and after challenge of EE[CCCC]C infected cells (SI of 0.01 to 0.02). In contrast, challenge of EE[EEEE]C- or EE[GGGG]C-infected cells resulted in SIs of >0.34 and 0.02 to 0.19, respectively. Therefore, strong reciprocal interference was observed between EE[CCCC]C and EE[ECCC]C, whereas weak interference was observed between EE[EEEE]C and EE[EECC]C.

61E (EE[EEEE]E) and EE[GGGG]E have been shown to strongly cross-interfere in vesicular stomatitis virus (VSV)-FeLV pseudotype assays, whereas preinfection with only EE[GGGG]E delayed and reduced the cytopathic effect of EE[CCCC]C superinfection in coinfection experiments (25). We therefore sought to determine whether FeT cells infected with an FeLV expressing [GGGG] SU behaved similarly in a PCR-based superinfection assay. Strong interference (SI of <0.06) equivalent to that against homologous virus was observed after EE[GGGG]C.MUT.NS challenge of FeT cells infected with any heterologous virus tested (EE[EEEE]C, EE[ECCC]C, and EE[CCCC]C).

Thus, these experiments demonstrated a spectrum of superinfection interference properties, with the extremes

FIG. 3. Generation of FeLV envelope protein-expressing single-cell clones. (A) Schematic diagram of the FeLV genome and the *Bgl*II and *Bam*HI restriction sites used to create most envelope protein expression vectors. Transcription regulatory sequences were provided by the LTRs, and the putative *env* splice donor (SD) and splice acceptor (SA) sites, as well as the initiation codon (AUG), were maintained. PCR primers JM.PCR.5 (1) and TR.PCR.4 (2) were used in PCRs to specifically detect challenge virus DNA. The probe used for Southern hybridization analysis of PCR products, exU3, is also shown (*). (B) FeT single-cell clones were stained with fluoresceinated anti-SU monoclonal antibody C11D8 (17) and analyzed for surface SU expression by flow cytometry. Representative histograms are shown; the y axis indicates relative cell number, and the x axis indicates arbitrary logarithmic fluorescence intensity units ranging from 10^{-1} to 10^3 .

represented by the parental noncytopathic EE[EEEE]C and cytopathic EE[CCCC]C viruses.

Superinfection interference properties of single-cell clones expressing FeLV envelope protein. We have shown that chronically infected FeT cells that have survived infection by cytopathic EE[CCCC]C are the descendants of a rare oligoclonal population (11) and that attenuation of the virus population occurs during passage of cytopathic virus (39). Combined, these data suggest that infrequently occurring changes in the virus population are associated with cell survival. We therefore generated single-cell clones expressing envelope protein to compare the abilities of envelope proteins to establish superinfection interference in the absence of virus replication versus chronic infection and its attendant selective features. FeT cells were coelectroporated with pSV2his and envelope protein expression vectors (Fig. 3A), and after selection in L-histidinol and single-cell cloning, cells were screened for envelope protein expression

by Western blot (immunoblot) analysis using the anti-SU monoclonal antibody C11D8 (data not shown). Cell clones expressing envelope protein were cloned a second time, and flow cytometric analyses showed that each represented a unimodal population and that >98% of the cells expressed envelope protein (Fig. 3B). As assayed by radioimmunoprecipitation, similar levels of protein were produced in cells infected with 61E and single-cell clones expressing 61E and 61C envelope proteins, whereas a higher level of expression was found in EECC-infected cells (data not shown; 37).

Envelope-expressing cell clones were challenged with FeLV at a multiplicity of infection of 0.005 and analyzed 1 week later for superinfection. For this analysis, we developed a second superinfection assay in which the presence of challenge virus DNA was detected by using PCR primers specific for exogenous FeLV LTR and *gag* sequences (Fig. 3A). This primer pair amplified DNA template generated by reverse transcription of the challenge virus genome but did not amplify endogenous FeLV sequences or transfected envelope expression vector sequences. The PCR products obtained were analyzed by Southern transfer and hybridization, and SIs were determined as described in Materials and Methods.

The interference patterns of cell clones expressing envelope protein formed approximately five groups (Table 1). Averaged SIs from 0.98 to 1.85 were obtained after superinfection of cells expressing [CCCC] or [CECE] SU regardless of which FeLV chimera was used as challenge virus (group 4), indicating that no interference was established. In contrast, cells expressing [EEEE], [EEEC], [EECE], or [EECC] SU yielded averaged indices from 0.45 to 1.25 after superinfection by EE[CCCC]C, EE[CECE]C, or EE[CEEC]C but yielded averaged SIs of 0.02 to 0.11 after superinfection by EE[EEEE]E, EE[EECE]C, or EE[EEEC]C (group 1).

The interference patterns of the [CEEE] and [CEEC] SU protein-expressing cell clones were intermediate between those of the 61C and 61E envelope protein-expressing cell clones, with averaged SIs of 0.04 to 0.28 after challenge with noncytopathic or weakly cytopathic viruses and averaged SIs of 0.08 to 0.68 after challenge with cytopathic viruses (group 3). The [CEEE] and [CEEC] SU proteins were unique in their abilities to establish at least partial interference to cytopathic viruses, a property not observed for SU protein derived from either noncytopathic or highly cytopathic viruses.

Superinfection of cell clones expressing [ECCC] SU yielded averaged SIs similar to those observed after superinfection of cells expressing group 1 SU proteins but was somewhat more permissive for the same group of viruses plus EE[EECE]C superinfection (group 2). [ECCC], [CCCC], and [CECE] SU-expressing cells all failed to interfere with homologous virus superinfection, establishing a correlation between the inability of an FeLV SU protein to establish interference to homologous challenge virus and the cytopathic potential of that virus.

Superinfection of cells expressing [GGGG] SU protein with either homologous virus, EE[EEEE]E or EE[ECCC]C, yielded averaged SIs of <0.07, indicating strong interference (group 5). In contrast, superinfection was observed with EE[CCCC]C (SI of 0.25), demonstrating the ability of EE[CCCC]C to partially overcome this interference. Thus, cell clones expressing [CEEE] or [GGGG] SU were the only ones to appreciably interfere with EE[CCCC]C superinfection.

In summary, cells expressing envelope protein derived

TABLE 1. Superinfection of FeT cells expressing envelope protein^a

Group	SU expressed in FeT cell clone	SI ^b							
		[EEEE]	[EEEC]	[EECE]	[EECC]	[CEEC]	[CECE]	[CCCC]	[GGGG]
1		[EEEE]	–	+	–	++	++	+++	–
			0.06	0.11	0.07	0.56	0.50	0.45	1.02
		[EEEC]	–	–	–	NT	+++	++	+++
			0.02	0.04	0.02		0.73	0.55	0.62
2		[EECE]	–	–	–	NT	+++	+++	NT
			0.05	0.07	0.09		0.92	0.86	0.86
		[EECC]	–	–	–	NT	+++	+++	+++
			0.04	0.08	0.02		1.02	1.25	1.06
3		[ECCC]	–	–	++	+++	+++	+++	–
			0.03	0.01	0.40	0.85	1.03	1.06	1.01
4		[CEEE]	–	+	+	NT	+	–	++
			0.04	0.13	0.17		0.18	0.08	0.22
		[CEEC]	–	++	++	NT	++	++	+++
			0.10	0.51	0.28		0.27	0.21	0.68
5		[CECE]	+++	+++	+++	NT	+++	+++	NT
			1.43	1.60	1.73		1.39	0.98	1.19
6		[CCCC]	+++	+++	+++	+++	+++	+++	+++
			1.20	1.44	1.28	1.14	1.25	1.34	1.27
7		[GGGG]	–	NT	NT	–	NT	NT	++
			0.01			0.07			0.25

^a FeT cells expressing the indicated SU were challenged with FeLV chimeras and analyzed for successful infection by PCR using primers JM.PCR.5 and TR.PCR.4 (see Fig. 3).

^b Determined as described in Materials and Methods. Averaged SIs are represented as follows: –, ≤0.10; +, 0.11 to 0.20; ++, 0.21 to 0.60; and +++, ≥0.61. Results are averages of a minimum of two experiments, each performed on a minimum of two single-cell clones. NT, not tested.

from cytopathic FeLVs failed to establish interference, whereas those expressing envelope protein derived from noncytopathic FeLVs established interference, although only against noncytopathic viruses. Cells expressing envelope protein derived from FeLVs of intermediate cytopathicity established interference with efficiencies intermediate between those of cytopathic and noncytopathic viruses.

Cell surface receptor recognition by 61E and 61C SU. Our current understanding of superinfection interference (53) would suggest that the results in Table 1 and Fig. 2, in which 61E and 61C envelope proteins do not cross-interfere, indicate that 61E and 61C use different receptors. To examine this latter point more directly, we performed competitive binding experiments using purified 61E and 61C SU. Binding of either ¹²⁵I-labeled 61E or 61C SU to FeT cell surfaces was blocked by preincubation of cells with a 100-fold excess of either unlabeled 61E SU or unlabeled 61C SU (Fig. 4), suggesting that both the 61E and 61C SU proteins utilize the same cell surface-binding protein(s). 61E SU was next used to block receptors on FeT cell surfaces prior to challenge with EE[EEEE]C.MUT.NS or EE[CCCC]C.MUT.NS. As shown in Fig. 5, PCR analysis of cells for the presence of challenge virus DNA revealed that 61E SU blocked EE[C CCC]C.MUT.NS infection, but less completely than it blocked EE[EEEE]C.MUT.NS infection. This result further suggested that 61E and 61C SU proteins utilize the same cell surface-binding protein during infection. Lastly, 61E SU has been shown to coprecipitate a 70-kDa protein, its putative receptor, from FeT cell surfaces (16). In the experiment shown in Fig. 6, 61C SU and 61E SU both specifically coprecipitated 70-kDa proteins from ¹²⁵I-labeled FeT cell

surfaces. Thus, competitive binding and receptor coprecipitation studies support the conclusion that 61E and 61C SU utilize a common receptor for binding on FeT cell surfaces.

FIG. 4. Competition of ¹²⁵I-labeled SU binding to FeT cells. ¹²⁵I-labeled 61E or 61C SU was bound to FeT cell surfaces either with or without unlabeled 61E or 61C SU as competitor. Washed cells were lysed, and lysates were separated by SDS-polyacrylamide (7.5%) gel electrophoresis under reducing conditions, the autoradiogram of which is shown. Molecular weight markers are in kilodaltons.

FIG. 5. Blockade of FeT cell infection with purified SU. Uninfected FeT cells were preincubated with 25 μ g of BSA and the indicated amounts of purified 61E or 61C envelope protein and then challenged with equivalent titers of EE[EEEE]C.MUT.NS or EE[CCCC]C.MUT.NS. Infection was analyzed by PCR (described in Materials and Methods). Results are presented as a normalized infection index which represents, after Southern hybridization and scintillation counting of hybridizing bands, (cpm of hybridizing probe in test sample band from cells preincubated with competitor SU/cpm of hybridizing probe in hybridization control band)/(cpm of hybridizing probe in control band from cells not preincubated with competitor SU/cpm of hybridizing probe in hybridization control band). Results are averages of two experiments, each done in triplicate.

DISCUSSION

We have shown here that the SU protein of cytopathic FeLV-61C fails to establish superinfection interference except in the rare circumstance of chronically EE[CCCC]C infected cells. The failure in establishment of interference by the 61C SU protein in single-cell clones might represent a situation analogous to the time early after infection with EE[CCCC]C, when high levels of UVD and cell killing occur (11). This suggests that there are differences in the ability of 61C SU protein to interact with its receptor(s), depending on whether it is on the surface of a virion or expressed within a cell. 61C SU does not have the ability to downregulate or otherwise interact with its receptor inside the cell in a manner sufficient to establish interference. However, a larger amount of SU protein is expressed in EE[CCCC]C-infected FeT cells than in FeT cell clones expressing 61C SU or 61E SU (Fig. 3) and in FeT cells chronically infected with EE[EEEE]C (37; data not shown). The larger amount of SU may occur as a result of the accumulation of proviruses in these rare cells. However, cell clones expressing roughly equivalent amounts of 61E SU compared with those expressing 61C SU were able to establish interference against

FIG. 6. Coprecipitation of iodinated FeT cell surface proteins by purified 61E and 61C SU. 61E or 61C SU was used to specifically coprecipitate 125 I-labeled FeT cell surface protein(s). Cell surface proteins complexed with the indicated SU were precipitated by the anti-SU monoclonal antibody C11D8 (17). Precipitated proteins were subjected to SDS-polyacrylamide (7.5%) gel electrophoresis, the autoradiogram of which is shown. Molecular weight markers are in kilodaltons.

homologous and certain heterologous virus challenges (Table 1), suggesting that the 61C interference defect is due to qualitative properties of SU. In the context of chronically EE[CCCC]C infected cells, survival is associated with attenuation of the virus population (39). Thus, the superinfection interference properties attributed to 61C SU might be due either to the generation of defective interfering particles or to the expression of an altered form of envelope protein.

The data compiled in Fig. 1 and Table 1 indicate that the determinants for the 61C SU interference defect are similar to those previously identified for T-cell killing (11). The more cytopathic a virus was, the less capable SU derived from it was at establishing interference. For example, EE[CECE]C and EE[CCCC]C had equivalent cytopathicity, and both failed to establish interference. SU chimeras derived from viruses of intermediate cytopathicity, such as [ECCC] and [CEEC], exhibited interference patterns intermediate between those of 61E and 61C SUs. In particular, EE[EC CC]C-infected cells were strongly resistant to EE[EEEE]C.MUT.NS superinfection and partially resistant to EE[CC CC]C.MUT.NS superinfection, whereas [ECCC] SU-expressing cell clones were resistant to superinfection by EE[EEEE]E but were susceptible to superinfection by virus bearing [EECE] or [ECCC] SU. Collectively, these data suggest that cell killing by cytopathic FeLV-FAIDS viruses is a direct consequence of the nearly complete failure of SU protein to establish superinfection interference. However, additional mechanisms are involved in chronic FeLV-FAIDS disease induction. This is illustrated by analysis of SU derived from the minimally cytopathic virus EE[EECE]C, which is able to cause an immunodeficiency syndrome in vivo after a long latency period (11, 38) but which exhibited interference properties similar to those of 61E SU. Furthermore, a virus of intermediate cytopathicity, EE[ECCC]C, caused immunodeficiency with short latency periods much like viruses bearing 61C SU but nevertheless established interference against 61E SU-bearing challenge viruses.

A number of biochemical properties determined by the SU protein may be responsible for the interference defect of cytopathic FeLV-FAIDS viruses. For example, reduced

rates and extents of 61C envelope protein precursor processing relative to 61E have been documented (36, 37). A major fraction of newly synthesized 61C SU may exist in a misfolded state and be retained within the endoplasmic reticulum (ER) as has been shown for immunoglobulin heavy chain and mutant influenza hemagglutinin (HA) (3, 15). Retention within the ER may also account for the posttranslational modification differences observed between 61C and 61E envelope proteins (36, 37), because these modifications would be expected to occur after transfer from the ER (24). If a functional receptor-binding site on 61C SU is determined in part by mature carbohydrate moieties, then retention within the ER would prevent the synthesis of receptor-binding-competent 61C SU, resulting in a failure to establish interference. Alternatively, failure of 61C SU to properly form oligomeric structures might result in its retention in the ER. Oligomerization has been shown to occur during biogenesis of numerous virus envelope proteins such as influenza virus HA, avian leukosis virus SU/TM, Semliki Forest virus surface glycoprotein, VSV G protein, and HIV SU/TM (5–7, 12, 13, 15, 56), and influenza virus HA oligomerization has been shown to be required for transport from the ER to the *cis*-Golgi (5).

In previous studies, we used VSV-FeLV pseudotype superinfection assays to examine virus entry-associated superinfection interference and analyzed the changes in kinetics and efficiency of cell killing by EE[CCCC]C as a result of prior virus infection with noncytopathic viruses (25). In these studies, VSV pseudotypes coated with 61E ([EEEE]) and FGA ([GGGG]) envelope proteins were interfered with strongly by homologous and reciprocal virus challenge. Equivalent results were obtained in the current assays.

In the VSV single-step assay of virus entry, prior infection with either 61E or FGA interfered with VSV ([CCCC]) virus challenge, although to a slightly lesser extent than against pseudotypes bearing homologous envelope protein (25). However, no interference (Fig. 2) or a low level of interference (Table 1) was noted in the current assays, which measured the amount of reverse-transcribed DNA following virus entry and spread in the culture. We cannot definitively reconcile these data; perhaps the small degree of infection inferred from the VSV assay translates to little or no blockade of cytopathic EE[CCCC]C virus growth in the current PCR-based assay.

In the VSV assays, prior infection with EE[CCCC]C only weakly blocked 61E and FGA virus entry. The current study confirms these results for 61E and for FGA when envelope protein-expressing cells are assessed (Table 1); however, a more substantial blockade of FGA was observed in cells chronically infected with EE[CCCC]C (Fig. 2). In addition, prior infection with the FGA chimera EE[GGGG]E, but not 61E, delayed and reduced cell killing by EE[CCCC]C, presumably by interference occurring at a step following virus entry (25). Furthermore, in the context of cell clones expressing envelope proteins, only the [GGGG] and [CEEE] SU proteins interfered at all with EECC superinfection (Table 1), and it is known that the latter protein in the context of the 61E genome renders the virus replication defective (11). Therefore, it may be that prior infection with EE[CCCC]C substantially interferes with FGA growth through some interaction between the viral envelope proteins, potentially heteromultimerization (25). Heteromultimerization of the evidently misfolded [CEEE] with [CCCC] might also contribute to interference of virus bearing the latter SU.

Thus, EECC entry and growth is partially inhibited by

prior infection with FGA, but its inability to establish homologous virus superinfection interference, and potentially disrupt interference by FGA SU through heteromultimerization or other interaction, might substantially overcome this blockade. In contrast, in cells that have survived to establish EE[CCCC]C superinfection interference and express high internal levels of viral envelope protein, the effect of interference on newly infecting FGA through interactions within the cell may dominate.

According to the assumption that superinfection interference is the result of FeLV SU-mediated downregulation or blockade of a single receptor protein that alone is sufficient to confer infectibility, neither a model hypothesizing a single common receptor nor a model hypothesizing distinct receptors for 61E and 61C entry would be completely consistent with our data. If they utilized the same single receptor protein for virus entry, we would expect either complete cross-interference or at least nonreciprocal interference, which would be indicative of differential affinities for the same receptor, and this was not observed. Conversely, if they utilized entirely different receptors, we would expect that their respective SU proteins would not block binding of the heterologous SU to FeT cell surfaces, but such blockade was observed. We also consider it unlikely that 61E and 61C are dualtropic. 61E behaved in these and previous superinfection experiments (25) as a subgroup A FeLV, indicating that it exclusively uses cell surface components required for FeLV-A infection of FeT cells. In addition, if they were dualtropic, their respective SU proteins might be expected to coprecipitate two proteins from the surfaces of FeT cells, which was not observed. If any of the viruses analyzed here could be considered dualtropic, it would be EE[ECCC]C because it interfered with superinfection by both 61E and 61C SU-bearing challenge viruses.

Superinfection interference might be the result of events other than blockade or downregulation of a single receptor protein. We have mentioned the potential for the intracellular interactions between different envelope proteins to affect the propagation of superinfecting virus. In addition, cell surface accessory factors required for virus entry, rather than the initial cell surface virus-binding proteins, might be blocked or downregulated. Evidence for the involvement of multiple cell surface factors for infection has been shown with ecotropic murine leukemia virus (52) as well as HIV-1 (27). For HIV-1, SU sequences that determine macrophage tropism also specify resistance to soluble CD4 neutralization, leading to the suggestion that a unique secondary factor is required for HIV-1 infection of macrophages (31). In addition, VSV pseudotypes bearing either HIV-1, HIV-2, or simian immunodeficiency virus envelope protein display unique infection profiles when tested on various CD4-expressing mammalian cells (4), suggesting that tissue-specific and species-specific secondary factors might be uniquely utilized by these viruses. Lastly, the observation that FeLV-A, -B, and -C SU protein-binding sites are available on FeT cell surfaces regardless of whether the cells are infected with FeLV-A, -B, or -C (8, 16) suggests that FeLV subgroup-specific interference most likely is not due to blockade or downregulation of the same primary virus-binding protein but rather blockade or downregulation of distinct forms of the receptor or other components required for efficient virus entry. Interestingly, SU purified from the subgroup C FeLV strain FSC also coprecipitates a 70-kDa protein from FeT cell surfaces (2).

EE[CCCC]C interfered with challenge viruses bearing SU proteins with efficiencies that decreased in the following

order: [CCCC] > [ECCC] > [GGGG] > [EEEE]. Conversely, EE[EEEE]C interfered with challenge viruses bearing the same SU proteins but with efficiencies that increased in that order. Therefore, despite binding to what appears to be the same cell surface protein, 61E SU and 61C SU have distinct interference properties. Because the order is reversed for 61E SU versus 61C SU, and because chimeric viruses establish interference with efficiencies between these two, it is plausible that a family of related receptors or secondary factors present at the cell surface can be differentially utilized during the processes of virus entry and establishment of interference. For example, the observation that [ECCC] SU exhibited interference against both 61E and 61C SU-bearing challenge viruses in an infected cell context suggests that [ECCC] SU interacts with both 61E-specific and 61C-specific secondary factors or members of a receptor family required for virus entry.

We have shown that the envelope protein of cytopathic FeLV-61C failed to establish superinfection interference when expressed in the absence of virus replication. We have also presented data suggesting that 61E and 61C use either distinct members of a closely related receptor family, differentially expressed or modified forms of the same receptor, or the same primary cell surface-binding protein but distinct secondary factors for infection of feline cells. Further resolution of these issues await the cloning and characterization of the FeLV receptor proteins.

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