JOURNAL OF VIROLOGY, Aug. 1997, p. 6174–6178 Vol. 71, No. 8
0022-538X/97/$04.00+0

Sensitization of Rhabdo-, Lenti-, and Spumaviruses to Human Serum by Galactosyl(α1–3)Galactosylation

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Received 14 January 1997/Accepted 9 May 1997

Vesicular stomatitis virus, human immunodeficiency virus type 2, and human foamy virus, which were produced by cell lines expressing galactosyl(α1–3)galactosyl (αGal) sugars, were found to be less stable in human serum than those from αGal-negative cells, indicating that galactosyl(α1–3)galactosylation sensitizes these viruses as well as mammalian type C oncoviruses (Rother et al., J. Exp. Med. 182:1345–1355, 1995; Takeuchi et al., Nature (London) 379:85–88, 1996) to complement killing via natural anti-αGal antibodies. Thus, virus killing mediated by anti-αGal antibodies may play a role as a barrier to animal-to-human infection of various enveloped viruses. Virus vectors for human in vivo gene therapy based on the viruses mentioned above should be produced from αGal-negative cells.

Most mammals express galactosyl(α1–3)galactosyl (αGal) terminal sugar structures on cell surface glycolipids and glycoproteins. However, this sugar structure is absent in humans, apes, and Old World monkeys because they lack a functional (α1–3)galactosyltransferase (αGT) gene (15, 20). Therefore, they are not immunologically tolerized and produce abundant natural antibodies against αGal sugar structure, probably stimulated by common bacteria of the gut (12, 13, 17). It has recently been shown that mammalian type C oncoviruses produced by cells expressing αGal sugar contain this sugar structure on their envelope proteins and are effectively inactivated by complement via natural anti-αGal antibodies when incubated in human serum (30, 39). This virus inactivation machinery was suggested to be a defense mechanism to prevent animal-to-human transmission of type C oncoviruses, because no infectious human type C oncovirus has been reported to date, although many strains of oncoviruses can infect human cells in vitro. Instability of viruses in human serum is likely to be a considerable impediment for retrovirus vectors to be used in human in vivo gene therapy (25, 38). Accordingly, type C retrovirus vectors produced by αGal-negative cells were predicted to be more stable in human serum than those produced by αGal-positive cells, and we have developed human packaging cells producing high-titer, complement-resistant, helper-free, type C retrovirus vectors (10).

Several strains of rhabdo-, lenti-, and spumaviruses can infect human cells. These viruses or their envelope glycoproteins are of interest for use as gene transfer vectors. The envelope protein VSV-G of a rhabdovirus, vesicular stomatitis virus (VSV), can be incorporated into retrovirus particles and direct entry of retrovirus cores into cells (2, 7, 22, 26, 40). Such VSV-pseudotyped retrovirus vectors have been shown to be stable during purification processes and have a wide host range (7). In contrast to oncoviruses, lentiviruses represented by human immunodeficiency viruses (HIVs) can infect nondividing cells, which are targets in many gene therapy settings and biological experiments (21, 26). A spumavirus, human foamy virus (HFV), which was isolated from an East African patient (1) but is now thought to be a variant of chimpanzee foamy virus transmitted as a zoonosis (4, 6, 35), has been used for development of gene transfer vectors (32, 34). Possible advantages of HFV vectors may be that they have a wide host range (18), they can infect stationary cells more efficiently than type C oncoviruses, although not as efficiently as HIV (5, 32), and they have a larger genome size and possibly a larger packaging capacity than other retroviruses (24). It was previously suggested that a number of enveloped viruses other than type C oncoviruses may also be sensitized to human serum when produced by αGal-positive cells (31, 39), but no direct evidence has been presented. In this study, we extended the observation of αGal-mediated virus inactivation by human serum to VSV, HIV-2, and HFV.

Different levels of expression of αGal sugar on cell lines. Cell lines with different levels of αGal expression were employed to produce viruses. Cells were stained with fluorescein isothiocyanate-labeled BS-IB4 lectin (Sigma), which specifically binds to the αGal structure, and then analyzed by fluorescence-activated cell sorter (FACS) with a FACScan (Becton Dickinson) as described previously (39). Mouse NIH 3T3 cells stained brightly, while only part of the mink Mv-1-Lu cell population weakly expressed αGal sugar (Fig. 1, panels 2 and 3), consistent with our previous observation that viruses produced from NIH 3T3 cells were more sensitive than those from Mv-1-Lu cells (38) and that a soluble homolog of the αGal structure, α1–3 galactobiose, blocked killing of amphotropic murine leukemia virus (MLV) from NIH 3T3 cells (39) but not the same virus from Mv-1-Lu cells (unpublished observations). BHK-21 cells, which have been reported to be deficient in αGal sugars (30, 31), were included as another example of αGal-deficient nonprimate cells (Fig. 1, panel 1). Cloning or FACS sorting of human HT1080 cells and TE671/RD cells transfected with an expression construct of pig cDNA for αGT (39) produced cell populations which are almost 100% positive for αGal expression (Fig. 1, panels 5, 8, and 9). Another αGal-positive clone derived from HT1080 cells, HTα4, was obtained

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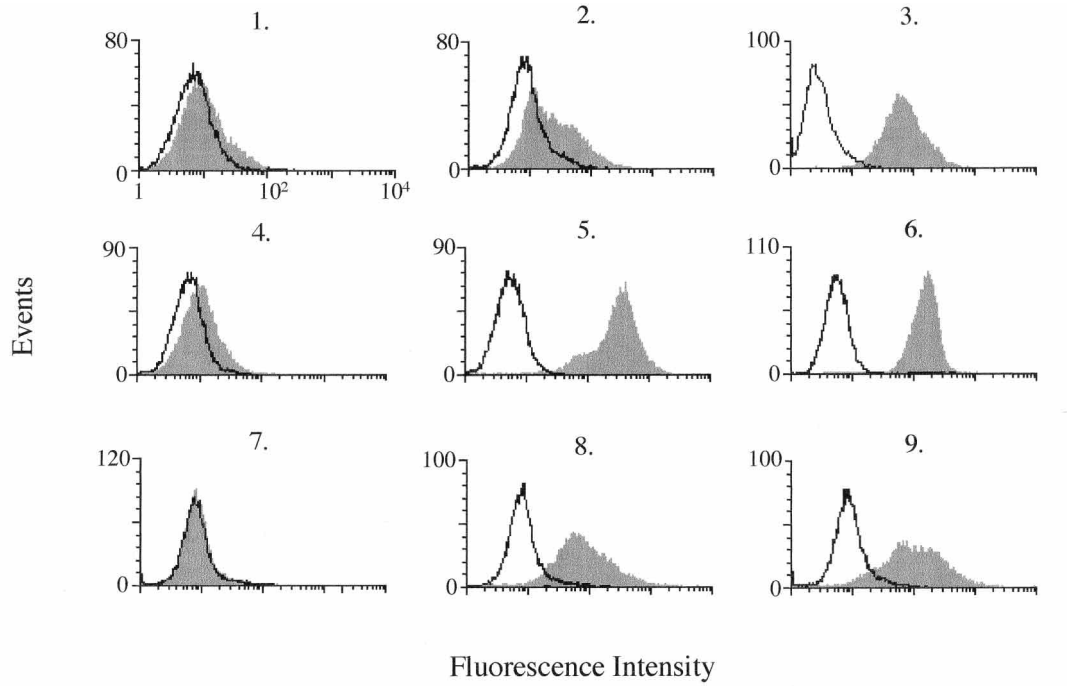

FIG. 1. Expression levels of αGal sugar structure on cell surface. Panels: 1, baby hamster kidney fibroblast BHK-21 cells; 2, mink lung epithelial Mv-1-Lu cells; 3 murine fibroblast NIH 3T3 cells; 4, human fibrosarcoma HT1080 cell derivatives, with the bulk of the population made up of transfectants of empty pcDNA3 vector (HT-neo); 5, clonal transfectants of a pig αGT expression vector, pXSA13GT2.1.1 (37) (HG13); 6, a clonal line transduced with a retroviral vector coding for pig αGT (HTα4); 7, human rhabdomyosarcoma TE671/RD cell derivatives persistently infected with HIV-2/ROD/B (8), with the bulk of the population made up of transfectants of empty pcDNA3 vector (TL-neo); 8, clonal transfectants of pXASA13GT2.1.1 (TG8); 9 FACS-sorted population of transfectants of pXASA13GT2.1.1 (TGPlus). Cells were stained with fluorescein isothiocyanate-labeled BS-IB4 lectin (shaded histograms) as previously described (39). Unstained cells are also shown (line histograms).

by transduction of a helper-free retrovirus vector coding for pig αGT followed by cloning by limiting dilution (27a) (Fig. 1, panel 6).

Serum sensitivity of VSV and MLV vector bearing VSV-G. Two million cells were seeded into T50 flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and incubated for 24 h at 37°C in 5% CO_2 and then challenged with 100,000 PFU of VSV for 1 h. Three milliliters of medium containing 2.5% FCS was added after washing of the cells, and cell supernatant was harvested 16 h later, filtered (0.45 μm-pore-diameter filter), and stored in aliquots at −70°C until used for protein analysis and the virus inactivation assay. The 50% tissue culture infective dose ($TCID_{50}$) of all harvests on Mv-1-Lu cells was between 10^9 and 10^{12}/ml.

α-Galactosylation of VSV-G envelope proteins was examined, because VSV-G has only two asparagine-linked sugar chains (29). VSV particles were pelleted by ultracentrifugation, lysed, and applied to Western blot analysis as described previously (9, 39). VSV-G protein was harvested from all cell supernatants tested, as demonstrated by binding to monoclonal anti-VSV-G antibody, P4D5 (Sigma). Viruses from αGal-positive NIH 3T3, HG13, and HTα4 cells, but not from αGal-negative HT-neo cells, contained a protein which migrates the same distance as VSV-G and binds to horseradish peroxidase-labeled BS-IB4 lectin (Fig. 2), indicating that VSV-G is α-galactosylated when produced in αGal-positive cells.

Wild-type VSV supernatant was 30-fold diluted with serum-free opti-MEM (GIBCO-BRL) and mixed with a serum sample at 1:1. This mixture was incubated for 1 h at 37°C and 4.65-fold (4.65^3 is approximately 100) serially diluted with DMEM with 10% FCS. Each dilution was plated in quadruplicate on Mv-1-Lu cells which had been seeded into 96 well plates at 8,000 cells/well 24 h earlier, and titer was determined 3 days later as $TCID_{50}$. To test inhibition of virus inactivation with soluble sugars, 10 mg of α1–3 or β1–4 galactobiose (Dextra Laboratories) per ml was added to the virus-serum mixture (39).

Figure 3 shows the results of serum sensitivity tests for VSV. While VSV titers varied after treatment with heat-inactivated serum and FCS less than 10-fold, reduction of titer by fresh human serum treatment was in most cases more than 10-fold, suggesting that VSV is susceptible to complement-mediated inactivation. Among the nonhuman cells, BHK-21, Mv-1-Lu, and NIH 3T3 cells produced the most-resistant, second-most-

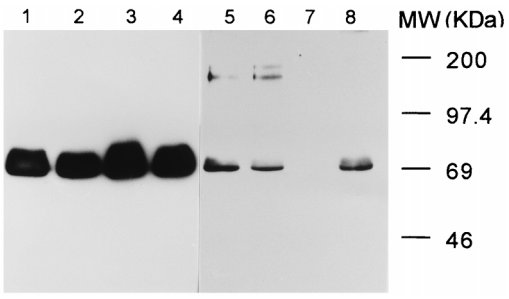

FIG. 2. α-Galactosylation of VSV-G protein. Virus particles were collected by ultracentrifugation from cell supernatants of HG13 (lanes 1 and 5), HTα4 (lanes 2 and 6), HT-neo (lanes 3 and 7), and NIH 3T3 (lanes 4 and 8) cells infected with VSV, lysed, and subjected to protein blot analyses. Protein blots were stained with a monoclonal anti-VSV-G antibody, P4D5 (lanes 1 to 4), and BS-IB4 lectin (lanes 5 to 8) as previously described (39). MW, molecular mass.

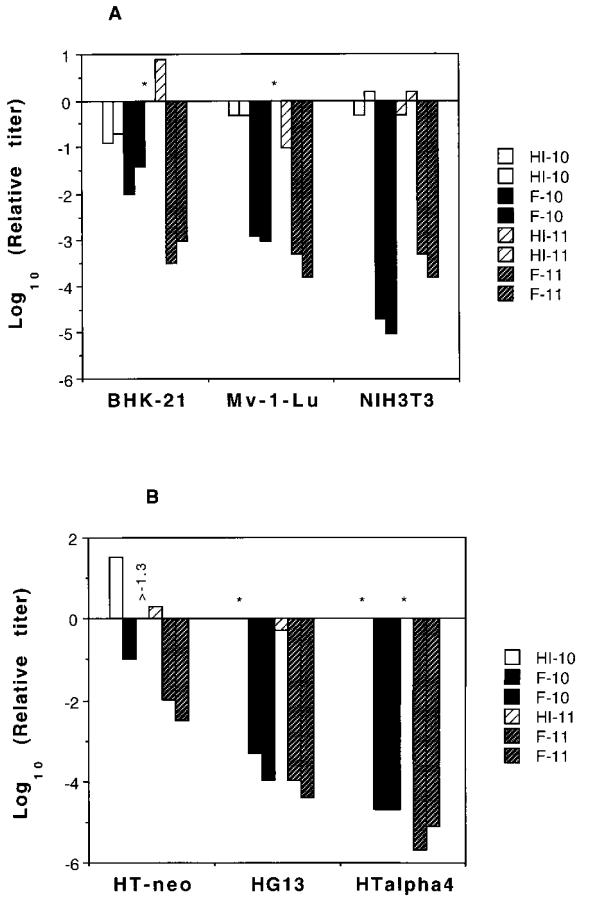

FIG. 3. Serum sensitivity of VSV from nonhuman (A) and human (B) cells. Results in [$\log_{10}$ (titer after test serum treatment) − $\log_{10}$ (titer after FCS treatment)] are shown for two normal human serum (NHS) samples from an independent source, NHS-10 (healthy laboratory worker [blood type AB+]) and NHS-11 (North London Blood Transfusion Centre [blood type AB+]. HI, heat-inactivated NHS; F, fresh NHS. One or two values of independent experiments are shown: *, value was 0; >−1.3, value was more than −1.3 toward 0.

resistant, and most-sensitive VSV, respectively, against fresh normal human serum 10 (NHS-10), although there was no significant difference in virus sensitivity against fresh NHS-11. The difference in sensitivity to NHS-10 may reflect the expression level of αGal sugar (Fig. 1, panels 1 to 3), while NHS-11 may have a major, unknown anti-VSV activity other than complement attack via anti-αGal antibodies. α-Galactosylated VSV from human HT1080 derivatives (HG13 and HTα4) was over 100 times more sensitive to both fresh NHS-10 and NHS-11 than αGal-negative VSV from HT-neo cells. Inactivation of VSV from HTα4 was partially blocked by α1–3, but not β1–4, galactobiose (Table 1). These results indicate that a rhabdovirus as well as oncoviruses (30, 39) can be sensitized to human serum inactivation by α-galactosylation.

Because VSV-G can be α-galactosylated and VSV particles bearing α-galactosylated VSV-G were more sensitive to human serum than αGal-negative VSV, similar to type C oncoviruses, it is likely that retrovirus vectors pseudotyped with VSV-G protein produced by αGal-negative cells are more stable than those from αGal-positive cells. Two αGal-negative retrovirus vectors were tested for serum sensitivity. A pseudotyped MLV vector encoding a *lacZ* chromogenic marker, MFGnlslacZ (VSV), at 10^7 infectious units (i.u.)/ml was harvested from a

TABLE 1. Block of virus inactivation by soluble sugars[a]

Virus (producer)	Log_{10} titer after treatment with[b]:			
	FCS	F-10	F-10+α1–3	F-10+β1–4
VSV (HTα4)	10.0 ± 0.4[c]	5.8 ± 0.2	8.8 ± 0.2	6.1 ± 0.2
HFV (HG13)	3.58 ± 0.09	1.78 ± 0	2.92 ± 0.30	1.93 ± 0.11

[a] HIV-2 was not tested, because HIV-2 was harvested in human serum and was likely to have already reacted with anti-αGal antibodies.
[b] FCS, FCS without sugar; F-10, fresh NHS-10 without sugar; F-10+α1–3, F-10 with 10 mg of α1–3 galactobiose per ml; F-10+β1–4, F-10 with 10 mg of β1–4galactobiose per ml.
[c] Standard errors for two experiments.

stable producer cell line in serum-free opti-MEM (10, 21a). LZRNL(G) at 2×10^8 to 5×10^8 i.u./ml after concentration by ultracentrifugation (42) was also tested. A 100-fold dilution of MFGnlslacZ(VSV) and a 2,000-fold dilution of LZRNL(G) with opti-MEM were tested for serum sensitivity as described previously (38). Table 2 shows that the MFGnlslacZ(VSV) produced by a TE671/RD-derived cell line, TELCeB/VSV-7, was more stable than type C oncoviruses produced by αGal-positive cell lines (38, 39), although it was variably inactivated by two independent serum samples, reminiscent of variable inactivation of type C viruses produced by TE671/RD cells (38). The LZRNL(G) transiently produced by a 293-derived cell line, 293GP, was only 1.5- to 3-fold reduced by fresh human serum treatment compared with control FCS treatment, consistent with recently published data about a vector produced by 293-derived packaging cells harboring an inducible VSV-G construct (27).

Serum sensitivity of HIV-2. TE671/RD cell derivatives TL-neo, TG8, and TGPlus were infected with an HIV-2 variant, ROD/B, which infects TE671/RD cells via the CXCR4 chemokine receptor independently of CD4 (8, 11). Persistently infected cell cultures were established 2 to 3 weeks after apparent cytopathic effect. Virus was harvested by incubation of confluent culture in DMEM supplemented with 10% heat-inactivated human serum (North London Blood Transfusion Centre) for 40 h because serum-free medium resulted in a low-titer harvest. FCS was not used for virus harvest in order to minimize contamination of αGal glycoconjugates present in FCS which may inhibit virus inactivation activity (28, 38). Virus harvests in 10% human serum at a titer of 2,000 to 4,000 i.u./ml were treated with serum samples in a 1:1 mixture for 1 h at 37°C, and surviving virus was titrated on TE671/RD-CD4 cells by in situ immunostaining as previously described (8). Table 3 shows that the titers of HIV-2 ROD/B produced from αGal-positive TG8 and TGPlus cells but not that from αGal-negative TL-neo cells were significantly reduced after treatment with fresh human serum compared with the titers after treatments with heat-inactivated human serum or FCS. This result dem-

TABLE 2. Serum sensitivity of MLV vector pseudotyped with VSV-G

Virus producer	Relative titer (%)[a]					
	HI-10		F-10		F-11	
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
TELCeB/VSV-7	ND	38	77	22	9	9
293gp/G/LXRNL	90	83	65	34	57	52

[a] [(Titer after test sample treatment) × 100/(titer after FCS treatment)]. HI-10, heat-inactivated NHS-10; F-10, fresh NHS-10; F-11, fresh NHS-11; ND, not determined.

TABLE 3. Serum sensitivity of HIV-2

Virus producer	Relative titer (%)[a]			
	HI-9	HI-10	F-9	F-10
TL-neo	65 ± 1	175 ± 5	86 ± 9	120 ± 5
TG8	133 ± 8	139 ± 10	4 ± 2	7 ± 2
TGPlus	117 ± 27	172 ± 27	5 ± 1	18 ± 4

[a] [(Titer after test sample treatment) × 100/(titer after FCS treatment)] ± standard error. Two normal human serum (NHS) samples from an independent source, NHS-9 (the same laboratory worker as for NHS-1 [38], blood type A+) and NHS-10 (blood type AB+), were used: heat-inactivated NHS (HI) and fresh NHS (F).

onstrates that lentivirus particles can be sensitized to virus inactivation mediated by anti-αGal antibody and complement.

Membrane-associated complement control proteins, CD55 (DAF) and CD59, have been reported to be incorporated in HIV-1 particles and protect viruses from complement-mediated inactivation (23, 33). A humoral negative regulator of complement activation present in serum, complement factor H (CFH), has also been shown to be involved in the virus protection by binding to HIV envelope proteins (36). Our HIV-2 virus particles may well contain CD55 and CD59 molecules, because TE671/RD cells express these two factors by antibody staining (data not shown). It is unlikely that all three of the human serum samples used for either virus harvest or killing assay are deficient in CFH. Although we have not examined their incorporation into HIV-2 ROD/B virus particles, the sensitization of virus by α-galactosylation might have overpowered the protection by CD55, CD59, and CFH in our experiment. Therefore, the resistance of human retroviruses to human complement in contrast to the sensitivity of the type C oncovirus originally reported (3, 19) may be due more to the absence of αGal sugars than to the presence of complement control factors.

Serum sensitivity of HFV. Cells were infected with HFV at a multiplicity of infection of 0.01 to 0.1 and then grown in DMEM supplemented with 10% FCS. When the cytopathic effect became apparent, medium was replaced with serum-free opti-MEM, and cell supernatant was harvested 24 h later, filtered, and stored as described above. After treatment of virus supernatant with serum samples at 1:1 for 1 h at 37°C, HFV titer was measured on 24-well plates containing 2×10^4 BHLL cells, which harbor the *lacZ* gene under transcriptional control of the HFV long terminal repeat as described previously (4). HFV produced by αGal-positive cells (NIH 3T3, HG13, and TGPlus cells) was inactivated by fresh human sera from two independent sources more effectively than that from αGal-deficient cells (BHK-21, HT-neo, and TL-neo) (Table 4). Inactivation of HFV from HG13 cells was partially blocked by α1–3, but not β1–4, galactobiose (Table 1), consistent with inactivation mediated by anti-αGal antibody. The titer of HFV from HG13 was also decreased about threefold by both heat-inactivated human sera, suggesting anti-αGal antibodies have weak neutralizing activity in the absence of complement (Table 4). Russell and Miller (32) previously reported that HFV vectors produced by BHK-21 cells were resistant to complement killing and concluded that "HFV is similar to known human retroviruses in its resistance to human complement." While we obtained a consistent result that HFV produced by BHK-21 cells was resistant, α-galactosylated HFVs were sensitive, as shown for HIV-2 above. Therefore, we conclude that HFVs as well as known human retroviruses are resistant to human complement when produced from α-Gal-negative cells but can be sensitized by α-galactosylation.

TABLE 4. Serum sensitivity of HFV

Virus producer	Relative titer (%)[a]			
	HI-9	HI-10	F-9	F-10
BHK-21	85 ± 2	87 ± 8	51 ± 3	79 ± 2
NIH 3T3	92 ± 4	57 ± 4	6.5 ± 2.5	12.5 ± 0.4
HT-neo	88 ± 5	93 ± 7	97 ± 4	93 ± 7
HG13	39 ± 4	34 ± 6	1.9 ± 0.3	0.8 ± 0
TL-neo	92 ± 8	82 ± 15	46 ± 2	67 ± 13
TGPlus	85 ± 5	51 ± 1	3.7 ± 0.4	4.6 ± 0.7

[a] See footnote *a* to Table 3 for details.

Our results demonstrate that VSV, HIV-2, and HFV produced by αGal-positive cells are more sensitive to human serum than those produced by αGal-negative cells, indicating that not only type C oncoviruses but also a broad range of enveloped viruses can be sensitized by α-galactosylation to virus inactivation mediated by human anti-αGal antibody and complement. Humans are exposed to many animal viruses, including those from α-Gal-positive mammals which can infect human cells at least in vitro. A few rhabdoviruses, including rabies virus, cause human diseases by animal-to-human infection. Some nonprimate lentiviruses may be able to enter human cells, because cat cells producing certain laboratory-adapted feline immunodeficiency virus strains can fuse to human cells via human CXCR4 chemokine receptor (41). Foamy viruses have been isolated from αGal-positive mammals, including New World monkeys, cats, and cattle as well as αGal-negative primates (14, 18). Such mammalian foamy viruses may have as wide a host range, including human cells, as HFV. Simian foamy virus 8, isolated from a spider monkey, grows in human embryonal lung fibroblast cells (16). Virus inactivation mediated by anti-αGal antibody and complement may play a role as a barrier to interspecies infection upon the exposure of humans to such animal viruses, although this defense may not be strong, as suggested by the occurrence of human diseases by zoonosis such as rabies and influenza. This defense would be effective only upon the initial infection, because once a single virion escapes the attack and replicates in a human host cell, the viral progeny would no longer be αGal positive. Even so, this virus killing may be helpful to prevent interspecies infection in collaboration with other defense systems, particularly when the concentration of antibody and complement is high at the initial infection site and the virus dose of exposure is low.

Finally, our results suggested that virus vectors based on rhabdo-, lenti-, and spumaviruses as well as type C oncoviruses will be more stable in human in vivo administration when produced by α-Gal-negative cells than when produced by αGal-positive cells. Therefore, lack of expression of the αGal epitope should be one of the properties of an ideal producer cell for many virus vectors.

We thank Mary Collins, Paul Clapham, and Kenth Gustafsson for reagents, suggestions, and discussions. Kenth Gustafsson supplied pig αGT cDNA under an agreement with BioTransplant Inc.

S.-H.L. has a Cancer Research Campaign studentship. This work was supported by the Medical Research Council.

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