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Resistance of HIV-1 to the broadly HIV-1-neutralizing, anti-carbohydrate antibody 2G12

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Abstract

The 2G12 mAb inhibits the infection of HIV-1 laboratory-adapted viruses at 50% inhibitory concentrations (IC_{50}) ranging from 0.02 to 0.2 μg/ml when evaluated in different cell-types. However, isolates from various HIV-1 subtypes (such as clade C, D, A/E, F and group O) were not inhibited by 2G12 mAb (IC_{50} >20 μg/ml). 2G12 mAb pressure in HIV-1 IIIB- and NL4.3-infected T cell cultures selected for resistant viruses containing only few (1 to 3 *N*-glycosylation) deletions in gp120. The 2G12-resistant viruses keep their full sensitivity to various mannose-specific lectins and other known HIV entry inhibitors. Moreover, we observed that the NL4.3-2G12-resistant virus, with the N295K mutation in gp120, became significantly more sensitive to several mannose-specific lectins. This is, to our knowledge, the first report showing that a resistant virus generated *in vitro* against a neutralizing mAb and containing a mutation in gp120, has increased sensitivity to another class of HIV entry inhibitors.

Keywords: 2G12; Resistance; Glycosylation; gp120; Mannose-specific lectins

Introduction

The necessity to lower or halt the world-wide spread of HIV infection is a serious problem that needs to be urgently addressed. The most obvious approach to combat the ongoing HIV pandemic is through the development of an effective vaccine. However, HIV vaccine design has faced numerous difficulties and especially the lacking of broadly neutralizing antibodies is a major drawback challenge (Burton et al., 1994; Burton and Montefiori, 1997; Connor et al., 1998; D'Souza et al., 1997; Kostrikis et al., 1996; Moog et al., 1997; Moore et al., 1996; Moulard et al., 2002; Parker et al., 2001; Parren et al., 1999; Roben et al., 1994).

So far, only very few broadly neutralizing monoclonal antibodies (mAbs) have been isolated from HIV-positive individuals (Burton et al., 1994; Burton, 1997; Moulard et al., 2002; Parker et al., 2001; Roben et al., 1994; Sattentau and Moore, 1995; Scanlan et al., 2002; Stiegler et al., 2001; Zwick et al., 2001a, 2001b) and have been shown to protect against HIV-1 infection *in vitro* and in animal models *in vivo* (Baba et al., 2000; Mascola et al., 1999, 2000a, 2000b; Shibata et al., 1999). One of these antibodies, 2G12 mAb, is directed against an epitope at gp120 that lies around the stem of the V3 loop and the V4 variable region. Interestingly, the recognized epitope of 2G12 mAb on gp120 contains high-mannose type glycans at several *N*-glycosylation sites (Calarese et al., 2003, 2005; Kunert et al., 1998; Kwong et al., 1998, 2000a, 2000b; Scanlan et al., 2002; Trkola et al., 1996; Wyatt et al., 1998; Wyatt and Sodroski, 1998; Zwick et al., 2001a). This is very unusual because glycosylated peptide epitopes often contain micro-heterogenic carbohydrates causing dilution of a single antigenic response.

The mAb 2G12 is unique among the studied antibodies in that it appears like a tightly packed dimer formed by an unprecedented V_H domain swapping (Calarese et al., 2003). It has been described that 2G12 mAb broadly and potently neutralized the majority of clade B primary and T cell line-adapted strains and that it also has neutralizing activity against

Abbreviations: CV-N, cyanovirin; HHA, *Hippeastrum hybrid* agglutinin; GNA, *Galanthus nivalis* agglutinin; CA, *Cymbidium* agglutinin; mAb, monoclonal antibody.

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viruses of clade A and other subtypes (Trkola et al., 1995, 1996). Some studies showed that 73% of clade B isolates were susceptible to neutralization with 2G12 mAb (Burton et al., 2004). Other studies showed that only 53%, 40% or 41% of the HIV-1 isolates were susceptible to 2G12 mAb (Binley et al., 2004; Mehandru et al., 2004; Rusert et al., 2005).

Poignard et al. (1999) evaluated high serum concentrations of a cocktail of the monoclonal antibodies 2G12, B12 and 2F5 for treatment of established HIV-1 infection in hu-PBL-SCID mice. They observed little sustained effect on viral load. Neutralization escape occurred after a few days of treatment.

The potency of 2G12 mAb (in combination with 2 other neutralizing mAbs 2F5 and 4E10) was recently evaluated in HIV-1-infected individuals. There was a delay in viral rebound in some of the patients (2 out of 8) and escape mutant analysis showed that the activity of 2G12 mAb was crucial for the *in vivo* effects of the neutralizing antibody cocktail (Trkola et al., 2005).

Trkola et al. (1996) and Scanlan et al. (2002) investigated mutations on gp120 at N295, N332, N339, N386 and N392 by N→A substitutions and demonstrated a marked decreased affinity for 2G12 mAb binding. Clade C isolates also do not react with 2G12 mAb (Binley et al., 2004; Chen et al., 2005), but reintroduction of a single glycosylation site at N295 recovered 2G12 mAb binding ability (Chen et al., 2005). Thus, whereas N339 and N386 proved less critical for 2G12 mAb binding, and may rather play an indirect role in maintaining the correct epitope conformation, N295 and N332 were described to be crucial for 2G12 mAb binding (Sanders et al., 2002; Scanlan et al., 2002; Chen et al., 2005).

The extensive activity profile of plant lectins and CV-N against numerous isolates and clades of HIV-1 and other lentiviruses supports the potential broad utility of these proteins as a microbicide to prevent the sexual transmission of HIV. Indeed, CV-N is shown to inhibit HIV infections in a vaginal transmission model (Tsai et al., 2003, 2004), suggesting that CV-N, but also mannose- and *N*-acetylglucosamine-specific plant lectins (Balzarini et al., 1991, 1992, 2004a, 2005a), are good candidates for testing in humans as a potential anti-HIV topical microbicide.

Here, we examined the antiviral activity of 2G12 mAb against various viruses, and examined the resistance profile of 2 HIV-1 strains (IIIB and NL4.3) that were exposed to 2G12 mAb. The nucleotide sequence of the gp120 envelope glycoprotein was characterized and the glycosylation patterns were derived thereof. Subsequently, the sensitivity to HIV entry inhibitors, with special emphasis on mannose-specific lectins such as CV-N, CA, HHA and GNA (Balzarini et al., 1991, 1992, 2004a, 2004b, 2005a, 2005b; Bewley, 2001; Bolmstedt et al., 2001; Botos et al., 2002; Boyd et al., 1997; Esser et al., 1999; Pashov et al., 2005; Tsai et al., 2003, 2004; Turville et al., 2005) was examined in detail.

Results

Antiviral activity of 2G12 mAb in cell cultures

The mAb 2G12, the α(1,3)- and/or α(1,6)-mannose-specific plant lectins GNA, HHA and CA, the α(1,2)-mannose-specific procaryotic lectin CV-N (Bolmstedt et al., 2001; Boyd et al., 1997), the polyanionic HIV adsorption inhibitor DS-5000 (Ito et al., 1987), the gp41 fusion entry inhibitor T-20 (Wild et al., 1994) and the CXCR4 antagonist AMD3100 (Schols et al., 1997) were evaluated for their antiviral activity against HIV-1 IIIB in CEM cells and against HIV-1 NL4.3 in MT-4 cells (Table 1). The 2G12 mAb proved to exert comparable antiviral activity against HIV-1 IIIB and NL4.3, a phenomenon which was also observed with T-20 and CA (Table 1). An IC_{50} of 0.26 μg/ml for 2G12 mAb was obtained against the clinical HIV-1 HE strain in MT-4 cells. Somewhat surprisingly was the observation that no activity of 2G12 mAb (IC_{50} >50 μg/ml) was observed when evaluated against several other HIV-1 laboratory strains of clade B such as RF, NDK and MN. AMD3100 and the mannose-specific lectins HHA and GNA showed a better antiviral activity against NL4.3 compared to IIIB, however, for CV-N and DS-5000 this was the opposite (Table 1).

Then we examined 2G12 mAb for its inhibitory potential against a wide variety of HIV-1 clade isolates in PBMCs (Table 2). Here also, 2G12 mAb showed variable antiviral activity from a strong inhibitory activity (IC_{50}: 0.04 μg/ml) to

Table 1
Sensitivity profile of HIV-1 wild-type and 2G12-resistant strains against viral entry inhibitors

Agent	IC_{50}[a] (μg/ml)					
	HIV-1 IIIB	HIV-1 IIIB-2G12res (#3)[b]	Fold resistance[c]	HIV-1 NL4.3	HIV-1 NL4.3-2G12res	Fold resistance/sensitivity[c]
2G12 mAb	1.4±0.4	>50	[>36]	0.98±0.42	>50	[>50]
HHA	0.29±0.19	0.55±0.35	[2]	0.092±0.033	0.006±0.001	[15]*
GNA	0.48±0.28	0.65±0.21	[1]	0.083±0.026	0.017±0.012	[5]*
CA	0.53±0.23	5.0±1.4	[9]	0.21±0.05	0.073±0.017	[3]*
CV-N	0.038±0.010	0.13±0.04	[4]	0.74±0.89	0.14±0.07	[5]*
DS-5000	0.33±0.12	1.25±0.35	[4]	2.32±0.18	1.62±0.43	[1]
AMD3100	0.05±0.03	0.2±0.1	[4]	0.003±0.001	0.002±0.001	[1]
T-20	0.010±0.002	0.04±0.01	[4]	0.012±0.002	0.009±0.004	[1]

[a] 50% Inhibitory concentration, or drug concentration required to inhibit virus-induced CPE in CEM cells for HIV-1 IIIB strains or MT-4 cells for HIV-1 NL4.3 strains by 50%.
[b] Comparable values were obtained with 2 other HIV-1 IIIB viruses that were generated in independent experiments under 2G12 mAb pressure (up to 50 μg/ml).
[c] Values between brackets represent the degree (fold) of resistance or sensitivity (indicated by an asterisk) of the test compounds, compared to wild-type virus. Values are mean±standard deviation from 3 to 4 separate experiments.

Table 2
Antiviral activity of 2G12 mAb and mannose-binding proteins in PBMCs against a variety of HIV-1 clade isolates, laboratory HIV-1 strains and HIV-2

Agent	IC_{50}[a] (μg/ml)											
	HIV-1 clades (with co-receptor preference)								HIV-2	HIV-1 laboratory strains		
	A	B	C	D	A/E	F	G	O				
	UG273	US2	ETH2220	UG270	ID12	BZ163	BCF-DIOUM	BCF06	BV-5061W	IIIB	NL4.3	BaL
	(R5)	(R5)	(R5)	(X4)	(R5)	(R5)	(R5)	(X4)	(X4)	(X4)	(X4)	(R5)
2G12 mAb	0.018	0.04	>20	>20	>20	>20	>20	>20	>20	0.18	0.08	0.09
HHA	29	5.4	44	4.9	12	4.6	41	1.2	8.9	1.1	0.36	6
GNA	27	17	≥100	>20	19	25	≥100	1.9	9.5	0.33	1.4	6.5
CA	5.5	2.9	12	5.3	1.2	6.3	9.2	3.3	1.1	0.36	0.22	2.4
CV-N	1.4	0.42	1.8	0.32	1.1	1.1	0.85	0.15	0.36	0.01	0.15	1.8

[a] 50% inhibitory concentration, or compound concentration required to inhibit viral p24 (for HIV-1) or p27 (for HIV-2) production by 50%.

no activity at all at 20 μg/ml. No activity of 2G12 mAb was observed against the HIV-2 strains BV-5061W, ROD and EHO or SIVmac251 (Table 2 and data not shown). Whereas the antiviral activity of CV-N only ranged between 0.15 and 1.8 μg/ml, the mannose-specific plant lectins showed greater variability in their virus-suppressive potential [i.e., 1.2 μg/ml (group O) to ≥100 μg/ml (clade G)] depending on the nature of the virus clade (Table 2).

Inhibition of 2G12 mAb binding to gp120 on HIV-infected MT-4 cells

Binding of CV-N to soluble gp120 inhibited subsequent interaction of 2G12 mAb with gp120, as shown previously by others (Boyd et al., 1997; Esser et al., 1999) using rgp120 coated on microtiter plates and measuring optical density. Here, we determined the IC_{50} value of CV-N to inhibit 2G12 mAb binding on HIV-1-infected MT-4 cells by flow cytometry (Fig. 1). The IC_{50} values of CV-N were 0.21 μg/ml for both HIV-1 IIIB and NDK and 0.20 μg/ml for HIV-1 MN. Also the mannose-specific plant lectins HHA and GNA inhibited the binding of 2G12 mAb with IC_{50} values in the lower microgram range (Fig. 1). HHA had IC_{50} values of 2.50 μg/ml, 1.46 μg/ml and 1.86 μg/ml and GNA had IC_{50} values of 4.76 μg/ml, 4.02 μg/ml and 1.08 μg/ml when using HIV-1 IIIB, MN and NDK, respectively. Interestingly, although the 2G12 staining of MN and NDK gp120 was comparable to that of HIV-1 NL4.3 and HE (Fig. 2), viral infection of MN and NDK was not inhibited by 2G12 mAb. We also performed staining with an anti-gp120 V3 loop mAb (9205) on these HIV-1 NL4.3-, HE-, MN- and NDK-infected cells. As can be seen in Fig. 2, 9205 mAb did not recognize HE- and NDK-infected cells and subsequently had also no antiviral activity against these two HIV-1 strains. However, although staining of 9205 mAb on HIV-1 MN-infected cells was less pronounced compared to the HIV-1 NL4.3-infected cells, the antiviral activity was not much different (IC_{50} was 0.10 μg/ml for HIV-1 NL4.3 and 0.16 μg/ml for HIV-1 MN).

In contrast, all these virus strains were equally well inhibited by the mannose-specific lectins HHA, GNA, CA and CV-N (Table 1; Balzarini et al., 2004a, 2004b, 2005a, 2005b; and unpublished data).

Determination of mutations in the genome of the 2G12-resistant virus isolates

The 2G12-resistant HIV-1 strains were analyzed for amino acid changes in the genome. When all amino acid changes were considered in gp160 of the HIV-1 NL4.3-2G12res (NL4.3-2G12-resistant) virus, only one specific and pure mutation occurred at an *N*-glycosylated asparagine, N295K. In addition, the mutation T297T/I was observed, a mutation in the T of the glycosylation motif which will also affect glycosylation of the gp120. For the NL4.3-2G12res virus, additional mutations were found at I194I/V, V270V/I and Q310H.

For the three IIIB-2G12res viruses, only mutations were found that indirectly affected the gp120 *N*-glycosylation motifs, i.e., mutation of T or S in the glycosylation motif. In these resistant HIV-1 IIIB viruses, mixtures of the wild-type and the mutated amino acids were noticed but no asparagines were directly affected. In all three HIV-1 IIIB-2G12res viruses was found the mutation T394[T, I]. HIV-1 IIIB-2G12res #1 and #3 had also the mutation T297[T, I] and T408[T, I] was only found in HIV-1 IIIB-2G12res #1.

Cross-resistance of 2G12-resistant viruses to other compounds

We also examined the effect of the mannose-specific lectins, the polyanion DS-5000, the CXCR4 antagonist AMD3100 and the gp41 fusion entry inhibitor T-20 on the replication of the wild-type virus and the 2G12res viruses (Table 1). Not only DS-5000, AMD3100 and T-20 kept their suppressive effects to the 2G12res strains, but also the mannose-specific lectins. Remarkably, the mannose-specific lectins were more inhibitory against the HIV-1 NL4.3-2G12res virus compared to the wild-type virus (especially HHA: ~15-fold), whereas this phenomenon was not observed against the 2G12res viruses of the HIV-1 IIIB strain. The 9205 mAb recognizing the V3 loop of gp120 was equally active against the wild-type and the 2G12res viruses. Also, the anti-CD4 mAb (RPA-T4) and soluble CD4 were equally active against the wild-type and the 2G12res viruses (data not shown). The wild-type virus that had been grown in MT-4 cells in parallel with the 2G12res virus (but in the absence of 2G12 mAb) was as sensitive as the original virus stock to 2G12 mAb (IC_{50}: 0.26 μg/ml).

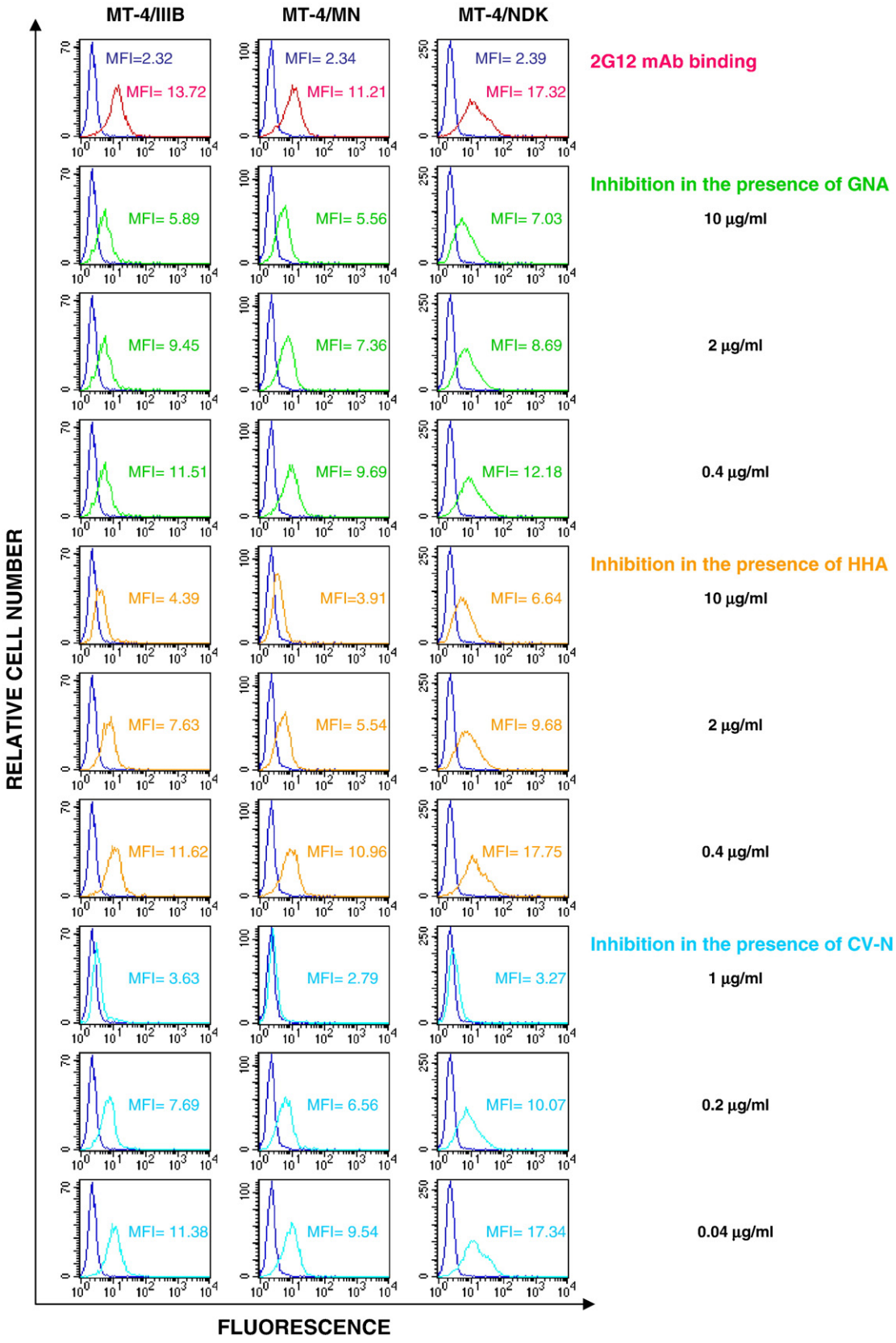

Fig. 1. Inhibition of the binding of 2G12 mAb to HIV-1-infected MT-4 cells in the presence of GNA, HHA and CV-N. MT-4 cells infected with HIV-1 strains IIIB (left panel), MN (middle panel) and NDK (right panel), were incubated with 2G12 mAb in the absence (red histograms) or presence of different concentrations of GNA (green histograms), HHA (orange histograms) and CV-N (light blue). The dark blue histograms show the background fluorescence. The mean fluorescence intensity (MFI) values are indicated in each histogram.

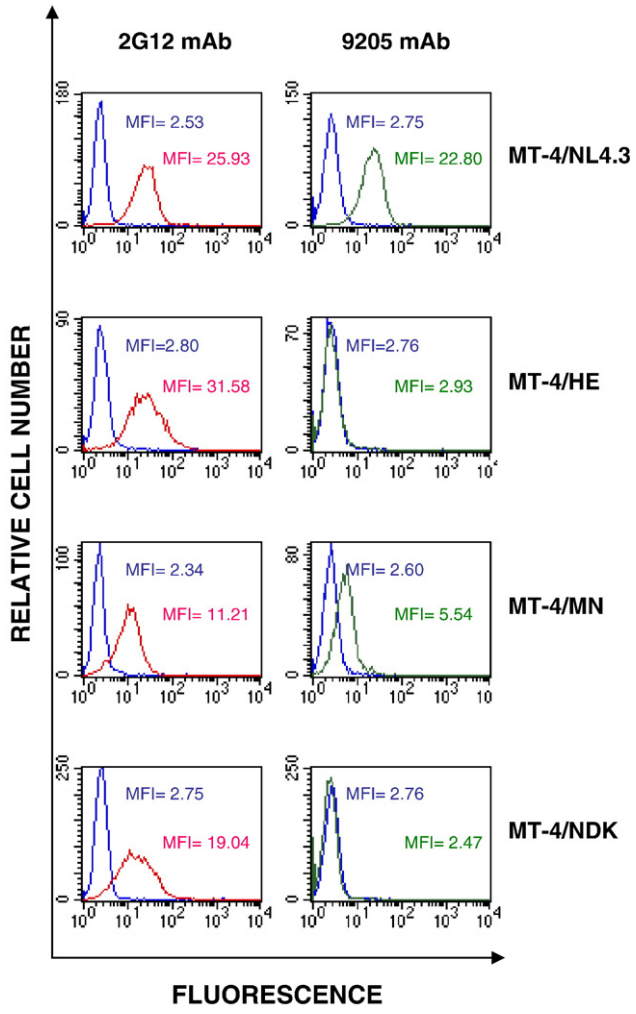

Fig. 2. Staining of 2G12 mAb and 9205 mAb on MT-4 cells 4 days after infection with HIV-1 strain NL4.3, HE, MN, and NDK. MFI of the background fluorescence (blue histograms), for the 2G12 mAb binding (red histograms) and for the 9205 mAb binding (green histograms) are indicated.

2G12 mAb staining of HIV-1-infected cells

When HIV-1 NL4.3-infected MT-4 cells were stained with the 2G12 mAb more than 99% of the cells were positive 3–4 days after infection (MFI: 28.96) (Fig. 3). However, when the staining was done on the HIV-1 NL4.3-2G12res-infected MT-4 cells, the MFI value decreased till 7.87 (73% decrease) (Fig. 3). As a control, staining with the 9205 mAb was included, a mAb recognizing the tip of the V3 loop of gp120 (Skinner et al., 1988). On wild-type and NL4.3-2G12res-infected MT-4 cells, the 9205 mAb staining was comparable demonstrating that on both cells gp120 was present in equal amounts (Fig. 3). When HIV-1 IIIB-infected MT-4 cells were stained with 2G12 mAb, the same fluorescence pattern was obtained as for HIV-1 NL4.3. When the staining was done on the MT-4 HIV-1 IIIB-2G12res (#1)-infected cells, the MFI decreased from 32.28 to 15.39 (~50% decrease) (Fig. 3). Comparable values were obtained for the 2 other HIV-1 IIIB-2G12res viruses (MFI of 16.31 and 23.59 for IIIB-2G12res #2 and #3, respectively). In contrast, no staining at all of 2G12 mAb was observed on HIV-1 IIIB-HHAres and on HIV-1 IIIB-GNAres viruses that were previously selected in the presence of HHA and GNA, respectively (Fig. 3). The MFI values of 2G12 mAb of the two mannose-specific lectin-resistant viruses decreased towards the level of the background staining. This observation is in line with earlier reports showing that none of these plant lectin-resistant viruses showed sensitivity to 2G12 mAb at a concentration up to 100 μg/ml (Balzarini et al., 2004b, 2005b). In contrast to the staining with the 2G12 mAb, staining with 9205 mAb was in all the virus-infected cells equally and consistently positive (Fig. 3, right panels).

Discussion

Many reports have documented the importance of glycosylation for infectivity and pathogenesis of HIV (Balzarini et al., 2005a; Balzarini, 2005; Turville et al., 2001; Wyatt et al., 1993). There may be about 20–29 potential *N*-linked glycosylation sites distributed on gp120 and the carbohydrate content constitutes about 50% of the mass of gp120 (Leonard et al., 1990). It was proposed that these carbohydrates act as a shield to protect the virion from the humoral immune response (Wyatt et al., 1998). Thus, compounds interacting with the carbohydrate domains on the virion envelope might provide effective ways to inhibit viral infection.

The 2G12 mAb interacts with very specific, highly conserved glycosylation sites on gp120 (i.e., N-295, N-332 and N-392), and its binding to HIV-1 gp120 is further influenced by the presence of the N-339 and N-386 glycans (Scanlan et al., 2002; Zhu et al., 2000). Here, we also observed that the single mutation of N295K rendered the NL4.3 virus insensitive to 2G12 mAb. Also the three 2G12-resistant HIV-1 IIIB strains showed the T394T/I (partial) mutation, deleting the N392 glycan. The *N*-glycosylation sites that were deleted under 2G12 mAb pressure were mapped on the three-dimensional structure of gp120 determined by Kwong et al. (1998) and appear to be clustered (Fig. 4, left panel). In contrast, the multiple deleted *N*-glycosylation sites appear to be scattered under the pressure of HHA and GNA (Fig. 4, right panel). Thus, whereas a large number of *N*-glycan deletions are required to afford a moderate decrease of mannose-specific lectins sensitivity against the mutated virus strains (Balzarini et al., 2004b, 2005b), one glycan deletion was already sufficient for 2G12 mAb to completely lose its antiviral activity. These observations explain why “broadly” neutralizing antibodies, such as 2G12 mAb, quickly generate resistant viruses, a characteristic which is obviously due to their specific binding interaction with gp120. Also, binding of 2G12 mAb to gp120 may be necessary, but not sufficient to exert its antiviral activity. As was shown in Fig. 2, 2G12 mAb binding of MN and NDK gp120 was comparable to that of NL4.3, HE and IIIB, but viral infection of MN and NDK was not at all inhibited by 2G12 mAb. It has been reported for another neutralizing mAb, 2F5, that high affinity binding by itself is not sufficient to be neutralizing, even when the epitope is at a critical position (Ou et al., 2006). When the HIV-1 NL4.3, NDK, MN and RF gp120 envelope was examined for the presence (or absence) of the

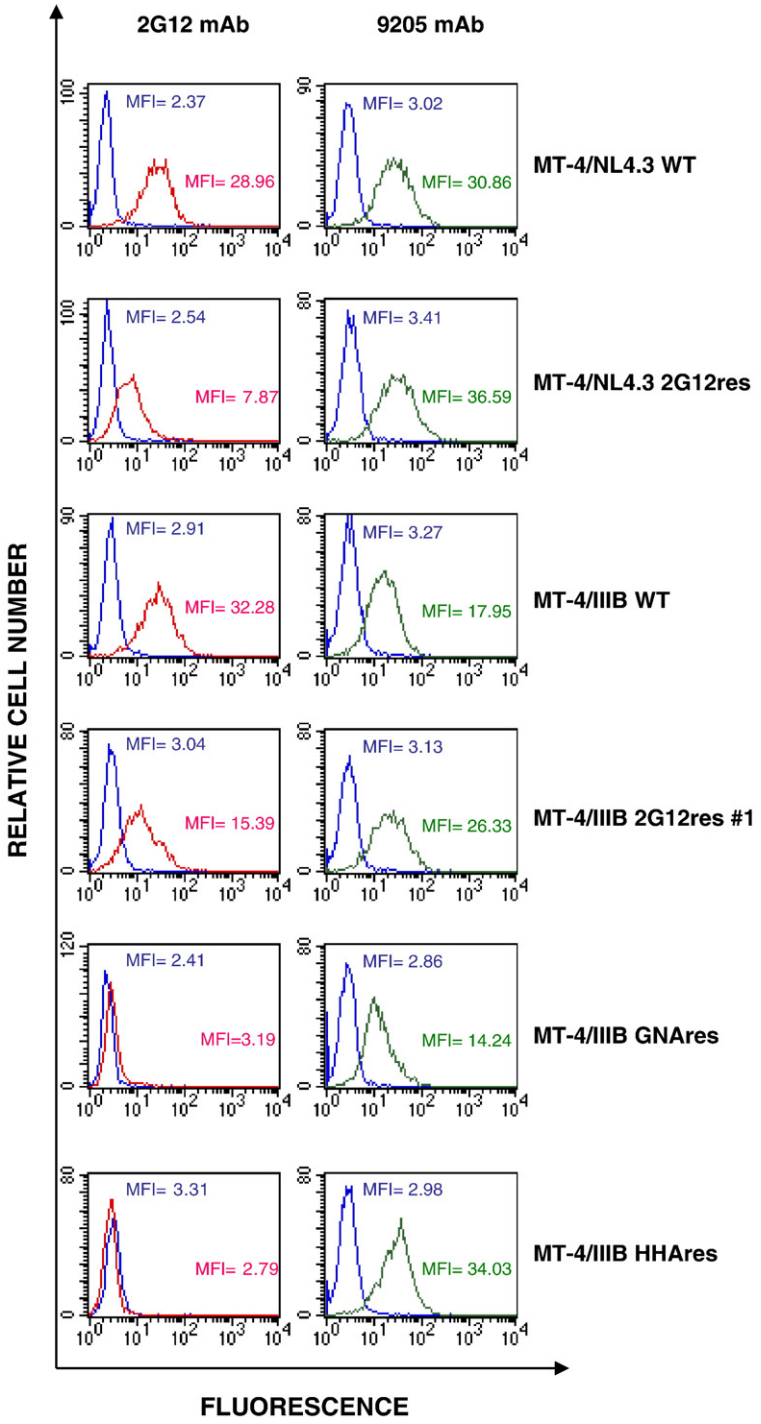

Fig. 3. Staining of 2G12 mAb (left panel) and 9205 mAb (right panel) on MT-4 cells 3–4 days after infection with HIV-1 NL4.3 and NL4.3-2G12res virus HIV-1 IIIB, IIIB-2G12res, IIIB-GNAres and IIIB-HHAres. The MFI of the background fluorescence (blue histograms), for the 2G12 mAb (red histograms) and for the 9205 mAb (green histograms) are indicated.

2G12 mAb-specific glycans (Table 3), no clear conclusions could be made to explain the inactivity of 2G12 mAb against several of these HIV-1 strains in cell culture. In fact, glycosylated N295, N392 and N386 were invariantly present in the wild-type HIV-1 IIIB, MN, NDK, NL4.3 and RF strains. However, N332 and N339 were absent in the NDK strain. Also the N406 glycan is lacking in HIV-1 NDK but not in the other HIV-1 strains. These observations cannot explain the inactivity of 2G12 mAb against the HIV-1 (MN or RF) strains. Similar observations have been made for several HIV-1 clade isolates. Clearly, there is a more complex situation ongoing, in which the genetic background of the gp120 envelope HIV-1 IIIB may also be important to determine the eventual phenotype of glycan deletions against 2G12 mAb. It also cannot be excluded that mutations in gp120 at amino acid sites that are not part of the glycosylation motifs, can afford subtle conformational changes,

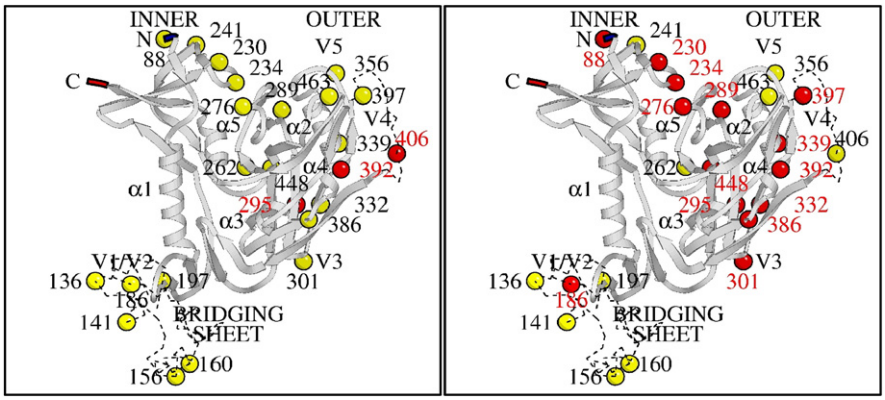

Fig. 4. Ribbon diagrams showing the *N*-glycosylation site mutations (red balls) in gp120 of 2G12 mAb-exposed HIV-1 strains (left panel, 3 mutations) and the mannose-binding plant lectin-exposed HIV-1 strains (right panel, 14 mutations). The left panel shows the 24 putative glycosylation sites represented by yellow circles and their accompanying amino acid number. The red circles indicate the deleted *N*-glycosylation sites that appear under 2G12 mAb in the 4 different HIV-1 isolates. The right panel shows the mutated *N*-glycosylation sites in gp120 of a variety of HIV-1 strains grown in the presence of the mannose-specific lectins HHA and GNA.

resulting in a less efficient recognition of the original epitope by the 2G12 mAb.

Resistance studies performed earlier in 2 subtype B viruses (92BR30 and S2/05) with 2G12 mAb showed that in none of the escape viruses a change in the N residues was found, except a change of N386K in one virus (Nakowitsch et al., 2005). Remarkably, the same authors found that HIV-1 IIIB was insensitive to 2G12 mAb inhibition, whereas Trkola et al. (1996) and our data showed that HIV-1 IIIB is quite sensitive to 2G12 mAb, adding even more complexity to the neutralizing potential of 2G12 mAb.

All five N positions of gp120 involved in 2G12 mAb binding were also found to be mutated in one or several of our plant lectin-resistant mutant virus strains (Balzarini et al., 2004b, 2005b). As it was also shown that the plant lectins competed with 2G12 mAb for the glycan interaction sites and prevents 2G12 mAb binding to this epitope on gp120 [whereas prior binding of 2G12 mAb to gp120 did not block subsequent binding of fluorescent GNA (data not shown)], it is logic to assume that these lectins may recognize this region of the gp120 molecule as well. These conclusions are also in line with the complete resistance of the mannose-specific lectin virus strains against the mAb 2G12.

Table 3
Presence of 2G12-related *N*-glycans in different HIV-1 strains

2G12-related *N*-glycan recognition [a]	HIV-1 strain				
	IIIB	MN	NL4.3	NDK	RF
N295[a]	+	+	+	+	+
N332[a]	+	+	+	−	+
N392[a]	+	+	+	+	+
N339[a]	+	+	+	−	+
N386[a]	+	+	+	+	+
N406 [b]	+	+	+	−	+

[a] *N*-glycans that play a direct (N295, N332, N392) or indirect (N339, N386) role in the recognition of gp120 by 2G12 mAb (according to Zhu et al., 2000 and Scanlan et al., 2002).

[b] *N*-glycan deletion observed to occur under selective pressure of 2G12 mAb against HIV-1 IIIB in CEM cell cultures.

Remarkable is the observation that the mannose-specific lectin HHA clearly gained more activity against the HIV-1 NL4.3-2G12res virus. This enhanced activity is also, although less, seen with the other mannose-specific lectins (GNA, CA and CV-N), but not with DS-5000, AMD3100, T-20 (Table 1), soluble CD4 or anti-CD4 mAb (data not shown). Enhanced activity (10-fold) was also observed with the mannose-specific plant lectin NPA (obtained from *Narcissus pseudonarcissus*), but not with the *N*-acetylglucosamine-binding protein from *Urtica dioica* (UDA) or with the non-peptidic carbohydrate binding agent pradimycin-A (data not shown). The enhanced activity was not only seen in MT-4 cells, but also in another T cell line (SUPT-1) and in PBMCs demonstrating that the enhanced antiviral effect is consistent and not due to the target cell used in our assays. How can mutations induced by a mAb and directed against the gp120 of HIV-1 NL4.3 enhances the antiviral activity of the mannose-specific lectins? Very likely the increased sensitivity towards HHA, GNA, CA and CV-N is not just due to the single N295 mutation, because in subtype clade C viruses the N295 glycosylation is consistently missing and no increase in antiviral activity of the mannose-specific lectins is observed (Table 2; Balzarini et al., 2005a, 2005b). Thus, the mechanism behind this phenomenon is more complex. We therefore performed several additional binding experiments on the 2G12-resistant NL4.3 virus. We observed that the binding of 2G12 mAb was significantly better inhibited by HHA and GNA in 2G12 mAb-resistant HIV-1 NL4.3 compared to the wild-type NL4.3 virus. We obtained IC_{50} values for HHA and GNA to inhibit 2G12 mAb binding on NL4.3 WT-infected cells of 0.89 μg/ml and 1.26 μg/ml, respectively, whereas on the 2G12 mAb-resistant HIV-1 NL4.3-infected cells this was 0.038 μg/ml (23-fold increase) and 0.064 μg/ml (20-fold increase). This observation confirms that, although one *N*-glycosylation site is missing, and mannose-specific lectins are expected to become less active, these agents showed an enhanced inhibition of viral infection. It is possible that in the envelope of the N-295 glycan mutated NL4.3 virus, various other mannose oligomers located on other glycan positions became better accessible to these relatively large tetrameric proteins (4 × 12.5 kDa). Reeves et al.

(2005) reported that enfuvirtide (T-20, fuzeon)-resistant viruses remained sensitive to other entry inhibitors such as anti-CD4 mAb, the CXCR4 antagonist AMD3100 and the CCR5 antagonist TAK-779. However, the authors also reported that some (but not all) enfuvirtide-resistant viruses showed an increased sensitivity to a subset of neutralizing mAbs such as B12, 2F5 and 4E10. They showed that mutations in the HR1 region of gp41 resulted in slower membrane fusion kinetics and it was therefore suggested that this could be a reason why some of the mAbs could have an enhanced neutralizing capacity. However, we observed that virus-induced CPE or viral infectivity as measured by p-24 Ag ELISAs were not significantly different between the wild-type and the 2G12 mAb-resistant NL4.3 virus.

Our studies now have shown that the "broadly" neutralizing mAb 2G12 might be endowed with less-broadly neutralizing properties than originally reported and anticipated and that it may become much more sensitive to activity loss due to one or a few amino acid mutations in the HIV envelope gp120. These properties should be taken into account in the eventual choice of a carbohydrate-binding agent to be introduced for HIV treatment or prevention as a microbicidal agent.

Materials and methods

Test agents

The mannose-specific plant lectins from *Galanthus nivalis* (GNA), *Hippeastrum hybrid* (HHA) and from *Cymbidium hybrid* (CA) were derived and purified, as described before (Van Damme et al., 1987, 1988, 1991, 1994). Cyanovirin (CV-N) was a kind gift of Dr. Michael Boyd (National Cancer Institute, Frederick) (Boyd et al., 1997). T-20 (enfuvirtide) was kindly provided by AIDS Research Alliance (Los Angeles, CA). The 2G12 mAb was purchased from Polymun Scientific (Vienna, Austria) and DS-5000 from Sigma (St. Louis, MO). AMD3100 was a kind gift of Dr. Gary Bridger (AnorMED, Langley, Canada). The anti-gp120 mAb (9205) [raised against a synthetic peptide representing amino acids 308 to 322 of the HIV-1 IIIB strain (RIQRGPGRAFVTIGK)] was purchased from Perkin Elmer (Zaventem, Belgium) (Skinner et al., 1988).

Primary leukocytes and cell line cultures

Buffy coat preparations from healthy donors were obtained from the Blood Bank in Leuven, Belgium. Peripheral blood mononuclear cells (PBMCs) were activated with PHA at 2 μg/ml (Sigma, Bornem, Belgium) for 3 days and cultured in the presence of 1 ng/ml IL-2 (Roche Molecular Biochemicals, Indianapolis, USA). Human T-lymphocytic CEM and SUPT-1 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI-1640 supplemented with 10% fetal bovine serum. The MT-4 T cell line was a kind gift of Dr. L. Montagnier (at that time at the Pasteur Institute, Paris, France).

Viruses

HIV-1 NL4.3, MN, RF, NDK and BaL were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH) (Adachi et al., 1986). HIV-1 HE was isolated from a Belgian AIDS patient in 1987 (Pauwels et al., 1990). Primary clinical isolates representing different HIV-1 clades and a HIV-2 isolate were all kindly provided by Dr. L. Lathey from BBI Biotech Research Laboratories, Inc., Gaithersburg, MD, and their co-receptor use (R5 or X4) was determined by us on the astroglioma U87.CD4 cell line transfected with either CCR5 or CXCR4.

Antiretrovirus assays in T cell lines

The methodology of the anti-HIV assays has been described previously (Balzarini et al., 2004b, 2005b). The IC_{50} value corresponds to the compound concentrations required to prevent syncytium formation/CPE by 50% in the virus-infected CEM cell cultures. For determining IC_{50} in MT-4 cells, MTT/MTS viability assays were performed, as described previously (Pauwels et al., 1988).

Antiviral testing of HIV isolates in PBMCs

The PHA-stimulated blasts were seeded at 0.5×10^6 cells per well into a 48-well plate containing varying concentrations of compound in medium containing IL-2 (25 U/ml, R & D Systems Europe, Abingdon, UK). The virus stocks were added at a final dose of 250 pg HIV-1 or HIV-2. Cell supernatant was collected at days 9–12 and HIV-1 core Ag in the culture supernatant was analysed by a p24 Ag ELISA kit (Perkin Elmer, Zaventem, Belgium). For HIV-2 p27 Ag detection the INNOTEST from Innogenetics (Temse, Belgium) was used.

Selection of 2G12 mAb-resistant HIV-1 strains

HIV-1 IIIB was added to CEM cell cultures ($3–4 \times 10^5$ cells/ml) in 48-well plates in the presence of 0.5 μg/ml of 2G12 mAb. Every 3 to 4 days, three independent drug-exposed cultures (#1, #2, #3) were subcultured by addition of 100 μl of the infected culture supernatant or 100 μl of the infected cell suspension to 900 μl of a suspension containing $3–4 \times 10^5$ uninfected CEM cells/ml. An increased drug concentration was given when extensive cytopathic effect (CPE) was obtained in the previous cell culture. Under these conditions, 9 to 11 cultivations were required to reach a concentration of 50 μg/ml 2G12 mAb that could be added to the three different virus-infected cell cultures. For HIV-1 NL4.3 resistance studies, MT-4 cells were infected with HIV-1 NL4.3 in medium containing 2G12 mAb at 0.1 μg/ml. Every 4 or 5 days, the replicating virus was passaged in fresh uninfected cells in the presence of 2G12 mAb at the same concentration as in the previous passage or at a two-fold higher concentration, depending on the amount of CPE observed. After 6 passages (~30 days), virus was recovered that could replicate in the presence of 100 μg/ml of 2G12 mAb,

and the induced CPE was similar to that of the wild-type virus (in absence of 2G12 mAb).

Genotyping of the HIV-1 env region

Proviral DNA or viral RNA was extracted using the QIAamp Blood Mini Kit or the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). A 3876-bp nucleotide fragment, encompassing the gp120 and the gp41 genes, was amplified in an outer PCR using the Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany) and outer primers KVL008 and KVL009. A 2346-bp nucleotide fragment of gp120 was amplified using the inner primers AV317 and AV323. A 2048-bp nucleotide fragment of gp41 was amplified using the inner primers AV318 and AV319 (Van Laethem et al., 2005). Amplification products were separated on a 1% agarose gel and visualized by ethidium bromide staining. The images were processed on a videoimager (ImageMaster VDS, Amersham Biosciences, Roosendaal, The Netherlands). PCR products for population sequencing were purified with Microspin S-400 (Amersham Biosciences, Roosendaal, The Netherlands). Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit and the reactions were run on an ABI3100 Genetic Analyzer (Applera, Nieuwerkerk a/d IJssel, The Netherlands). The sequences were analyzed using Sequence Analysis version 3.7 and SeqScape version 2.0 (Applera, Nieuwerkerk a/d IJssel, The Netherlands).

Flow cytometric analyses

MT-4 cells were infected with various HIV-1 strains and analyzed when cytopathicity (CPE) started to occur (3–4 days after infection). Briefly, after washing with PBS containing 2% FCS, cells were pre-incubated with or without agents at different concentrations for 30 min, washed and then incubated with 2G12 mAb or 9205 mAb for 30 min at 4 °C. Then the cells were washed and incubated with Rabbit-anti-Human (RaH) IgG-FITC (DakoCytomation, Denmark) or Goat-anti-mouse (GaM) IgG-PE (Caltag Laboratories, Burlingame) for 30 min at 4 °C for 2G12 mAb and 9205 mAb, respectively. As a control for aspecific background staining, cells were stained in parallel with RaH-IgG-FITC or GaM-IgG-PE only. Then the cells were washed, fixed with 1% aqueous formaldehyde solution and analyzed by flow cytometry with a FACScalibur (BD Biosciences, San Jose, CA). Data were acquired and analyzed with CellQuest software (BD Biosciences). For the calculations of the mAb binding, the mean fluorescence intensity (MFI) of each sample was expressed as percentage of the MFI of control cells (after subtracting the MFI of the background staining). Finally, the IC_{50} values of the compounds were calculated.

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