A Novel Mechanism of Retrovirus Inactivation in Human Serum Mediated by Anti–α–Galactosyl Natural Antibody

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Summary

Type C retroviruses endogenous to various nonprimate species can infect human cells in vitro, yet the transmission of these viruses to humans is restricted. This has been attributed to direct binding of the complement component C1q to the viral envelope protein p15E, which leads to classical pathway–mediated virolysis in human serum. Here we report a novel mechanism of complement-mediated type C retrovirus inactivation that is initiated by the binding of "natural antibody" [Ab] (anti–α-galactosyl Ab) to the carbohydrate epitope Galα1-3Galβ1-4GlcNAc-R expressed on the retroviral envelope. Complement-mediated inactivation of amphotropic retroviral particles was found to be restricted to human and other Old World primate sera, which parallels the presence of anti–α-galactosyl natural Ab. Blockade or depletion of anti–α-galactosyl Ab in human serum prevented inactivation of both amphotropic and ecotropic murine retroviruses. Similarly, retrovirus was not killed by New World primate serum except in the presence of exogenous anti–α-galactosyl Ab. Enzyme-linked immunosorbent assays revealed that the α-galactosyl epitope was expressed on the surface of amphotropic and ecotropic retroviruses, and Western blot analysis further localized this epitope to the retroviral envelope glycoprotein gp70. Finally, down-regulation of this epitope on the surface of murine retroviral particle producer cells rendered them, as well as the particles liberated from these cells, resistant to inactivation by human serum complement. Our data suggest that anti–α-galactosyl Ab may provide a barrier for the horizontal transmission of retrovirus from species that express the α-galactosyl epitope to humans and to other Old World primates. Further, these data provide a mechanism for the generation of complement-resistant retroviral vectors for in vivo gene therapy applications where exposure to human complement is unavoidable.

The inactivation of nonprimate-derived type C retroviruses in human serum has been described as an Ab-independent mechanism involving activation of the classical complement pathway through direct binding of C1q to the retroviral surface protein p15E (1–4). This mechanism of complement activation has also been described on the surface of cells coinfected with human T cell lymphotropic virus I and III, although these retroviruses are not inactivated by human serum (5–7). The serum inactivation of type C retroviruses is primarily restricted to Old World primates, as these viruses survive exposure to sera from other mammalian species, including goat, guinea pig, rabbit, rat, and mouse (1, 5, 8, 9). Considering that nonprimate type C retroviruses are known to infect and transform human cells in vitro, it has been proposed that a species-restricted interaction between human C1q and viral p15E limits the transmission of retroviruses from nonprimate species to humans (8, 10–13).

The Galα1-3Galβ1-4GlcNAc-R (α-galactosyl) epitope is a glycosidic structure that has been identified on the surface of cells from mammalian species excluding Old World primates (14, 15). The absence of this epitope in Old World primates results from two frameshift mutations present in the α1-3galactosyl transferase gene (16–18). Cross-reactive α-galactosyl–like epitopes exist on the surface of bacteria constituting the normal human intestinal flora as well as certain human pathogens (19). As much as 1% of the total circulating IgG in humans targets this glycosidic structure, apparently because of continuous antigenic stimulation from the normal flora (19–24). Recent evidence suggests that anti–α-galactosyl natural Ab may provide an important immunological barrier against patho-

genic organisms that express the α-galactosyl epitope. For example, the binding of anti–α-galactosyl Ab to the surface of *Trypanosoma cruzi* has been shown to induce complement-mediated lysis of this organism (25). In contrast, other studies suggest that binding of anti–α-galactosyl Ab to the surface of some bacteria may serve to protect them from complement-mediated damage by blocking alternative complement pathway activation (23, 24).

In this study, we investigated the role of anti–α-galactosyl natural Ab in the inactivation of type C retrovirus by human serum complement. We show that inactivation of amphotropic and ecotropic retroviral particles is dependent on anti–α-galactosyl Ab and that the α-galactosyl epitope is present on the viral envelope glycoprotein gp70. Our data reveal a novel mechanism of complement-mediated retrovirus killing by Old World primate sera and suggest that activation of complement by anti–α-galactosyl Ab may provide an immunological barrier for the horizontal transmission of retroviruses or other enveloped viruses to humans from species that express the α-galactosyl epitope.

Materials and Methods

Retrovirus Titer Assay. The retroviral vector pLXSN (26), containing the neomycin resistance gene for selection, was used to examine the ability of type C retrovirus to survive in human serum. Retroviral particles were generated from the amphotropic packaging cell line PA317, which contains the amphotropic murine leukemia virus (MLV)[1] *gag, pol,* and *env* genes (26), or the ecotropic packaging cell line GP+E-86, which contains the ecotropic Moloney murine leukemia virus (MMLV) *gag, pol,* and *env* genes (27). Titers of infectious particles were determined by serial dilutions on NIH/3T3 cells. Briefly, 2.5 × 10⁴ cells were plated per well in six-well plates with 2 ml of DMEM containing 10% fetal bovine serum (D10). The medium in each well was replaced with 2 ml of D10 containing 8 μg/ml of polybrene, and retroviral particles were added. Tenfold dilutions were made from the original well, and plates were incubated for 24 h at 37°C. Medium was removed, and 2 ml of D10 containing 500 μg/ml of G418 (active weight) was added. The cells were maintained under selection for 7 d with two changes of medium during this period. Finally, medium was removed, and colonies were stained with methylene blue–saturated methanol for 15 min followed by a brief rinse in water. Wells containing <100 colonies were counted to determine titers.

Retrovirus Killing Assay in Primate Sera. Retroviral particles from PA317, GP+E-86, or PA317/H-transferase cells (~500 CFU) were incubated for 30 min at 37°C in 100 μl of 40% serum diluted in HBSS and were subsequently titered on NIH/3T3 cells to assess retrovirus survival as described above. The different primate sera included human (Diamedix Corp., Miami, FL), chimpanzee (Southwest Foundation for Research, San Antonio, TX), baboon, squirrel monkey, owl monkey, and tamarin (all from the

New England Regional Primate Research Center, Southborough, MA). Data were calculated as percent retrovirus survival in the various sera relative to the number of input CFU (determined by incubation of virus in 40% heat-inactivated human serum). In experiments examining the ability to block inactivation of retroviral particles, human serum was preincubated with galactose α1-3galactose (Galα1-3Gal; Dextra Laboratories, Reading, UK), D(+) glucose, D(+) galactose, α-L(−) fucose, maltose, or sucrose (all from Sigma Chemical Company, St. Louis, MO) for 30 min at 37°C before the addition of retrovirus. In the anti–α-galactosyl Ab depletion experiments, retroviral particles were preincubated with either anti–α-galactosyl Ab (90 μg/ml) or buffer alone (PBS; BioWhittaker, Inc., Walkersville, MD) for 30 min at 37°C before the addition of either 40% human serum, 40% human serum depleted of anti–α-galactosyl Ab, or 40% squirrel monkey serum for an additional 30 min incubation. Retrovirus survival was then assessed as described above.

Retrovirus Purification. Retroviral particles were isolated from the amphotropic packaging cell line PA317 via centrifugation. Briefly, a 24-h supernatant was harvested from confluent flasks of transduced PA317 cells. The supernatant was centrifuged at 40,000 g in an SW28 rotor for 90 min. Supernatant was removed, and the retroviral pellet was resuspended in HBSS. A 24-h supernatant from confluent NIH/3T3 cells was treated similarly as a no-virus control for the Western blot analysis.

Anti–α-Galactosyl Ab Purification. Anti–α-galactosyl Ab was purified from human serum using a Galα1-3Gal-polyacrylamide-glass (B$_{di}$-sorbent) column (Syntesome, Munich, Germany). Briefly, 10 ml of human serum was diluted 1:2 in PBS, pH 7.5, and passed over a 5-ml B$_{di}$-sorbent column three times. The final column flow through was collected as a source of anti–α-galactosyl Ab–depleted serum. After washing the column extensively with PBS, Ab was eluted with immunopure IgG elution buffer (Pierce, Rockford, IL) and immediately neutralized by adding 1.0 M Tris, pH 8, to a final concentration of 0.1 M. Fractions containing protein were combined and dialyzed against PBS overnight.

ELISA. Capture ELISAs were performed using flat-bottomed 96-well microtiter plates. The plates were coated for 1 h with Fab generated from an anti-gp70 mAb (purified from MLV gp70 hybridoma 715; National Institutes of Health AIDS Research and Reference Reagent Program, Rockville, MD) in 0.1 M sodium carbonate buffer, pH 9.6. After blocking the plates in Tris-buffered saline (20 mM Tris, pH 7.5, 150 mM NaCl) containing 1% BSA for 2 h, either undiluted PA317/LXSN or GP+E-86/LXSN viral supernatant was added and incubated for 30 min. In the next step, serially diluted purified anti–α-galactosyl Ab (starting at 70 μg/ml) or biotinylated *Griffonia simplicifolia* (GS)–IB₄ lectin (starting at 20 μg/ml; E. Y. Laboratories, Inc., San Mateo, CA) was added for a 30-min incubation. Horseradish peroxidase (HRP)-conjugated streptavidin or HRP-conjugated anti–human IgG Ab (Zymed Laboratories, Inc., San Francisco, CA) was added for 30 min, and plates were developed using peroxidase substrate. All incubations were performed at 37°C, and plates were washed between steps with Tris-buffered saline containing 1% BSA. Absorbance values were measured on a microplate reader (Bio-Rad Laboratories, Hercules, CA) at 492 nm and corrected for background absorbance using identically treated wells in the absence of retroviral particles.

Western Blot Analysis. Purified amphotropic retrovirus in HBSS or mock-purified supernatant was size fractionated via SDS-PAGE on a 12% gel. Proteins were transferred to nitrocellulose, and the membrane was incubated for 1 h in blocking solution (500 mM NaCl, 35 mM Tris, pH 7.4, 0.5 mM CaCl₂, 10%

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[1]*Abbreviations used in this paper:* Galα1-3Gal, galactose α1-3galactose; GS, *Griffonia simplicifolia*; HRP, horseradish peroxidase; LCMV, lymphocytic choriomeningitis virus; MLV, murine leukemia virus; MMLV, Moloney murine leukemia virus.

nonfat dry milk [Carnation Co., Los Angeles, CA], and 0.2% Tween 20). Identical blots were incubated for 45 min in 5 μg/ml of HRP-conjugated GS-IB₄ lectin, 5 μg/ml HRP-conjugated *Ulex europaeus* agglutinin (UEA) lectin (Sigma Chemical Co.), or a 1:1,000 dilution of anti-gp70 mAb (provided by Dr. James Mason, Alexion Pharmaceuticals, Inc., New Haven, CT), all diluted in fresh blocking solution. Blots were washed twice with Ab wash solution (500 mM NaCl, 35 mM Tris, pH 7.4, 0.5 mM CaCl₂, 0.1% SDS, 1.0% NP-40, and 0.5% deoxycholic acid). The blot previously reacted with anti-gp70 mAb was incubated for 20 min in fresh blocking solution containing 1.0% NP-40 and HRP-conjugated goat anti–rat IgG (1:2,000 dilution) (Zymed Laboratories, Inc.). Finally, blots were washed twice in Ab wash solution, incubated for 1 min in enhanced chemiluminescence Western blot reagent, and exposed to enhanced chemiluminescence Hyperfilm (Amersham Corp., Arlington Heights, IL).

Generation and Analysis of H-Transferase/PA317 Trans-duc-tants. The full-length human α1-2fucosyl transferase (H-transferase) cDNA was obtained by PCR amplification of reverse-transcribed cDNA from human epitheliod carcinoma cells using primers that flanked the start and stop codons of the molecule. The cDNA was subcloned as an EcoRI fragment into the pLXSN retroviral vector. Amphotropic retroviral particles were produced through the intermediate ecotropic packaging cell line GP+E-86 (27). Briefly, GP+E-86 cells were transfected with pLXSN containing H-transferase or pLXSN alone using the calcium phosphate method (28) followed by selection in D10 medium containing 500 μg/ml G418. Transfectants were pooled, and a 24-h supernatant was harvested from cells at 90% confluency. The ecotropic virus stock was used to transduce the amphotropic packaging cell line PA317, which was also selected as a pool in G418. The transduced PA317 cells were screened for surface expression of the α-galactosyl epitope or H-antigen by fluorescence staining using purified anti–α-galactosyl Ab, GS-IB₄ lectin, or UEA lectin. Cell-surface staining was performed on 2.5 × 10⁵ cells with 20 μg/ml of anti–α-galactosyl Ab or 10 μg/ml of FITC-conjugated GS-IB₄ or FITC-conjugated UEA in 1× PBS with 2% fetal bovine serum. FITC-conjugated goat anti–human IgG (Zymed Laboratories, Inc.) was used as a secondary Ab for cells incubated with anti–α-galactosyl Ab. Fluorescence was measured by FACS® using a FACSort® (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Complement-mediated Dye Release Assay. Complement-mediated killing of PA317 cells transduced with H-transferase or the pLXSN vector alone was assessed by measuring the release of the cytoplasmic indicator dye, Calcein AM (Molecular Probes, Inc., Eugene, OR) after exposure to human serum. PA317 cells were grown to confluency in 96-well plates, washed twice with HBSS containing 1% BSA, and incubated with 10 μM Calcein AM at 37°C for 30 min. Cells were again washed twice before the addition of human serum for a 30-min incubation at 37°C. Dye release was measured on a plate reader (Cytofluor 2350; Millipore Corp., Bedford, MA; excitation, 490 nm; emission, 530 nm). Dye retained, represented as percent cell survival, was calculated from the percent dye released relative to total cell–associated dye (determined from dye released from cells treated with 1% SDS). Dye release from cells not subjected to serum treatment allowed the determination of background fluorescence and nonspecific dye release. In the anti–α-galactosyl Ab depletion experiment, Calcein AM–loaded cells were incubated for 30 min at 37°C with either anti–α-galactosyl Ab (90 μg/ml) or buffer alone (PBS), and unbound Ab was removed with two washes in HBSS containing 1% BSA. Cells were then exposed to either 20% human serum,

20% anti–α-galactosyl Ab–depleted serum, or 20% squirrel monkey serum for 30 min at 37°C, and percent cell survival was determined as described above.
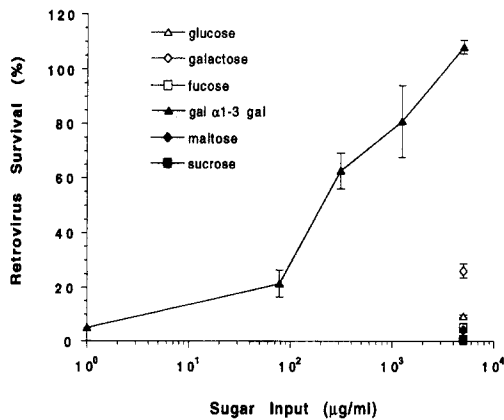
Results

Survival of Retrovirus in Sera of Old and New World Primates. Previous studies have demonstrated that many type C retroviruses are effectively inactivated in Old World primate sera but not in sera from various other mammalian species (1, 5, 8, 9). Additionally, recent studies have shown that Old World, but not New World, primates produce anti–α-galactosyl Ab (14, 15). To investigate the potential role of this Ab in the inactivation of type C retroviruses, retroviral particles containing the MLV-derived amphotropic envelope were incubated in sera from various Old World (human, baboon, and chimpanzee) and New World (squirrel monkey, owl monkey, and tamarin) primate species. This retrovirus has been previously shown to be effectively inactivated in human serum via activation of the classical complement pathway in a manner dependent on the formation of the terminal membrane attack complex (C5b-9) (29). All primate sera were shown to have similar functional complement levels in a classical pathway-mediated chicken erythrocyte hemolytic assay (data not shown). Sera from Old World primate species effectively inactivated the retrovirus with a mean survival of 7.5% (Fig. 1). In contrast, the retrovirus was resistant to inactivation in New World primate sera with a mean survival of 95%. The survival of amphotropic retroviral particles in New World,
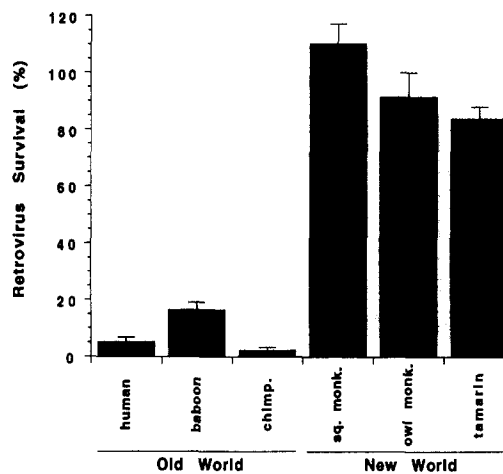
Figure 1. Retrovirus survival in Old World versus New World primate sera. Amphotropic retroviral particles were incubated in sera from Old World primate species, including human, baboon, or chimpanzee (*chimp.*), or with sera from New World primate species, including squirrel monkey (*sq. monk.*), owl monkey (*owl monk.*), or tamarin. After exposure to 40% serum, retrovirus was titered on NIH/3T3 cells to assess survival (see Materials and Methods). Bars represent the percentage of infectious particles remaining relative to the number of input virus (determined from survival in 40% heat-inactivated human serum). Data represent duplicate determinations of a single experiment, one of three so performed. Error bars denote SEM.

but not Old World, primate sera parallels the presence of anti–α-galactosyl Ab, suggesting that this natural Ab may play a role in complement-mediated inactivation of type C retroviruses.

*Galα1-3Gal Blocks Retrovirus Inactivation in Human Serum.* Synthetic Galα1-3Gal has been shown to specifically bind anti–α-galactosyl Ab (19). Furthermore, it has been demonstrated that this carbohydrate serves as an effective inhibitor of anti–α-galactosyl Ab–initiated complement lysis of cells expressing the α-galactosyl epitope (30). To further investigate the role of anti–α-galactosyl Ab in retrovirus inactivation, human serum was incubated with soluble Galα1-3Gal before the addition of retroviral particles. Total serum complement activity was unaffected by pretreatment with the various carbohydrates as assessed in a classical complement-mediated chicken erythrocyte hemolytic assay (data not shown). Preincubation of human serum with Galα1-3Gal successfully inhibited retrovirus inactivation in a dose-dependent manner (Fig. 2). The addition of 5 mg/ml Galα1-3Gal completely blocked complement-mediated virolysis. In contrast, D(+) glucose, α-L(−) fucose, maltose, or sucrose added to the serum at a concentration of 5 mg/ml did not affect retrovirus survival. Preincubation of serum with D(+) galactose (5 mg/ml), which has been shown to partially block anti–α-galactosyl Ab reactivity (20, 31, 32), inhibited ~25% of retrovirus inactivation. Similar results were obtained when retroviral particles were challenged with serum from other Old World primates, including baboon and chimpanzee (data not shown). Taken together, these data demonstrate that anti–α-galactosyl Ab plays a major role in the complement-mediated inactiva-

tion of murine-derived type C amphotropic retroviral particles in human serum.

Contribution of Anti–α-Galactosyl Ab to Retrovirus Inactivation by Human Serum. Previous studies have described type C murine retrovirus inactivation by human serum complement as an Ab-independent event initiated by the direct binding of C1q to the retroviral envelope (1–4). In the present study, we have demonstrated that complement activation on the surface of type C retroviral particles is initiated through an Ab-dependent mechanism involving natural Ab directed against the α-galactosyl epitope. To determine the direct contribution of anti–α-galactosyl Ab to retrovirus inactivation, human serum was selectively de-
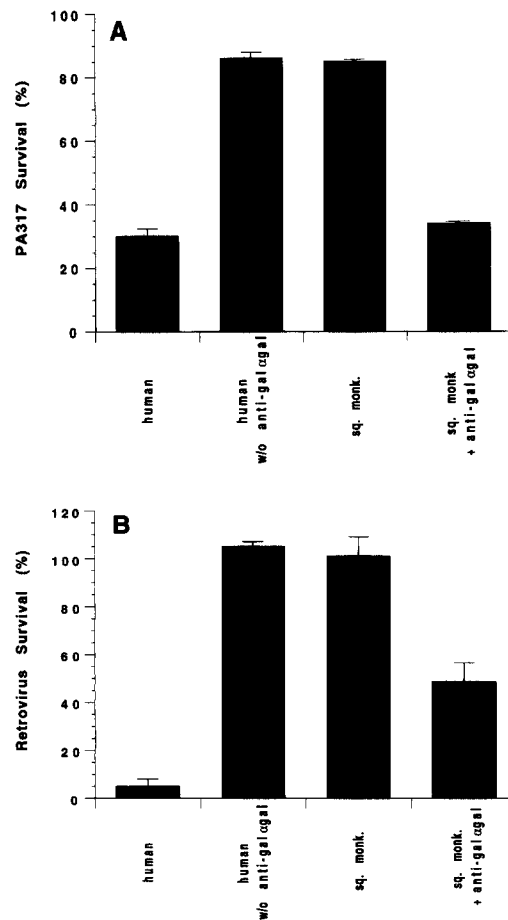
Figure 3. The role of anti–α-galactosyl Ab in complement-mediated killing of PA317 producer cells and retrovirus. In *A*, Calcein AM dye-loaded PA317 producer cells were incubated with 20% human serum (*human*), 20% anti–α-galactosyl Ab–depleted human serum (*w/o anti-galα gal*), 20% squirrel monkey serum (*sq. monk.*), or 20% squirrel monkey serum containing purified human anti–α-galactosyl Ab (+ *anti-galαgal*), and dye release was measured. Bars represent percent survival of PA317 cells calculated as the percent dye retained relative to total cell-associated dye. In *B*, the LXSN amphotropic retroviral particles generated from the PA317 producer cells were incubated with the same sera listed above at a concentration of 40%, and retrovirus survival was determined (see Fig. 1). Bars represent the percentage of infectious particles remaining relative to input virus. Data represent duplicate determinations from a single experiment, one of two so performed. Error bars denote SEM.

Figure 2. Inhibition of retrovirus inactivation in human serum using soluble Galα1-3Gal. Amphotropic retroviral particles were incubated in 40% human serum in the presence of soluble carbohydrates, including D(+) glucose (*glucose*), D(+) galactose (*galactose*), α-L(−) fucose (*fucose*), Galα1-3Gal, maltose, or sucrose. After exposure to serum, retrovirus survival was assessed (see Fig. 1). The curve represents the percentage of infectious particles remaining at various concentrations of input Galα1-3Gal relative to virus survival in 40% heat-inactivated human serum. Single points indicate retrovirus survival in the presence of various other carbohydrates at a concentration of 5 mg/ml. Data represent duplicate determinations from a single experiment, one of three so performed. Error bars denote SEM.

pleted of this Ab before incubation with either PA317 retroviral particle producer cells or retroviral particles liberated from these cells. It has been shown that depletion of anti–α-galactosyl natural Ab from human serum eliminates the ability of that serum to mediate killing of cells expressing the α-galactosyl epitope (32). After anti–α-galactosyl Ab depletion, the absorbed serum retained normal levels of complement activity as determined in a classical complement-mediated chicken erythrocyte hemolytic assay (data not shown). PA317 cells producing LXSN amphotropic retroviral particles were loaded with the cytoplasmic dye Calcein AM and exposed to human serum or anti–α-galactosyl Ab–depleted serum. While only 30% of PA317 cells survived exposure to human serum, 85% of cells survived in the depleted human serum (Fig. 3 A). Similarly, 85% of cells incubated with squirrel monkey serum, which does not contain anti–α-galactosyl Ab, survived, indicating that anti–α-galactosyl Ab had been effectively removed from the depleted human serum. Conversely, addition of the purified anti–α-galactosyl Ab to squirrel monkey serum resulted in killing of the PA317 cells. These data demonstrate that anti–α-galactosyl Ab plays a critical role in the activation of complement on the surface of PA317 retroviral particle producer cells.

To determine whether anti–α-galactosyl Ab also plays a major role in the killing of retroviral particles generated from the PA317 producer cells, retrovirus was incubated with either human serum or anti–α-galactosyl Ab–depleted human serum. Only 5% of input retrovirus survived in human serum, while retrovirus survival in depleted serum was 100% (Fig. 3 B). As was shown for the PA317 cells, incubation of retroviral particles with untreated squirrel monkey serum had no effect on retrovirus survival, while squirrel monkey serum in the presence of purified anti–α-galactosyl Ab effectively inactivated the retrovirus. The inability to achieve 100% retrovirus inactivation in squirrel monkey serum could reflect insufficient concentrations of the anti–α-galactosyl Ab. Although the final concentration of purified anti–α-galactosyl Ab in the squirrel monkey serum (~50 μg/ml) was similar to that previously reported in human serum (20), the binding of anti–α-galactosyl Ab to α-galactosyl epitopes associated with squirrel monkey serum proteins may decrease Ab available for binding to the retroviral envelope. Taken together, these data indicate that type C amphotropic retroviral particle inactivation by human serum complement is initiated by anti–α-galactosyl natural Ab.
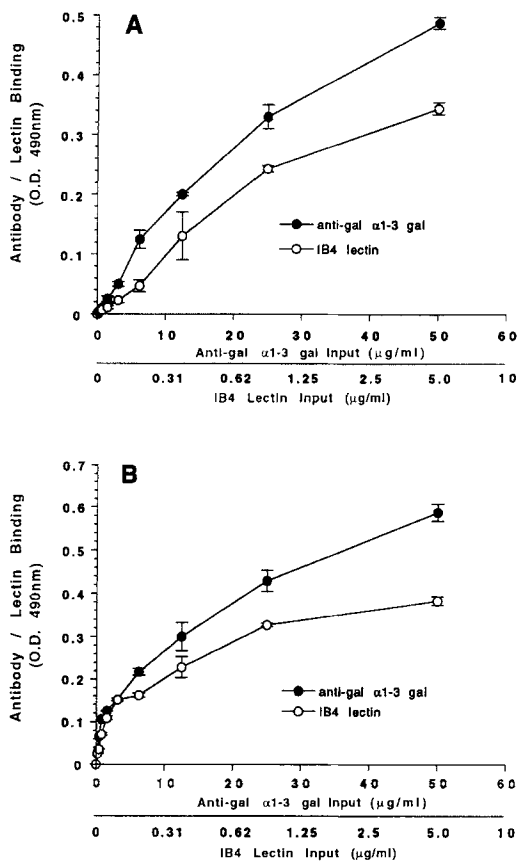
Serum Survival of Retrovirus Containing an Ecotropic Envelope. Previous studies have suggested that the ecotropic MMLV envelope binds complement component C1q directly, thus initiating complement-mediated destruction of the virus in the absence of antibody (2–4). In the present study, we have demonstrated that retrovirus containing the amphotropic MLV envelope is inactivated in human serum primarily through an antibody-dependent mechanism. To determine if this mechanism of retrovirus inactivation extends to other type C retroviruses, the role of anti–α-galactosyl Ab in the inactivation of retrovirus containing the MMLV envelope (27) was investigated. Retrovirus gener-
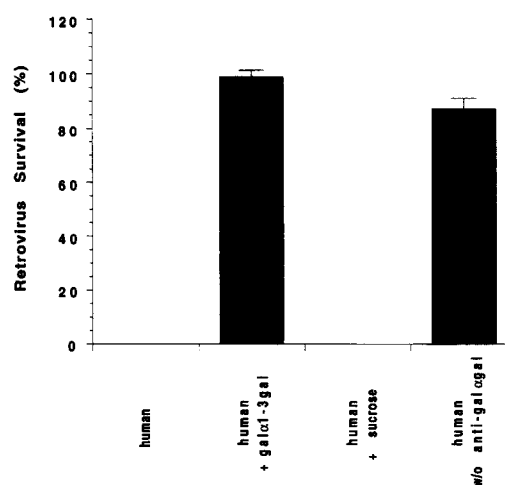
Figure 4. Contribution of anti–α-galactosyl natural Ab to the serum inactivation of retrovirus containing the MMLV ecotropic envelope. Ecotropic retroviral vector particles were incubated with 40% human serum (*human*), 40% serum containing 5 mg/ml soluble Galα1-3Gal (*human + galα1-3gal*), 40% serum containing 5 mg/ml sucrose (*human + sucrose*), or 40% anti–α-galactosyl Ab–depleted human serum (*human w/o anti-galαgal*). After exposure to serum, retrovirus survival was determined (see Fig. 1). Bars represent the percentage of infectious particles remaining relative to the number of input virus. Data represent duplicate determinations of a single experiment, one of three so performed. Error bars denote SEM.

ated from the ecotropic GP+E-86 producer cell line was exposed to serum in the presence or absence of soluble Galα1-3Gal or to serum depleted of anti–α-galactosyl Ab. As was demonstrated for the amphotropic retrovirus, the MMLV-derived ecotropic retrovirus was inactivated in human serum, while virus survival approached 100% with the addition of soluble Galα1-3Gal (Fig. 4). Similarly, the ecotropic retrovirus was not effectively inactivated in serum depleted of anti–α-galactosyl Ab. The addition of sucrose to the serum had no effect on survival of the retrovirus. Taken together, these data demonstrate that the inactivation of retroviral vector particles containing two distinct envelopes is primarily mediated by the anti–α-galactosyl natural Ab.
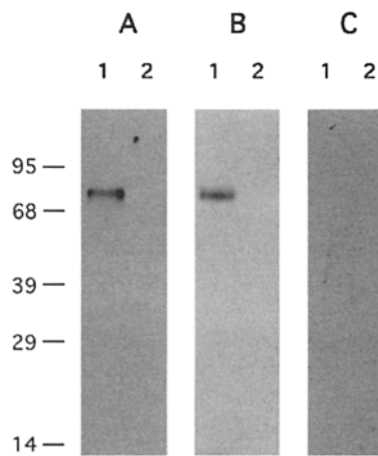
Expression of the α-Galactosyl Epitope on the Viral Envelope. We have shown that blockade or removal of anti–α-galactosyl Ab in human serum prevents inactivation of both amphotropic and ecotropic retroviral vector particles. To confirm the presence of the α-galactosyl epitope on the surface of the retroviral envelope, a capture ELISA was performed. Plates were coated with Fab directed against the retroviral envelope protein gp70, and retroviral particles were captured from supernatants of amphotropic (PA317) or ecotropic (GP+E-86) producer cells. Plates were then exposed to affinity-purified anti–α-galactosyl Ab or GS-IB4 lectin. This lectin has been shown to specifically interact with the α-galactosyl epitope (33, 34). Anti–α-galactosyl Ab and GS-IB4 lectin bound to the amphotropic (Fig. 5 A) and ecotropic (Fig. 5 B) retrovirus in a dose-dependent manner. These data establish that the α-galactosyl epitope is expressed on the surface of type C retroviral par-

Figure 6. Western blot analysis of α-galactosyl epitope expression on purified retrovirus. Purified amphotropic retroviral particles (lane *1*) or mock-purified supernatant from cells not producing retrovirus (lane *2*) were subjected to SDS-PAGE and transferred to nitrocellulose. Individual blots were then subjected to Western blot analysis using either anti-gp70 mAb (*A*), HRP-conjugated GS-IB$_4$ lectin (*B*), or HRP-conjugated UEA lectin (*C*) (see Materials and Methods). Protein size in kilodaltons is indicated to the left of the figure.

Figure 5. Analysis of α-galactosyl epitope expression on retrovirus by ELISA. Amphotropic (*A*) or ecotropic (*B*) supernatants were added to plates coated with Fab directed against the gp70 envelope protein and were subsequently reacted with either anti-α-galactosyl Ab or biotinylated IB$_4$ lectin (*amounts indicated on the abscissa*). Binding of Ab and lectin (*indicated on the ordinate*) was determined after development in the appropriate HRP-conjugated secondary reagent (see Materials and Methods). Absorbance values were corrected for background absorbance using identically treated wells in the absence of retroviral particles. Data represent duplicate determinations from a single experiment, one of two so performed. Error bars denote SEM.

ticles containing two distinct envelopes and that anti–α-galactosyl natural Ab binds directly to this epitope.

*Association of the α-Galactosyl Epitope with the Viral gp70 Envelope Protein.* A recent study has demonstrated that the α-galactosyl epitope is associated with the envelope glyco-proteins E1 and E2 of the eastern equine encephalitis virus (34). We have shown that the α-galactosyl epitope is present on the surface of amphotropic and ecotropic retroviral particles. To determine if the α-galactosyl moiety on the retroviral surface is associated with a particular envelope protein, Western blot analysis was performed. Protein extracts from purified amphotropic retroviral particles were assayed for reactivity with GS-IB$_4$ lectin, UEA lectin, or anti-gp70 mAb. The GS-IB$_4$ lectin specifically recognized a molecule at ~70 kD (Fig. 6). This molecular weight corresponds with that of the MLV major envelope glycoprotein, gp70. The anti-gp70 mAb recognized a molecule at the same position on the blot, indicating that the α-galac-

tosyl epitope is associated with gp70. UEA lectin, which recognizes a different glycosidic structure (see below), did not react with gp70 or any other molecule on the blot. Purified supernatants from NIH/3T3 cells did not react with lectins or the mAb, confirming that the 70-kD band was of viral origin. These results demonstrate that the α-galactosyl epitope expressed on the surface of retroviral particles is associated with the envelope glycoprotein gp70.
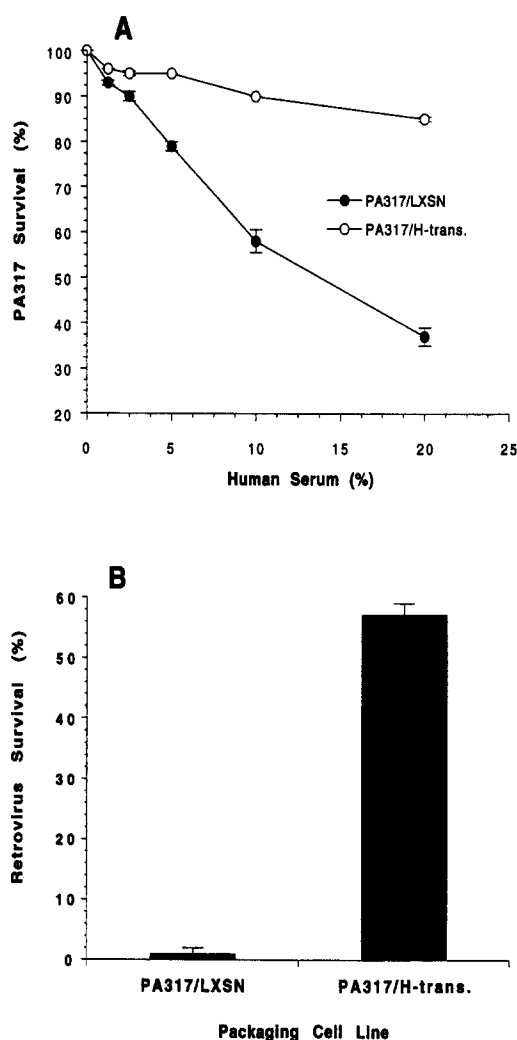
Down-regulation of the α-Galactosyl Epitope on PA317 Producer Cells Results in the Production of Serum-resistant Retrovirus. We have recently shown that overexpression of H-transferase in porcine cells (LLC-PK1) drastically reduces the expression of the α-galactosyl epitope because of substrate competition between the H-transferase and α1-3galactosyl transferase enzymes (Sandrin, M. S., W. L. Fodor, E. Mouhtouris, S. A. Rollins, E. R. Guilmette, E. Setter, S. P. Squinto, and I. F. G. McKenzie, manuscript submitted for publication). Furthermore, down-regulation of the α-galactosyl epitope on these cells resulted in decreased sensitivity to human serum killing. The H-transferase enzyme transfers a fucose residue to an *N*-acetyl lactosamine acceptor to generate fucosylated *N*-acetyl lactosamine (H-antigen), a glycosidic structure that is not recognized by anti–α-galactosyl Ab (35). To investigate the effect of α-galactosyl epitope down-regulation on the serum sensitivity of retrovirus, H-transferase was expressed in the PA317 retroviral particle producer cells. PA317 producer cells were transduced with H-transferase (PA317/H-transferase) or the pLXSN vector alone (PA317/LXSN) and selected as pools in G418. Transduced cells were reacted with GS-IB$_4$ lectin, UEA lectin (which recognizes the H-antigen), or anti–α-galactosyl Ab and analyzed by FACS®. PA317/LXSN cells expressed high levels of the α-galactosyl epitope, while expression of H-antigen in

Log Fluorescence Intensity
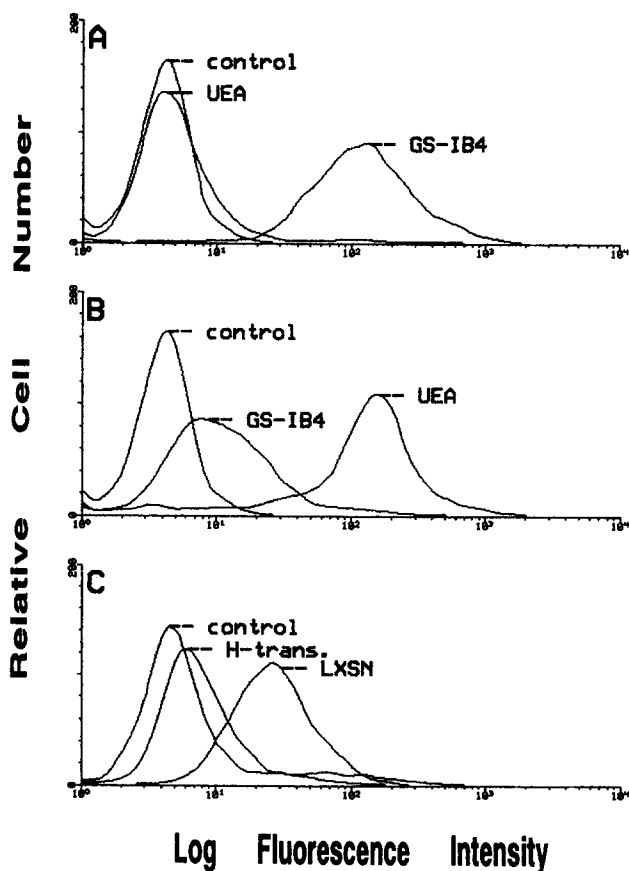
Figure 7. FACS® analysis of α-galactosyl epitope expression in PA317/ H-transferase transductants. The PA317 amphotropic packaging cell line was transduced with either H-transferase or the pLXSN vector alone and selected with G418. Transduced cells were reacted with GS-IB₄ lectin, UEA lectin, or anti–α-galactosyl Ab and analyzed by FACS® (see Materials and Methods). UEA lectin staining (*UEA*) indicates expression levels of the H-transferase product (H antigen), while GS-IB₄ lectin staining (*GS-IB₄*) indicates α-galactosyl epitope expression. *A* and *B* show lectin binding to PA317 cells transduced with the LXSN vector alone or with H-transferase, respectively. Unstained cells are indicated in each panel (*control*). *C* shows the reactivity of PA317 cells transduced with H-transferase (*H-trans.*) or LXSN vector alone (*LXSN*) with the purified anti–α-galactosyl Ab. H-transferase cells incubated with secondary Ab alone are also shown (*control*).

these cells was low (Fig. 7 *A*). Conversely, PA317/H-transferase cells showed an increase in H–antigen expression, while α-galactosyl epitope expression was reduced by >90% (Fig. 7 *B*). Similarly, binding of purified anti–α-galactosyl Ab to the PA317/H-transferase producer cells was greatly reduced (Fig. 7 *C*). These results show that expression of H-transferase in the PA317 producer cells drastically reduces α-galactosyl epitope expression.

In an attempt to correlate α-galactosyl epitope expression with human serum killing, the sensitivity of both the PA317/H-transferase producer cells and the retroviral particles liberated from these cells was investigated. The producer cells were incubated with human serum, and their survival was assessed in a dye release assay. PA317/H-transferase cells showed a marked increase in survival relative to

Figure 8. Serum sensitivity of PA317/H-transferase producer cells and retrovirus after α-galactosyl epitope down-regulation. (*A*) PA317 cells transduced with either H-transferase (*PA317/H-trans.*) or the LXSN vector alone (*PA317/LXSN*) were subjected to a complement-mediated dye release assay (see Materials and Methods). Curves represent PA317 cell survival at increasing concentrations of human serum calculated as the percent dye retained relative to total cell-associated dye. (*B*) Retroviral particles liberated from PA317 cells transduced with H-transferase (*PA317/H-trans.*) or the LXSN vector alone (*PA317/LXSN*) were subjected to 40% human serum, and retrovirus survival was determined (see Fig. 1). Bars represent the percentage of infectious vector particles remaining relative to input virus. Data represent duplicate determinations of a single experiment, one of two so performed. Error bars denote SEM.

PA317/LXSN cells after exposure to human serum (Fig. 8 *A*). These results indicate that the level of α-galactosyl epitope expression inversely correlates with the survival of PA317 retroviral particle producer cells in human serum. Concomitantly, 56% of retrovirus generated from the PA317/H-transferase producer cells survived exposure to human serum, while only 1% of retrovirus from the PA317/LXSN producer cells survived (Fig. 8 *B*). These data indicate that down-regulation of α-galactosyl epitope expression on producer cells results in the release of retro-

viral particles that are resistant to inactivation by human serum complement.

Discussion

In this study, we show that inactivation of murine type C amphotropic and ecotropic retroviral particles by human serum complement is initiated by the binding of natural Ab to α-galactosyl epitopes on the retrovirus envelope. This mechanism of inactivation requires that the retrovirus originates from cells that encode a functional α1-3galactosyl transferase (i.e., cells derived from mammalian species other than Old World primates). Consistent with these observations are reports in the literature demonstrating that enveloped viruses propagated in cells from various species differ in their sensitivity to human serum killing. For example, vesicular stomatitis virus is resistant to inactivation by human serum when propagated in human cells (Hep-2) as compared with vesicular stomatitis virus passaged through rat, hamster, or dog cells (36). Additionally, the lymphocytic choriomeningitis virus (LCMV) is less susceptible to inactivation by human serum when propagated through human cells (HeLa) in comparison with the same virus liberated from mouse L cells (37). Sera from agammaglobulinemic patients fails to inactivate LCMV grown in mouse L cells, indicating that lysis of this virus is Ab dependent. Interestingly, LCMV is resistant to human serum inactivation when passaged through baby hamster kidney cells, which have been reported to lack expression of the α-galactosyl epitope (37, 38). More recently, the inactivation of various type C retroviruses in human serum was shown to be dependent on both the viral genome and producer cell type (39).

In addition to reports on the decreased sensitivity of viruses to human serum after passage through human cells, some studies have shown that certain viruses passaged through human cells remain sensitive to lysis by human serum (1, 5, 39, 40). There are several possible explanations for viral inactivation in the absence of the α-galactosyl epitope, including activation of complement by specific antiviral Ab or Ab-independent activation of complement through the alternative or classical pathway. Other studies have reported that sera from agammaglobulinemic patients were able to lyse retrovirus (8, 37). However, the possibility that these sera contained sufficient concentrations of anti–α-galactosyl Ab to activate complement on the surface of the virus cannot be ruled out. For example, it has been shown that a patient with Bruton type agammaglobulinemia maintained 20% of the anti–α-galactosyl Ab reactivity when compared with an age-matched individual (20). It has also been reported that guinea pig serum added to heat-inactivated human serum failed to inactivate retrovirus, again suggesting that killing of retrovirus is antibody independent (1). We have demonstrated that sensitization of retrovirus with purified anti–α-galactosyl Ab before the addition of squirrel monkey serum can induce complement-mediated virolysis. One plausible explanation for this discrepancy is that, in the previous experiments, heat-inac-

tivated human serum was added to guinea pig serum before the addition of virus. Under these conditions the anti–α-galactosyl Ab may bind guinea pig serum proteins that express the α-galactosyl epitope and effectively block their ability to bind to the retroviral envelope. Indeed, in the present study, it was critical to add the purified anti–α-galactosyl Ab to the virus before the addition of squirrel monkey serum to achieve effective retrovirus inactivation. We have also demonstrated that the addition of heat-inactivated human serum to the anti–α-galactosyl Ab–depleted serum restores the ability of that serum to inactivate retrovirus, again indicating that retroviral killing in human serum is an antibody-dependent event (data not shown). In any case, our results clearly demonstrate that the α-galactosyl epitope on the surface of murine-derived retroviral particles plays a critical role in their inactivation in human serum.

The host range subgroups of MLV include ecotropic, xenotropic, polytropic, and amphotropic, which are determined by the recognition of cell-surface receptors by the gp70 envelope protein (41). In the present study, we show that human serum inactivation of retroviral particles expressing the MLV amphotropic envelope (26) is mediated by anti–α-galactosyl natural Ab and complement. These retroviral particles were chosen because of their broad host range, including mouse, rat, chicken, cat, dog, monkey, and human cells (42). We also demonstrate that MMLV-derived ecotropic retroviral particles (27) express the α-galactosyl epitope and are inactivated in human serum by anti–α-galactosyl Ab. Involvement of the α-galactosyl epitope in the human serum sensitivity of other type C retroviruses or of enveloped viruses in general seems likely. The α-galactosyl epitope has been detected on the surface of the eastern equine encephalitis virus and the Friend murine leukemia virus (34, 43), although the role of this epitope in the serum activation of these viruses has not been determined. In addition, studies have demonstrated that naturally occurring human antibodies that develop soon after birth recognize unidentified envelope glycoproteins of several type C retroviruses (44–46). These antibodies bind retrovirus propagated in cells from various species but fail to recognize retrovirus grown in Old World primate cells, suggesting that they recognize determinants of cellular rather than viral origin. These data are in agreement with the species-restricted expression of the α-galactosyl epitope (14, 15, 19) and support the potential role of anti–α-galactosyl Ab in restricting the horizontal transmission of type C retroviruses to humans.

In this report, we demonstrate that the α-galactosyl epitope is associated with the retroviral envelope glycoprotein gp70. However, our experiments do not rule out the possibility that this epitope is also acquired as part of the lipid bilayer when the retrovirus passes through the host cell membrane. Indeed, the α-galactosyl glycosidic structure has been reported to be expressed on glycosphingolipids associated with the cell membrane (22). In any event, our results indicate that the level of α-galactosyl epitope expression on the surface of the retroviral particles is suffi-

cient for Ab sensitization leading to inactivation by human complement.

Nonprimate type C retroviruses have been shown to induce T cell neoplasms in rodents (47–49) as well as to infect and transform human cells in vitro (10–13). However, the pathogenic potential of these retroviruses in humans and other Old World primates is controversial. For example, amphotropic MLV infused into rhesus monkeys is rapidly cleared from the bloodstream and fails to cause disease (9). Conversely, stem cells from rhesus monkeys transduced ex vivo with MLV induce fulminant lymphomas when transplanted back into the donor (50). Our findings offer a plausible explanation for these data in that retrovirus derived from murine producer cells expressing the α-galactosyl epitope would be rapidly inactivated in Old World primate sera. However, ex vivo transduction of stem cells derived from Old World primates in the absence of serum complement would allow the retrovirus to survive and replicate. Consequently, retrovirus liberated from these cells would not express the α-galactosyl epitope and would therefore escape natural Ab-mediated destruction after transplantation back into primates.

In addition to the potential importance of this natural Ab-dependent mechanism of virolysis as an immunological barrier, it may also have important implications in the field of gene therapy. The majority of vector systems used today in retroviral-mediated gene transfer experiments are derived from MLV (51–53). Although these vectors have provided useful tools for in vitro gene transfer, experiments performed in vivo have been primarily restricted to nonprimate species. The inactivation of murine-derived retroviral particles in human serum represents a formidable barrier for many potential applications of in vivo retroviral-mediated gene therapy. We have previously reported that pretreatment of human serum with functionally blocking mAbs that target terminal complement components effectively protects retroviral particles from inactivation (29). In the present study we demonstrate that retroviral particles can be protected from complement-mediated inactivation by the addition of soluble Galα1-3Gal. These reagents could be valuable for certain applications of in vivo gene therapy in humans where exposure to complement is unavoidable.

Additionally, the generation of retroviral vector packaging cell lines that do not express the α-galactosyl epitope would allow for the production of retroviral particles that survive complement-mediated inactivation in human serum. Indeed, our data show that down-regulation of this epitope in PA317 producer cells results in the production of retroviral particles that are resistant to human complement. Other potential strategies include the use of packaging cell lines derived from Old World primates or certain Chinese hamster ovary or baby hamster kidney cell lines, all of which apparently do not express the α-galactosyl epitope (14, 15, 38).

Previous studies have suggested that complement-mediated inactivation of murine type C retroviruses in human serum is initiated via direct binding of C1q to the MMLV envelope protein p15E (2–4). Several findings in the present study bring into question the relative contribution of this Ab-independent mechanism to the inactivation of type C retroviruses. These include the following: (a) Type C amphotropic retroviral particles were inactivated by primate sera that contain anti–α-galactosyl Ab (Old World primates) but not by sera from primates that do not produce such Ab (New World primates); (b) addition of purified human anti–α-galactosyl Ab to New World primate serum resulted in retrovirus inactivation; (c) the inactivation of retroviral particles containing either the amphotropic MLV or the ecotropic MMLV envelope was completely abrogated by the selective removal of anti–α-galactosyl Ab from or the addition of soluble Galα1-3Gal to human serum; (d) the α-galactosyl epitope was demonstrated on the surface of both the amphotropic and ecotropic retroviral envelopes in association with the glycoprotein gp70; (e) down-regulation of α-galactosyl epitope expression on amphotropic PA317 retroviral particle producer cells resulted in the production of retrovirus that was resistant to inactivation by human serum complement. Taken together, these data suggest that the predominant mechanism of type C retrovirus inactivation in human serum is mediated by anti–α-galactosyl natural Ab. Furthermore, this study suggests that this Ab provides a barrier to the horizontal transmission of retroviruses from species that express the α-galactosyl epitope.

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