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Mechanisms of HIV-1 subtype C resistance to GRFT, CV-N and SVN

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ABSTRACT

We examined the ability of HIV-1 subtype C to develop resistance to the inhibitory lectins, griffithsin (GRFT), cyanovirin-N (CV-N) and scytovirin (SVN), which bind multiple mannose-rich glycans on gp120. Four primary HIV-1 strains cultured under escalating concentrations of these lectins became increasingly resistant tolerating 2 to 12 times their 50% inhibitory concentrations. Sequence analysis of gp120 showed that most had deletions of 1 to 5 mannose-rich glycans. Glycosylation sites at positions 230, 234, 241, 289 located in the C2 region and 339, 392 and 448 in the C3-C4 region were affected. Furthermore, deletions and insertions of up to 5 amino acids in the V4 region were observed in 3 of the 4 isolates. These data suggest that loss of glycosylation sites on gp120 as well as rearrangement of glycans in V4 are mechanisms involved in HIV-1 subtype C escape from GRFT, CV-N and SVN.

Introduction

The surface of the HIV-1 envelope is populated with glycans that play an important role in protecting neutralization sensitive epitopes, promoting gp120 structural integrity and mediating interaction with cellular receptors (Geijtenbeek and Gringhuis, 2009; Li et al., 1993; Lin et al., 2003; Liu et al., 2004; Losman et al., 2001; Lue et al., 2002; Wei et al., 2003; Zhu et al., 2000). The majority of glycans on the HIV-1 envelope trimer are mannose-rich comprising 7 to 9 terminal mannose residues (Bonomelli et al., 2011; Doores et al., 2010) although the precise number and location remains undetermined. Complex glycans with terminal sialic acid residues are likely also present (Leonard et al., 1990). Mannose-rich glycans are targets for lectins or carbohydrate binding agents (CBAs) such as griffithsin (GRFT), cyanovirin-N (CV-N) and scytovirin (SVN) isolated from naturally occurring algae (Bokesch et al., 2003; Boyd et al., 1997; Mori et al., 2005; Moulaei et al., 2007; Ziolkowska and Wlodawer, 2006). These lectins show potent and broad anti-HIV-1 activities in vitro and are, therefore, being investigated for use in HIV-1 prevention, mostly in the form of microbicides (Balzarini, 2005; Ferir et al., 2012; O'Keefe et al., 2009; Tsai et al., 2003, 2004).

Since the neutralization activity of lectins involves interaction with glycans, one potential mechanism of HIV-1 escape from these compounds is the deletion of glycosylation sites. Indeed studies on HIV-1 subtype B have shown deletion of mannose-rich glycans is a mechanism of resistance to CV-N (Balzarini et al., 2006; Hu et al., 2007). More specifically, a loss of mannose-rich glycans at positions 230, 289, 295, 332, 339, 386, 392 and 448 was associated with resistance in the laboratory-adapted strains HIV-1_{IIIB} and HIV-$1_{NL\text{-}4.3}$ (Balzarini et al., 2006). In another study, the deletion of these glycans excluding those at positions 230 and 386 in HIV-1_{IIIB} cultured under escalating concentrations of CV-N, resulted in resistance to the lectin (Hu et al., 2007). In addition, HIV-1 resistance to the lectins *Galanthus nivalis* agglutinin and *Hippeastrum* hybrid agglutinin, was reported to occur via a partial loss of glycans on the envelope (Balzarini et al., 2004, 2005). Resistance to the broadly neutralizing antibody 2G12, that targets glycans on gp120, also involves the deletion of mannose-rich glycans. This is supported by the fact that most subtype C viruses are resistant to this antibody due to their lack of the 295 glycosylation site (Binley et al., 2004; Chen et al., 2005; Gray et al., 2007; Manrique et al., 2007).

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The glycosylation pattern on the HIV-1 subtype C envelope differs from subtype B (Zhang et al., 2004), and the ability of these viruses to develop resistance to lectins is unknown. In the current study we describe the mechanism of resistance to CV-N among four subtype C primary viruses, which while similar to subtype B showed some differences. This involved the deletion of mannose-rich glycans on gp120 as well as 4–5 amino acids deletions or insertions in the V4 region. In addition, we studied HIV-1 escape from two other lectins, GRFT and SVN, and showed that it followed a similar pathway to CV-N although patterns varied between the lectins. Thus, changes of glycosylated and non-glycosylated amino acid sequences suggest multiple mechanisms of escape from these three lectins.

Results

Replication of HIV-1 subtype C isolates in the presence of sub-inhibitory concentrations GRFT, CV-N and SVN

HIV-1 subtype B has previously been shown to develop resistance after repeated passages in the presence of escalating concentrations of CV-N (Balzarini et al., 2004, 2005; Hu et al., 2007; Witvrouw et al., 2005). Since the glycosylation pattern of HIV-1 envelope differs by subtype (Zhang et al., 2004), we determined the ability of viruses from subtype C to develop resistance to CV-N and two other lectins, GRFT and SVN. Four subtype C primary isolates were cultured in CD8 depleted PBMC in the presence of increasing lectin concentrations for 11 to 22 weeks, starting with the concentration equal to the IC_{50} for each compound (Table 1). Viral growth was measured weekly by p24 antigen ELISA. When the p24 levels in the lectin-containing cultures were lower than the control cultures (containing no lectin), the lectin concentrations were reduced in order to facilitate ongoing replication (Fig. 1). Of all four isolates, Du179 showed the highest levels of resistance, tolerating at least 10 times the starting concentration of each lectin (Table 1). The other 3 viruses, Du151, Du422 and COT9 grew at 3 times the starting concentrations of GRFT and SVN and 5 times the starting concentration of CV-N. Altogether, these data showed that the continuous growth of these four HIV-1 subtype C viruses under lectin selective pressure resulted in their ability to tolerate higher concentrations suggesting a level of resistance to these compounds.

Lectin-selected isolates showed decreased sensitivity and cross-resistance

We next determined whether viruses cultured in the presence of GRFT, CV-N and SVN showed reduced sensitivity to these compounds in the PBMC neutralization assay, using an 80% neutralization (IC_{80}) cut-off (Bures et al., 2002; Fenyo et al., 2009). Du179/GRFT.R, Du179/CV-N.R and Du179/SVN.R showed at least 5-fold increase in IC_{80} compared to the control viruses, passaged in the absence of the lectin (Fig. 2A). For Du151 and Du422 there was an increase in IC_{80} that ranged from 2 to 4 fold for all 3 lectins (Fig. 2B and C), while for COT9, the GRFT resistant virus showed a ~2 fold increase in IC_{80} (Fig. 2D). However, there was no change in IC_{80} for COT9 cultured under CV-N and SVN, despite the ability to grow under increased concentrations of the two lectins.

We next investigated whether viruses that were resistant to each compound also showed cross-resistance or decreased sensitivity to the other two lectins. We used Du179 for this study as this virus developed the greatest resistance to all three lectins. Du179/GRFT.R that was 10 fold resistant to GRFT showed a ~3 fold increase in resistance to CV-N and SVN neutralization (Fig. 3). A similar pattern was observed for Du179/CV-N.R and Du179/SVN.R. These data suggest that resistance to one lectin confers cross-resistance to the other; however resistance to the selecting agent was always the strongest.

Table 1
IC_{50} values of GRFT, CV-N and SVN for the neutralization of HIV-1 isolates.

Virus	Pre-selection[a] IC_{50} (nM)			Fold increase after selection		
	GRFT	CV-N	SVN	GRFT	CV-N	SVN
Du179	37.8	102.2	134.1	10	10	12
Du151	40.3	41.2	128.5	3	5	3
Du422	39.5	82.1	215.6	3	5	2
COT9	85.8	77.1	449.5	3	4	2

[a] 50% Inhibitory concentration.

Resistance was associated with amino acid changes and deletions at and around mannose-rich glycosylation sites

The full envelope sequences of both wild-type (viruses passaged in the absence of the lectin) and the corresponding resistant viruses were compared. Wild-type Du179, Du151 and COT9 each had 10 intact mannose-rich glycosylation sites while Du422 had nine. All four viruses lacked the 295 glycosylation site as is common among subtype C viruses (Zhang et al., 2004). Of the 12 selected viruses (four for each lectin), nine had deletions of glycans on gp120 with no changes in gp41 glycosylation patterns. Seven of the 11 glycosylation sites on gp120 that have been confirmed to contain mannose-rich glycans (Leonard et al., 1990), were involved in resistance to GRFT, CV-N and SVN (Fig. 4). Deletions of the 230, 392 and 448 glycans were observed among viruses selected by all three lectins with the loss of the 448 glycan observed in 6 out of the 12 selected viruses. Except for position 289, deletions at the seven sites occurred in response to more than one lectin.

Examination of glycan changes to individual lectins showed that the greatest number of deleted glycans was conferred by GRFT selection (Table 2). The loss of the glycan at position 339 occurred in three out of four GRFT selected viruses (Table 2 and Fig. 4), with those at position 230 and 234 occurring in two. The GRFT resistant Du179 also deleted the 442 glycan, predicted to be complex in studies conducted with monomeric gp120 (Kwong et al., 1998; Leonard et al., 1990). The loss of sensitivity to GRFT in Du422 restored the glycan at position 386 that was absent in the wild type virus (Table 2). For CV-N resistant viruses, the loss of the 448 glycan was the most common, occurring in half of these viruses (Table 2). However, we did not observe any changes in COT9 and Du422 sequences that accompanied their increased resistance to CV-N. Three out of four SVN selected viruses had the 448 deletion while two out four had the 339 glycan loss (Table 2). As with GRFT, selection with SVN restored the 386 glycan in Du422. Lastly, similar to CV-N, COT9 resistance to SVN was not associated with any apparent changes in glycans.

In addition to loss of glycans on gp120, we observed deletions and insertions of amino acid sequences near and within mannose-rich glycosylation sites located in the fourth variable (V4) region. GRFT resistance was associated with the deletion of four amino acids at position 400–403 and 396–399 in Du179 and COT9, respectively (Fig. 5A and D). However, in Du422 resistance to GRFT resulted in the insertion of five amino acids at position 398–402 (Fig. 5C). Similarly, CV-N resistance led to the deletion of four amino acids in Du179 at position 392–395 (Fig. 5A) that resulted in the loss of the 392 glycan. In Du422, SVN resistance resulted in the insertion of five amino acids at position 398–402, this was similar to GRFT (Fig. 5C). We observed no deletions or insertions of amino acids in Du151 under the selective pressure of any of the three lectins. In conclusion, our data using 4 subtype C primary isolates