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Monoclonal Antibody 667 Recognizes the Variable Region A Motif of the Ecotropic Retrovirus CasBrE Envelope Glycoprotein and Inhibits Env Binding to the Viral Receptor

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Monoclonal antibody (MAb) 667 is a neutralizing mouse monoclonal antibody recognizing the envelope glycoprotein (Env) of the ecotropic neurotropic murine retrovirus CasBrE but not that of other murine retroviruses. Since 667 can be used for preclinical studies of antiviral gene therapy as well as for studying the early events of retroviral infection, we have cloned its cDNAs and molecularly characterized it in detail. Spot technique-based experiments showed that 667 recognizes a linear epitope of 12 amino acids located in the variable region A of the receptor binding domain. Alanine scanning experiments showed that six amino acids within the epitope are critical for MAb binding. One of them, D_{57}, is not present in any other murine retroviral Env, which suggests a critical role for this residue in the selectivity of 667. MAb 667 heavy- and light-chain cDNAs were functionally characterized by transient transfection into Cos-7 cells. Enzyme-linked immunosorbent assays and Biacore studies showed that the specificities as well as the antigen-binding thermodynamic and kinetic properties of the recombinant 667 MAb (r667) produced by Cos-7 cells and those of the parental hybridoma-produced MAb (h667) were similar. However, h667 was shown to contain contaminating retroviral and/or retrovirus-like particles which interfere with both viral binding and neutralization experiments. These contaminants could successfully be removed by a stringent purification protocol. Importantly, this purified 667 could completely prevent retrovirus binding to target cells and was as efficient as the r667 MAb produced by transfected Cos-7 cells in neutralization assays. In conclusion, this study shows that the primary mechanism of virus neutralization by MAb 667 is the blocking of the retroviral receptor binding domain of CasBrE Env. In addition, the findings of this study constitute a warning against the direct use of hybridoma cell culture supernatants for studying the initial events of retroviral cell infection as well as for carrying out in vivo neutralization experiments and suggest that either recombinant antibodies or highly purified antibodies are preferable for these purposes.

CasBrE is a simple murine ecotropic retrovirus which causes a spongiform encephalopathy primarily affecting the motor center of the brain and the spinal cord. The virus was originally isolated from wild mice (17), and its neurovirulence is determined by the envelope glycoprotein (Env) sequence (13, 46, 47). Due to the poor replication ability of the virus in the brain, long periods of incubation (3 to 6 months) after neonatal infection are required for the onset of neuropathology. However, when the Env of CasBrE is introduced into the neuroinvasive but nonneuropathogenic Friend murine leukemia virus (MLV) strain FB29 in place of its natural Env, the resulting virus, $FrCas^E$, induces a rapidly progressing, noninflammatory spongiform neurodegenerative disease with an incubation period of only 2 weeks.

Retroviral envelope glycoproteins are synthesized as precursors which are proteolytically processed into two components. The resulting heterodimer contains a surface moiety (SU; also called gp70 in MLVs), responsible for virus binding to its cellular receptor, and a transmembrane part [TM; also called p15(E) in MLVs], anchoring Env in the viral envelope and carrying a latent fusion activity that is activated upon the virus binding to its cognate receptor (22, 26). Monoclonal antibody (MAb) 667 is an IgG2a/κ neutralizing mouse MAb that binds to the SU Env subunit of CasBrE but not to the subunits of other ecotropic retroviruses (31). The neutralization effect in vitro of MAb 667, in the absence of complement, has been described for both the CasBrE retrovirus (31) and $FrCas^E$ (45). It has also been reported that, when mixed with retroviral particles prior to the infection of susceptible mice, 667 prevents all manifestations of $FrCas^E$-induced neurodegeneration, most probably because of in vitro neutralization of the viral inoculum (45). More recently, Pelegrin et al. have shown that intravenous injection of 667 can also protect mice from $FrCas^E$, demonstrating that 667 can exert an antiviral effect in vivo, even in animals with established infections (42). However, its mechanism(s) of action still remains to be elucidated.

The rapid onset, the clear symptomatology, the highly predictable clinical course, and the 100% incidence of the neurodegeneration induced by $FrCas^E$ make it a unique model for studying a virus-induced chronic neurological disease. More-

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over, combined with the use of MAb 667, it is an invaluable tool for the optimization of antiviral antibody-based gene or cell therapy approaches. The development of such new therapeutic approaches is justified by the fact that intravenous infusion of purified antibodies, despite its apparent simplicity, is not applicable for the long-term treatment of patients suffering from severe or life-threatening diseases. The reasons for this (reviewed in references 41 and 43) include the high cost of treatments, side effects, and possible anti-idiotypic responses associated with the injection of massive doses of antibodies in addition to variations in the bioavailability of the therapeutic agent. In recent years, Noël et al. have shown that the grafting of ex vivo genetically modified cells of various types (35, 36, 38), as well as direct in vivo gene transfer (37) and implantation of antibody-producing cells encapsulated in an immunoprotective device (40, 42), permits the long-term in vivo production and systemic delivery of MAbs in living organisms. Pelegrin et al. have shown that mice lethally infected with the neurodegeneration-inducing $FrCas^E$ retrovirus are efficiently protected from developing the illness through the control of viremia by the 667 MAb produced by hybridoma cells encapsulated in cellulose sulfate capsules, thus demonstrating the therapeutic value of the in vivo production of MAbs (42).

As 667 is not only an important instrument for preclinical studies aiming at developing and optimizing antibody-based gene or cell therapies but also one of the rare MAbs which have been shown to have neutralizing activity in vivo against a specific murine retrovirus, it was of interest to clone its cDNAs as well as to characterize its mode of action through the identification of its cognate epitope. Here, we report that 667 acts primarily by directly blocking cell infection through the recognition of the viral receptor binding domain, and we propose a molecular explanation for the selectivity of 667 towards CasBrE Env. Moreover, endogenous retroviral particles were detected in the antibody preparations produced from the hybridoma cells, suggesting that highly purified MAbs, or recombinant MAbs, produced by appropriately engineered cells should be the preferred source of antibodies for studying the early events of retroviral infection in vitro and neutralization experiments in vivo.

MATERIALS AND METHODS

Cell lines, culture conditions, antibodies, antibody purification, and virus. TelCeb6 cells were derived from the TE671 human rhabdomyosarcoma cell line (10). They produce retroviral particles, lacking Env, and carry a nuclear localization sequence β-galactosidase reporter gene. 3A2B6 is a stable cell line derived from TelCeb6 cells by transfection of a Moloney murine leukemia virus (MoMLV) ecotropic Env expression vector. Mouse NIH-Akv cells have been previously described (23). FLYA cells (10) are HT1080-based packaging cell lines which produce MoMLV cores with envelope glycoproteins of amphotropic MLV (4070A). TelCeb6, 3A2B6, NIH-Akv, FLYA, BALB/c, and 293 cells as well as *Mus dunni* fibroblasts were grown in Dulbecco's modified Eagle's medium. The 667 (31), 83A25 (15), 709 (31), R187 (8), Tg10 (34, 44), and G8P2B5 (44) hybridomas were grown in RPMI 1640 (Gibco-BRL). Both media were supplemented with 10% fetal calf serum, 100 U of streptomycin/ml, 100 U of penicillin/ml, and 2 mM L-glutamine. The 667 antibody obtained from hybridoma cell culture supernatants (h667) was purified by affinity chromatography on protein A-Sepharose, essentially as described by Harlow and Lane (19). However, an additional washing step with buffer containing 0.1% sodium dodecyl sulfate (SDS) was included to remove all contaminating retroviral or retrovirus-like particle proteins. The chimeric $FrCas^E$ virus was originally created as a molecular clone by replacing the *env* gene of the Friend MLV clone FB29 with that of the neurovirulent wild-mouse ecotropic virus CasBrE (46). Culture supernatants of *M. dunni* fibroblasts, transfected with the $FrCas^E$ clone and cultured as described previously (42), were used as virus stocks. They were aliquoted and kept at −70°C until use.

Cloning procedures, nucleotide sequencing, and cell transfection. $Poly(A)^+$ RNA from h667 cells was prepared with Dynabeads (Dynal) according to the supplier's specifications and used for constructing a cDNA library with the Superscript plasmid system (Gibco-BRL) and the pSPORT1 vector (Gibco-BRL). The selection of MAb 667 heavy- and light-chain DNA clones was carried out by using specific α-^{32}P-labeled oligonucleotide probes prepared as described by Chardes et al. (7). Nucleotide sequencing was carried out with the ABI PRISM automatic sequencer and, according to Kabat's classification (24), indicated that (i) the Vκ chain belongs to the Vκ4/5 gene family, (ii) the Jκ fragment used was Jκ5, (iii) the VH region belongs to the VH8/3609 gene family, (iv) the D gene belongs to the DS gene family, and (v) the JH gene used was JH4. Comparison with published sequences confirmed the IgG2a(κ) nature of 667 and permitted the precise delineation of complementarity-determining regions. For expression in mammalian cells, MAb 667 light- and heavy-chain full-length cDNAs were recloned into the pcDNA3 vector (Invitrogen) to give clones PM514 and PM524, respectively. Transient transfection of Cos-7 or 293 cells was carried out with FuGene transfection reagent (Roche) according to the supplier's recommendations.

ELISA and immunoblotting assays of MAb 667. MAb 667 was assayed with the concanavalin A (ConA) enzyme-linked immunosorbent assay (ELISA) developed by Cole et al. (9) and modified as described by Pelegrin et al. (42) by using a two-step procedure for microtiter plate coating with viral antigens. In the first step, plates are coated with ConA, a lectin that binds glycoprotein, and, in the second step, they are coated with the retroviral Env purified by ultracentrifugation as previously described (42). For the immunoblotting experiments, protein extracts from monolayer cultures of either 293 or Cos-7 cells were prepared 3 days posttransfection by detergent lysis as described by Harlow and Lane (19). For the detection of MLV Env proteins, 1.2 ml of 667 hybridoma culture supernatants was adjusted to 10 mM $CaCl_2$ and incubated at room temperature for 30 min. The samples were then spun down at 15,000 × *g* at 4°C for 1 min and resuspended directly in 10 μl of electrophoresis loading buffer. Viral and cell protein samples were fractionated through 10% polyacrylamide gels containing SDS and transferred to Protran nitrocellulose membranes (Schleicher & Schuell). Immunodetection of mouse light- and heavy-chain antibodies was conducted with a biotinylated anti-mouse antibody goat antiserum (Amersham) and peroxidase-conjugated streptavidin (Amersham), as determined with a Renaissance chemoluminescence kit from NEN Life Science Products according to the supplier's recommendations. Immunodetection of viral particles in 667 hybridoma supernatants was carried out with rabbit polyclonal Rauscher leukemia virus antiserum (NCI/BCB Repository, Camden, N.J.); mouse MAb 709, which recognizes only the CasBrE and Friend virus gp70 proteins (31); rat MAb 83A25, which recognizes a wide range of MLV gp70s (15); or MAb R187, which recognizes the $p30^{gag}$ protein of a wide range of MLVs (8).

Determination of kinetic association and dissociation constants of MAb 667. Real-time analysis of the interaction between MAb 667 and $FrCas^E$ viral particles was performed with technology from Biacore AB (Uppsala, Sweden). Bald MoMLV-derived retroviral particles produced by TelCeb6 cells (10) were used as negative controls. They were purified in parallel with $FrCas^E$ viruses by ultracentrifugation (10) and immobilized on two adjacent flow cells of a CM5 sensor chip as described by the supplier (Biacore AB). Several concentrations (82 to 330 nM) of recombinant or parental 667 antibody were injected at a flow rate of 30 μl/min simultaneously onto both viral particles and onto a third flow cell, which was free of particles and used as a blank. HBS (HEPES-buffered saline, pH 7.4, containing 3.4 mM EDTA and 0.05% Biacore surfactant) was used both for sample and running buffers. Regeneration of the sensor chip was performed with 15 μl of 100 mM HCl. After blank subtraction, the sensorgrams were analyzed with the BIAevaluation 3.0 software and the so-called global method (27).

Virus and MAb 667 neutralization activity assay. Virus titers in cell culture supernatants were determined with the focal immunofluorescence assay (FIA) described by Sitbon et al. (50). Briefly, cell culture supernatants were incubated on *M. dunni* fibroblast monolayers at 25% confluence in the presence of 8 μg of Polybrene/ml (Sigma). Cell-to-cell spread of replication-competent retroviruses was allowed to proceed for 2 days, and focus-forming units (FFU) were visualized by means of an indirect immunofluorescence assay in which anti-Env antibodies (MAb 667 or rat 83A25 MAb [15]) were used as primary antibodies to detect the envelope glycoprotein expressed at the surfaces of infected cells. For the virus neutralization assay, 4×10^2 $FrCas^E$ FFU was diluted in a 1:1 ratio with either 667 from transfected cells, 667 hybridoma cells, or purified 667 (p667) cells and incubated with 2×10^4 cells per well in 12-well culture plates at 37°C for 1 h.

When the cells had reached confluence, an FIA was performed. The irrelevant IgG2a(κ) G8P2B5 anti-human thyroglobulin MAb directed against human thyroglobulin (44) was used as a negative control in experiments conducted in parallel.

Spot multiple-peptide synthesis and antibody assay. A total of 217 overlapping 15-mer amino acid peptides frameshifted by three residues and representing the complete CasBrE Env sequence (48) were synthesized on a cellulose membrane (Abimed GmbH) with the spot technique (32) and the ASP 412 spotter from Abimed GmbH. Membrane-bound peptides were probed with 30 ml of cell culture supernatant containing MAb 667. MAb binding was revealed by means of an alkaline phosphatase-conjugated rabbit anti-mouse antiserum (Sigma) as previously described (32). When necessary, membranes were recycled for further probing by removing the precipitated dye by the method of Molina et al. (32).

Binding assays and flow cytometry analysis. Binding experiments were performed as described by Lavillette et al. (29). Briefly, 5×10^5 BALB/c target cells were incubated at room temperature for 10 to 30 min with soluble Env from a supernatant cleared of virus corresponding to a titer of 5×10^4 FrCasE FFU in the presence of various quantities of 667 MAb produced by either transfected or hybridoma cells, as well as in the presence of 667 purified by protein A-Sepharose affinity chromatography (see above). Cells were washed twice with PBA (2% fetal calf serum, 0.1% sodium azide in phosphate-buffered saline [PBS]) and stained for 45 min at 4°C with 83A25 hybridoma supernatant. Following two washes with PBA, the cells were incubated for 45 min at 4°C in the dark with an anti-rat Ig fluorescein isothiocyanate-conjugated antibody (Sigma). The cells were counterstained for 5 min with 20 μg of propidium iodide/ml and washed twice in PBA. The fluorescence of living cells was analyzed with a FACScalibur fluorescence-activated cell sorter from Becton Dickinson. A similar protocol was used with MAbs 667 and 709 to detect infected cells or viral binding to BALB/c cells, where the secondary antibody was replaced with an anti-mouse Ig fluorescein isothiocyanate-conjugated antibody (Sigma).

Nucleotide sequence accession numbers. Heavy- and light-chain cDNA sequences are available in the EMBL database under accession numbers AJ421677 and AJ421676, respectively.

RESULTS

Cloning and functional characterization of the recombinant 667 antibody. The full-length cDNAs encoding MAb 667 light and heavy chains were cloned into the mammalian expression vector pcDNA3 and sequenced as described in Materials and Methods, giving rise to plasmids PM514 and PM524, respectively. The plasmids were transiently transfected, either individually or in combination, into simian Cos-7 or human 293 cells. The presence of the recombinant antibody (r667) specific for CasBrE Env was assayed in culture supernatants with a modified version of the ConA ELISA described by Cole et al. (9). For the sake of experimental convenience, $FrCas^E$ viral particles produced by infected *M. dunni* cells were preferred over CasBrE as a source of Env in this assay. Equivalent quantities of vectors expressing the light and heavy chains of the Tg10 mouse MAb (34) directed against human thyroglobulin as well as the empty pcDNA3 vector were used as negative controls. Following the immobilization of $FrCas^E$ Env on ELISA plates, only the combination of PM514 and PM524 resulted in detection of Env-binding antibody in transfected cell culture supernatants (Fig. 1A), establishing that r667 light and heavy chains undergo functional synthesis and assembly in transfected cells. The absence of binding under other conditions was not due to the lack of release of antibody light and heavy chains in culture supernatants, as demonstrated by the immunoblotting experiments presented in Fig. 1C, suggesting that both chains are necessary for antigen recognition. This situation differs from that of many MAbs, such as Tg10 (34), whose heavy chain can bind to the antibody cognate epitope on its own, although often with lower affinity. It was also important to confirm that r667 retained the same antigen specificity as the hybridoma-produced 667 MAb. To this end, r667 produced by transfected cells and the MAb contained in hybridoma cell culture supernatant (h667) were tested in the ConA ELISA for their ability to recognize envelope glycoproteins, encoded by three different MLVs, including the ecotropic MoMLV obtained from 3A2B6 cells, the amphotropic 4070A MLV-A, and the ecotropic Akv, derived from NIH-Akv cells. MoMLV-derived retroviral particles expressing no Env (obtained from TelCeb6 cells) were also included as a negative control in these experiments. The data presented in Fig. 1B indicate that h667 and r667 MAbs recognize only the retroviral particles displaying the CasBrE Env. These results, on the one hand, are in agreement with those of McAtee and Portis (31) showing that 667 did not bind to Akv Envs, and, on the other hand, extend the notion of selectivity for 667 since it can recognize neither 4070A amphotropic nor ecotropic MoMLV Envs.

Finally, the association (k_{on}) and dissociation (k_{off}) kinetic and equilibrium ($K_D = k_{off}/k_{on}$) constants of r667 and the parental h667 antibody were determined by using the Biacore technology, which permits the determination of kinetic constants independently of both the concentration and the purity of the antibody (at least when the protocol adopted for the present study is used; see Materials and Methods). As shown in Table 1, the affinity of r667 for viral Env is only slightly less (approximately threefold) than that of the parental h667. This decrease is accounted for by an accelerated dissociation rate and not by a reduction in the rate of binding of r667 to CasBrE Env. Taken together, these data indicate that r667 is functionally assembled in transfected cells and retains an affinity comparable to that of the parental antibody.

Identification of the epitope recognized by the 667 MAb. As a first step to elucidate the mechanisms whereby 667 exerts its neutralizing effect, we wanted to identify the viral determinant(s) recognized by the antibody in an epitope-mapping experiment. The 667 antibody can bind to denaturated and reduced CasBrE SU in an immunoblotting assay (data not shown), suggesting that the attachment to the epitope is not conformation dependent. This finding prompted us to use the spot method (16, 32) in an attempt to identify the 667 epitope. A set of 217 overlapping peptides spanning the entire 661-amino-acid sequence of the CasBrE Env precursor (Fig. 2A) was thus synthesized onto a cellulose membrane and probed with h667. Four spots were reproducibly recognized (Fig. 2B), with the strongest signal from spots 28 and 29. This indicates that the motif is the 12-amino-acid continuous epitope T_{52}HW GLDNHPPYS, located in the N-terminal region of the Env SU subunit, with amino acid 1 corresponding to the N-terminal amino acid of SU after removal of the signal sequence (Fig. 2A). This direct recognition of a peptide motif also suggested that recognition by 667 is not dependent on CasBrE Env glycosylation (although it might potentially be modulated by this posttranslational modification). In line with this observation, the epitope bound by 667 does not include any of the putative Env glycosylation sites (Fig. 2A). Interestingly, CasBrE Env amino acid sequence analysis indicated that the 667 epitope resides in the variable region A (VRA) region which, together with the so-called VRB domain, is one of the most variable regions among the different MLV Envs (Fig. 2D) and is implicated in the retroviral receptor choice (4, 11, 20).

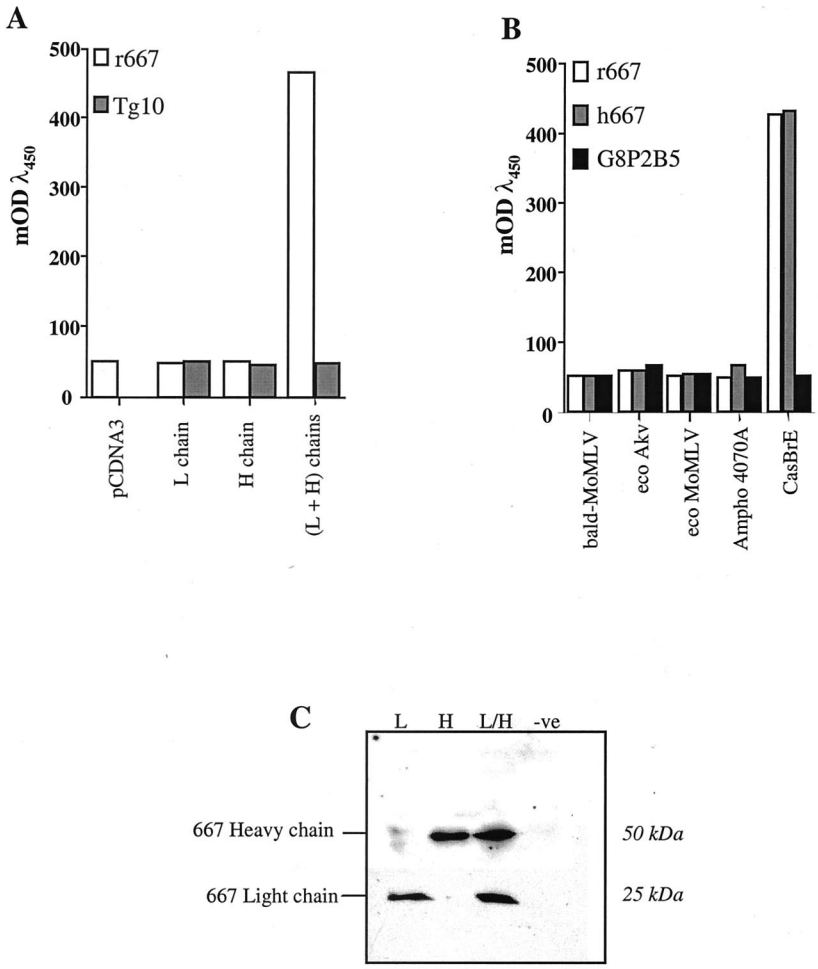

FIG. 1. Functional characterization of r667 heavy- and light-chain cDNAs. (A) Binding of r667 heavy and light chains to CasBrE Env. Cells were transiently transfected with PM514 and PM524 expression vectors coding for r667 light and heavy chains, respectively, either individually or in combination. The void pcDNA3 vector as well as PM117 and PM124 coding for the Tg10 anti-human thyroglobulin light- and heavy-chain cDNAs, respectively, were used as negative controls. Culture supernatants were then tested by ConA ELISA using purified $FrCas^E$ particles as a source of CasBrE Env. (B) Specificity of recognition of r667. Cells were transiently cotransfected with PM514 and PM524 vectors, and cell culture supernatants were used in ConA ELISA with various sources of retroviruses to coat the ELISA plates. h667 and G8P2B5 antibodies were used as positive and negative controls, respectively. (C) Expression of r667 light and heavy chains in transfected cells. Cell culture supernatants from transiently transfected cells were processed for immunoblotting analysis, and r667 light and heavy chains were detected by using a specific anti-mouse-γ and anti-mouse-κ antibodies as described in Materials and Methods. L, light; H, heavy; eco, ecotropic; Ampho, amphotropic; −ve, negative control; $mOD\lambda_{450}$, milli-optical density units at 450 nm.

The contribution to antigen recognition by MAb 667 by each residue of the peptide sequence was investigated by an Ala scanning analysis in which all amino acids were separately replaced by an L-alanine residue. Mutated 15-mer peptides were synthesized with the spot method, and membranes were probed with either h667 (Fig. 2C) or r667 (data not shown) MAbs, with similar outcomes. Three categories of amino acids could be identified: (i) those (W_{54}, G_{55}, L_{56}, and D_{57}) whose mutation led to total loss of binding and which thus make an essential contribution to the contact surface between CasBrE Env and the antigen recognition site of 667, (ii) those (H_{53} and Y_{62}) whose mutation led to partial inhibition of binding and which thus contribute marginally to the epitope, and (iii) those (T_{52}, N_{58}, H_{59}, P_{60}, P_{61}, and S_{63}) whose change had no effect and which thus contribute minimally, if at all, to the epitope.

MLV and/or MLV-like particles are produced by 667 hybridoma cells. Contaminating endogenous retroviruses have already been reported to be released by a number of myeloma cell lines and hybridomas (3, 49, 51). Because we observed a high batch-to-batch variability in h667 neutralization activity

TABLE 1. Kinetic and equilibrium constants of MAbs r667 and h667[a]

Antibody	Cell origin	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (M)
h667	667 hybridoma	5.6×10^4	5.1×10^{-4}	0.93×10^{-8}
r667	Cos-7	4.2×10^4	14.2×10^{-4}	3.4×10^{-8}

[a] Kinetic constants were determined using the Biacore technology, as described in Materials and Methods, with Cos-7 and 667 hybridoma cell culture supernatants.

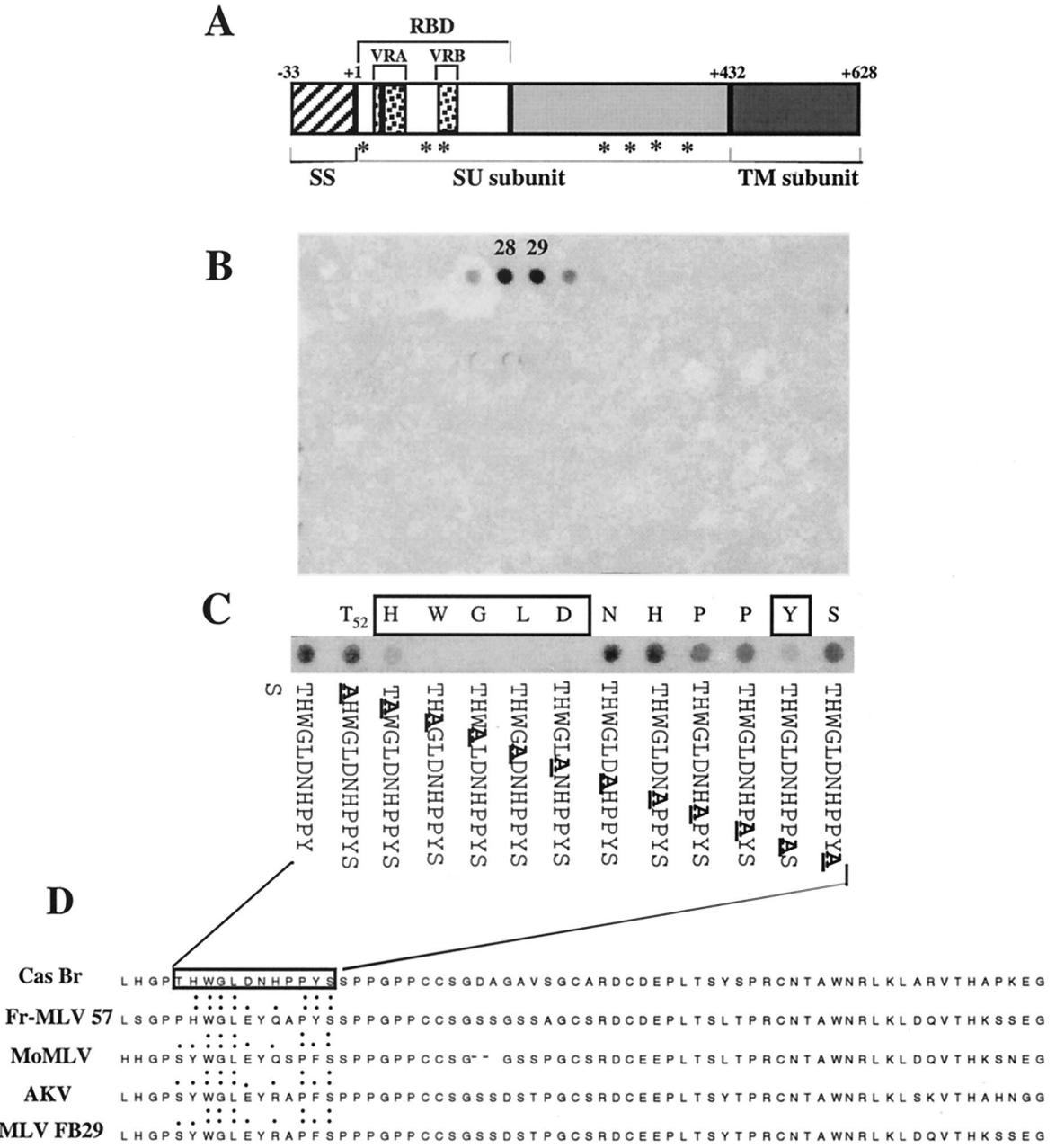

FIG. 2. Characterization of the 667 epitope. (A) Structure of the CasBrE Env precursor. CasBrE is synthetized as a gp85 precursor which is proteolytically processed into a gp70 surface protein (SU) and a p15(E) transmembrane protein (TM). RBD, receptor binding domain; SS, signal sequence. VRA and VRB are the two most variable domains among the different Envs. Numbers indicate amino acid positions. Asterisks indicate CasBrE Env putative glycosylation sites. The black box represents the location of the epitope recognized by MAb 667. (B) Spot analysis of CasBrE Env. A total of 217 overlapping 15-mer oligopeptides were synthetized on a cellulose membrane and probed with a 667 MAb-containing hybridoma cell culture supernatant as described in Materials and Methods. (C) Alanine scanning of the 667 epitope. A series of twelve 15-mer oligopeptides, each containing one amino acid change into alanine at a different position, was synthetized on a cellulose membrane and probed with MAb 667. (D) Amino acid comparison of various ecotropic MLV Envs. The VRA regions from five ecotropic MLV Envs are compared. The 667 epitope is enclosed in a box. Single and double points indicate amino acids homologous and identical to CasBrE Env amino acids, respectively.

(data not shown), we investigated whether viral contaminants could be produced by 667 hybridoma cells as well. Consistent with this idea, a high level of reverse transcriptase activity was detected in 667 hybridoma cell culture supernatants (data not shown). The possible binding of retroviral particles to murine cells was also assessed. To this end, BALB/c cells were first incubated with either h667 or $FrCas^E$ virus as a positive control. Then, the presence of cell-associated gp70 was tested in a flow cytometry assay with MAb 83A25, which reacts with nearly all members of the polytropic, xenotropic, and amphotropic classes of MLVs (15). Incubation with h667 alone led to quantitative cell labeling, suggesting that MLV- or MLV-like particles were present in the h667 supernatant and could bind to murine cells (Fig. 3A), which was not the case with r667

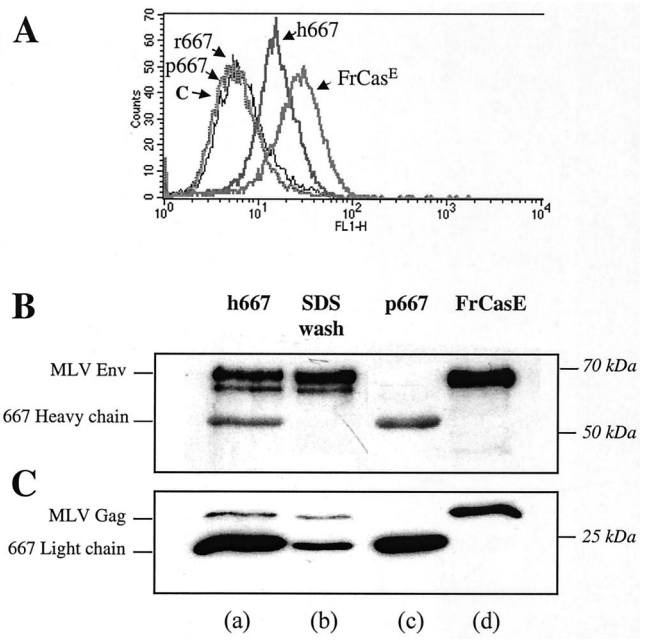

FIG. 3. Detection and clearance from MAb 667 of contaminating retroviral particles contained in 667 hybridoma cell culture supernatant. (A) Flow cytometry analysis of retroviral contaminants contained in 667 hybridoma cell culture supernatants. A total of 10^5 BALB/c cells were incubated with soluble Env obtained from a supernatant cleared of virus corresponding to a titer of 5×10^4 FrCas^E FFU, 10 μg of h667, 10 μg of r667, or 10 μg of p667 and subsequently stained with the anti-MLV Env MAb 83A25. C, control cells. (B) Immunoblotting analysis of h667 and p667 MAbs for the presence of Env antigens. h667 was purified by affinity chromatography on protein A-Sepharose, as described in Materials and Methods, following a protocol including washing protein A-Sepharose-bound MAb with a buffer containing SDS. h667, proteins contained in the SDS-containing buffer wash (SDS wash), and p667 were analyzed in parallel by immunoblotting with the anti-Rauscher MLV antiserum. Purified FrCasE particles were included as positive controls. The rabbit anti-goat Ig antiserum used as secondary antibody also detects the 667 heavy chain, though weakly. (C) Immunoblotting analysis of h667 and p667 MAbs for the presence of Gag antigens. The same samples as in panel B were probed with the R187 anti-Gag MAb. The rabbit anti-rat Ig antiserum used as a secondary antibody also detects the 667 light chain. Note that a fraction of the 667 light chain is eluted in the SDS wash. The heavy chain is not detected in the luminogram exposure presented in panel B because cross-detection of murine heavy chain with the anti-goat Ig antiserum is poor.

(Fig. 3A). This result demonstrated that the signal observed with h667 is due to contaminants in h667 detected by MAb 83A25 rather than the result of nonspecific binding of MAb 667 to cells. In addition, the presence of replication-competent retroviruses in 667 hybridoma cell culture supernatants was confirmed by an FIA (see Materials and Methods) in which the formation of the foci of infected cells was visualized by using either MAb 83A25 or MAb 667. A total of 4×10^2 FFU/ml was scored in the first case and 10 FFU/ml was scored in the second, clearly demonstrating the presence of infectious viral particles in the samples analyzed. However, it must be stressed that the FIA used probably underestimates contamination to a large extent because (i) physical retroviral particles are usually much more abundant than infectious ones in a given retrovirus preparation and (ii) detection of replication-competent viruses is limited by the availability of appropriate antibodies. Immunoblotting analysis aimed at the detection of MLV Env and Gag proteins was also conducted with a polyclonal antiserum initially raised against the Rauscher retrovirus (which recognizes a variety of MLV SU), MAb 709 (which recognizes only CasBrE and Friend virus SU), or MAb R187 (which recognizes the p30^{gag} protein of a number of MLVs). Large amounts of Env and Gag were detected in h667 with the anti-Rauscher antiserum (Fig. 3B, lane a) and R187 (Fig. 3C, lane a), respectively. However, no Env signal was seen when MAb 709 was used, though control experiments with purified FrCas^E particles were positive (data not shown). Thus, our data are consistent with the idea that 667 hybridoma cells produce a heterogenous population of retroviral or retrovirus-like particles. A majority of the detected contaminants are not related to CasBrE, whereas a minority are related to it at the level of the 667 epitope. These viruses are, however, unlikely to be actual CasBrE viruses because they are not recognized by MAb 709.

After standard affinity chromatography purification of h667 on protein A-Sepharose (19), we observed that the Env and Gag proteins were still present in the antibody preparation (data not shown). Moreover, in gel filtration-based size fractionation experiments, viral Env proteins were also detected in association with a population of antibody molecules (data not shown), which is indicative of a physical association between virus and antibody. Finally, we modified the affinity chromatography purification protocol on protein A-Sepharose. An additional step, consisting of a supplementary wash cycle with buffer containing 0.1% SDS with the protein A-Sepharose-bound 667, was added. This led to the quantitative elimination of contaminating particles from the antibody preparation (called p667 hereafter) (Fig. 3B and C, lanes c). Also, the flow cytometry binding assay was repeated, and no background signal was observed when p667 was used (Fig. 3A), confirming that the signal observed with h667 is not a result of nonspecific binding of MAb 667 to cells.

TABLE 2. In vitro neutralization[a] of FrCas^E by r667 and p667

Antibody	% Inhibition at:				
	0 ng/ml	10 ng/ml	100 ng/ml	1 μg/ml	2.5 μg/ml
r667	0	55	52.1	64.8	81
p667	0	42.5	63.4	75.4	81.7

[a] Neutralization is presented as a percentage of inhibition of infection with FrCas^E virus and *M. dunni* target cells. Values are averages of results of two independent experiments.

Neutralization activity of p667 and r667 MAbs. It was important to verify that the cloned r667 displayed neutralization activity, a property which might have been altered upon production by a non-B cell. As h667 contained contaminating particles, which might have been the cause for variations in its activity, neutralization experiments were performed with p667 in parallel as a control for successful infection inhibition. To this end, we used an FIA, in which mouse fibroblasts were infected with FrCas^E viral particles previously incubated with various amounts of either p667 or r667 MAbs for 1 h. The Tg10 MAb (34) was used as a negative control. As shown in Table 2, the specific activity of r667 was comparable to that of p667 with 50% neutralization achieved with MAb concentrations between 10 and 100 ng/ml. However, total neutralization was not

obtained in the antibody concentration range used (10 ng/ml to 2.5 μg/ml) (Table 2) and only a twofold-better neutralization was obtained when the antibody concentration was increased 250-fold (10 ng/ml to 2.5 μg/ml). This result was surprising because immunoblotting experiments conducted in parallel involving internal standards (data not shown) indicated that at a concentration of 2.5 μg/ml, MAb 667 was present in a molar excess several hundredfold greater than that of Env. Possible explanations for this result are found in Discussion. In conclusion, our data demonstrate that p667 and r667 are equally effective at virus neutralization.

MAb 667 inhibits binding of CasBrE Env to the viral receptor. Binding of MAb 667 to the VRA domain of CasBrE Env strongly suggests that a primary mechanism for neutralization is the direct inhibition of virus attachment to its receptor. To address this issue, we used two complementary approaches.

In the first step, we aimed at demonstrating that the 667 epitope is no longer accessible to MAb 667 when Env is bound to the viral receptor. Retroviral particles expose multiple Envs, some of which might not be engaged in interactions with the receptor. As this might interfere with our analysis, we used soluble Env as a binding agent. The simple elimination of viral particles from retrovirus-producing cell culture supernatants was accomplished by ultracentrifugation. Mouse cells were incubated in the presence of Env, stained with MAbs r667, 83A25, and 709, and analyzed by flow cytometry. The data presented in Fig. 4A show that r667 is less efficient at detecting cell-associated SU than are MAbs 83A25 and 709, both of which produced comparable signals. This was due neither to limiting amounts of r667, as experiments carried out with different concentrations of antibody gave similar outcomes, nor to a lower recognition efficiency of r667, as r667 is more efficient than MAb 709 at detecting cell surface-exposed Env on infected cells under our experimental conditions (Fig. 4B).

In the second step, we investigated whether 667 could prevent the binding of CasBrE Env to target cells. The multiple Envs exposed at the surfaces of virions may induce agglutination by antibodies under certain conditions and, thus, flaw the interpretations of virus-to-cell binding experiments. We thus preferred to analyze the binding inhibition of monomeric SU. A culture supernatant corresponding to 5×10^4 FFU of $FrCas^E$, but cleared of viral particles by ultracentrifugation, was incubated in the presence of various concentrations of p667, and Env binding to BALB/c cells was assessed by using MAb 83A25 for detection. Almost no binding inhibition was detected in the presence of 10 μg/ml of p667, whereas nearly total inhibition was observed in the presence of 100 μg/ml. It is noteworthy that the latter concentration corresponds to an Env/antibody ratio comparable to the ratio that was most effective in the neutralization assays (Table 2) (a supernatant that is approximately 100-fold more concentrated is required in the flow cytometry assay; see Materials and Methods). In conclusion, these data indicate that MAb 667 can inhibit binding of Env to the viral receptor.

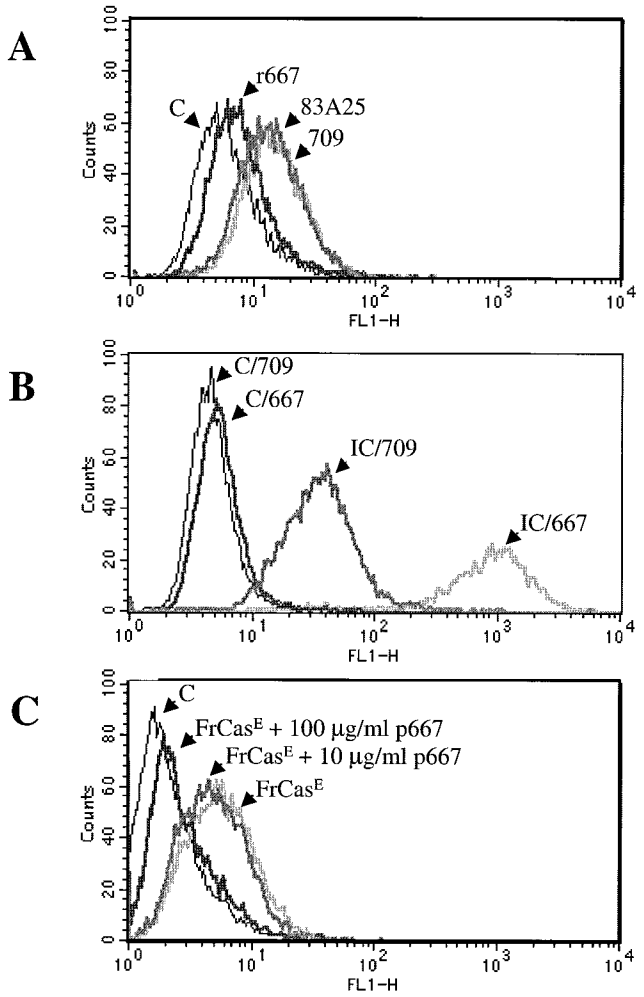

FIG. 4. Inhibition of Env binding to the viral receptor by 667. (A) Masking of the 667 epitope on cell-bound CasBrE Env. A total of 5×10^5 BALB/c cells were incubated with soluble Env from a supernatant cleared of virus corresponding to a titer of 5×10^4 $FrCas^E$ FFU. They were subsequently subjected to flow cytometry analysis with the r667, 83A25, and 709 MAbs as described in Materials and Methods. C, control cells. (B) Detection of Env on $FrCas^E$-infected *M. dunni* cells by r667 or 709. A total of 10^5 control (C/709 and C/r667) or *M. dunni* cells infected with $FrCas^E$ as in panel A (IC/709 and IC/r667) were incubated with 5 μg of 709 or r667 MAb per ml, respectively, for flow cytometry analysis. (C) Binding of $FrCas^E$ Env to BALB/c cells in the presence of p667. Soluble Env obtained from a viral supernatant corresponding to 5×10^4 $FrCas^E$ FFU was incubated with 100 or 10 μg of p667/ml or with no MAb and added to 10^5 BALB/c cells for flow cytometry analysis with MAb 83A25 for detection. Noninfected BALB/c cells (C) were used as negative controls.

DISCUSSION

To our knowledge, no epitope recognized by MAbs on murine retroviral envelope glycoproteins has ever been precisely delineated and characterized. At best, they have been mapped with some precision by using related Envs, one recognized by the antibody and the other not, either in immunoassays involving chimeric molecules or through amino acid sequence comparison followed by site-directed mutagenesis. In the present study, we have used the spot technique to identify the cognate epitope of MAb 667 on CasBrE Env. Because of its simplicity and efficiency, we believe that this approach, possibly coupled to Ala scanning analysis, will be an invaluable tool for future delineations of epitopes for other neutralizing anti-Env MAbs and, thereby, for the elucidation of the variety of mechanisms used by antibodies to inhibit MLV infection.

The 667 epitope consists of a stretch of 12 amino acids, of which 4 are absolutely required for recognition and 2 contribute to a lesser degree. Interestingly, the epitope is located within the VRA motif of the receptor binding domain of CasBrE Env. As VRA is one of the two domains involved in viral receptor recognition (5, 11, 20), a likely explanation for the neutralization activity of MAb 667 is steric interference with virus attachment to this receptor. Consistent with this interpretation, we showed that $FrCas^E$ binding to mouse cells is inhibited by 667, which suggests a mechanistic explanation for neutralization. Although this neutralization mechanism is simple and straightforward, it is worth underlining that the mechanism does not apply to all neutralizing MAbs. For example, the 83A25 MAb also used in this study inhibits the in vitro infection of a wide range of MLVs and binds to a conserved epitope lying in the Env C-terminal moiety (15), i.e., a region which is, a priori, not involved in attachment to MLV receptors. Indeed, it was recently demonstrated that, in this case, neutralization most probably occurs by blocking the intramolecular signaling which is activated upon binding to the receptor to unveil the fusogenic activity of Env (6). Along the same line, a number of antibodies directed against a variety of viruses have been shown to inhibit fusion with target cells, while others operate at unidentified postentry levels (14, 39).

The Ala scanning analysis of the 667 epitope not only allowed the identification of the amino acid residues important for MAb binding but also explained at the molecular level why MAb 667 neutralizes only retroviruses displaying CasBrE Env, since there are several sequence differences between the 667 12-mer epitope motif in CasBrE Env and its homologous regions in the other ecotropic MLV Envs (Fig. 2D). The D_{57} residue deserves specific attention here. Among the amino acids of the 667 epitope that vary among the different MLV Envs, D_{57} is the only one which is indispensable for recognition by 667, and, strikingly, it is replaced by a homologous glutamic acid residue in all other known ecotropic Envs (Fig. 2D). This fact suggests that in addition to specifying the recognition by 667, the acidic residue present at that position of Env is also important for retroviral receptor recognition. It is thus tempting to speculate that CasBrE Env-displaying retroviruses that would escape the neutralizing effect of MAb 667 have a high probability of having their D residues at position 57 turned into E.

An interesting issue relates to the number of MAb 667 molecules per viral particle that are necessary for neutralizing CasBrE Env-expressing retroviruses in vitro versus in vivo. In vitro, it was surprising to see that 100% neutralization was never reached even in the presence of MAb in a molar excess several hundredfold greater than that of Env. There might be several nonexclusive explanations for this finding. First, the affinity of 667 for its cognate antigen is relatively low (Table 1), which facilitates antibody-antigen dissociation. Second, the local concentration of receptor molecules at the surfaces of indicator cells might be sufficiently high to promote Env-667 dissociation, which would be even more efficient as the affinity of 667 for Env is modest. Third, by using MoMLV-derived vectors expressing various amounts of Env, we have recently shown that, in vitro, a small number of Env molecules is sufficient for achieving efficient cell infection (2). It is thus plausible that efficient in vitro neutralization requires the binding of 667 to the vast majority, if not to all, Env molecules displayed at the viral surface. Finally, most Env molecules (approximately 95%, as estimated by immunoblotting) found in virus-containing supernatants are not virus associated but free because of shedding from both retroviral particles and retrovirus-producing cells. The Env molecules certainly compete for antibody molecules and, thereby, also contribute to impose high antibody concentrations for total neutralization.

In vivo, the situation is probably more complex. In fact, it very likely that other mechanisms also operate, meaning that the amounts of 667 required might be much less than those required in vitro. MAb 667 is an IgG2a, and this mouse isotype has been reported to be the most efficient IgG isotype at fixing complement (33), at binding to Fc receptors on macrophages (21, 52) and NK cells (28), and at being the most effective isotype in mediating antibody-dependent cellular cytotoxicity and opsonization in vivo (12, 25). Moreover, the passive administration of IgG2a appears to be pivotal in antiviral immunity, as evidenced by the fact that monoclonal IgG2a protects mice from both influenza virus (18) and Ebola virus infections (53). Thus, a first point to consider is the likely antibody-dependent cellular cytotoxicity occurring against CasBrE Env-expressing cells and, therefore, the limitation in viral particle production. Second, antibody effector function-linked mechanisms of virus clearance, such as opsonization, complement-mediated destruction, and elimination by phagocytic cells, most probably act in addition to both the inhibition of cell infection and the intrinsic instability of retroviruses in biological fluids. Finally, if one assumes that blocking only a fraction of Envs by 667 mimics the reduction in Env density, it is possible that a limited amount of this MAb also decreases the celerity of retroviral entry into cells, as we have shown for low-density Env-expressing MLVs (2). This feature should provide antibody effector function-linked systems of virus clearance with more time for action. Experiments are currently under way to formally establish whether 667 actually achieves all of these functions.

Our experiments have demonstrated that p667 and r667 have comparatively high levels of neutralization activity. In contrast, h667 showed more variable activity. On the one hand, Pincus et al. (45) have reported that an h667 concentration of several tenths microgram per milliliter is necessary for efficient neutralization in vitro under experimental conditions similar to ours. On the other hand, certain of our own h667 preparations showed an activity level comparable to that observed by Pincus et al., whereas other samples demonstrated activities that were close to the efficacy of p667 and r667 (data not shown). A plausible explanation for this result is that the retroviral and/or retrovirus-like particles detected in h667 were responsible for the reduced activity of the MAb and that batch-to-batch variations simply reflected different extents of contamination, possibly accounted for by differences in cell culture conditions. It is also clear from our analysis that the contaminating particles, though not characterized in detail, are of several types. It is worth underlining that such contaminations are not a unique finding, as production of retroviral or retrovirus-like particles by hybridoma cells (3, 49, 51) and even hamster cells such as CHO cells (the most frequently used cell line for the production of recombinant antibodies) (1, 30) have already been reported. Nevertheless, in the context of a MAb such as 667,

which can be used for probing and studying the initial events of retroviral binding to cells, this finding indicates that interpretations of experiments can be considerably flawed, as illustrated by the flow cytometry analysis presented in Fig. 3A. With regard to in vivo neutralization experiments in mice, it must also be considered that administration of a MAb contaminated with retroviral or retrovirus-like particles can possibly induce an antiviral immune response on its own, which may partly influence the outcomes of virus challenge experiments. Hence, our observation constitutes an even stronger warning that contaminants are not eliminated efficiently with standard antibody purification methods and suggests that either a recombinant antibody produced by nonmurine cells or a MAb subjected to stringent purification is preferable in this type of investigation. Finally, it is possible that a number of MAbs with potential neutralizing activities may have been missed during screenings simply because neutralization assays were performed with hybridoma cell culture supernatants containing viral antigens which could have decreased the activity of the antibody.

It was mentioned in the introduction that MAb 667 has been used to validate the therapeutic interest in in vivo MAb production for the treatment of severe viral diseases through implantation of cellulose sulfate capsules containing hybridoma cells (42). Even though the pores of the capsules are, in principle, too small to let retroviral particles pass through them, we cannot formally exclude the possibility that 667 cell-produced viruses may escape from the capsules and thus may contribute to viremia in $FrCas^E$-infected animals. This reservation does not question the overall conclusion of our original work (42) but may have partially altered the efficacy of the anti-FrCasE virus treatment.

It is also worth stressing here that CasBrE Env can pseudotype virtually all MLVs. Hence, the paired MAb 667-CasBrE Env can potentially be used in a wide variety of retroviral diseases with different clinical manifestations to optimize our gene or cell therapy approach. To avoid possible bias induced by 667 hybridoma cell-produced retroviruses, we feel that in vivo production of r667 MAbs by appropriately modified nonmurine cells is preferable to MAbs produced by encapsulated hybridoma cells in future studies (this would also hold true for other anti-MLV Env MAbs). Viral vectors and cell lines are currently being engineered with this goal in mind.

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