

^1H NMR (500 MHz, CDCl_3) δ 1.44 (t, $J = 7.00$ Hz, 3H), 4.43 (q, $J = 7.00$ Hz, 2H), 7.44 (d, $J = 8.50$ Hz, 1H), 7.49–7.51 (m, 1H), 7.87, 2.35 (d, $J = 2.50$ Hz, 1H); ^{13}C NMR (125 MHz, CDCl_3) δ 14.0, 64.0, 119.0, 121.5, 129.0, 130.8, 133.2, 135.7, 153.9, 160.5; HRMS (ESI), m/z calcd for $\text{C}_{10}\text{H}_{10}\text{Cl}_2\text{NO}_3$ (MH^+) 262.0038, found 262.0031.

4.1.3. Ethyl 2-((4-chloro-3-methylphenyl)amino)-2-oxoacetate (6c)

By use of a procedure similar to that described for the preparation of compound **6b**, the aniline **4c** (3.34 g, 24.0 mmol) was converted into the title compound **6c** (4.63 g, 96% yield) as white powder.

^1H NMR (500 MHz, CDCl_3) δ 1.43 (t, $J = 7.00$ Hz, 3H), 2.38 (s, 3H), 4.42 (q, $J = 7.00$ Hz, 2H), 7.33 (d, $J = 8.50$ Hz, 1H), 7.43–7.46 (m, 1H), 7.51–7.54 (m, 1H), 8.82 (s, 1H); ^{13}C NMR (125 MHz, CDCl_3) δ 14.0, 20.2, 63.8, 118.5, 122.0, 129.7, 130.9, 134.8, 137.1, 153.8, 160.9; HRMS (ESI), m/z calcd for $\text{C}_{11}\text{H}_{13}\text{ClNO}_3$ (MH^+) 242.0578, found 242.0568.

4.1.4. Ethyl 2-((3-fluoro-4-methylphenyl)amino)-2-oxoacetate (7a)

By use of a procedure similar to that described for the preparation of compound **6b**, the aniline **5a** (3.00 g, 24.0 mmol) was converted into the title compound **7a** (4.24 g, 94% yield) as white powder.

^1H NMR (500 MHz, CDCl_3) δ 1.43 (t, $J = 7.20$ Hz, 3H), 2.25 (s, 3H), 4.42 (q, $J = 6.80$ Hz, 2H), 7.12–7.21 (m, 2H), 7.48–7.56 (m, 1H), 8.83 (s, 1H); ^{13}C NMR (125 MHz, CDCl_3) δ 14.2 (2C), 63.8, 107.1 (d, $J = 27.5$ Hz), 115.0 (d, $J = 10.0$ Hz), 122.3 (d, $J = 17.5$ Hz), 131.6 (d, $J = 6.25$ Hz), 135.3 (d, $J = 13.8$ Hz), 153.8, 160.8, 161.1 (d, $J = 243.8$ Hz); HRMS (ESI), m/z calcd for $\text{C}_{11}\text{H}_{13}\text{FNO}_3$ (MH^+) 226.0879, found 226.0878.

4.1.5. Ethyl 2-((3-chloro-4-methylphenyl)amino)-2-oxoacetate (7b)

By use of a procedure similar to that described for the preparation of compound **6b**, the aniline **5b** (3.40 g, 24.0 mmol) was converted into the title compound **7b** (5.19 g, 94% yield) as white powder.

^1H NMR (500 MHz, CDCl_3) δ 1.43 (t, $J = 7.00$ Hz, 3H), 2.35 (s, 3H), 4.42 (q, $J = 7.00$ Hz, 2H), 7.22 (d, $J = 8.50$ Hz, 1H), 7.41–7.43 (m, 1H), 7.71 (d, $J = 2.00$ Hz, 1H), 8.83 (s, 1H); ^{13}C NMR (125 MHz, CDCl_3) δ 14.0, 20.0, 63.8, 118.0, 120.3, 131.2, 133.3, 134.7, 135.0, 153.8, 160.8; HRMS (ESI), m/z calcd for $\text{C}_{11}\text{H}_{13}\text{ClNO}_3$ (MH^+) 242.0584, found 242.0573.

4.1.6. Ethyl 2-((3-bromo-4-methylphenyl)amino)-2-oxoacetate (7c)

By use of a procedure similar to that described for the preparation of compound **6b**, the aniline **5c** (4.47 g, 27.0 mmol) was converted into the title compound **7c** (6.24 g, 96% yield) as white powder.

^1H NMR (500 MHz, CDCl_3) δ 1.43 (t, $J = 7.00$ Hz, 3H), 2.38 (s, 3H), 4.42 (q, $J = 7.00$ Hz, 2H), 7.23 (t, $J = 8.50$ Hz, 1H), 7.48–7.53 (m, 1H), 7.83–7.90 (m, 1H), 8.80 (s, 1H); ^{13}C NMR (125 MHz, CDCl_3) δ 14.0, 22.4, 63.9, 118.7, 123.4, 125.0, 131.0, 135.0, 135.2, 153.7, 160.8; HRMS (ESI), m/z calcd for $\text{C}_{11}\text{H}_{13}\text{BrNO}_3$ (MH^+) 286.0079, found 286.0068.

4.1.7. N^1 -(4-Chloro-3-fluorophenyl)- N^2 -(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide (8a)

To a solution of compound **6a** (70.0 mg, 0.286) in EtOH (2.9 mL) were added Et_3N (0.200 mL, 1.45 mmol) and 2,2,6,6-tetramethylpiperidin-4-amine (0.150 mL, 0.870 mmol). The reaction mixture was stirred for 3 h at 150 °C under microwave irradiation. After being concentrated in vacuo, the residue was extracted with CHCl_3 ,

and washed with saturated NaHCO_3 and brine, then dried over MgSO_4 . Concentration under reduced pressure to provide the title compound **8a** (34.6 mg, 34% yield) as white powder.

^1H NMR (500 MHz, CDCl_3) δ 0.99–1.50 (m, 15H), 1.92 (dd, $J = 3.50, 9.00$ Hz, 2H), 4.20–4.32 (m, 1H), 7.21–7.25 (m, 1H), 7.34–7.41 (m, 1H), 7.69–7.73 (m, 1H), 9.31 (br, 1H); ^{13}C NMR (125 MHz, CDCl_3) δ 28.4, 34.8, 43.8, 44.5, 51.0, 108.3 (d, $J = 26.3$ Hz), 115.8 (d, $J = 3.75$ Hz), 117.1 (d, $J = 17.5$ Hz), 130.8, 136.2 (d, $J = 8.75$ Hz), 157.6, 158.1 (d, $J = 247.5$ Hz), 158.4; HRMS (ESI), m/z calcd for $\text{C}_{17}\text{H}_{24}\text{ClFN}_3\text{O}_2$ (MH^+) 356.1536, found 356.1548.

4.1.8. N^1 -(3,4-Dichlorophenyl)- N^2 -(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide (8b)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **6b** (261.0 mg, 1.00 mmol) was converted into the title compound **8b** (520.0 mg, 70% yield) as white powder.

^1H NMR (500 MHz, CDCl_3) δ 1.07 (t, $J = 12.0$ Hz, 2H), 1.16 (s, 6H), 1.28 (s, 6H), 1.90–1.93 (m, 2H), 4.20–4.32 (m, 1H), 7.26 (m, 1H), 7.40–7.48 (m, 2H), 7.88 (s, 1H), 9.33 (s, 1H); ^{13}C NMR (125 MHz, CDCl_3) δ 28.5 (2C), 34.9 (2C), 43.8, 44.6 (2C), 50.9 (2C), 119.0, 121.4, 128.7, 130.8, 133.1, 135.8, 157.7, 158.5; HRMS (ESI), m/z calcd for $\text{C}_{17}\text{H}_{22}\text{Cl}_2\text{N}_3\text{O}_2$ (MH^-) 370.1095, found 370.1105.

4.1.9. N^1 -(4-Chloro-3-methylphenyl)- N^2 -(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide (8c)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **6c** (482.0 mg, 2.00 mmol) was converted into the title compound **8c** (364.0 mg, 49% yield) as white powder.

^1H NMR (500 MHz, CDCl_3) δ 1.07 (t, $J = 12.0$ Hz, 2H), 1.15 (s, 6H), 1.28 (s, 6H), 1.86–1.94 (m, 2H), 4.15–4.31 (m, 1H), 7.21–7.24 (m, 1H), 7.32–7.38 (m, 2H), 7.74 (s, 1H), 9.24 (s, 1H); ^{13}C NMR (125 MHz, CDCl_3) δ 19.6, 28.5 (2C), 34.9 (2C), 43.7, 44.7 (2C), 50.9 (2C), 117.9, 120.2, 131.2, 133.1, 134.7, 135.1, 157.5, 158.8; HRMS (ESI), m/z calcd for $\text{C}_{18}\text{H}_{25}\text{ClN}_3\text{O}_2$ (MH^-) 350.1641, found 350.1656.

4.1.10. N^1 -(3-Fluoro-4-methylphenyl)- N^2 -(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide (9a)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **7a** (225.0 mg, 1.00 mmol) was converted into the title compound **9a** (161.0 mg, 48% yield) as white powder.

^1H NMR (500 MHz, CDCl_3) δ 1.07 (t, $J = 12.5$ Hz, 2H), 1.15 (s, 6H), 1.28 (s, 6H), 1.92 (dd, $J = 12.5, 3.50$ Hz, 2H), 2.26 (s, 3H), 4.12–4.32 (m, 1H), 7.12–7.20 (m, 2H), 7.30–7.37 (m, 1H), 7.48–7.54 (m, 1H), 9.27 (s, 1H); ^{13}C NMR (125 MHz, CDCl_3) δ 14.2, 28.5 (2C), 34.9 (2C), 43.7, 44.7 (2C), 50.9 (2C), 107.1 (d, $J = 26.3$ Hz), 115.0, 121.8 (d, $J = 17.5$ Hz), 131.6, 135.4 (d, $J = 15.0$ Hz), 157.5, 158.8, 161.1 (d, $J = 242.5$ Hz); HRMS (ESI), m/z calcd for $\text{C}_{18}\text{H}_{25}\text{FN}_3\text{O}_2$ (MH^-) 334.1936, found 334.1942.

4.1.11. N^1 -(3-Chloro-4-methylphenyl)- N^2 -(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide (9b)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **7b** (482.0 mg, 1.00 mmol) was converted into the title compound **9b** (448.0 mg, 48% yield) as white powder.

^1H NMR (500 MHz, CDCl_3) δ 1.09 (t, $J = 12.5$ Hz, 3H), 1.18 (s, 6H), 1.30 (s, 6H), 1.93–1.95 (m, 2H), 2.41 (s, 3H), 4.20–4.34 (m, 1H), 7.30–7.37 (m, 2H), 7.44–7.46 (m, 1H), 7.53 (d, $J = 2.50$ Hz, 1H), 9.25 (s, 1H); ^{13}C NMR (125 MHz, CDCl_3) δ 20.3, 28.5 (2C), 34.9 (2C), 43.7, 44.7 (2C), 50.9 (2C), 118.5, 122.0, 130.0, 130.7, 134.8, 137.1, 157.5, 158.8; HRMS (ESI), m/z calcd for $\text{C}_{18}\text{H}_{25}\text{ClN}_3\text{O}_2$ (MH^-) 350.1641, found 350.1636.

4.1.12. *N*¹-(3-Bromo-4-methylphenyl)-*N*²-(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide (9c)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **7c** (285.0 mg, 1.00 mmol) was converted into the title compound **9c** (157.0 mg, 40% yield) as white powder.

¹H NMR (500 MHz, CDCl₃) δ 1.07 (t, *J* = 12.5 Hz, 3H), 1.15 (s, 6H), 1.28 (s, 6H), 1.91 (dd, *J* = 8.00, 4.00 Hz, 2H), 2.38 (s, 3H), 3.70–3.75 (m, 1H), 7.22 (d, *J* = 8.50 Hz, 1H), 7.30–7.37 (m, 1H), 7.43–7.45 (m, 1H), 7.90 (d, *J* = 2.50 Hz, 1H), 9.25 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 22.4, 28.5 (2C), 34.9 (2C), 43.7, 44.7 (2C), 50.9 (2C), 118.6, 123.4, 125.0, 131.0, 134.9 (2C), 157.5, 158.8; HRMS (ESI), *m/z* calcd for C₁₈H₂₅BrN₃O₂ (MH⁺) 394.1136, found 394.1158.

4.1.13. Amine (12)

The compound **11** was prepared according to the reported procedure.¹⁴ To a stirred solution of piperidone **11** (247.8 mg, 1.05 mmol) in MeOH (2.10 mL) was added *p*-methoxybenzylamine (0.41 mL, 3.15 mmol). After being stirred at room temperature for 23 h, sodium cyanoborohydride was added and stirred at room temperature for 48 h. The reaction mixture was poured into saturated NaHCO₃ and extracted with EtOAc, then dried over MgSO₄. After concentration under reduced pressure, the residue was treated with 1 M TMS in THF (4.8 mL). The mixture was stirred at 0 °C for 14 h. Concentration under reduced pressure followed by short chromatography with CHCl₃/MeOH gave the PMB-protected amine. To a solution of the above amine (584.0 mg, 1.64 mmol) in CH₃CN/H₂O (13.1 mL, v:v = 2:1) was added CAN (2.74 g, 8.2 mmol). The mixture was stirred at room temperature for 14 h. The reaction mixture was diluted with 0.5 M HCl and washed with CH₂Cl₂. The water layer was alkalinized and extracted with EtOAc, then dried over Na₂SO₄. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc–EtOH (4:1) to give the title compound **12** (175.5 mg, 71% yield) as a yellow oil.

¹H NMR (500 MHz, CDCl₃) δ 1.15–1.85 (m, 24H), 2.95–3.05 (m, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 22.2 (2C), 22.8 (2C), 26.2 (2C), 37.3 (2C), 42.3 (2C), 43.6 (2C), 47.0, 53.2 (2C); HRMS (ESI), *m/z* calcd for C₁₅H₂₉N₂ (MH⁺) 237.2325, found 237.2321.

4.1.14. *N*¹-((4-Chloro-3-fluorophenyl)-*N*²-(2,6-dicyclohexylpiperidin-4-yl)oxalamide (13a)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **6a** (36.8 mg, 0.150 mmol) was converted into the title compound **13a** (7.6 mg, 12% yield) as yellow powder.

¹H NMR (400 MHz, CDCl₃) δ 0.71–2.28 (m, 24H), 2.03–2.20 (m, 2H), 4.02–4.16 (m, 1H), 7.13–7.18 (m, 1H), 7.27–7.33 (m, 1H), 7.62–7.66 (m, 1H), 9.25 (br, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 14.1, 22.0 (2C), 22.6 (2C), 25.8 (2C), 29.3, 29.7 (2C), 31.9, 70.5, 108.3 (d, *J* = 26.3 Hz), 115.8, 117.1 (d, *J* = 18.8 Hz), 130.8, 136.2 (d, *J* = 10.0 Hz), 157.6, 158.1 (d, *J* = 247.5 Hz), 158.6; HRMS (ESI), *m/z* calcd for C₂₃H₃₂ClFN₃O₂ (MH⁺) 436.2162, found 436.2156.

4.1.15. *N*¹-(4-Chlorophenyl)-*N*²-(2,6-dicyclohexylpiperidin-4-yl)oxalamide (13b)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **6b** (31.3 mg, 0.120 mmol) was converted into the title compound **13b** (28.0 mg, 52% yield) as white powder.

¹H NMR (400 MHz, CDCl₃) δ 0.96 (t, *J* = 12.5 Hz, 2H), 1.10–1.84 (br, 20H), 2.05–2.19 (m, 2H), 4.08–4.21 (m, 1H), 7.23–7.33 (br, 1H), 7.39–7.46 (m, 2H), 7.88 (t, *J* = 1.00 Hz, 1H), 9.34 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 14.1, 22.1 (2C), 22.7 (2C), 26.1 (2C), 31.6, 37.2 (2C), 42.6, 43.0, 43.6, 52.6 (2C), 119.0, 121.4, 128.7,

130.8, 133.1, 135.8, 157.7, 158.5; HRMS (ESI), *m/z* calcd for C₂₃H₃₂Cl₂N₃O₂ (MH⁺) 452.1872, found 452.1865.

4.1.16. *N*¹-((4-Chloro-3-methylphenyl)-*N*²-(2,6-dicyclohexylpiperidin-4-yl)oxalamide (13c)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **6c** (121.0 mg, 0.500 mmol) was converted into the title compound **13c** (15.1 mg, 7% yield) as white powder.

¹H NMR (500 MHz, CDCl₃) δ 0.87–1.88 (br, 22H), 2.09–2.20 (m, 2H), 2.38 (s, 3H), 4.09–4.22 (m, 1H), 7.32–7.33 (m, 1H), 7.41–7.43 (m, 1H), 7.51 (d, *J* = 2.00 Hz, 1H), 7.73 (m, 1H), 9.24 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 20.2, 22.1 (2C), 22.7 (2C), 26.0 (2C), 29.7, 37.0, 42.3 (2C), 42.8 (2C), 43.4, 52.9 (2C), 118.4, 122.0, 130.0, 130.6, 134.8, 137.1, 157.5, 158.9; HRMS (ESI), *m/z* calcd for C₂₄H₃₅ClN₃O₂ (MH⁺) 430.2267, found 430.2264.

4.1.17. *N*¹-(3-Fluoro-4-methylphenyl)-*N*²-(2,6-dicyclohexylpiperidin-4-yl)oxalamide (14a)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **7a** (225.0 mg, 1.00 mmol) was converted into the title compound **14a** (27.5 mg, 7% yield) as white powder.

¹H NMR (500 MHz, CDCl₃) δ 0.971 (t, *J* = 12.5 Hz, 2H), 1.18–1.86 (m, 20H), 2.13–2.16 (m, 2H), 2.26 (s, 3H), 4.09–4.21 (m, 1H), 7.13–7.18 (m, 2H), 7.33 (d, *J* = 8.00 Hz, 1H), 7.50–7.53 (m, 1H), 9.27 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 14.2, 22.2 (2C), 22.8 (2C), 26.1 (2C), 37.2 (2C), 42.2 (2C), 43.3 (2C), 43.5, 52.6 (m, 2C), 107.0 (d, *J* = 27.5 Hz), 115.0 (d, *J* = 3.75 Hz), 121.8 (d, *J* = 17.5 Hz), 131.6 (d, *J* = 6.25 Hz), 135.4 (d, *J* = 10.0 Hz), 157.5, 158.9, 161.3 (d, *J* = 242.5 Hz); HRMS (ESI), *m/z* calcd for C₂₄H₃₃FN₃O₂ (MH⁺) 414.2554, found 414.2562.

4.1.18. *N*¹-(3-Chloro-4-methylphenyl)-*N*²-(2,6-dicyclohexylpiperidin-4-yl)oxalamide (14b)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **7b** (120.5 mg, 0.500 mmol) was converted into the title compound **14b** (12.9 mg, 6% yield) as white powder.

¹H NMR (500 MHz, CDCl₃) δ 0.973 (t, *J* = 12.5 Hz, 2H), 1.18–1.86 (br, 20H), 2.11–2.19 (m, 2H), 2.35 (s, 3H), 4.09–4.21 (m, 1H), 7.20–7.22 (m, 1H), 7.30–7.32 (m, 1H), 7.35–7.37 (d, *J* = 2.50 Hz, 1H), 7.73 (m, 1H), 9.22 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 19.6, 22.1 (2C), 22.7 (2C), 26.0 (2C), 29.7, 37.0, 42.1 (2C), 42.7 (2C), 43.2, 53.3 (2C), 118.0, 120.3, 131.2, 133.0, 134.7, 135.1, 157.5, 158.8; HRMS (ESI), *m/z* calcd for C₂₄H₃₃ClN₃O₂ (MH⁺) 430.2267, found 430.2257.

4.1.19. *N*¹-(3-Bromo-4-methylphenyl)-*N*²-(2,6-dicyclohexylpiperidin-4-yl)oxalamide (14c)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **7c** (142.0 mg, 0.500 mmol) was converted into the title compound **14c** (11.5 mg, 5% yield) as white powder.

¹H NMR (500 MHz, CDCl₃) δ 0.67–2.07 (br, 22H), 2.28 (br, 2H), 2.38 (s, 3H), 4.09–4.21 (m, 1H), 7.22 (d, *J* = 8.00 Hz, 1H), 7.28–7.38 (br, 1H), 7.43 (dd, *J* = 4.50, 2.50 Hz, 1H), 7.90 (d, *J* = 2.50 Hz, 1H), 9.21 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 14.1, 22.1 (2C), 22.4 (2C), 22.7 (2C), 25.9, 30.0, 31.6, 36.9 (2C), 42.7 (3C), 52.7, 52.9, 118.6, 123.4, 125.0, 131.0, 134.9, 135.1, 157.4, 158.8; HRMS (ESI), *m/z* calcd for C₂₄H₃₃BrN₃O₂ (MH⁺) 474.1762, found 474.1746.

4.2. Antiviral assay and cytotoxicity assay

Anti-HIV activity and cytotoxicity measurements in PM1/CCR5 cells (Yoshimura et al., 2010) were based on viability of cells that

had been infected or not infected with 100 TCID₅₀ of an R5 primary isolate YTA48P exposed to various concentrations of the test compound. After the PM1/CCR5 cells were incubated at 37 °C for 7 days. The 50% inhibitory concentration (IC₅₀) values and the 50% cytotoxic concentration (CC₅₀) were then determined using the Cell Counting Kit-8 assay (Dojindo Laboratories). All assays were performed in duplicate or triplicate.

4.3. FACS analysis

JR-FL (R5, Sub B) chronically infected PM1 cells were pre-incubated with 0.5 µg/mL of sCD4 or 100 µM of a CD4 mimic for 15 min, and then incubated with an anti-HIV-1 mAb, 4C11, at 4 °C for 15 min. The cells were washed with PBS, and fluorescein isothiocyanate (FITC)-conjugated mouse anti-human IgG antibody was used for antibody-staining. Flow cytometry data for the binding of 4C11 (green lines) to the Env-expressing cell surface in the presence of a CD4 mimic are shown among gated PM1 cells along with a control antibody (anti-human CD19: black lines). Data are representative of the results from a minimum of two independent experiments. The number at the bottom of each graph shows the mean fluorescence intensity (MFI) of the antibody 4C11.

4.4. Molecular modeling

Dockings of compounds **3** and **13a** were performed using Molecular Operating Environment modeling package (MOE 2008. 10, Canada), into the crystal structure of gp120 (PDB, entry 3TGS).

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Supplementary data

Supplementary data (NMR charts of compounds) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmc.2013.02.041>.

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Conformational Epitope Consisting of the V3 and V4 Loops as a Target for Potent and Broad Neutralization of Simian Immunodeficiency Viruses

Takeo Kuwata,^a Kaori Takaki,^a Kazuhisa Yoshimura,^b Ikumi Enomoto,^a Fan Wu,^c Ilmour Ourmanov,^c Vanessa M. Hirsch,^c Masaru Yokoyama,^d Hironori Sato,^d Shuzo Matsushita^a

Center for AIDS Research, Kumamoto University, Kumamoto, Japan^a; AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan^b; Laboratory of Molecular Microbiology, NIAID, NIH, Bethesda, Maryland, USA^c; Pathogen Genomics Center, National Institute of Infectious Diseases, Tokyo, Japan^d

Inducing neutralizing antibodies (NAb) is the key to developing a protective vaccine against human immunodeficiency virus type 1 (HIV-1). To clarify the neutralization mechanism of simian immunodeficiency virus (SIV), we analyzed NAb B404, which showed potent and broad neutralizing activity against various SIV strains. In 4 SIVsmH635FC-infected macaques, B404-like antibodies using the specific VH3 gene with a long complementarity-determining region 3 loop and λ light chain were the major NABs in terms of the number and neutralizing potency. This biased NAb induction was observed in all 4 SIVsmH635FC-infected macaques but not in 2 macaques infected with a SIV mix, suggesting that induction of B404-like NABs depended on the inoculated virus. Analysis using Env mutants revealed that the V3 and V4 loops were critical for B404 binding. The reactivity to the B404 epitope on trimeric, but not monomeric, Env was enhanced by CD4 ligation. The B404-resistant variant, which was induced by passages with increasing concentrations of B404, accumulated amino acid substitutions in the C2 region of gp120. Molecular dynamics simulations of the gp120 outer domains indicated that the C2 mutations could effectively alter the structural dynamics of the V3/V4 loops and their neighboring regions. These results suggest that a conformational epitope consisting of the V3 and V4 loops is the target for potent and broad neutralization of SIV. Identifying the new neutralizing epitope, as well as specifying the VH3 gene used for epitope recognition, will help to develop HIV-1 vaccines.

Neutralizing antibodies (NAb) against human immunodeficiency virus type 1 (HIV-1) protect against viral challenge in nonhuman primate models (1–5), suggesting that NAb induction may be an important key to the development of vaccines against HIV-1. The role of NABs in prevention of infection and control of viral replication has been suggested in several studies using candidate vaccines (6–8). However, the difficulties in inducing NABs, especially those that are broadly reactive to various HIV-1 strains, have hampered the development of such vaccines (9–11). Monoclonal antibodies (MAb) with broad neutralizing activity that were recently isolated from HIV-1-infected patients have been characterized to understand the specificities and mechanisms of broad neutralization (12–16). The epitopes of these potent and broad NABs, such as PG9, PGT128, VRC01, and 10E8, have been determined precisely (17–19) and provide an opportunity for structure-based vaccine design to develop antibody-based vaccines for HIV-1 (11, 20–23).

Nonhuman primate models of simian immunodeficiency virus (SIV) infection are commonly used to develop vaccines against HIV-1 (6, 8, 24). Various immunogens, vectors, and regimens have been evaluated by challenge infection with SIV. Moreover, immune factors associated with prevention of infection have been explored in the SIV model. However, epitopes for potent and broad neutralization of SIV remain unclear because few MABs that neutralize a wide range of SIV strains have been available. Recently, we isolated MABs from a rhesus macaque infected with SIVsmH635FC, which was isolated from a rapid progressor macaque (25). Infection with SIVsmH635FC, a highly neutralization-sensitive molecular clone, resulted in a vigorous and potent antibody response in all the infected macaques together with viral mutations to escape antibody recognition (26, 27). MAb B404

bound to a conformational epitope on gp120 of various SIV strains and did not react to overlapping peptides of SIV Env. The V3 region was shown to be important by competition enzyme-linked immunosorbent assay (ELISA) with anti-V3 antibodies (25). The neutralizing activity of B404 against homologous neutralization-sensitive SIVsmH635FC, genetically divergent SIVmac316, and neutralization-resistant SIVsmE543-3 was observed.

In this study, we analyzed the epitope of B404 and the induction of B404-like NABs in SIV-infected macaques. Analysis of more than 400 anti-Env MABs demonstrated that B404-like NABs with the same gene usage and specificity were mainly induced in 4 SIVsmH635FC-infected macaques. The B404 epitope was mapped to a conformational epitope consisting of the V3 and V4 loops exposed on a trimeric Env structure after CD4 binding. The identification of the new neutralizing epitope and vigorous antibody response to this epitope in SIV-infected macaques will help us to understand broad neutralization in a macaque model of SIV infection.

MATERIALS AND METHODS

Cells and viruses. PM1 (28) and PM1/CCR5 (29) cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS). TZM-bl

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Address correspondence to Shuzo Matsushita, shuzo@kumamoto-u.ac.jp.

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(30–33) and 293T (34) cells were maintained in Dulbecco's modified Eagle medium containing 10% FBS. Infectious molecular clones, SIVsmE543-3 (35), SIVsmH635FC (27), SIVmac239 (36), SIVmac316 (37), SIVsmE660FL14, SIVsmH805-24w-3, and SIVsmH807-24w-4 (38) were transfected into 293T cells. After 2 days, the supernatants were filtered (0.45 μ m) and stored at -80°C as virus stocks.

Construction of Fab libraries from SIV-infected macaques. The Fab library from SIVsmH635FC-infected rhesus macaque H723 was described previously (25). The Fab libraries from SIV-infected rhesus macaques H704, H709, H714, H711, and H725 (26, 27, 39) were similarly constructed using the pComb3X system according to the instructions of Barbas et al. (40). Four macaques, H723, H704, H709, and H714, were infected with SIVsmH635FC. H711 was infected with a combination of SIVsmE543-3 and SIVsmH635FC. H725 was infected with plasma samples from 2 SIVsmH445-infected macaques, H631 and H635. Rhesus macaques of Indian origin were used in this study. RNA was extracted from lymphocytes from the lymph nodes of these macaques using an RNeasy minikit (Qiagen, Hilden, Germany) and used for subsequent RT-PCR using oligo(dT)20 primer, ReverTra Ace (Toyobo, Osaka, Japan), and Platinum high-fidelity *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Two libraries, κ and λ light chains, were constructed for each macaque to examine the frequency of NAb in each population, although only one library, containing both κ and λ light chains, was constructed for H723. Immunoglobulin (Ig) genes were inserted into pComb3X, and the ligation mix was used for transformation of XL1-Blue (Stratagene, La Jolla, CA) by electroporation. Transformed cultures were incubated in superbroth medium with 50 $\mu\text{g}/\text{ml}$ carbenicillin, 10 $\mu\text{g}/\text{ml}$ tetracycline, and 1.4 $\mu\text{g}/\text{ml}$ kanamycin overnight at 37°C after addition of VCSM13 helper phage (Stratagene). Library phage stock was obtained from the culture medium by polyethylene glycol 8000–NaCl precipitation. Library size was determined by assessing the number of CFU after infection of XL1-Blue with a diluted phage sample.

Biopanning to obtain anti-Env antibodies. Biopanning was performed using SIV antigen (Ag), which was prepared by infection of PM1 cells with SIVsmE543-3 as previously described (25). To obtain Fab clones against Env, we selected Fab clones from the H723 library using a 96-well plate in which Env was conjugated with anti-Env Fab clones B404, B408, and H301, which recognize gp120 (conformational), gp41 cluster I and gp120 V1, respectively (25). A MaxiSoap 96-well plate (Thermo Fisher Scientific, Waltham, MA) was incubated with 100 μl of 1.25 $\mu\text{g}/\text{ml}$ B404, 0.625 $\mu\text{g}/\text{ml}$ B408, and 10 $\mu\text{g}/\text{ml}$ H301 for 1 h at 37°C . The wells were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) and blocked with 5% skim milk (Wako Pure Chemical Industries, Osaka, Japan) in PBS (MPBS) for 1 h at 37°C . After the blocking solution was discarded, the wells were incubated with 100 μl 40-fold-diluted SIV Ag for 1 h at 37°C , washed with PBS-T, and used for panning. After incubation with 50 μl of phage library for 2 h at 37°C , the wells were washed 5 times with PBS-T, and bound phage was eluted with 50 μl 100 mM glycine (pH 2.2). Amplified phage was used for the next round of panning, and 3 or 4 rounds of panning were performed. To isolate Fab clones specific to Env, we transformed phagemid DNA into TOP10F' *Escherichia coli* cells (Invitrogen), and supernatants from isopropyl- β -D-thiogalactopyranoside (IPTG; Wako Pure Chemical Industries)-induced cultures were screened for reactivity to SIV Env using ELISA. Fab clones were purified using a His GraviTrap column (GE Healthcare, Buckinghamshire, United Kingdom), as described previously (25).

Construction of a single-chain variable fragment (scFv) form of B404. B404 Fab was previously converted into complete rhesus IgG produced from a stable cell line carrying heavy- and light-chain plasmids pHCG-B404 and pLL-B404 (25). From these plasmids, B404 scFv was constructed using the pComb3X system (40). The heavy-chain variable region (VH) was amplified using pHCG-B404 as a template and primers HSCVH35-FL (5'-GGT GGT TCC TCT AGA TCT TCC TCC TCT GGT GGC GGT GGC TCG GGC GGT GGT GGG GAG GTG CAG CTG GTG SAG TCT GG-3') and RhSCG404-B (5'-CCT GGC CGG CCT GGC CAC

TAG TGA CCG ATG GGC CCT TGG TGG AGC C-3'). The light-chain λ variable region (VL) was amplified from pLL-B404 using primers HSCCLam3 (5'-GGG CCC AGG CGG CCG AGC TCG AGC TGA CTC AGC CAC CCT CAG TGT C-3') and RhSCJLam404 (5'-GGA AGA TCT AGA GGA ACC ACC GCC TAG GAC GGT CAG CCG GGT CCC-3'). The amplified products were combined by overlapping PCR using primers RSC-F (5'-GAG GAG GAG GAG GAG GAG GCG GGG CCC AGG CGG CCG AGC TC-3') and RSC-B (5'-GAG GAG GAG GAG GAG GAG CCT GGC CGG CCT GGC CAC TAG TG-3'), digested with *Sfi*I, and inserted into pComb3X in a manner similar to that of Fab construction. The resultant plasmid had the B404 VL and VH regions, which were connected with an 18-amino-acid linker, a histidine tag, and a hemagglutinin (HA) tag. This plasmid was transformed into Rosetta 2 (Merck, Darmstadt, Germany), and B404 scFv was purified from the cell pellet using a His GraviTrap column.

ELISA. ELISA was performed to detect antibodies specific to SIV Ag as previously described (25, 41). Briefly, a MaxiSoap 96-well plate was coated with PBS containing 50 ng/ml concanavalin A (Sigma, St. Louis, MO) for 1 h at 37°C , and SIV Env was conjugated by incubation with 50 $\mu\text{l}/\text{well}$ 10-fold diluted SIV Ag for 1 h at 37°C . Samples were added to each well at 50 $\mu\text{l}/\text{well}$ with 50 μl of MPBS, and the plate was incubated for 1 h at 37°C . When the enhancement effect of soluble CD4 (sCD4) was examined, 25 μl of sample, 25 μl of sCD4, and 50 μl MPBS were added to each well. Fabs specific to SIV Env were detected with anti-HA-peroxidase (1:1,000 dilution; 3F10, Roche Molecular Biochemicals, Mannheim, Germany) and ABTS [2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid)] solution (Roche Molecular Biochemicals).

Competition ELISA was performed similarly using B404 IgG as a competitor. Ag-coated wells were incubated with 50 μl MPBS and 25 μl serial dilutions of B404 IgG for 1 h at 37°C . After incubation with 25 μl saturating concentrations of Fab clones, Fab clone binding was detected by anti-HA-peroxidase (1:1,000) and ABTS solution.

Analysis of neutralizing antibody titers. The neutralizing capability of Fab samples was measured as the reduction in luciferase activity after infection of TZM-bl cells with various SIV strains (6, 25). In addition to Fab samples, plasma samples from SIVsmH635FC-infected macaque H704 (26) and SIVmac239-infected macaque MM324 (42) and MAb M318T (43), which recognizes the V2 region of SIV Env, were used to examine the sensitivity of SIV variants to antibody-mediated neutralization. Briefly, 100- μl portions of serially diluted samples in duplicate were incubated with 50 μl containing 200 50% tissue culture infectious doses (TCID₅₀) of virus in a 96-well plate. After incubation for 1 h at 37°C , 100 μl containing 1×10^5 TZM-bl cells/ml with 37.5 $\mu\text{g}/\text{ml}$ DEAE dextran was added. Infected cultures were incubated for 2 days, but cultures infected with SIVsmH635FC were incubated for 3 days. After incubation, cells were lysed with 30 μl cell lysing buffer (Promega, Madison, WI) for 15 min at room temperature (RT), and 10 μl cell lysate was transferred to a 96-well black solid plate (OptiPlates-96F; Perkin-Elmer, Boston, MA) for measurements of luminescence using a GloMax 96 microplate luminometer (Promega) and a luciferase assay system (Promega). The 50 and 90% inhibitory concentrations (IC₅₀ and IC₉₀, respectively) were calculated with nonlinear regression using PRISM5 and defined as the concentration that caused 50 and 90% reductions in luciferase activity, respectively, compared to that in virus control wells after the subtraction of background.

Construction of Env mutants. The *env* gene was amplified by PCR using primers SRev-F (5'-GGT TTG GGA ATA TGC TAT GAG-3') and SEnv-R (5'-CCT ACT AAG TCA TCA TCT T-3') and SIVsmE543-3 plasmid as a template. The PCR product was inserted into pcDNA3.1/V5-His-TOPO vector (Invitrogen). After *Xba*I digestion, the plasmid was ligated with an *Nhe*I-*Xba*I fragment from pLP-IRES2-EGFP (Clontech Laboratories Inc., Mountain View, CA) to generate a plasmid designated RE543-EGFP that expressed both enhanced green fluorescent protein (EGFP) and Env. Mutants were constructed from RE543-EGFP using PCR mutagenesis. Deletion mutants Δ V1, Δ V2, Δ V3, and Δ V4 were created by

deleting amino acid residues 115 to 149 in the V1 loop, 153 to 209 in the V2 loop, 313 to 342 in the V3 loop, and 404 to 430 in the V4 loop and replacing them with Gly-Ala-Gly, Gly, Gly-Ala, and Gly-Ala-Gly, respectively. These mutations were introduced into RE543-EGFP using primers DV1F (5'-ATG TAA TGG AGC CGG CTC TTG CAT AAA AAA-3') and DV1R (5'-AAG AGC CGG CTC CAT TAC ATC TCA TTG CTA-3') for Δ V1, DV2F (5'-ATA GGA GCC GGC CAT TGT AAC ACC AGT-3') and DV2R (5'-ACA ATG GCC GGC TCC TAT GCA AGA ATC ACC-3') for Δ V2, DV3F (5'-TGT AGA GGA GCC GGC TGG TGC CGG TTT GGA-3') and DV3R (5'-GCA CCA GCC GGC TCC TCT ACA TTT CAT TGT-3') for Δ V3, and DV4F (5'-AAG AAT TCT TAT ACT GCA AAG GAG CCG GCC CAT GTC ATA TTA GAC AAA-3') for Δ V4. Mutant Δ Gly was constructed using primers N306AFw2 (5'-TAT TAT GCT CTA ACA ATG AAA TGT AG-3'), N306ARv (5'-CAT TGT TAG AGC ATA ATA CTT-3'), N316AFw (5'-AGA CCA GGA GCT AAG ACA GTT-3'), N316ARv (5'-AAC TGT CTT AGC TCC TGG TCT-3'), N349AFw (5'-GGT TTG GAG GAG CCT GGA GCG-3'), and N349ARv (5'-CGC TCC AGG CTC CTC CAA ACC G-3') to introduce the mutations N306A, N316A, and N349A at potential N-linked glycosylation sites. Mutant D385R was constructed using primers S-D368RFw (5'-CCA GCA GGA GGA CGT CCA GAA GTC AC-3') and S-D368RRv (5'-TTC TGG ACG TCC TCC TGC TGG AGC TGT-3'). The D385R substitution in SIVsmE543-3 corresponds to D368R in HIV-1, which interferes with CD4 binding site (CD4bs) antibodies (13, 44, 45). Mutant I434R was constructed using primers S-I420RFw (5'-GCC ATG TCA TCG TAG ACA AAT AAT CAA C-3') and S-I420RRv (5'-GAT TAT TTG TCT ACG ATG ACA TGG CAC-3'). The I434R substitution in SIVsmE543-3 corresponds to I420R in HIV-1, which interferes with CD4-induced (CD4i) antibodies (13, 45, 46). Amino acid numbering of Env was based on that of SIVmac239, the reference sequence of SIV, and the HIV-2 sequence in the Los Alamos HIV databases (<http://www.hiv.lanl.gov/>).

Flow-cytometric analysis. Plasmids to express wild-type and mutant Env were transfected into 293T cells using X-tremeGENE 9 DNA transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. After incubation for 2 days, the transfected cells were detached with PBS containing 0.05% trypsin and 0.53 mM EDTA and adjusted to 1×10^7 cells/ml in PBS containing 0.2% bovine serum albumin (BSA). To examine the reactivity of Fab, we incubated 50 μ l cells with 10 μ l 50 ng/ μ l Fab for 40 min at RT. After washing with PBS containing 0.2% BSA, the cells were incubated with 50 μ l anti-HA antibody (1:200; 3F10; Roche Molecular Biochemicals) for 20 min at RT, followed by incubation with 50 μ l allophycocyanin (APC)-conjugated AffiniPure goat anti-rat IgG (H+L) F(ab')₂ fragment (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 20 min at RT. When enhancement with sCD4 was examined, cells were resuspended in PBS containing 0.2% BSA in the presence or absence of 2 μ g/ml sCD4 at 1×10^7 cells/ml before staining. After incubation with sCD4 for 15 min at RT, 20 μ l of cells was mixed with 10 μ l 25 ng/ μ l Fab and stained with anti-HA and anti-rat antibodies. Murine MA6 KK46 (1:200) was used as a control antibody against the linear V3 epitope (47). KK46-incubated cells were stained by APC-conjugated goat anti-mouse Ig (1:200; BD Biosciences, Franklin Lakes, NJ). The stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). The reactivity of Fab to Env was determined by comparison with an unstained control after gating EGFP⁺ cells. Data analysis was performed using FlowJo (TreeStar, San Carlos, CA).

Isolation of B404-resistant variants from SIVmac316. The selection of B404-resistant variants from SIVmac316 was performed as described previously (48, 49). Briefly, 5,000 TCID₅₀ SIVmac316 was incubated with 5 ng/ml Fab B404 for 30 min at 37°C. Then, 5×10^4 PM1/CCR5 cells were added to the virus-Fab mixture. After incubation for 5 h, cells were washed with PBS and resuspended in RPMI 1640 medium supplemented with 10% FBS without Fab B404. The culture supernatant was harvested on day 7 and used to infect fresh PM1/CCR5 cells for the next round of culture in the presence of increasing concentrations of Fab B404. A B404-

resistant virus, P26B404, was recovered from the cell culture supernatant at passage 26 at 400 μ g/ml Fab B404. SIVmac316 was also passaged for the same period in PM1/CCR5 cells in the absence of Fab B404, and the resulting virus was designated P26C. Proviral DNA samples were extracted from PM1/CCR5 cells infected with P26B404 and P26C using a QIAamp DNA blood minikit (Qiagen). The gp120 region was amplified by PCR using primers SEnv-F (5'-ATG GGA TGT CTT GGG AAT CAG C-3') and SER1 (5'-CCA AGA ACC CTA GCA CAA AGA CCC-3'), cloned using a TA cloning kit (Invitrogen), and subjected to sequencing.

Nucleic acid sequence analysis. The Ig variable regions were sequenced using the primers ompseq and pelseq (40), and analyzed with V-QUEST in the International Immunogenetics Database (IMGT; <http://www.imgt.org/>) (50). The germ line sequence of the VH gene, from which B404 originated, was determined using the genome database of rhesus macaque (51). Sequences were aligned and phylogenetically analyzed using Molecular Evolutionary Genetics Analysis version 5 (MEGA5) (52).

The gp120 region from P26B404 and P26C was sequenced using primers M13F and M13R in the vector and SE1 (5'-ATA ATA CAG TCA CAG AAC A-3'). Predicted amino acid sequences were aligned using CLC Sequence Viewer 6 (CLC Bio, Aarhus, Denmark), together with other SIV sequences.

Molecular dynamics (MD) simulation of gp120 from B404-resistant variants. MD simulations of the gp120 outer domain of SIVmac316 and the mutants with a F277V or N295S substitution were performed essentially as described for MD simulations of the HIV-1 gp120 outer domain (53). SIV gp120 outer domain structures with various V3 regions were constructed using the homology modeling technique with the Molecular Operating Environment (MOE) 2011.10 (Chemical Computing Group Inc., Montreal, Quebec, Canada). The modeling template was the crystal structure of HIV-1 gp120 containing the entire V3 region at a resolution of 3.30 Å (PDB code, 2QAD [54]) and the SIV gp120 core at a resolution of 4.00 Å (PDB code, 3FUS [55]). The 195 amino-terminal and 7 carboxyl-terminal residues were deleted to construct the gp120 outer domain structures. Glycans were added to the gp120 outer domain structures using Online Glycoprotein Builder (56). MD simulations were performed using the SANDER module in the AMBER 10 program package (57, 58) and the AMBER force field (59) and GLYCAM06 (60) with the TIP3P water model (61). Bond lengths involving hydrogen were constrained with SHAKE (62), and the time step for all MD simulations was set to 2 fs. A nonbonded cutoff of 12 Å was used. After heating calculations for 20 ps until 310 K using the NVT ensemble, the simulations were executed using the NPT ensemble at 1 atm and at 310 K for 50 ns. To map structurally fluctuating sites in the gp120 outer domain, we calculated the root mean square fluctuation (RMSF) of the main chains of individual amino acid residues as described previously (53). Briefly, the RMSF were calculated using the 90,000 snapshots obtained from MD simulations of 5 to 50 ns. The average structures during these MD simulations were used as reference structures for the calculation of the RMSF using the ptraj module in AMBER 10.

Nucleotide sequence accession numbers. Sequence data for Ig clones obtained from SIVsmH635FC-infected macaques were submitted to GenBank under accession numbers JF925337 to JF925378 and JF925380 to JF926116.

RESULTS

Potent and broad neutralizing activity of NAb B404 against various SIV strains. SIV-specific Fab clones were previously isolated from the Fab library from SIVsmH635FC-infected macaque H723 through panning against whole SIV Ag (25). Four Fab clones specific to gp120, represented by B404, showed similar gene usage, epitope specificity, and neutralizing activity that covered homologous and heterologous SIV strains. To define the neutralizing potency of B404 further, IgG, Fab, and scFv with B404 variable regions were constructed and examined for their neutralizing ac-

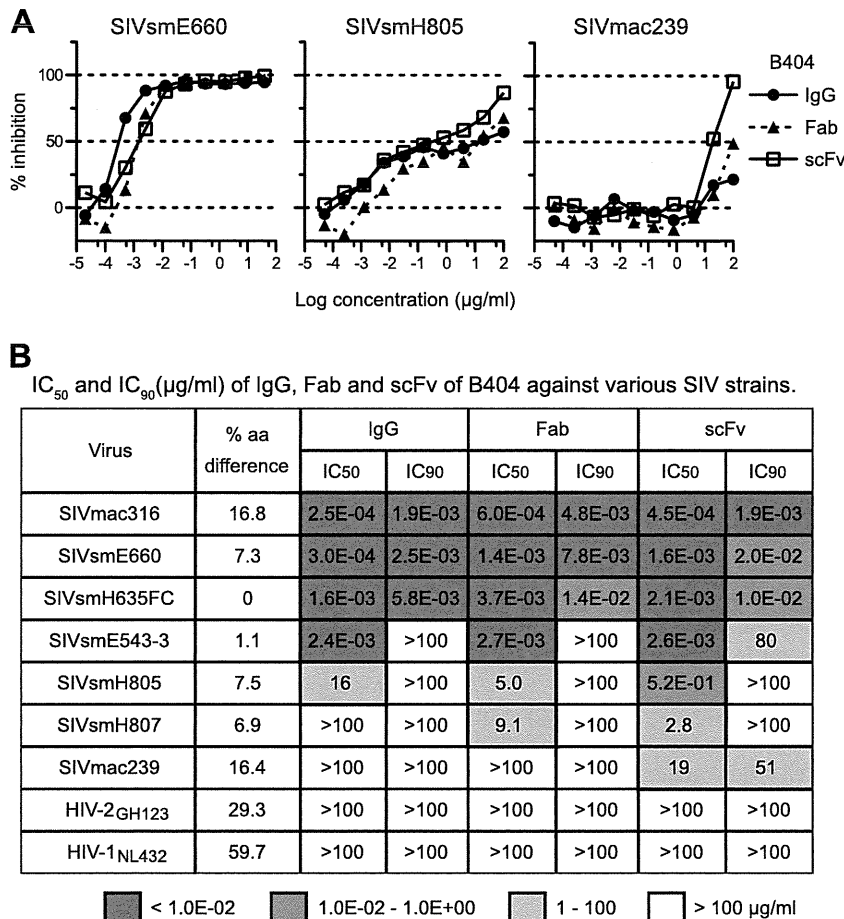


FIG 1 Potent and broad neutralization by monoclonal antibody B404 from a SIVsmH635FC-infected macaque. (A) The neutralizing potencies of IgG, Fab, and scFv of B404 are shown by inhibition kinetics against SIVsmE660FL14, SIVsmH805-24w-3, and SIVmac239. (B) The neutralizing potencies of IgG, Fab and scFv of B404 are shown by IC₅₀ and IC₉₀ (µg/ml). Seven SIV strains, HIV-2_{GH123} and HIV-1_{NL432} were examined for their neutralizing sensitivities against B404 in TZM-bl cells. The IC₅₀ and IC₉₀ values are shown in dark gray (<1.0 × 10⁻² µg/ml), medium gray (1.0 × 10⁻² to 1.0 × 10⁰ µg/ml), light gray (1 to 100 µg/ml) and white (>100 µg/ml). Percent amino acid differences were calculated by pairwise comparison with SIVsmH635FC.

tivity against 7 SIV strains (Fig. 1). These SIV strains were classified into 2 lineages, lineage 1 (SIVsmE660, SIVsmH635FC, SIVsmE543-3, SIVsmH805, and SIVsmH807) and lineage 8 (SIVmac316 and SIVmac239), according to the phylogenetic analysis by Apetrei et al. (63), which identified nine divergent lineages in SIVsm/mac corresponding to HIV-1 subtypes. Sensitivity to neutralization was known to be high in SIVmac316, SIVsmE660, and SIVsmH635FC (25, 38). Infection with these neutralization-sensitive SIV strains was almost completely blocked by low concentrations of all forms of B404. Although the potency to inhibit infection was similar among IgG, Fab, and scFv, B404 IgG was slightly more effective against these SIV strains, as shown by the neutralizing kinetics of SIVsmE660 (Fig. 1A). Neutralization of SIVsmE543-3, SIVsmH805, and SIVsmH807 reached a plateau at 10 to 100 ng/ml IgG B404 and Fab B404, as represented by neutralization kinetics against SIVsmH805 (Fig. 1A). These viruses were moderately sensitive to B404-mediated neutralization, although the IC₅₀s were variable among these SIV strains (Fig. 1B). Interestingly, B404 scFv was more effective at high concentrations than B404 IgG and B404 Fab in neutralization of these moderately neutralization-sensitive viruses and SIVmac239, which is a highly neutralization-resistant strain

(Fig. 1). Infection with SIVmac239 was unaffected by the presence of any form of B404 at a concentration of less than 1 µg/ml but was inhibited more than 90% by 100 µg/ml B404 scFv. Neutralization of 7 of 7 SIV strains, including genetically diverse, neutralization-resistant SIVmac239, by B404 scFv indicates that B404 is a potent and broad NAb against SIVsm/mac strains.

Isolation of Env-specific Fab clones from SIVsmH635FC-infected macaques. To analyze the induction of B404-like antibodies in SIV-infected macaques, Env-specific Fab clones were isolated from 4 SIVsmH635FC-infected macaques: H723, H704, H709, and H714 (26, 39). Env-specific Fab clones from H723 were isolated from the previously constructed phage library (25) through panning against Env, which was conjugated by coating plate wells with the anti-Env Fab clones B404 (anti-gp120 conformational), B408 (anti-gp41 cluster I), and H301 (anti-gp120 V1). Together with anti-Env Fab clones from the previous study, 98 anti-Env Fab clones, including 33 NAb (33.7%), were obtained from H723. From 3 other SIVsmH635FC-infected macaques, κ and λ light-chain phage libraries were separately constructed, and 2 panning series were performed using B404 and H301 to conjugate Env. After 4 series of panning in each macaque, we obtained 155, 102, and 53 independent Fab clones from H704, H709, and

TABLE 1 Preferential gene usage and competition with B404 of NAb

Inoculated virus	Animal	Frequency (%) of NAb ^a	No. of NAb	% of NAb with:		Avg CDRH3 length ^b	Competition (%) with B404 ^c
				VH3 genes	λ light chains		
SIVsmH635FC	H723	33.7	33	81.8	93.9	20.0	81.8
	H704	30.3	47	95.7	97.9	18.7	100
	H709	7.8	8	75.0	100	17.8	100
	H714	52.8	28	96.4	92.9	18.7	100
SIV mix ^d	H711	17.2	15	26.7	40.0	15.2	86.7
	H725	2.2	1	0.0	100	12	100

^a Frequencies of Fab clones with the VH3 gene and λ light chain are shown as percentages of NAb.

^b Average number of amino acids in CDRH3.

^c Competition ELISA with 2 μg/ml B404 IgG was performed. The frequency of NAb showing more than 50% inhibition is shown as a percentage of NAb.

^d H711 was inoculated with a mixture of SIVsmE543-3 and SIVsmH635FC. H725 was inoculated with plasma samples from 2 SIVsmH445-infected macaques, H631 and H635.

H714, respectively. Neutralizing activities were observed in 47 clones (30.3%) from H704, 9 clones (8.8%) from H709, and 28 clones (52.8%) from H714 (Table 1). Phylogenetic analysis of VH genes revealed that 105 NAb formed a major NAb cluster with B404 (Fig. 2). The remaining NAb were separated into 3 minor clusters containing 3 or 4 NAb. Fab clones in the major group, designated the B404 group, were isolated from all 4 macaques analyzed.

Although Fab clones in the B404 group were genetically similar to one another, several small clusters were observed in the B404 group, suggesting multiple B cell origins generated by VDJ recombination in these B404-like NAb. Sequence analysis using the International Immunogenetics Database (50) indicated that the VH genes of Fab clones in the B404 group were close to human pseudogene IGHV3-h (approximately 90% identity), but no

functional VH gene showed >90% identity. Analysis using the genome database of the rhesus macaque (51) revealed a significant relationship (>95% identity) between Fab clones in the B404 group and the rhesus macaque VH3 gene in the chromosome 7 scaffold (GenBank accession number NW_001122023). These results suggest that a major group of NAb in SIVsmH635FC-infected macaques preferentially use the same rhesus VH3 germ line that lacks a human counterpart.

Bias in gene usage of NAb from SIVsmH635FC-infected macaques. The genetic features of anti-Env Fab clones are summarized in Fig. 3 and Table 1. As mentioned above, a major population of NAb from SIVsmH635FC-infected macaques used the same VH3 gene as B404, resulting in a high rate of NAb using the VH3 gene (Fig. 3A and Table 1). A high occupancy of λ light chains was also characteristic of NAb from SIVsmH635FC-infected macaques (Fig. 3B and Table 1). Moreover, a long complementarity-determining region 3 loop of the heavy chain (CDRH3) was characteristic of the NAb (Fig. 3C and Table 1). CDRH3 of most NAb had 19 or more amino acids, although the length of CDRH3 was usually less than 18 amino acids in nonneutralizing Fab clones. These results clearly showed that B404-like NAb with the VH3 gene-encoded heavy chain with a long CDR3 and λ light chain are the main NAb population in SIVsmH635FC-infected macaques. In contrast, Fab clones from macaques H711 and H725 lacked these remarkable features of B404-like NAb (Fig. 3, bottom; Table 1). These 2 macaques were inoculated with a mixture of SIVs (SIV mix). H711 was infected with a combination of SIVsmE543-3 and SIVsmH635FC. H725 was infected with plasma samples from 2 SIVsmH445-infected macaques, H631 and H635, which SIVsmH635FC was isolated from. Although anti-Env Fab clones were similarly isolated from these macaques, the frequency of NAb from H711 (17.2%) and H725 (2.2%) was lower than that from SIVsmH635FC-infected macaques (8.8 to 52%). NAb from H711 and H725 preferentially used VH1 gene-encoded heavy chains with a short CDRH3 and κ light chains, but NAb from these macaques showed a genetic variation, similarly to those in HIV-1-infected patients (64). These results suggested that B404-like NAb are induced exclusively in SIVsmH635FC-infected macaques.

Potent neutralizing activity and the same specificity of NAb in the B404 group. To analyze the epitopes recognized by these NAb, we first separated Fabs with neutralizing activity into 2 groups according to the results of competition ELISA with B404 IgG. All the NAb in the B404 group and group II (Fig. 2) com-

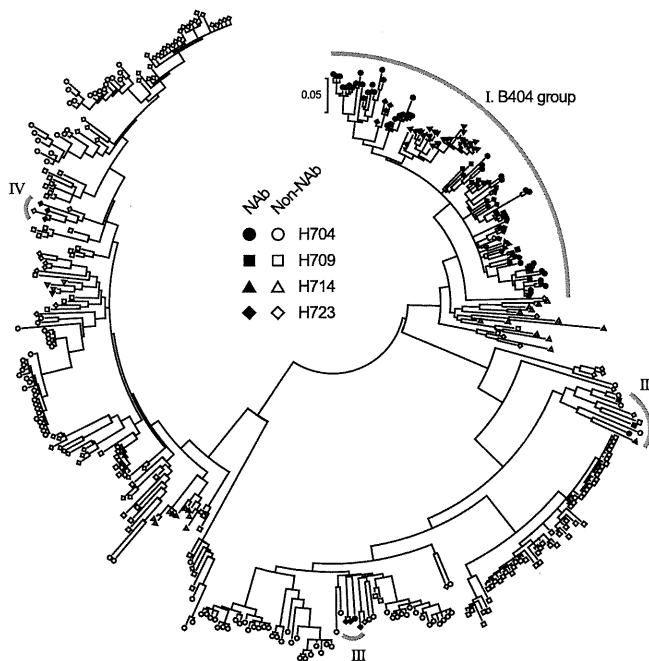


FIG 2 B404-like NAb formed a major group in anti-Env antibodies from 4 SIVsmH635FC-infected rhesus macaques. NAb were separated into 4 groups in the phylogenetic tree, which was generated using MEGA5 (70) from heavy-chain genes of 98, 155, 102, and 53 Fab clones from H704, H709, H714, and H723, respectively.

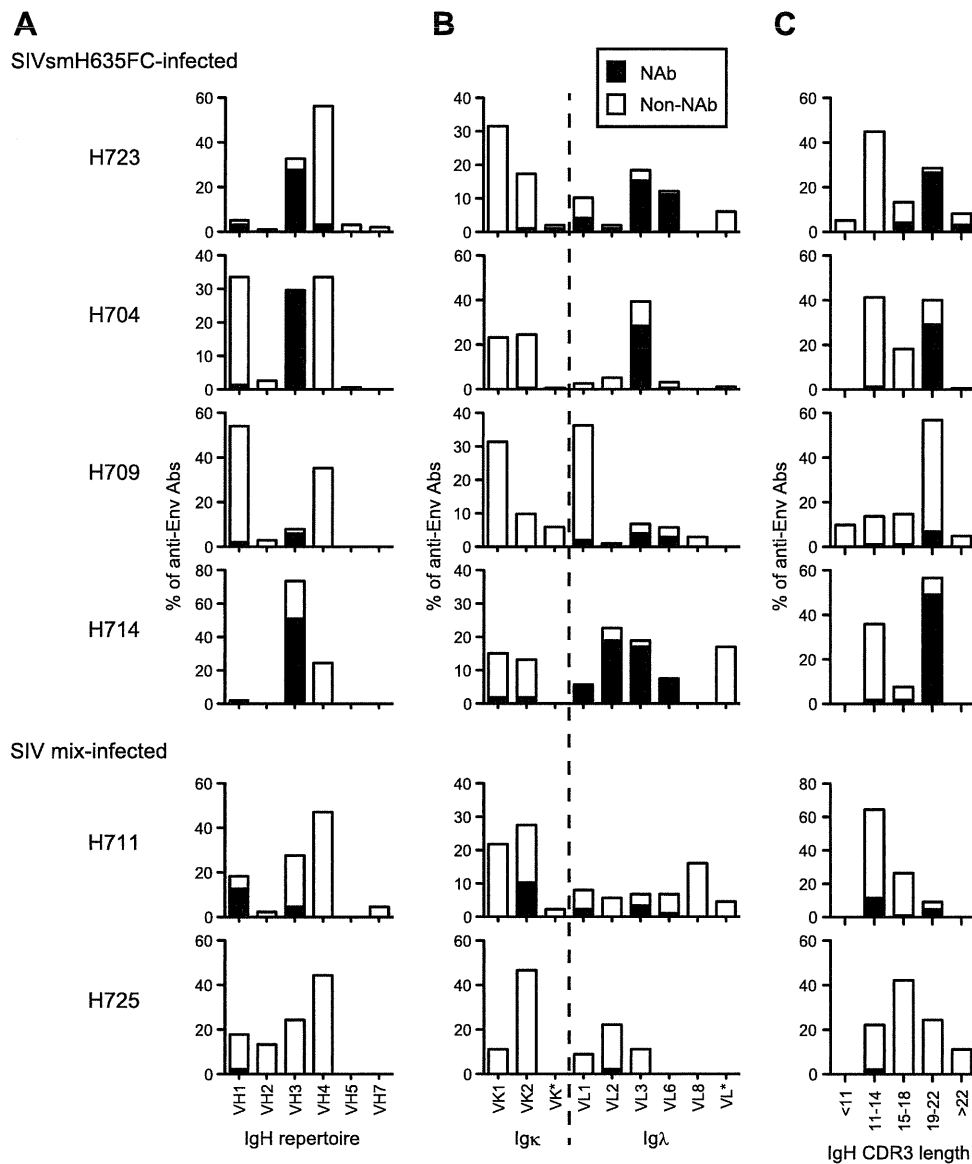


FIG 3 Bias in the Ig gene usage and CDR3 length of neutralizing Fab clones from SIVsmH635FC-infected macaques. The proportions of IgH (A) and Ig λ (B) repertoires and heavy-chain CDR3 length (C) are shown as percentages of NABs and non-NABs. In addition to Fabs isolated from 4 SIVsmH635FC-infected macaques, 87 Fab clones from H711, which was infected with a combination of SIVsmE543-3 and SIVsmH635FC, and 45 Fab clones from H725, which was infected with plasma samples from 2 SIVsmH445-infected macaques, H631 and H635, were similarly analyzed. Usage of Ig genes was analyzed using V-QUEST in the International Immunogenetics Database (44).

peted with B404 IgG (Fig. 4A), suggesting that epitopes for these NABs overlap or are close to that for B404. Despite the differences in gene usage, competition with B404 IgG was also observed in most NABs from macaques infected with SIV mix (Table 1). NABs belonging to groups III and IV, with the exception of 1 Fab in group III, did not compete with B404 IgG (Fig. 4A). The binding ability of these Fabs was even enhanced by the addition of B404. Competition of the Fabs in groups III and IV with biotinylated K8 in group III suggested that the Fabs in group III and IV share the same epitope (data not shown). The neutralizing activity of these Fabs was examined against the genetically divergent SIVmac316 and the neutralization-resistant SIVsmE543-3 (Fig. 4B). All of the Fabs tested showed at least 50% inhibition against both viruses, and the B404 group included Fabs with potent neutralizing activ-

ity that showed efficient inhibition at low concentrations. Accordingly, the IC_{50} s of the 4 Fabs in the B404 group ranged from 0.8 to 316 ng/ml (average IC_{50} , 79 ng/ml) against SIVmac316, indicating the presence of NABs comparable to B404 (IC_{50} against SIVmac316, 0.6 ng/ml) (Fig. 1). In contrast, IC_{50} against SIVmac316 ranged between 32 and 908 ng/ml (average IC_{50} , 243 ng/ml) in groups III and IV. This result suggests that B404-like NABs are the main NAB population in terms of number and neutralizing potency.

Epitope mapping of NAB B404. To define the region of the Env targeted by B404, we examined reactivity against mutants of SIVsmE543-3 Env. Because the V3 loop has been shown to be important for B404 binding (25), mutants with deletions in the V1, V2, V3, and V4 loops (Δ V1, Δ V2, Δ V3, and Δ V4) and a mu-

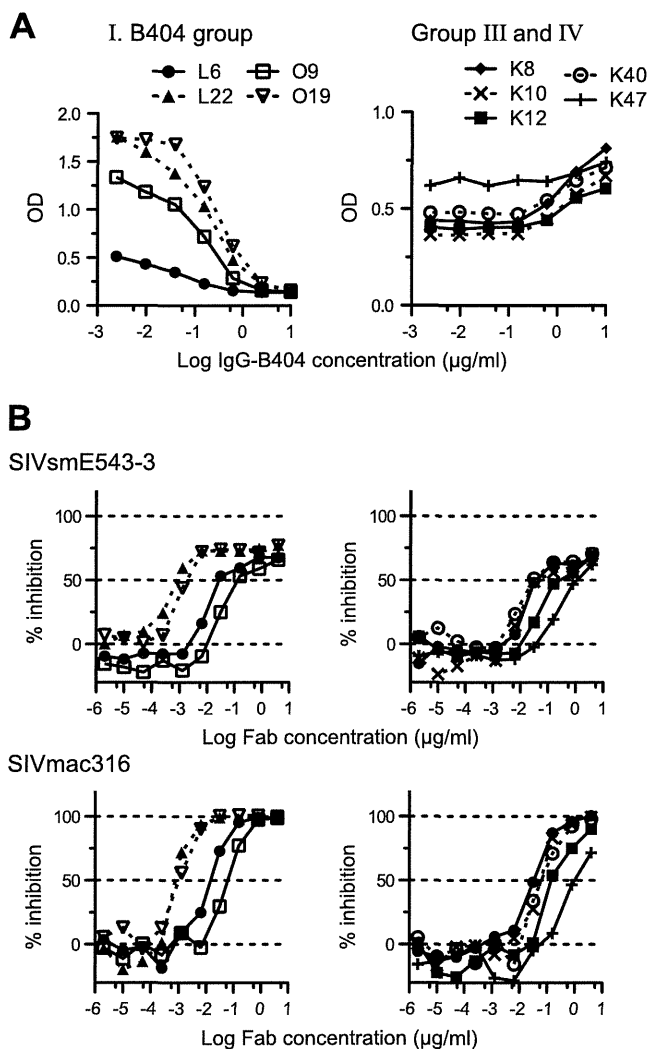


FIG 4 The specificity and potency of Fab clones in the B404 group are similar to those of B404. (A) Competition ELISA was performed using serially diluted B404 IgG as a competitor. B404 IgG significantly inhibited the binding of the Fabs in the B404 group (L6, L22, O9, and O19). In contrast, B404 IgG did not compete with the Fabs in groups III (K8 and K10) and IV (K12, K40, and K47) and even enhanced the binding of these Fabs. (B) Neutralization potencies of Fabs in the B404 group (left) and groups III and IV (right) are shown by inhibition of infection to TZM-bl cells with neutralization-resistant SIVsmE543-3 and genetically divergent SIVmac316.

tant lacking 3 glycosylation sites flanking the V3 loop (Δ Gly) were constructed. In addition, mutants carrying single mutations in the CD4bs (D385R) and CD4i (I434R) sites, corresponding to D368R and I420R in HIV-1 gp120 (13, 44–46), were examined to clarify the relationship of the B404 epitope to the CD4bs and CD4i sites. Flow cytometry analysis using cells expressing these Env mutants revealed that the reactivity of B404 was completely lost in Δ V3 and Δ V4 mutants, though B404 bound to other mutants even better than it did to the wild type (Fig. 5A). These results suggested that B404 recognizes a conformational epitope consisting of the V3 and V4 loops.

The reactivity of another Fab, K8, which targets an epitope other than that of B404 (Fig. 4A), was lost in Δ V4 and I434R mutants (Fig. 5A). No reactivity to I434R strongly suggested that

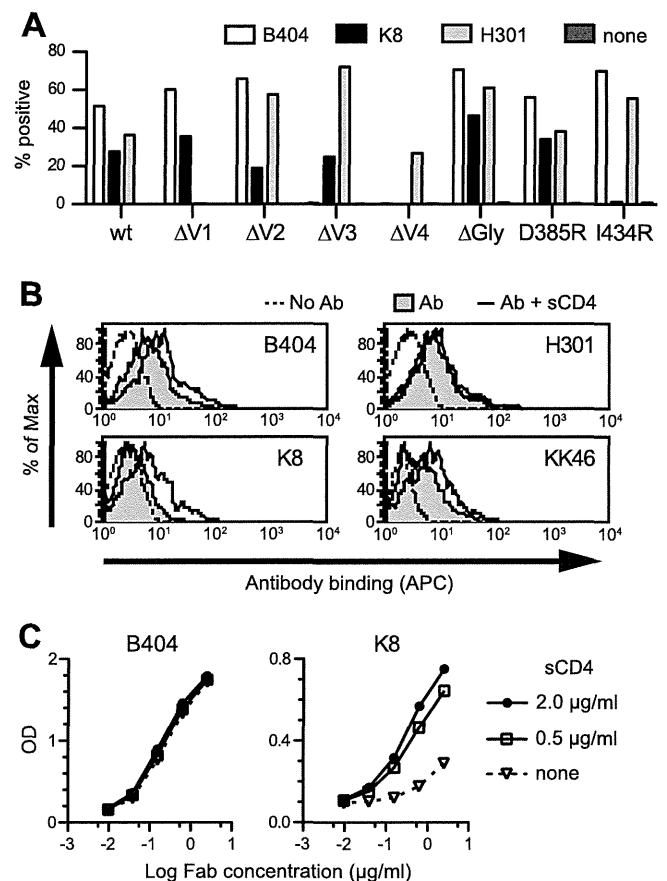


FIG 5 B404 recognizes a conformational epitope, including the V3 and V4 loops, and sCD4 enhances the exposure of the epitope in trimeric Env. (A) Reactivity of B404, K8, and H301 (anti-V1 Fab) to Env mutants was examined using 293T cells transfected with plasmids to express SIVsmE543-3 Env (wild-type), mutants with deletions in the V1 (Δ V1), V2 (Δ V2), V3 (Δ V3), and V4 (Δ V4) loops, an N306A/N316A/N349A mutant lacking glycosylation sites near the V3 loop (Δ Gly), a D385R mutant interfering with CD4bs antibodies (D385R), and an I434R mutant interfering with CD4i antibodies (I434R). The transfected cells were stained with Fabs B404, K8, and H301, and the reactivity of Env mutants was analyzed using flow cytometry. The percentage of Fab⁺ cells is shown. (B) Reactivity of Fabs B404, K8, and H301, and murine anti-V3 MAb KK46 to sCD4-treated trimeric Env on the cell surface. Cells transfected with the plasmid to express SIVsmE543-3 Env were incubated with 2 μg/ml sCD4 for 15 min, and the reactivities of antibodies were similarly examined. The tinted histogram represents cells stained by antibody in the absence of sCD4. The dotted line shows the unstained control. (C) Reactivity of Fab clones B404 and K8 to sCD4-treated monomeric Env. The reactivity of serially diluted Fab to Env was examined by ELISA using SIVsmE543-3 as an antigen in the absence or presence of 0.5 or 2.0 μg/ml sCD4.

K8 is a CD4i antibody. Therefore, the effect of sCD4 ligation on antibody binding to Env trimers and monomers was examined using flow cytometry and ELISA. The reactivity of B404, K8, and KK46 (murine anti-V3 MAb) to Env on the cell surface was enhanced by the addition of sCD4, although no effect was observed in anti-V1 Fab H301 (Fig. 5B). This suggested that epitopes for B404, K8, and KK46 are exposed in the open conformation of the Env trimer triggered by CD4 binding. Consistent with the analysis of mutant Envs, the reactivity of K8 to Env monomer was enhanced by the addition of sCD4, but B404 showed no enhancement of reactivity (Fig. 5C).

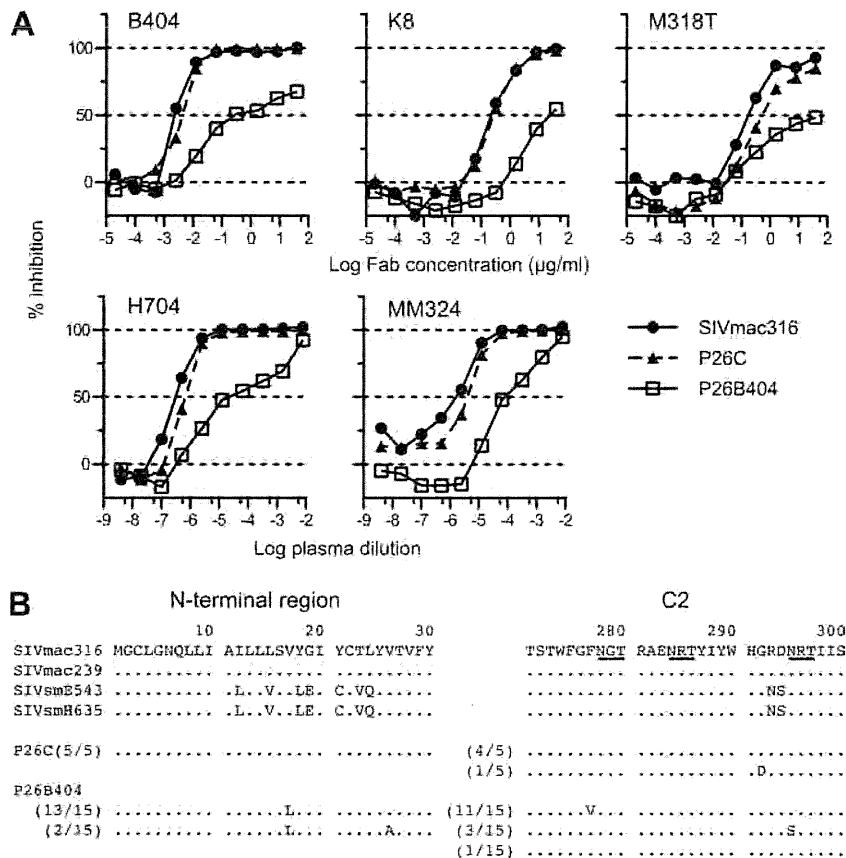


FIG 6 Isolation of variants resistant to B404 and amino acid substitutions in gp120. The B404-resistant variant was induced from SIVmac316 by passages of viruses in PM-1/CCR5 cells with increasing concentrations of B404 Fab. A B404-resistant variant, P26B404, was obtained from the supernatant of passage 26. P26C was obtained after 26 passages without B404. (A) The sensitivities of P26B404, P26C, and parental SIVmac316 to neutralization are shown by inhibition of infection of TZM-bl cells. B404 and K8 Fabs, murine MAb M318T, which recognizes the V2 of gp120, and plasma samples from SIVsmH635FC-infected macaque H704 and SIVmac239-infected macaque MM324 were used for the neutralization assay. (B) Amino acid sequences of the N-terminal and C2 regions of gp120 from P26C and P26B404 are aligned with those of parental SIVmac316 and SIV strains, SIVmac239, SIVsmE543-3 and SIVsmH635FC. The number of clones per total number of clones is given in parentheses. Identical amino acids are shown as dots, and potential glycosylation sites in SIVmac316 are indicated with underlining.

The enhancement by sCD4 of reactivity to both monomeric and trimeric Env and the interference in binding by the I434R (I420R in HIV-1) mutation in Env, which are features of so-called CD4i antibodies against HIV-1 (13, 45, 46), indicate that K8 targets the CD4i epitope. The enhanced reactivity of B404 by sCD4 to trimeric but not monomeric Env is analogous to the reactivity of anti-V3 antibodies (65). These results suggest that B404 recognizes a conformational epitope consisting of the V3 and V4 loops, which are intensely exposed on the Env trimer after CD4 ligation.

Selection of variants resistant to NAb B404. To select B404-resistant variants *in vitro*, we passaged SIVmac316, which is the most sensitive to B404 of the SIV strains tested (Fig. 1), in PM1/CCR5 cells in the presence of increasing concentrations of B404. As a control, passage under the same conditions without B404 was also performed to monitor spontaneous changes during infection in PM1/CCR5 cells. The concentration of B404 was increased from 5 ng/ml to 400 $\mu\text{g/ml}$ at passage 26. Viruses recovered at passage 26 in the presence and absence of B404, which were designated P26B404 and P26C, respectively, were examined for their sensitivity to antibodies and plasma samples from SIV-infected macaques (Fig. 6A). The IC₅₀ for B404 against SIVmac316, P26C, and P26B404 were 2.8, 4.1, and 240 ng/ml, respectively, showing

an 86-fold resistance of P26B404 to B404 compared with that of wild-type SIVmac316. P26B404 was also resistant to neutralization by MAbs K8 (CD4i) and M318T (V2), which target epitopes other than that of B404, and plasma samples from SIV-infected macaques (Fig. 6A). These results suggested that P26B404 acquired resistance to antibody-mediated neutralization comparable to that observed in neutralization-resistant SIV strains, such as SIVmac239 and SIVsmE543-3. Sequence analysis of gp120 revealed 3 amino acid substitutions specific to P26B404: V17L in the N-terminal region and F277V and N295S in the C2 region (Fig. 6B). Of these substitutions, the two in the C2 region were highly conserved among SIVsm/mac and HIV-2 strains. These substitutions were independently observed, and no variant with both F277V and N295S was found in the 15 clones sequenced.

MD simulation of gp120 outer domains from B404-sensitive and B404-resistant variants. To address structural impacts of the 2 mutations in the C2 region, we performed MD simulation of unliganded gp120 outer domains from B404-sensitive (SIVmac316) and B404-resistant (F277V and N295S) variants. To map the sites at which structural dynamics were influenced by C2 mutations, we calculated the RMSF of the main chains of individual amino acid residues using 90,000 snapshots from 5 to 50 ns of

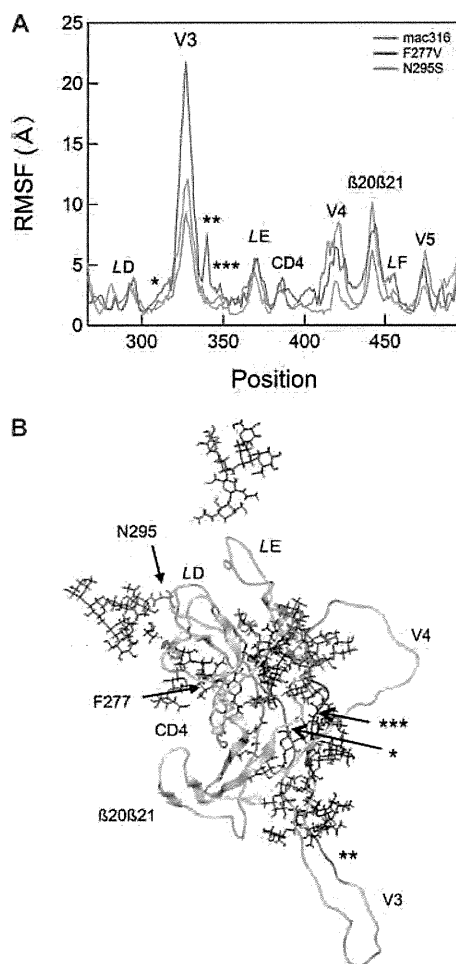


FIG 7 Effects of F277V and N295S mutations on the structural dynamics of the gp120 outer domain. (A) Distribution of RMSF in the gp120 outer domain. MD simulations of gp120 outer domains of SIVmac316, F277V, and N295S were carried out at 1 atm and 310 K for 50 ns as described in Materials and Methods. The RMSF values, which indicate the atomic fluctuations of the main chains of individual amino acids during MD simulations, were calculated using 90,000 snapshots from 9 to 50 ns of each MD simulation. The numbers on the horizontal axes indicate amino acid positions in gp120. The RMSF values of the mutants are significantly different from those of parental SIVmac316 at the V3 loop, the V3 flanking regions (indicated by asterisks), the V4 loop, the $\beta 20\beta 21$ /LF loop, and the V5 loop regions. (B) A structure at 50 ns of MD simulation of the SIVmac316 gp120 outer domain is shown as a representative to indicate the steric location of mutation sites and various loops. The regions that are proximal to the V3/V4 loops and displayed fluctuations that differed from those of parental SIVmac316 (Fig. 7A) are highlighted in orange (*), blue (**), and purple (***) . Green sticks indicate glycans.

each MD simulation (Fig. 7A). RMSF values provide key information about the atomic fluctuations of the individual amino acids of a protein in solution (57). These values were maximal at the tip of the V3 loop and prominent at other loop regions, including LD, LE, CD4 binding, V4, $\beta 20\beta 21$ /LF, and V5 (Fig. 7A), suggesting that these loops fluctuate in solution. Notably, the F277V and N295S mutations were found to induce changes in RMSF values mainly at the V3, V4, $\beta 20\beta 21$ /LF, and V5 loop regions (Fig. 7A, blue and green lines, respectively). Interestingly, these regions are located far from the C2 mutation sites compared with the locations of other loops, such as LD, LE, and CD4 binding, which had

RMSF values that were similar among wild-type and B404-resistant variants (Fig. 7B; F277V in LD and N295S in the proximal region of LD). In particular, RMSF changes by the C2 mutations were the most prominent at the V3/V4 loops and their neighboring regions (Fig. 7). These results suggested that the F277V and N295S mutations could alter the structural dynamics of V3/V4 loops and their neighboring regions in solution. The structural alterations would lead to changes in entropy of the regions, which affect the binding affinity to B404. The finding is consistent with the results of an epitope mapping study of B404.

DISCUSSION

Potent and broadly neutralizing MAbs have recently been isolated from HIV-1-infected patients and analyzed to understand the mechanism of neutralization against a broad spectrum of HIV-1 strains and to design vaccines against their neutralizing epitopes (12–16). Although the SIV-macaque model has been used as an animal model for HIV-1 infection for vaccine development (6, 8, 24), no potent and broadly neutralizing monoclonal antibody against SIV was available. Therefore, the epitopes and mechanism for broad neutralization of SIV remained uncertain. Many monoclonal antibodies against SIVsm/mac were isolated from SIV-infected macaques (66, 67) and mice immunized with SIV Env (43, 47, 68), but few of them showed neutralizing activity against various SIV strains, including highly neutralization-resistant SIVmac239 (68). In comparison with these monoclonal antibodies against SIV identified so far, B404 apparently has a broadly neutralizing activity, which enables it to neutralize multiple, diverse SIV isolates, and can be defined as the first generation of broadly NABs against SIV. The broad and potent neutralizing activity of B404 shown in this study indicates that B404 can be used to analyze broad neutralization against SIV. The B404 epitope, the newly identified broadly neutralizing epitope against SIV, will further understanding of the mechanism of broad neutralization effective for protection from SIV infection.

The infection of rhesus macaques with SIVsmH635FC, a highly neutralization-sensitive clone, was chosen for this study because this SIV strain induced a vigorous and potent antibody response in all the infected macaques and acquired many viral mutations to escape antibody recognition (25, 26). The kinetics of B404 neutralization against various SIV strains were similar to those observed in the plasma sample of the macaque from which B404 was isolated, suggesting that B404-like NABs are representative of the neutralizing activity in SIVsmH635-infected macaque H723 (25). Consistent with this observation, B404-like NABs were shown to be a major group in NABs genetically and functionally. Most of the NABs in the 4 SIVsmH635FC-infected macaques analyzed had the same features, including the use of a specific VH3 germ line and λ light chains, a long CDRH3 loop, and competition with B404. The bias in the specificity and gene usage may be partially enhanced by the screening process, because B404-like NABs were predominantly isolated from λ light-chain libraries by panning against H301-conjugated Env. In addition, isolating antibodies against quaternary epitopes constituted by the Env trimer through panning using monomeric Env was difficult. However, the presence of many independent B404-like NABs strongly suggests that B404-like NABs compose a significant fraction of NABs in 4 SIVsmH635FC-infected macaques. Moreover, the vigorous induction of B404-like NABs in SIVsmH635FC-infected macaques was also supported by the multiple B cell origins apparent

from several subgroups in B404-like NAb (Fig. 1). These subgroups originated from distinct B cell precursors generated by VDJ recombination, because they were often distinguished by the length and nucleotide sequences of CDRH3.

The observation of few B404-like NAb from macaques infected with the SIV mix clearly indicates that induction of B404-like NAb depends on infection with SIVsmH635FC. The exclusive induction of B404-like NAb only in SIVsmH635FC-infected macaques also raises the possibility that Env from SIVsmH635FC is a highly immunogenic protein that induces antibodies against the B404 epitope. The use of Env from SIVsmH635FC for vaccination may be advantageous for the induction of broadly neutralizing antibodies, because the Env from an HIV-1-infected patient with broadly neutralizing antibodies induced cross-reactive anti-HIV-1 NAb in an animal model (69). The Env in this vaccination study is capable of mediating CD4-independent infection. Since the Env of SIVsmH635FC has a mutation in the CD4-binding region (D385N), NAb induction may be affected by CD4 independence of Env. The development of vaccines aimed at inducing B404-like NAb in rhesus macaques will be useful in establishing models for development of antibody-based vaccines targeting specific epitopes for broad neutralization.

Biased usage of a specific VH3 germ line gene and λ light chain are remarkable genetic features of B404-like NAb. The induction of NAb with specific germ line genes, such as VH1-69 for CD4i (70) and VH5-51 for V3 (64), is frequently observed in HIV-1-infected patients. A close relationship between VH germ line genes and target epitopes suggests the importance of Ig gene usage in the induction of broadly neutralizing antibodies. Therefore, rational design of vaccines has been undertaken based on reactivity to antibodies with the germ line genes used by known broadly neutralizing antibodies (11, 71, 72). Unfortunately, the VH3 germ line gene of B404 is divergent from all human VH3 germ line genes, suggesting the absence of a human counterpart. This may partially explain why B404-like NAb have not been identified in HIV-1-infected humans, although the structure of HIV-1 Env, which is different from that of SIV, significantly affects immunogenicity of the B404 epitope. Rhesus macaque-specific germ line genes were also used by NAb against the quaternary epitope of HIV-1 Env from simian and human immunodeficiency virus (SHIV)-infected macaques, but their germ line genes were different from the VH3 germ line gene used by B404 (73). Even in the presence of a human VH gene counterpart, the antibody response to a neutralizing epitope may differ between rhesus macaques and humans (74). In addition to the genetic diversity of germ line genes, the rhesus macaque CDRH3 repertoire differs from that of humans, resulting in species-specific antibody repertoires (75). This species specificity in antibody induction is a problem in the evaluation of HIV-1 vaccines in animal models, especially those designed for specific neutralizing epitopes of HIV-1. Conversely, K8, the CD4i NAb from a SIV-infected macaque, used the rhesus VH1 germ line gene, an analog of human VH1-69 frequently used by CD4i NAb in HIV-1-infected humans (70). Thus, the mechanism of induction of CD4i NAb with VH1-69 may be common in both humans and rhesus macaques. To analyze vaccine candidates properly in nonhuman primates, similarities and differences in antibody response between rhesus macaques and humans should be considered.

B404 recognizes a conformational epitope consisting of the V3 and V4 loops. The enhanced exposure of the epitope in trimeric

Env by sCD4 and efficient neutralization of neutralization-resistant SIV strains by the scFv form of B404 suggests that the B404 epitope is sterically masked by the V1/V2 loops and glycans, analogous to CD4i and V3 epitopes (46, 65, 76). Consistent with this interpretation, B404 reacted more intensely to Env mutants lacking the V1 and V2 loops than to the wild-type Env. MD simulation supported these observations by indicating changes in the structural dynamics of V3/V4 loops and their neighboring regions in gp120 of resistant variants with F277V and N295S mutations in the C2 region. The acquisition of resistance to the broadly neutralizing antibodies b12, PG9, and PG16 due to C2 mutations far from the target epitopes was also observed in HIV-1 CRF01_AE (77, 78). MD simulation is a powerful computational method for analyzing structural dynamics of proteins in solution on the basis of theoretical and empirical principles in physical chemistry (79) and has been applied to research on viruses (80). The structural dynamics of protein surfaces in solution plays a key role in protein interactions, and MD simulation is advantageous in speculation about interactions between proteins containing flexible regions, such as V3 and V4 loops. Analysis of fluctuation changes in mutant proteins is useful to identify the regions which affect protein-protein interaction.

Although the V3 and V4 loops are known to contain linear epitopes for antibody-mediated neutralization in SIV infection, no conformational MAb against SIV with characteristics similar to those of B404 has been reported (43, 47, 66–68). Several MAbs to conformational epitopes that include the V3 region, such as PGT antibodies, represented by PGT128 (15, 19), 3BC176, and 3BC315 (81), were isolated from HIV-1-infected patients. The PGT128 epitope consists of the short segment of the V3 loop and 2 neighboring glycans (19), but the binding of B404 is independent of these glycans near the V3 region. The epitope of 3BC176 and 3BC315 is close to the V3 loop, and their binding is partially enhanced by CD4 binding, similar to that of B404 (81). However, in contrast to B404, 3BC176 and 3BC315 do not bind to monomeric Env and compete with CD4i antibodies, suggesting that their epitope is different from that of B404. Although we have not determined the precise B404 epitope, the characteristics of B404 that are similar to those of CD4i and V3 antibodies suggest that B404 recognizes a conserved region important for binding to the CCR5 coreceptor (46, 65, 76, 82).

Despite recent progress in understanding the broad neutralization of HIV-1, epitopes for potent and broad neutralization have not been analyzed in an SIV model because NAb analysis using SIV cannot be directly applied to HIV-1. The main disadvantage of SIV is the antigenicity difference relative to HIV-1, which makes examination of neutralizing epitopes of HIV-1 impossible. Although the use of SHIV expressing HIV-1 Env enables the evaluation of vaccine candidates designed for target epitopes of HIV-1 in nonhuman primates, an SIV model was more predictive of vaccine efficacy than a SHIV model in clinical trials of a T-cell-based vaccine (83, 84). For comprehensive assessment of immunity induced by vaccine candidates, proof-of-concept trials using the SIV model should be considered before further efficacy trials. The identification of B404 with its potent and broad neutralizing activity against SIV will be a useful adjunct for evaluating the mechanism of neutralization in an SIV-macaque model and will contribute to the development of HIV-1 vaccines.

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Impact of antiretroviral pressure on selection of primary human immunodeficiency virus type 1 envelope sequences *in vitro*

Shigeyoshi Harada,^{1,2} Kazuhisa Yoshimura,^{1,2} Aki Yamaguchi,¹ Samatchaya Boonchawalit,^{1,2} Keisuke Yusa³ and Shuzo Matsushita¹

Correspondence
Kazuhisa Yoshimura
ykazu@nih.go.jp

¹Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan

²AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

³Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, 1-18-1 Kami-youga, Setagaya-ku, Tokyo 158-8501, Japan

The initiation of drug therapy results in a reduction in the human immunodeficiency virus type 1 (HIV-1) population, which represents a potential genetic bottleneck. The effect of this drug-induced genetic bottleneck on the population dynamics of the envelope (Env) regions has been addressed in several *in vivo* studies. However, it is difficult to investigate the effect on the *env* gene of the genetic bottleneck induced not only by entry inhibitors but also by non-entry inhibitors, particularly *in vivo*. Therefore, this study used an *in vitro* selection system using unique bulk primary isolates established in the laboratory to observe the effects of the antiretroviral drug-induced bottleneck on the integrase and *env* genes. Env diversity was decreased significantly in one primary isolate [KP-1, harbouring both CXCR4 (X4)- and CCR5 (R5)-tropic variants] when passaged in the presence or absence of raltegravir (RAL) during *in vitro* selection. Furthermore, the RAL-selected KP-1 variant had a completely different Env sequence from that in the passage control (particularly evident in the gp120, V1/V2 and V4-loop regions), and a different number of potential *N*-glycosylation sites. A similar pattern was also observed in other primary isolates when using different classes of drugs. This is the first study to explore the influence of anti-HIV drugs on bottlenecks in bulk primary HIV isolates with highly diverse Env sequences using *in vitro* selection.

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INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) shows a high degree of genetic diversity owing to its high rates of replication and recombination and the high mutation rate of the HIV-1 reverse transcriptase (Nájera *et al.*, 2002). Even in a single infected individual, the virus can best be described as a population of distinct, but closely related, genetic variants or 'quasi-species' (Eigen, 1993; Nijhuis *et al.*, 1998). The quasi-species behaviour of viruses is recognized as a key element in our understanding and modelling of viral evolution and disease control (Vignuzzi *et al.*, 2006).

The GenBank/EMBL/DDBJ accession numbers for the *env* sequences of HIV-1 KP-1, KP-2 and KP-4, are AB640872–AB640881, AB641341–AB641351 and AB641335–AB641340, respectively.

Two supplementary figures are available with the online version of this paper.

Combination antiretroviral (ARV) therapy results in a contraction of the viral population, which represents a potential genetic bottleneck (Charpentier *et al.*, 2006; Delwart *et al.*, 1998; Ibáñez *et al.*, 2000; Kitrinis *et al.*, 2005; Nijhuis *et al.*, 1998; Nora *et al.*, 2007; Sheehy *et al.*, 1996; Zhang *et al.*, 1994). Whilst this bottleneck has a direct effect on the region that is being targeted by the drugs (e.g. protease or reverse transcriptase), it also affects other regions of the viral genome. Indeed, the effect of the drug-induced genetic bottleneck on the population dynamics of the envelope (Env) regions has been addressed in several *in vivo* studies (Charpentier *et al.*, 2006; Delwart *et al.*, 1998; Ibáñez *et al.*, 2000; Kitrinis *et al.*, 2005; Nijhuis *et al.*, 1998; Nora *et al.*, 2007; Sheehy *et al.*, 1996; Zhang *et al.*, 1994).

Virus bottleneck evolution of the HIV-1 *env* gene might be important when choosing the optimal drugs to treat a particular patient. Indeed, a CCR5 antagonist (maraviroc, MVC) and a fusion inhibitor (enfuvirtide, T-20) have now

been approved for use as HIV-1 entry inhibitors. Analysing the dynamics of drug-induced genetic bottlenecks and studying drug-resistant mutation profiles in response to HIV-1-specific ARV drugs are both important if we are to understand fully HIV-1 drug resistance and pathogenesis.

The aim of the present study was to understand better the effect of *in vivo* drug-induced genetic bottlenecks. *In vitro* selection of different primary HIV-1 isolates was performed using the recently approved HIV integrase inhibitor raltegravir (RAL) (Steigbigel *et al.*, 2008). Two R5-, one X4-, one dual- and one mixed R5/X4-tropic isolates were passaged through a RAL-induced genetic bottleneck. We also performed *in vitro* selection of the R5/X4 isolate using lamivudine (3TC), saquinavir (SQV) and MVC, and compared the results with those from the RAL-selected isolate.

RESULTS

Genotypic profiles of the HIV-1 primary isolates

Four genetically heterogeneous HIV-1 primary isolates (KP-1–4) from Japanese drug-naïve patients were used to assess the extent to which RAL affected the selection of bulk primary viruses *in vitro*. A laboratory isolate, strain 89.6, was also used in the study (rather than a molecular clone) to allow escape mutants to be selected from each quasi-species pool and to be generated *de novo*. First, the sequences of the integrase (IN) regions of the four primary isolates were determined. Table 1 shows the detailed evaluation of the R5/X4 mixture subtype B (KP-1), R5-CRF08_BC (KP-2), R5 subtype B (KP-3) and X4-CRF01_AE (KP-4) primary isolates, and the dual-tropic subtype B laboratory virus (89.6). Although some naturally occurring polymorphisms were observed within the IN regions of these isolates compared with the subtype B consensus sequence available from the Los Alamos National Laboratory HIV sequence database, we did not identify any primary resistant mutations to RAL. Three baseline viruses (KP-1, KP-4 and 89.6) were sensitive to RAL, with IC_{50} values ranging from 1.2 to 4 nM, which are comparable with those reported previously (Kobayashi *et al.*, 2008). However, KP-2 and KP-3 showed minor resistance to RAL, with IC_{50} values of 16 and 32 nM, respectively. These two isolates contained amino acid mutations at positions 72, 125 and 201 within the IN region [previously reported as L-870,810 and S-1360 resistance mutations (Hombrouck *et al.*, 2008; Rhee *et al.*, 2008), but not as RAL-resistance mutations]. KP-2 also contained a unique insertion at position 288 (NQDME) at the C-terminal end of the IN region.

In vitro selection of variants of the primary isolates and 89.6 using RAL

To induce RAL-selected HIV-1 variants *in vitro*, PM1/CCR5 cells, a T-cell line expressing high levels of CCR5, were exposed to the four primary isolates and strain 89.6.

The viruses were then serially passaged in the presence of RAL. As a control, each isolate was passaged under the same conditions, but without RAL, to allow monitoring of spontaneous changes occurring in the viruses during prolonged PM1/CCR5 cell passage (the passage control). The selected viruses were initially propagated at a RAL concentration equal to each IC_{50} value. The RAL concentrations were then increased from 20 to 85 nM during the course of the selection procedure (Table 1).

Only small shifts in the IC_{50} to RAL were observed in four of the five isolates (KP-1, KP-2, KP-4 and 89.6), with fold changes in IC_{50} values of 3.4, 6.5, 16 and 9.2, respectively. KP-3 did not show resistance to RAL. IC_{50} values in all the passage controls were comparable with those of the baseline viruses (Table 1).

IN region sequences in RAL-selected variants

The full-length IN genes were amplified and cloned to determine the genetic basis of selection in the presence or absence of RAL. Ten to 12 clones from each sample were sequenced.

Substitutions within IN were observed at passages 30 (G189R) and 29 (T210I) in two RAL-selected isolates (KP-2 and KP-4, respectively). Neither of these has been reported as IN inhibitor-resistant mutations. No substitutions in the IN regions of KP-3 and 89.6 were found. However, A125T and V180I substitutions were observed in the KP-3 and 89.6 control variants at the last passage. No previously reported mutations were identified in the IN region of KP-1 (an R5/X4 mixture isolate) after 17 passages. However, four amino acids (K7/K111/H216/D278) were selected by RAL from the baseline quasi-species, whereas different amino acids (R7/R111/Q216/N278) were selected in the control-passage variants (Table 1).

Taken together, these findings showed that RAL-induced selection pressure causes adaptation within the IN regions of bulk primary viruses during *in vitro* passage in the target cells, and confirmed that this system can be used to analyse drug-selected variants *in vitro*.

Comparison of *env* gene sequences in RAL-selected and passage-control isolates

A highly diverse gp120 region was observed in the baseline R5/X4 mixture isolate, KP-1; however, the viral diversity of variants passaged in the presence or absence of RAL decreased significantly during *in vitro* selection (overall mean distance after RAL selection of 0.056 at baseline to 0.007 after passage 17; mean overall distance in the passage control of 0.01 after 20 passages, Table 2). Moreover, the RAL-selected and control variants utilized CCR5 to enter the target cell; neither variant used CXCR4 (Table 3).

Interestingly, the low-diversity RAL-selected variant contained a completely different Env sequence from that of the passage-control variant (Fig. 1a). Different regions spanning

Table 1. Susceptibility of HIV-1 isolates to RAL and distinct differences in IN region sequences between RAL-selected and control-passaged viruses

Isolate	Subtype	Tropism	Passage no.	Concn (nM)	RAL-selected variant*		Passage control	
					IN sequence	RAL IC ₅₀ (nM)	IN sequence	RAL IC ₅₀ (nM)
KP-1	B	Mix	0	0	<i>K/R7, K/R111, Q/H216, D/N278</i>	4	<i>K/R7, K/R111, Q/H216, D/N278</i>	4
			8	20	K111, H216, D278	31 (7.8)	R7, R111, Q216, N278	4.5 (1.2)
			17†	20	K7, K111, H216, D278	26 (6.5)	R7, R111, Q216, N278	0.4 (0.1)
KP-2	CRF08_BC	R5	0	0	<i>I201, ins289NQDME</i>	16	<i>I201, ins289NQDME</i>	16
			18	40	<i>G189G/R, I201, ins289NQDME</i>	32 (2)	<i>I201, ins289NQDME</i>	16 (1)
			30	85	<i>G189R, I201, ins289NQDME</i>	55 (3.4)	<i>I201, ins289NQDME</i>	25 (1.6)
KP-3	B	R5	0	0	<i>V72, A125</i>	32	<i>V72, A125</i>	32
			11	25	<i>V72, A125</i>	25 (0.78)	<i>V72, A125</i>	33 (1)
			22	27.5	<i>V72, A125</i>	37 (1.2)	<i>V72, A125T</i>	13 (0.41)
KP-4	CRF01_AE	X4	0	0	–	2.1	–	2.1
			8	40	–	33 (16)	R166R/K, D279N	4.4 (2.1)
			29	40	T210I	22 (10)	G163E, R166R/K, D279N/S	4.1 (2)
89.6	B	R5X4	0	0	–	1.2	–	1.2
			8	15	–	34 (28)	–	4.4 (3.7)
			34	20	–	11 (9.2)	V180I	1.2 (1)

*Amino acid changes in each passage variant are shown. Italicized letters represent mutations relative to the consensus subtype BC or B present in the baseline isolates. Bold letters represent amino acids selected out of the quasi-species cloud. The fold increase in RAL IC₅₀ values is shown in parentheses for *in vitro*-selected variants compared with those in the baseline isolates.

†The RAL variant selected after 17 passages was compared with the control selected after 20 passages.

Table 2. Comparison of amino acid length and number of PNGs between RAL-selected and control-passage KP-1 variants

Passage no.	Genetic diversity*	Mean ENV ₁₋₄₇₄ length (range)†	Mean V1/V2 length (range)	Mean V3 length (range)	Mean V4 length (range)	Mean PNGs (range)
Baseline						
0	0.056	472 (461-480)	69 (60-74)	34 (33-34)	30 (29-31)	24 (22-28)
RAL-selected virus						
2	0.038	479 (472-480)‡	74 (71-74)‡	34 (33-34)‡	31 (29-31)‡	27 (25-28) ‡
8	0.0070	480	74	34	31	28 (26-29)
17	0.0070	480	74	34	31	27 (26-27)§
Passage control						
2	0.045	464 (461-466)‡	64 (60-74)‡	34 (33-34)‡	29 (29-31)‡	24 (22-27)‡
8	0.0070	463 (462-463)	62	34	29	23 (22-23)
10	0.0080	462 (459-463)	62	34	29	23 (22-23)
20	0.010	463	62	34	29	23 (22-23)§
P value		<0.0001‡	<0.0001‡	0.91‡	0.0048‡	0.0019‡ <0.0001§

*Overall mean distance.

†Sequence from gp120 SP to the V5 region (aa 1-474).

‡, § P values were calculated using the homoscedastic *t*-test between the RAL-selected and the passage-control variants indicated by the same symbols above.

the whole envelope sequence [from the signal peptide (SP) to V5] were compared in the RAL-selected and passage-control viruses. The results showed that, after only two passages, the gp120, V1/V2 and V4-loop regions within RAL-selected variants were longer than those in the control variants, and the number of putative *N*-linked glycosylation sites (PNGs) was significantly higher than that in the control-passage viruses (Table 2). This phenomenon was seen consistently in two independent experiments.

We also analysed the gp120 sequences in the other four isolates. Although the number of positional differences between the RAL-selected and passage-control variants for these four isolates was lower than that in KP-1 (between three and nine, compared with >40), there was a similar pattern of separation between the Env sequences (Fig. 1). In three of the four isolates (KP-2, KP-3 and KP-4), positional differences were observed in SP, C1 and all the variable regions of gp120 (Fig. 1b-d). In strain 89.6, differences were observed in the C2, C3 and V4 regions (Fig. 1e).

These results suggested that RAL treatment of target cells causes a decrease in viral diversification within quasi-species Env regions via a route different from that in untreated target cells.

In vitro induction of RAL-selected V3-loop library virus variants

To investigate further the effects of RAL on viral Env sequences, we used the V3-loop library virus (JR-FL-V3Lib) developed by Yusa *et al.* (2005), which carries a set of random combinations from zero to ten substitutions (27 648 possibilities) in the V3 loop (residues 305, 306, 307, 308, 309, 317, 319, 322, 323 and 326; V3 loop from Cys²⁹⁶ to Cys³³¹). The variants contained in the library were polymorphic mutations derived from 31 R5 clinical isolates (Yusa *et al.*, 2005). PM1/CCR5 cells were exposed to the JR-FL-V3Lib and serially passaged in the presence of RAL. After two passages, the V3 sequence within the RAL-selected variant was completely different from that in the passage control (Fig. 1f). This suggested that, under pressure from RAL, the infectious clone harbouring different V3 region sequence from the passage control had adapted to the target cells, despite containing the same IN sequences.

Phylogenetic analysis of the Env regions after passage with or without RAL

To confirm the temporal and spatial differences observed in each of the RAL-selected and passage-control viruses, phylogenetic analyses were conducted using complete SP-V5 sequences. The neighbour-joining phylogenetic tree showed a clear and distinct branching between RAL-selected and passage-control KP-1 viruses (Fig. 2a). We also identified a similar pattern in all the other isolates tested (Fig. 2b-e).