Short Communication: Potential Risk of Replication-Competent Virus in HIV-1 Env-Pseudotyped Virus Preparations

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Abstract

Env-pseudotyped viruses are valuable reagents for studies of HIV-1 neutralizing antibodies. It is often assumed that all pseudovirus particles are capable of only a single round of infection, making them a safe alternative to work with live HIV-1. In this study, we show that some Env-pseudotyped virus preparations give rise to low levels of replication-competent virus. These levels did not compromise results in the TZM-bl neutralization assay; however, their presence highlights a need to adhere to the same level of biosafety when working with Env-pseudotyped viruses that are required for work with replication competent HIV-1.

Keywords: HIV, viral fitness, HIV envelope glycoproteins, retroviral vectors, envelope

STUDIES OF NEUTRALIZING ANTIBODIES in HIV-1-infected individuals and in preclinical and clinical trials of candidate vaccines place a heavy reliance on the use of Env-pseudotyped viruses.^{1,2} These pseudotyped viruses are produced by cotransfecting 293T or other suitable cell lines with an Env expression vector and an Env-defective backbone vector. One popular backbone vector, pSG3Δenv, is a full-length HIV-1 subtype B genome in plasmid pTZ19U that contains a four nucleotide insertion that inactivates the *env* gene (NIH AIDS Reagent Program, catalogue #11051; contributed by Drs. John C. Kappes and Xiaoyun Wu). Because most of *env* is preserved in this backbone, potential exists for recombination with the cotransfected functional *env* gene to produce replication-competent virus (RCV). The presence of RCV could compromise the neutralization assay by representing an unintended viral target for neutralization. It would also highlight a safety concern for pseudoviruses that are presumed to be replication incompetent and therefore low risk. We tested a large number of assay stocks of HIV-1 Env-pseudotyped viruses made with the SG3Δenv backbone for the presence of RCV. In addition, we tested a subset of these Env-pseudotyped viruses made with HIV-1 subtype A backbone, Q23Δenv, containing a 431 base pair deletion of the V1-C2 region of gp120.³ We also tested a small subset of Env-pseudotyped viruses made with an alternate HIV-1 subtype B backbone, NL4-3.Luc.R E⁻.⁴ Both backbone vectors are available from the NIH AIDS Reagent Program (Q23Δenv catalogue #11548, contributed by Dr. Julie Overbaugh; NL4-3.Luc.R E⁻

catalogue #3418, contributed by Dr. Nathaniel Landau). All Env-pseudotyped viruses were prepared as described.⁵

Phytohemagglutinin-stimulated human peripheral blood mononuclear cells (PHA-PBMCs) were inoculated with undiluted Env pseudovirus preparations in 96-well round-bottom culture plates in the presence of IL-2.⁶ PHA-PBMCs were washed after 1 day to remove the virus inoculum and the medium was replaced. Fresh PHA-PBMCs were added on day 7. Cultures were incubated for 12–14 days with regular changing of the medium every 3 days. Culture fluids from days 7, 10, and the final day of incubation were tested for the presence of p24 Gag antigen (PerkinElmer Life Sciences, Inc., Boston, MA). Culture fluids on the final day of incubation were further tested for infectious virus by transferring 50 μl to 96-well plates containing TZM-bl cells in a total volume of 250 μl/well (this transfer was made before collecting culture fluid for p24 assay). Luciferase reporter activity was measured after two days of incubation as described.⁵ Samples were considered RCV positive if elevated levels of both p24 Gag antigen and relative luminescence units (RLU) were detected. All assays used the same pool of donor PBMC, which was prescreened and found to be negative for HIV-1.

As seen in Table 1, 17% (20/117) of Env-pseudotyped virus preparations tested positive for RCV. Four independent preparations of the subtype B pseudovirus, SF162.LS, were positive when made with the SG3ΔEnv backbone. One of two preparations of this pseudovirus was positive when made with the subtype A backbone, Q23Δenv, whereas two preparations made

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TABLE 1. TEST FOR REPLICATION-COMPETENT VIRUS IN HIV-1 ENV-PSEUDOTYPED VIRUS PREPARATIONS

Env-pseudotype	Backbone	Clade	p24 (ng/ml)			RLU in TZM-bl d12-14 PBMC supernatant
			Day 7	Day 10	Day 12-14	
Q168.a2	Q23Δenv	AD	2.1	1.2	2.2	2061
Q168.a2	Q23Δenv	AD	1.7	0.1	0.1	1317
Q168.a2	SG3ΔEnv	AD	0.6	0.2	0.2	1889
Q23.17	Q23Δenv	A	39.7	47.2	123	141010
Q23.17	SG3ΔEnv	A	5.4	0.2	0.1	1558
Q259.d2.17	Q23Δenv	A	3.5	1.9	3.6	1859
Q259.d2.17	SG3ΔEnv	A	1.8	0.5	0.5	2033
Q461.e2	Q23Δenv	AD	7.2	4.1	6.6	2615
Q461.e2	SG3ΔEnv	A	1.2	0.4	0.4	2054
Q769.d22	Q23Δenv	A	7.8	4.3	8.2	31689
Q769.d22	SG3ΔEnv	A	3.2	0.1	0.1	1271
Q842.d12	Q23Δenv	A	3.5	1.6	3.1	1596
Q842.d12	SG3ΔEnv	A	0.7	0.1	0.1	1278
235-47	SG3ΔEnv	AG	1.7	0.1	0.1	1773
252-7	SG3ΔEnv	AG	2.6	0.2	0.1	1942
263-8	SG3ΔEnv	AG	1.3	0.1	0.1	1758
271-11	SG3ΔEnv	AG	1.4	0.1	0.1	1675
T251-18	SG3ΔEnv	AG	5.4	0.2	0.1	1736
T255-34	SG3ΔEnv	AG	0.6	0.1	0.1	2243
T257-31	SG3ΔEnv	AG	0.1	0.1	0.1	1519
T266-60	SG3ΔEnv	AG	0.8	0.1	0.1	1412
T280-5	SG3ΔEnv	AG	0.8	0.1	0.1	1584
6101.1	SG3ΔEnv	B	3.4	0.5	2.2	33183
6535.3	Q23Δenv	B	0.1	0.1	0.1	1628
6535.3	SG3ΔEnv	B	0.3	0.1	0.1	1600
62357.14.D3.4589	SG3ΔEnv	B	3.3	7.2	7.5	119276
6240.08.TA5.4622	SG3ΔEnv	B	1	0.4	0.3	2118
6244.13.B5.4576	SG3ΔEnv	B	3.7	41.4	61.1	110315
6535.3 ID	SG3ΔEnv	B	0.1	0.1	0.1	2057
700010040.C9.4520	SG3ΔEnv	B	2.2	0.4	0.4	2069
9021-14.B2.4571	SG3ΔEnv	B	3.4	0.9	0.8	2034
AC10.0.29	SG3ΔEnv	B	2.4	0.2	0.1	2422
AC10.0.29	SG3ΔEnv	B	0.9	0.3	0.4	2062
AC10.0.29	SG3ΔEnv	B	0.4	0.2	0.6	1829
Bal.26	SG3ΔEnv	B	4.0	0.2	0.1	5586
BB-1006-11.C3.1601	SG3ΔEnv	B	0.4	0.2	0.2	2081
BB-1012-11.TC21	SG3ΔEnv	B	0.7	0.3	0.2	1952
BB-1054-07.TC4.1499	SG3ΔEnv	B	3.3	20.5	32	98849
BB-1056-10.TA11.1826	SG3ΔEnv	B	0.5	0.3	0.2	2107
BZ167.12	SG3ΔEnv	B	8.6	19.9	21.3	16897
CAAN5342.A2	SG3ΔEnv	B	1.1	0.4	0.5	2118
CAAN5342.A2	SG3ΔEnv	B	1.1	0.8	2.5	3349
CAAN5342.A2	SG3ΔEnv	B	0.4	0.2	0.5	1816
H022.7	SG3ΔEnv	B	3.2	0.1	3.3	9824
H030.7 (T2)	SG3ΔEnv	B	2.2	0.1	0.1	1808
H035.18	SG3ΔEnv	B	2.2	0.1	0.1	1483
H077.31 (T1)	SG3ΔEnv	B	2.0	0.1	0.1	1341
H079.2 (T1)	SG3ΔEnv	B	0.3	0.1	0.1	1238
PRB926-04.A9.4237	SG3ΔEnv	B	3.3	29.3	27	119652
PVO.4	Q23Δenv	B	2.4	1.4	2.4	1795
PVO.4	SG3ΔEnv	B	0.8	0.3	0.3	2030
QH0692.42	Q23Δenv	B	5.4	2.9	5.1	1529
QH0692.42	SG3ΔEnv	B	18.1	42.2	91.9	187048
QH0692.42	SG3ΔEnv	B	6.0	142.5	198	92427
QH0692.42	SG3ΔEnv	B	2.2	0.7	0.8	1940
QH0692.42	SG3ΔEnv	B	2.1	2	3.1	10815
QH0692.42	SG3ΔEnv	B	3.5	2	3.3	1989
REJO4541.67	SG3ΔEnv	B	1.0	0.2	0.3	2000
REJO4541.67	SG3ΔEnv	B	1.9	1.3	2.1	1761
RHPA.7	SG3ΔEnv	B	0.7	0.4	0.7	1664
RHPA4259.7	SG3ΔEnv	B	0.6	0.2	0.3	2028
SC05.8C11.2344	SG3ΔEnv	B	1.2	0.4	0.3	1988

(continued)

TABLE 1. (CONTINUED)

Env-pseudotype	Backbone	Clade	p24 (ng/ml)			RLU in TZM-bl d12–14 PBMC supernatant
			Day 7	Day 10	Day 12–14	
SC422661.8	SG3ΔEnv	B	1.3	0.4	0.5	1923
SC422661.8	SG3ΔEnv	B	1.9	0.8	1.7	1280
SF162.LS	NL4-3.Luc.R ⁺ E ⁻	B	0.4	0.3	0.5	2108
SF162.LS	NL4-3.Luc.R ⁺ E ⁻	B	0.1	0.1	0.2	1066
SF162.LS	Q23Δenv	B	11.7	29.9	57.1	125593
SF162.LS	Q23Δenv	B	1.2	0.7	0.5	1894
SF162.LS	SG3ΔEnv	B	343.5	122.2	70.4	221969
SF162.LS	SG3ΔEnv	B	64.7	266.1	343.3	132423
SF162.LS	SG3ΔEnv	B	17.4	19.9	63.2	81844
SF162.LS	SG3ΔEnv	B	4.5	38.4	132.2	197350
89.6P.18	NL4-3.Luc.R ⁺ E ⁻	B	0.1	0.1	0.1	1847
89.6P.18	NL4-3.Luc.R ⁺ E ⁻	B	0.01	0.1	0.1	1849
89.6P.18	Q23Δenv	B	1.5	0.9	1.5	1932
SF162P3.5	SG3ΔEnv	B	2.1	4.4	8.1	109604
SF162P3.5	SG3ΔEnv	B	0.8	0.3	0.3	2082
SS1196.1	SG3ΔEnv	B	1.2	0.1	0.1	2892
THRO4156.18	SG3ΔEnv	B	23.6	45.6	58.3	94297
THRO4156.18	SG3ΔEnv	B	1.1	0.4	0.5	1878
THRO4156.18	SG3ΔEnv	B	1	0.6	1.1	1737
TRJO4551.58	SG3ΔEnv	B	0.6	0.2	0.3	1763
TRJO4551.58	SG3ΔEnv	B	0.1	0.1	0.1	1890
TRO.11	SG3ΔEnv	B	0.6	0.2	0.3	2080
TRO.11	SG3ΔEnv	B	0.2	0.2	0.2	1797
WEAU-d15.410.787	SG3ΔEnv	B	2.4	0.5	0.5	2095
WITO4160.33	Q23Δenv	B	1.1	0.8	1.4	1826
WITO4160.33	SG3ΔEnv	B	0.3	0.2	0.2	1984
CH038.12	SG3ΔEnv	BC	1.4	0.3	0.4	8835
CH070.1	SG3ΔEnv	BC	2.1	0.1	0.1	1675
CH110.2	SG3ΔEnv	BC	2.7	0.2	0.1	1644
CH115.12	SG3ΔEnv	BC	0.3	0.1	0.1	2036
CH119.10	SG3ΔEnv	BC	1.5	0.1	0.1	1657
CH181.12	SG3ΔEnv	BC	2.4	0.1	0.1	1705
97ZA012.12	Q23Δenv	C	0.7	0.4	0.8	1817
CAP210.2.00.E8	SG3ΔEnv	C	0.3	0.1	0.2	1935
CAP45.2.00.G3	SG3ΔEnv	C	0.3	0.2	0.2	1993
Du151.2	SG3ΔEnv	C	4.5	0.2	0.1	1522
Du156.12	SG3ΔEnv	C	0.5	0.2	0.2	2008
DU172.17	Q23Δenv	C	0.1	0.1	0	1747
Du172.17	SG3ΔEnv	C	0.8	0.1	0.1	1821
Du422.1	SG3ΔEnv	C	1.8	0.1	0.1	1099
HIV-0013095-2.11	SG3ΔEnv	C	0.6	0.1	0.1	1554
HIV-00836-2.5	SG3ΔEnv	C	0.8	0.1	0.1	1303
HIV-16845-2.22	SG3ΔEnv	C	1.2	0.1	0.1	1594
HIV-25710-2.43	SG3ΔEnv	C	1.4	0.1	0.1	1631
HIV-25925-2.22	SG3ΔEnv	C	2.4	0.2	0.1	1759
ZM106F.PB9	SG3ΔEnv	C	5.0	0.2	0.1	1566
ZM109F.PB4	SG3ΔEnv	C	0.4	0.2	0.2	1994
ZM135M.PL10a	SG3ΔEnv	C	0.2	0.1	0.1	2036
ZM197M.PB7	SG3ΔEnv	C	7.6	0.2	0.1	1413
ZM197M.PB7	SG3ΔEnv	C	0.5	0.2	0.2	1973
ZM214M.PL15	SG3ΔEnv	C	0.7	0.3	0.2	2074
ZM233M.PB6	Q23Δenv	C	0.8	0.6	1.2	1296
ZM233M.PB6	SG3ΔEnv	C	0.2	0.2	0.2	2057
ZM249M.PL1	SG3ΔEnv	C	0.4	0.2	0.2	1941
ZM53M.PB12	SG3ΔEnv	C	1.3	10.3	15.8	56045

RLU, values >9,000 were considered positive and are *bolded*; p24 values >3 ng/ml were considered positive and are *bolded*. Env-pseudotyped viruses that tested positive for both RLU and p24 are also *bolded*.

PBMC, peripheral blood mononuclear cell; RLU, relative luminescence units.

with the NL4-3.Luc.R⁺ backbone were both negative. Among six different preparations of subtype B pseudovirus QH0692.42 made with the SG3Δenv backbone, three tested positive for RCV. One of three different preparations of the subtype B pseudovirus THRO4156.18 made with SG3Δenv backbone was positive. In other cases, multiple preparations of the same pseudoviruses made with the SG3Δenv backbone were consistently negative (e.g., AC10.0.29, CAAN5342.A2, REJO4541.67, SC422661.8, TRJO4551.58, TRO.11, and ZM197M.PB7 Envs). Thus, certain Envs appear to have a greater tendency to generate RCV than others. This tendency may also be related to the backbone vector. Thus, nonsubtype B viruses made with the subtype B backbone SG3Δenv were rarely RCV positive (1/41 cases positive, where the only positive was ZM53M.PB12, a subtype C Env). Among seven subtype A pseudoviruses made with the subtype A backbone Q23Δenv, one tested positive and this single positive was an Env that was isogenic to the vector (Q23.17). Overall, of the 20 RCV-positive tests, only two occurred when the subtype of the Env and backbone were mismatched (SF162.LS made with Q23Δenv; ZM53M.PB12 made with SG3Δenv). These results indicate that the potential to generate RCV may be greater when using a backbone that is matched to the subtype of the Env clone. That being said, use of a nonsubtype-matched backbone vector is no guarantee that RCV will be avoided.

To confirm that recombination did in fact occur, we repeated a set of PBMC infection assays with three transfection stocks of Env pseudoviruses that tested positive for RCV and determined the Env sequences in the infected PBMC. These repeat tests were performed with the SG3Δenv backbone pseudotyped with BZ167.12 and 62357.14.D3.4589 Envs and with the Q23Δenv backbone pseudotyped with SF162.LS Env. All three transfection stocks of pseudoviruses once again tested positive for p24 Gag antigen after 10 days of PBMC infection.

Bulk gp160 sequencing of PBMC DNA was performed as described previously.⁷ Sequence analysis revealed the presence of recombinant gp160 genes in all three cases, where the defective region of the backbone gp160 was replaced with an overlapping region of the pseudotype gp160 (Fig. 1).

We next tested whether RCV impacted the outcome of neutralization assays performed in TZM-bl cells. Any impact would depend on where the RCV arose (i.e., during transfection or after infection) and how much RCV was present. Any RCV that arose after infection in TZM-bl cells would be of little consequence for an assay that is not incubated long enough to detect multiple rounds of infection. In contrast, if recombination occurred during transfection, the resulting virus would be a mixed population containing the intended Env and a recombinant Env containing elements of the backbone vector. In this latter case, levels of RCV that are sufficient to register in the assay might complicate the results by measuring neutralization of the RCV rather than solely the intended virus.

For our tests, we selected two separate stocks of SF162.LS and one stock of QH0692.42 that scored among the highest RCV values. These transfection stocks of Env-pseudotyped viruses that tested positive for RCV were assayed in parallel with a transfection stock of each Env-pseudotyped virus that tested negative for RCV. All Env-pseudotyped viruses were made with the SG3Δenv backbone except for the RCV-negative stock of SF162.LS, which was made with the NL4-3.Luc.R⁺ backbone. Assays were performed in TZM-bl cells⁵ using 13 HIV-1 plasma samples, soluble CD4, and the monoclonal antibodies IgG1b12, 2G12, 2F5, and 4E10. Equivalent neutralization results were obtained with all reagents regardless of RCV status (<3-fold difference in ID50 neutralization titers between RCV positive and negative pairs of Env-pseudotyped viruses, data not shown). These results indicate that the level of RCV present in these Env-pseudotyped

FIG. 1. Genetic recombination in replication-competent virus. Shown are the recombination events that occurred between backbone and pseudotyped *env* genes detected in peripheral blood mononuclear cell DNA 10 days after infection with three different transfection stocks of Env-pseudotyped viruses. Backbone Env segments are shaded gray. Pseudotyped Env segments are not shaded. Nucleotide residues at the borders of mutations (backbone vectors) and regions of recombination are shown above each Env.

viral preparations had no measurable effect on the TZM-bl neutralization assay.

Regardless of the lack of measurable impact of RCV on the results of neutralization assays, the real potential for RCV to be present at low levels in Env-pseudotyped virus preparations should raise awareness for laboratory personnel who may consider Env-pseudotyped viruses to be less of a risk and, therefore, require a lower level of biosafety than work with replication-competent HIV-1. We strongly recommend that all laboratory procedures with HIV-1 Env-pseudotyped viruses be conducted using the same level of biosafety that is required for work with replication competent HIV-1. At the very least, rigorous testing for RCV should be performed on all pseudovirus preparations before initiating work at a lower biosafety level.

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Author Disclosure Statement

No competing financial interests exist.

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