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Refined Identification of Neutralization-Resistant HIV-1 CRF02_AG Viruses

Rajesh Abraham Jacob,[a,b] Fatima Abrahams,[a] Marcel Tongo,[c,d] Michael Schomaker,[e] Paul Roux,[f,g] Eitel Mpoudi Ngole,[d] Wendy A. Burgers,[c,h] and Jeffrey R. Dorfman[a,b]

International Centre for Genetic Engineering and Biotechnology, Cape Town,[a] Division of Immunology[b] and Division of Medical Virology,[c] Department of Clinical Laboratory Sciences, University of Cape Town, Centre de Recherche sur les Maladies Émergentes et Réémergentes, Yaoundé, Cameroon,[d] Centre for Infectious Disease Epidemiology and Research, University of Cape Town,[e] School of Child and Adolescent Health, University of Cape Town,[f] Groote Schuur Hospital, Cape Town,[g] and Institute of Infectious Diseases and Molecular Medicine, University of Cape Town,[h] South Africa

We studied neutralization of CRF02_AG HIV-1-infected plasma samples. In contrast to previous reports, these samples neutralized CRF02_AG viruses better than other viruses. This included six of eight CRF02_AG viruses previously designated resistant (tier 2/3 or 3). Only viruses 253-11 and 278-50 remained highly resistant, but they were sensitive to membrane-proximal external region (MPER)-specific monoclonal antibodies, suggesting neutralization targets for even these viruses. We propose using high-neutralizing-within-subtype samples for evaluation of neutralization resistance of viruses.

Human immunodeficiency virus type 1 (HIV-1) is an inefficiently transmitted virus (34) and thus depends upon survival within the host for extended periods of time. To persist, it must survive in the face of intense and sustained immune responses (23, 26). In part, this persistence is accomplished by its error-prone replication process and high recombination rate (42), which generate substantial diversity early in infection (16, 25). This diversity, in turn, likely ensures that viruses resistant to particular antibody responses are almost always present, even if at a very low frequency (21), and that neutralizing antibodies select them (7, 8, 10, 13, 19, 22, 29, 32, 33, 44).

There is evidence that induction of neutralizing antibodies to HIV-1 may be a fruitful approach for vaccine development. Passive immunization with neutralizing antibodies can prevent infection in primate models (15, 24, 41, 46) and also protects neonatal primates (35), even at low doses of antibody (14), all in cases in which the antibodies are able to neutralize the challenge virus. It thus appears likely that vaccine-induced antibodies will be able to protect a vaccinee from infection by viruses that they neutralize. The vaccine-induced prophylactic antibodies would have to be broadly neutralizing because of the great diversity of the pool of HIV against which vaccinees would have to be protected (45). Nonetheless, even a vaccine that gives rise to neutralizing antibodies with highly broad but less than 100% coverage of HIV-1 isolates may be able to prevent many infections. About three-quarters of heterosexual HIV-1 infections (1, 17, 36) can be traced back to a single virus. Neutralization by vaccine-induced antibody of one or a few infecting viruses is presumably a protective event.

In the case of less than 100% strain coverage of a vaccine, a worrisome prospect is the possibility that such a vaccine might select for difficult-to-neutralize HIV-1 viruses. Viruses differ substantially in their neutralization resistance. A recent large study (40) classified 107 viruses into 4 ordered categories, or tiers: tier 1A and 1B viruses were most sensitive, and tier 3 viruses the most resistant. Here, we report our work in which we have refined how highly neutralization-resistant viruses may be better identified by testing within-subtype neutralization, and we apply this principle to a set of CRF02_AG viruses.

Anonymous blood samples found to be HIV-1-infected were obtained from Yaoundé Central Hospital Blood Service, Yaoundé, Cameroon ($n = 64$) between December 2006 and August 2007 and were subtyped by sequencing of *gag* and *nef* (data not shown). Twenty-two samples were subtyped CRF02_AG for both genes. We selected 12 samples from subjects likely to be HIV infected for >5.5 months, by using the BED HIV-1 incidence test kit (Calypte Biomedical, Portland, OR) (31) (data not shown), because broad neutralizers are more frequent among individuals infected for longer time periods (2, 11, 27, 38). The median age of the donors of the 12 samples was 29 (interquartile range (IQR), 27 to 32); 33% (4/12) of donors were female; median viral load was 94,200 copies/ml (IQR, 53,000 to 231,000), and median CD4 count was 464 cells/μl (IQR, 316 to 770).

A pseudovirus panel ($n = 27$) representative of the global HIV-1 pandemic was assembled, with CRF02_AG highly represented and screened for sensitivity to our CRF02_AG plasma samples (Fig. 1a). Pseudoviruses were chosen based upon subtype diversity, neutralization resistance (3, 40; R. A. Jacob, unpublished data), within-subtype sequence diversity, and geographic diversity of origin. All references to tier designations are according to those reported by Seaman et al. (40). Viruses are described as "tier 2/3" if they were between the clusters of tiers 2 and 3.

The relatively high neutralization resistance of CRF02_AG viruses has been reported previously, with several fitting into tier 3 or tier 2/3 categories (40). CRF02_AG viruses were more likely to

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Address correspondence to Jeffrey R. Dorfman, jeffrey.dorfman@icgeb.org.

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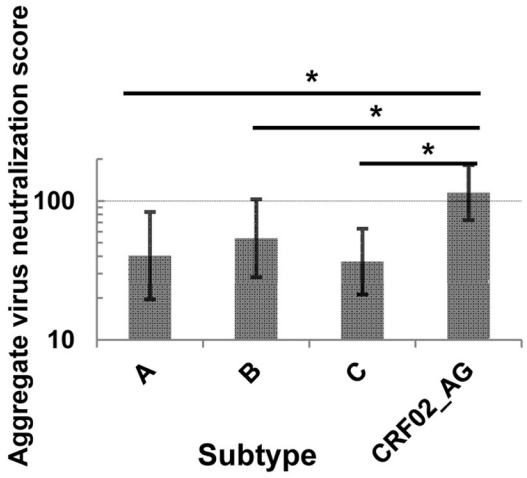
a

	Controls		27 Virus panel																												
			Subtype A Kenya				Subtype B, various locations					Subtype C southern Africa				Subtype C India			G	CRF02_AG											
			Tier 2	unk	unk	Tier 2/3	Tier 2				Tier 3	Tier 2			Tier 2/3	Tier 2/3	Tier 2		Tier 2	Tier 2		Tier 2/3			Tier 3					
	MLV	SF162	Q168 .a2	QG984 .21M .ENV .A3	QH343 .21M .ENV .A10	Q461 .e2	TRO11	RHPA 4259.7	REJO 4541 .67	SC 422661 .8	PVO.4	ZM249 M.PL1	CAP45 .2.00 .G3	Du151 .2	Du422 .1	001428 -2.42	26191 -2.48	16936 -2.21	252-7	269-12	255-34	928-28	211-9	250-4	257-31	251-18	33-7	278-50	253-11			
BS47	4	95	84	52	69	57	48	34	68	59	39	64	56	74	70	90	56	82	42	80	95	71	97	83	89	52	73	84	72		
BS06	6	96	63	97	28	39	70	40	71	66	54	48	51	84	53	95	66	85	61	75	85	84	89	94	74	69	96	50	53		
BS77	23	99	98	100	22	75	82	55	67	87	90	63	49	30	43	47	62	38	5	84	88	71	68	67	63	54	98	12	27		
BS73	7	91	52	66	67	25	38	77	59	47	62	57	62	62	46	23	26	38	60	61	78	59	94	93	86	51	23	39	30		
BS50	0	72	18	60	29	16	60	44	60	45	21	22	17	57	50	11	14	25	53	86	79	71	88	84	82	63	58	61	51		
BS66	1	99	69	87	49	22	26	88	82	75	36	39	49	88	39	12	16	35	36	75	65	65	83	87	49	46	25	23	35		
BS39	3	96	22	28	22	19	31	38	55	40	26	34	43	36	34	9	24	37	43	52	86	51	85	76	80	54	35	35	36		
BS22	2	83	30	23	20	24	31	40	53	35	24	31	47	21	40	10	29	37	46	64	76	52	73	67	70	53	47	35	40		
BS45	13	86	22	21	29	28	21	28	51	48	34	37	45	0	35	11	23	31	40	53	73	63	71	57	56	46	32	31	33		
BS01	0	83	54	0	12	15	34	29	36	10	7	0	0	0	24	0	15	0	16	28	69	28	71	72	52	30	32	17	0		
BS55	5	99	28	0	21	9	0	0	33	2	0	0	0	48	22	17	6	0	43	41	65	47	61	65	43	25	28	17	14		
BS43	5	48	21	0	12	13	0	0	27	26	20	30	24	0	10	4	11	0	0	19	64	22	28	30	20	16	0	9	20		

>90%
70-90%
50-70%

b



c

CRF02_AG plasma

Virus	clade	Tier	Neutralization score (95% PI)
Most Sensitive			
255-34	CRF02_AG	2	402 (191-845)
211-9	CRF02_AG	2/3	371 (176-778)
250-4	CRF02_AG	2/3	314 (149-659)
257-31	CRF02_AG	3	180 (86-378)
269-12	CRF02_AG	2	142 (67-298)
928-28	CRF02_AG	2/3	139 (66-292)
REJO 4541.67	B	2	106 (50-222)
QG984.21M.ENV.A3	A	unk	71 (34-150)
33-7	CRF02_AG	3	67 (32-141)
SC 422661.8	B	2	64 (30-134)
Q168.a2	A	2	63 (30-133)
Du151.2	C (Afr)	2	60 (29-126)
251-18	CRF02_AG	3	53 (25-112)
RHPA 4259.7	B	2	48 (23-101)
CAP45.2.00.G3	C (Afr)	2	41 (20- 87)
TRO11	B	2	41 (20- 86)
252-7	G	2	41 (19- 85)
Du422.1	C (Afr)	2/3	41 (19- 85)
ZM249 M.PL1	C (Afr)	2	38 (18- 79)
16936-2.21	C (Ind)	2	38 (18- 79)
PVO.4	B	3	34 (16- 72)
253-11	CRF02_AG	3	29 (14- 61)
QH343.21M.ENV.A10	A	unk	27 (13- 56)
001428-2.42	C (Ind)	2/3	26 (12- 55)
26191-2.48	C (Ind)	2	24 (11- 50)
278-50	CRF02_AG	3	23 (11- 49)
Q461.e2	A	2/3	22 (11- 47)
Least Sensitive			

d

ID50 values:

CRF02_AG plasma & tier 3 CRF02_AG viruses

	33-7	251-18	278-50	253-11	257-31
BS01	<50	<50	<50	<50	95
BS06	1307	140	89	156	260
BS22	88	77	<50	<50	223
BS39	54	77	<50	62	374
BS43	<50	<50	<50	<50	<50
BS45	<50	52	<50	<50	118
BS47	300	93	352	369	693
BS50	173	167	105	179	384
BS55	<50	<50	<50	<50	92
BS66	<50	70	<50	<50	165
BS73	68	74	75	54	621
BS77	3838	149	<50	<50	188

<50
50-99
100-299
300-999
>1000

TABLE 1 Differences in neutralization sensitivities between viruses or groups of viruses[a]

Virus(es) or virus group	Fold difference (95% CI)[b]	Wald χ^2	*P* value
CRF02_AG viruses	1.00	Reference	
Group A	2.84 (1.95–4.13)	29.92	<0.00005
Group B	2.13 (1.51–3.02)	18.36	<0.00005
Group C	3.14 (2.30–4.28)	51.68	<0.00005
Group G	2.83 (1.46–5.48)	9.42	0.0021
278-50 and 253-11	1.00	Reference	
33-7, 253-11, and 257-31	3.33 (1.95–5.67)	19.55	<0.00005
33-7	2.59 (1.34–5.00)	8.02	0.0046
251-18	2.05 (1.06–3.96)	4.57	0.0326
257-31	6.93 (3.59–13.39)	33.23	<0.00005

[a] Statistical comparisons were made for the neutralization sensitivities of the indicated viruses or groups of viruses to the indicated reference group.
[b] Fold differences shown are ratios of geometric means of all measurements in each comparison group. The 95% confidence interval of the fold difference was obtained from a mixed regression model.

fit into one of these categories than other viruses (8/17 versus 20/90; $\chi^2 = 4.565$; $P = 0.033$). In addition, a CRF02_AG-infected plasma pool was unable to preferentially neutralize within-subtype viruses, including the viruses used in this study (5, 40). In contrast, we observed substantial within-subtype neutralization with our CRF02_AG-infected samples (Fig. 1). Collectively, the CRF02_AG viruses were 2- to 3-fold more sensitive to the CRF02_AG-infected plasma samples than the subtype A, B, or C virus groups or the lone subtype G virus (Fig. 1b; Table 1). All of the six most sensitive viruses to the CRF02_AG-infected plasma samples were CRF02_AG viruses, and two others were moderately sensitive (Fig. 1a and c). Strikingly, three tier 3 CRF02_AG viruses (251-18, 33-7, and 257-31) were moderately or highly sensitive (Fig. 1a, c, and d). These three were significantly and substantially (overall, 3.33-fold) more sensitive to neutralization by our CRF02_AG-infected plasma than the two resistant CRF02_AG viruses (253-11 and 278-50) (Table 1). Two of these sensitive viruses (251-18 and 33-7) ranked among the three most resistant viruses of all 107 that were previously tier ranked (40). The most parsimonious explanation for this discrepancy may be that the CRF02_AG pools used in the previous studies did not contain high levels of heterologous neutralizing antibody, including antibody specific for within-subtype CRF02_AG viruses.

An understanding of the vulnerability of CRF02_AG viruses characterized monoclonal antibodies (MAbs) could provide information for vaccine design. Thus, we assessed the neutralization of the panel viruses to four commonly used MAbs. Nine of 10 CRF02_AG viruses were resistant to b12 (9) (recognizes the CD4 binding site [37]) and 2G12 (recognizes a cluster of α1→2-linked mannose residues on gp120 [39, 43]). On the other hand, CRF02_AG viruses were sensitive to the anti-gp41 membrane-proximal external region (MPER)-recognizing MAbs (48). All CRF02_AG viruses were sensitive to 4E10 (6), and 8/10 were sensitive to 2F5 (30)(Fig. 2). We conclude that it may be possible to neutralize even highly resistant CRF02_AG viruses, such as 278-50 and 253-11, with antibodies directed at the MPER. This may be important for development of an HIV-1 vaccine effective for a wide variety of HIV-1 strains, including neutralization-resistant strains such as 278-50 and 253-11.

Our studies highlight 253-11 and 278-50 as highly neutralization-resistant viruses and also demonstrate the utility of using pools or panels of within-subtype samples selected for good neutralizers to identify such viruses selectively. It is striking that such antibodies specific for 253-11 and 278-50 occur rarely, even among CRF02_AG-infected donors. Neutralizing anti-MPER antibodies in plasma samples previously were found to be rare in a North American cohort (47), but they were less rare in South Africa-sourced plasma samples (15/50, 30%) (12). Based on preliminary data from a panel of South Africa-sourced samples, we found that 17/68 contained neutralizing antibodies to a subtype C consensus MPER sequence, yet only 3/17 (18%) sera neutralized 253-11 and only 9/17 (53%) neutralized 278-50 (R. A. Jacob and J. R. Dorfman, unpublished data), suggesting that 253-11 and, to a lesser extent, 278-50, are resistant to most anti-MPER antibodies. Although the MPER is already targeted in vaccine efforts, we argue that study of neutralization of 253-11 and similar viruses remains important for vaccine development.

It is important that viruses with high neutralization resistance be defined as rigorously as possible. Based upon our study, we propose that procedures for selection of highly neutralization-resistant viruses include within-clade neutralization using samples selected for good neutralizers. Identification and study of these viruses is important because (i) epitopes from resistant viruses may be desirable in a vaccine and (ii) resistant viruses should be included in panels to evaluate candidate vaccines.

FIG 1 (a) Sensitivity of panel viruses to 12 plasma samples from CRF02_AG-infected study subjects. The percent neutralization of the indicated pseudovirus by the indicated plasma at a screening dilution of 1/100 is shown. Plasma samples are ranked by number of viruses neutralized at >50%; ties were broken by ranking the number of viruses neutralized at 70% and then at 90%. Plasma samples that neutralized ≥16 of the 24 viruses (at ≥50% neutralization) are indicated in bold. Neutralization assays were performed as described previously (28). Samples were tested against murine leukemia virus (MLV) as a negative control and against the highly neutralization-sensitive subtype B SF162.2 as a positive control. unk, tier unknown; virus not analyzed in reference 40. (b) Graphic depiction of the aggregate sensitivity of viruses grouped by subtype to the CRF02_AG plasma samples. The vertical axis represents the aggregate virus neutralization score for all viruses belonging to a particular subtype. Error bars represent the 95% confidence intervals from a linear mixed regression model. *, $P < 0.0005$. The virus neutralization score is the marginal prediction of a linear mixed model and is equal to the geometric mean of the 50% inhibitory dilution (ID_{50}) values of all plasma samples neutralizing that virus. The ID_{50} value was directly measured for samples 33-7, 251-18, 278-50, 253-11, 257-31, and 928-28, or is an estimated ID_{50} based upon the percent neutralization at 1/100 for other viruses (R. A. Jacob et al., unpublished data). (c) Relative sensitivity rankings of individual viruses to the subtype CRF02_AG plasma samples. Viruses were ranked by the same virus neutralization scores used for panel b; 95% prediction intervals from the model are also shown. (d) Measured ID_{50} values for tier 3 CRF02_AG viruses neutralized by individual CRF02_AG plasma samples.

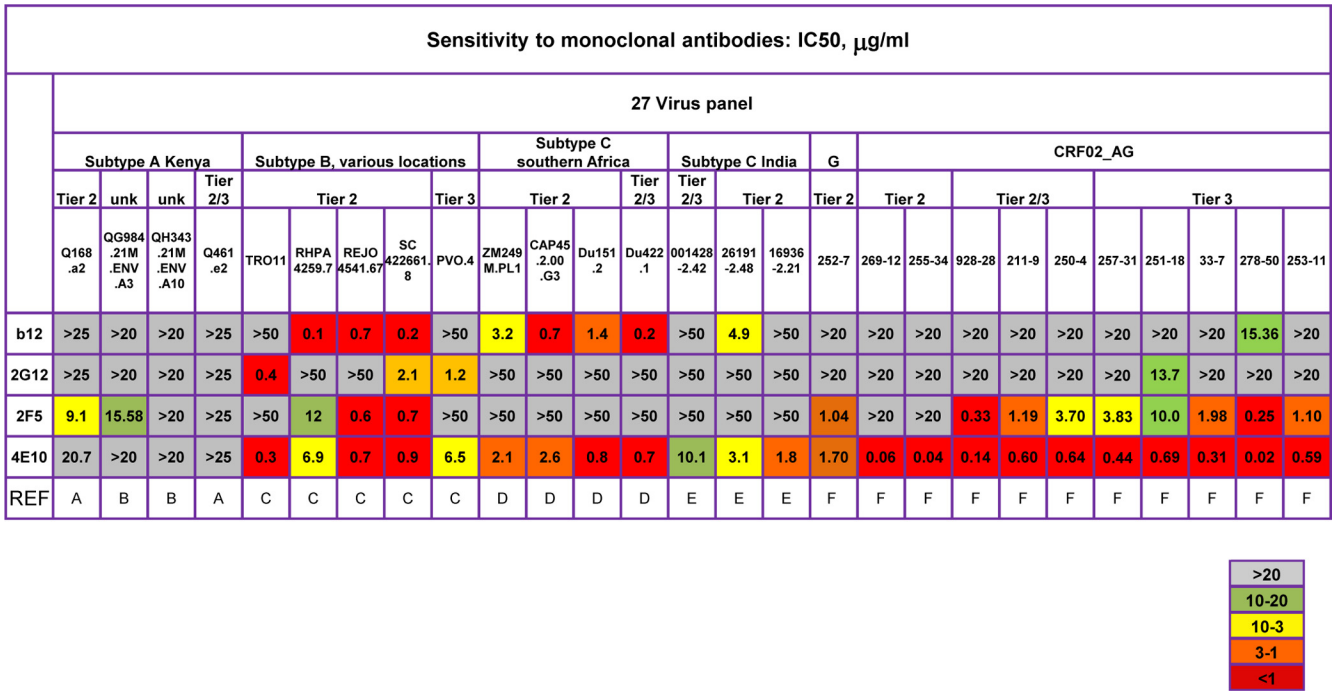


FIG 2 Fifty percent inhibitory concentration (IC_{50}) titers for monoclonal antibodies against panel viruses. The "REF" row refers to previously reported IC_{50} values, as follows (reference indicated in parentheses): A (4); B (3); C (20); D (19); E (18); F (this study). unk, tier unknown; virus not analyzed in reference 40.

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