H-6b), 2.59 (dd, 1 H, H-3b_{eq}), 2.19–1.44 (m, 19 H, 6 Ac, H-3b_{ax}); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 170.7, 170.6, 170.3, 170.2, 170.0, 168.0, 165.7, 165.3, 155.4, 151.3, 133.2, 133.0, 130.1, 130.0, 129.7, 128.3, 128.3, 118.9, 114.2, 101.1, 96.7, 71.6, 71.1, 70.8, 69.3, 67.6, 67.4, 66.4, 62.3, 62.0, 55.4, 53.0, 48.7, 37.2, 23.2, 21.3, 20.7, 20.1; MALDI MS: *m/z*: calcd for $C_{49}H_{55}O_{22}NNa$: 1,032.31; found: 1,032.38 [M + Na] $^+$.

*{Methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate-(2→3)}-4-O-acetyl-2,6-di-O-benzoyl-β-D-galactopyranosyl Trichloroacetimidate (***13***)* To a solution of compound **11** (164 mg, 162 μmol) in mixed solvent (MeCN/PhMe/H_2O= 3.5:2.9:1.7 ml) was added diammonium cerium(IV) nitrate (CAN; 445 mg, 812 μmol). The mixture was stirred for 5 h at ambient temperature, as the proceeding of the reaction was monitored by TLC ($CHCl_3$/MeOH=20:1). The reaction mixture was extracted with $CHCl_3$, and the organic layer was washed with H_2O, sat. $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated. The residue was purified with column chromatography on silica gel ($CHCl_3$/MeOH= 65:1) to give **12** (147 mg). To a solution of compound 12 in CH_2Cl_2 (5.0 ml) were added trichloroacetonitrile (410 μl, 407 μmol) and 1,8-diazabicyclo[5.4.0]-7-undecene (DBU; 4.9 μl, 33.0 μmol). The mixture was stirred for 2 h at 0°C, as the proceeding of the reaction was monitored by TLC ($CHCl_3$/MeOH=20:1). The reaction mixture was concentrated and the residue was purified with column chromatography on silica gel ($CHCl_3$/MeOH=75:1) to give **13** (132 mg, 78%).; $[\alpha]_D$=+ 18.6° (*c* 0.8, $CHCl_3$); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.67 (s, 1 H, C=NH), 8.10–7.41 (m, 10 H, 2 Ph), 6.20 (d, 1 H, $J_{1,2}$=8.3 Hz, H-1a), 5.60–5.56 (m, 2 H, H-2a, H-8b), 5.22–5.20 (m, 2 H, H-4a, H-7b), 4.98 (d, 1 H, $J_{5,NH}$=10.3 Hz, NH-b), 4.93 (dd, 1 H, H-3a), 4.87 (m, 1 H, H-4b), 4.49 (q, 1 H, H-6'a), 4.34–4.29 (m, 3 H, H-5a, 6a, 9'b), 3.93 (dd, 1 H, H-9b), 3.85–3.77 (m, 4 H, H-5b, OMe), 3.60 (dd, 1 H, H-6b), 2.58 (dd, 1 H, H-3b_{eq}), 2.19–1.43 (m, 19 H, 6 Ac, H-3b_{ax}); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 170.8, 170.7, 170.6, 170.2, 170.2, 170.0, 168.0, 165.7, 165.1, 161.1, 133.2, 130.1, 129.9, 129.7, 129.7, 128.3, 128.3, 96.8, 96.4, 90.3, 77.2, 71.8, 71.5, 71.1, 70.0, 69.4, 67.6, 67.4, 66.5, 62.4, 61.5, 53.1, 48.8, 37.3, 29.7, 23.1, 21.4, 20.8, 20.7, 20.2; MALDI MS: *m/z*: calcd for $C_{44}H_{49}O_{21}N_2Cl_3Na$: 1,069.18; found: 1,069.41 [M + Na] $^+$.

*Benzyl 2,3-di-O-benzoyl-4,6-O-benzylidene-β-D-glucopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside (***18***)* To a solution of compound **16** (970 mg, 1.70 mmol) and **17** (762 mg, 1.41 mmol) in CH_2Cl_2 (31 ml) was added molecular sieves 4 Å (1.70 g). The suspension was stirred for 2 h and cooled to 0°C. To the mixture were added *N*-iodosuccinimide (NIS; 765 mg, 3.40 mmol) and trifluoromethanesulfonic acid (TfOH) (30 μl, 0.34 mmol) and stirring was continued for 1.5 h. Completion of the reaction was confirmed by TLC (EtOAc/hexane=1:3). The reaction mixture was filtered through Celite. The combined filtrate and washings was extracted with $CHCl_3$, and the organic layer was washed with sat. Na_2CO_3, sat. $Na_2S_2O_3$ and brine, dried over Na_2SO_4 and concentrated. The residue was purified with column chromatography on silica gel (EtOAc/hexane=1:5) to give **18** (1.26 g, 90%).; $[\alpha]_D$=-9.3° (*c* 1.0, $CHCl_3$); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 7.95–7.13 (m, 35 H, 7 Ph), 5.75 (t, 1 H, $J_{2,3}$=8.8 Hz, $J_{3,4}$=8.6 Hz, H-3f), 5.55 (s, 1 H, >C*H*Ph), 5.52 (t, 1 H, $J_{1,2}$=8.2 Hz, $J_{2,3}$=8.8 Hz, H-2f), 4.91–4.86 (m, 3 H, H-1f, 2 CH*H*Ph), 4.77–4.65 (m, 4 H, 4 CH*H*Ph), 4.49–4.39 (m, 4 H, H-1e, 6f, 2 CH*H*Ph), 4.14 (d, 1 H, J_{gem}= 11.0 Hz, H-6e), 3.99 (t, 1 H, $J_{3,4}$=8.6 Hz, $J_{4,5}$=9.6 Hz, H-4f), 3.89 (br t, 1 H, J_{gem}=10.3 Hz, $J_{5,6}$=9.3 Hz, H-6'f), 3.73–3.63 (m, 2 H, H-6'e, 5f), 3.57 (t, 1 H, $J_{2,3}$=8.4 Hz, H-3e), 3.45–3.40 (m, 3 H, H-2e, 4e, 5e); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 165.7, 165.2, 138.6, 138.5, 138.1, 137.5, 137.0, 133.3, 133.2, 129.9, 129.8, 129.5, 129.3, 129.1, 128.5, 128.4, 128.3, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 126.3, 102.5, 101.6, 101.4, 84.7, 82.2, 78.8, 77.8, 75.7, 75.0, 74.9, 74.7, 72.7, 72.3, 71.1, 68.8, 68.4, 67.2, 66.6, 29.8; MALDI MS: *m/z*: calcd for $C_{61}H_{58}O_{13}Na$: 1,021.38; found: 1,021.49 [M + Na] $^+$.

*Benzyl 2,3-di-O-benzyl-4,6-O-benzylidene-β-D-glucopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside (***20***)* To a solution of compound **18** (1.25 g, 1.25 mmol) in mixed solvent (MeOH/THF=15:7.5 ml) was added sodium methoxide (28% in MeOH; 24 mg). The mixture was stirred for 7.5 h at ambient temperature, as the proceeding of the reaction was monitored by TLC ($CHCl_3$/MeOH= 50:1). The reaction mixture was neutralized with Dowex (H^+) and filtered through cotton. The combined filtrate and washings was concentrated under diminished pressure. To a solution of the residue in DMF (12.5 ml) were added sodium hydride 60% (200 mg, 5.00 mmol) and benzyl bromide (594 μl, 5.00 mmol). The mixture was stirred for 3 h at ambient temperature, as the proceeding of the reaction was monitored by TLC (toluene/EtOAc= 12:1). Triethylamine and ammonium chloride were added to the reaction mixture. The reaction mixture was washed with H_2O and brine, dried over Na_2SO_4 and concentrated. The residue was purified with column chromatography on silica gel (toluene/EtOAc=40:1) to give **20** (1.07 g, 88%).; $[\alpha]_D$=−21.7° (*c* 1.1, $CHCl_3$); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 7.49–7.21 (m, 35 H, 7 Ph), 5.56 (s, 1 H, >C*H*Ph), 4.96–4.69 (m, 10 H, 10 CH*H*Ph), 4.59 (d, 1 H, $J_{1,2}$=8.2 Hz, H-1f), 4.54–4.45 (m, 3 H, H-1e, 2 CH*H*Ph), 4.33 (dd, 1 H, J_{gem}=9.3 Hz, $J_{5,6}$=4.8 Hz, H-6f), 4.16 (d, 1 H, J_{gem}=11.0 Hz, H-6e), 3.79–3.72 (m, 2 H, H-6'e, 6'f), 3.68–3.64 (m, 3 H, H-2f, 4f, 5f), 3.57 (t, 1 H, $J_{2,3}$=8.5 Hz, $J_{3,4}$=9.0 Hz, H-3e), 3.50–3.47 (m, 2 H, H-2e, 2f), 3.44 (t,

1 H, $J_{3,4}$=9.6 Hz, $J_{4,5}$=9.6 Hz, H-4e), 3.35 (m, 1 H, H-5e); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 138.6, 138.6, 138.8, 138.1, 137.6, 137.5, 129.1, 128.7, 128.5, 128.4, 128.3, 128.3, 128.3, 128.2, 128.1, 128.0, 128.0, 127.9, 127.8, 127.8, 127.7, 127.6, 126.1, 104.3, 102.7, 101.3, 84.8, 82.4, 82.1, 81.6, 81.0, 78.3, 77.3, 75.8, 75.4, 75.2, 75.1, 74.9, 71.3, 68.9, 66.1, 29.8; MALDI MS: *m/z*: calcd for $C_{61}H_{62}O_{11}Na$: 993.42; found: 993.50 $[M + Na]^+$.

*Benzyl 2,3,6-tri-O-benzyl-β-D-glucopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside (**21**)* To a solution of compound **20** (82 mg, 84.5 μmol) in CH_2Cl_2 (845 μl) were added triethylsilane (162 μl, 1.01 mmol) and boron trifluoride diethyl etherate (BF_3·OEt_2; 21.4 μl, 169 μmol). The mixture was stirred for 1.5 h at ambient temperature, as the proceeding of the reaction was monitored by TLC (toluene/EtOAc=12:1). The reaction mixture was diluted with $CHCl_3$ and washed with sat. $NaHCO_3$, H_2O and brine, dried over Na_2SO_4 and concentrated. The residue was purified with column chromatography on silica gel (toluene/EtOAc=20:1) to give **21** (70 mg, 85%).; $[\alpha]_D$=−12.9° (*c* 1.0, $CHCl_3$); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 7.35–7.21 (m, 35 H, 7 Ph), 5.01–4.69 (m, 10 H, 10 CH*H*Ph), 4.59–4.51 (m, 3 H, H-1f, 2 CH*H*Ph), 4.46 (d, 1 H, $J_{1,2}$=9.6 Hz, H-1e), 4.19 (d, 1 H, J_{gem}=11.0 Hz, H-6e), 3.74–3.58 (m, 6 H, H-6′e, 3f, 4f, 5f, 6f, 6′f), 3.50–3.39 (m, 5 H, H-2f, 2e, 3e, 4e, 5e), 2.54 (s, 1 H, -OH); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 138.9, 138.7, 138.5, 138.5, 138.2, 138.0, 137.6, 128.6, 128.5, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 128.0, 127.9, 127.8, 127.7, 104.1, 102.7, 84.8, 84.2, 82.4, 81.6, 78.4, 77.3, 75.8, 75.4, 75.3, 75.1, 74.9, 74.8, 74.1, 73.8, 71.8, 71.3, 68.8, 29.8; MALDI MS: *m/z*: calcd for $C_{61}H_{64}O_{11}Na$: 995.43; found: 995.38 $[M + Na]^+$.

*Benzyl {methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate-(2→3)}-4-O-acetyl-2,6-di-O-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-benzyl-β-D-glucopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside (**22**)* To a solution of compound **13** (107 mg, 102 μmol) and **21** (200 mg, 206 μmol) in CH_2Cl_2 (5.0 ml) was added molecular sieves 4 Å (1.00 g). The suspension was stirred for 1 h and cooled to 0°C. To the mixture was added trimethylsilyl trifluoromethanesulfonate (TMSOTf; 3.7 μl, 20 μmol) and stirring was continued for 1 h. Completion of the reaction was confirmed by TLC ($CHCl_3$/MeOH=20:1). The reaction mixture was filtered through Celite. The combined filtrate and washings was extracted with $CHCl_3$, and the organic layer was washed with sat. Na_2CO_3 and brine, dried over Na_2SO_4 and concentrated. The residue was purified with column chromatography on silica gel ($CHCl_3$/MeOH=75:1) to give **22** (170 mg, 86%).; $[\alpha]_D$=+ 2.0° (*c* 0.5, $CHCl_3$); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.24–7.15 (m, 45 H, 9 Ph), 5.66 (m, 1 H, H-8b), 5.31 (t, 1 H, $J_{1,2}$=8.0 Hz, $J_{2,3}$= 9.7 Hz, H-2a), 5.18 (dd, 1 H, H-7b), 5.13 (d, 1 H, $J_{1,2}$=8.0 Hz, H-1a), 5.06 (d, 1 H, $J_{3,4}$=3.4 Hz, H-4a), 4.99 (d, 1 H, CH*H*Ph), 4.92–4.66 (m, 12 H, H-3a, 4b, NH, 9 CH*H*Ph), 4.49–4.36 (m, 7 H, H-6′a, 1e, 1f, 4 CH*H*Ph), 4.29 (d, 1 H, H-9′b), 4.13–3.88 (m, 6 H, H-5a, 6a, 5b, 9b, H-6′ of Glc units), 3.77 (q, 1 H, H-5b), 3.71 (s, 1 H, OMe), 3.67–3.35 (m, 10 H, H-6b, Glc units), 3.22 (m, 1 H, H-5 of Glc units), 2.52 (dd, 1 H, J_{gem}=12.6 Hz, $J_{3eq,4}$=4.6 Hz H-3b$_{eq}$), 2.13–1.43 (m, 19 H, 6 Ac, H-3b$_{ax}$) ^{13}C-NMR (125 MHz, $CDCl_3$) δ 170.8, 170.7, 170.3, 170.2, 170.1, 168.0, 165.4, 165.1, 139.1, 138.6, 138.4, 138.0, 137.6, 133.3, 133.0, 130.3, 130.0, 129.8, 129.7, 128.6, 128.4, 128.3, 128.3, 128.2, 128.1, 128.1, 127.9, 127.9, 127.7, 127.6, 127.4, 127.3, 127.2, 127.1, 103.7, 102.7, 100.4, 96.9, 84.7, 82.9, 82.3, 81.6, 78.2, 76.3, 75.7, 75.2, 75.1, 74.9, 74.8, 74.8, 74.4, 72.8, 71.7, 71.5, 71.2, 70.4, 69.4, 69.0, 68.5, 67.4, 67.0, 66.5, 62.5, 61.2, 53.0, 48.8, 37.3, 29.7, 23.2, 21.3, 20.8, 20.7, 20.7, 20.3; MALDI MS: *m/z*: calcd for $C_{103}H_{111}O_{31}NNa$: 1,880.70; found: 1,880.96 $[M + Na]^+$.

*Benzyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-galactopyranosyl-(1→4)-{methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate-(2→3)}-2,6-di-O-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-benzyl-β-D-glucopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside (**23**)* To a solution of compound **14** (58 mg, 43 μmol) and **21** (84 mg, 86 μmol) in CH_2Cl_2 (2.0 ml) was added molecular sieves 4 Å (165 mg). The suspension was stirred for 1 h at ambient temperature and cooled to 0°C. To the mixture was added TMSOTf (1.6 μL, 8.6 μmol) and stirring was continued for 3.5 h. Completion of the reaction was confirmed by TLC ($CHCl_3$/MeOH=15:1). Triethylamine was then added to quench the reaction. The reaction mixture was filtered through Celite. The combined filtrate and washings was extracted with $CHCl_3$, and the organic layer was washed with sat. $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated. The residue was purified with column chromatography on silica gel ($CHCl_3$/MeOH=50:1) to give **23** (70 mg, 76%).; $[\alpha]_D$=−11.0° (*c* 0.76, $CHCl_3$); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.01–7.15 (m, 45 H, 9 Ph), 5.97 (d, 1 H, NH-c), 5.49 (dd, 1 H, $J_{3,4}$=2.7 Hz, H-3c), 5.40 (m, 1 H, H-8b), 5.37 (d, 1 H, $J_{3,4}$=2.7 Hz, H-4c), 5.34 (t, 1 H, $J_{1,2}$= 10.2 Hz, H-2a), 5.25 (d, 1 H, H-7b), 5.14 (br d, 1 H, NH-b), 5.06 (d, 1 H, $J_{1,2}$=8.9 Hz, H-1c), 5.00 (dt, 1 H, $J_{3eq,4}$= 4.8 Hz, H-4b), 4.95–4.88 (m, 4 H, 4 CH*H*Ph), 4.82–4.80 (m, 3 H, 3 CH*H*Ph), 4.79 (d, 1 H, $J_{1,2}$=10.2 Hz, H-1a), 4.72 (t, 2 H, 2 CH*H*Ph), 4.67 (d, 1 H, CH*H*Ph), 4.62 (q, 1 H, H-6c), 4.51 (d, 1 H, CH*H*Ph), 4.47 (d, 1 H, CH*H*Ph), 4.43 (d, 1 H, CH*H*Ph), 4.40 (d, 1 H, $J_{1,2}$=8.2 Hz, H-1f), 4.37 (d, 1 H, $J_{1,2}$= 8.2 Hz, H-1e), 4.28 (d, 1 H, CH*H*Ph), 4.19 (t, 1 H, H-5a), 4.15–3.96 (m, 10 H, H-3a, 4a, 6a, 6′a, 5b, 9b, 9′b, 2c, 6′c, 5e), 3.95 (t, 1 H, H-4f), 3.83–3.81 (m, 4 H, OMe, H-6b), 3.64

(t, 1 H, H-5c), 3.63–3.59 (m, 2 H, H-3e, 6e), 3.53 (t, 1 H, H-6'e), 3.50–3.49 (m, 2 H, H-6f, 6'f), 3.47 (t, 1 H, H-3f), 3.44 (t, 1 H, H-4e), 3.39 (t, 1 H, H-2f), 3.36 (t, 1 H, H-2e), 3.14 (m, 1 H, H-5f), 2.22 (dd, 1 H, J_{gem}=13.7 Hz, $J_{3eq,4}$=4.8 Hz, H-3b_{eq}), 1.93 (t, 1 H, H-3b_{ax}), 2.19–1.75 (9 s, 27 H, 9 Ac); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 170.7, 170.4, 170.3, 170.2, 169.7, 169.6, 168.0, 165.8, 164.1, 138.8, 138.6, 138.5, 138.4, 138.3, 137.9, 137.5, 133.2, 133.1, 129.9, 129.4, 128.4, 128.3, 128.3, 128.2, 128.2, 128.2, 128.1, 128.1, 128.0, 127.8, 127.8, 127.6, 127.5, 127.5, 127.3, 127.0, 103.7, 102.6, 101.1, 100.0, 98.6, 84.6, 82.6, 82.2, 81.6, 78.2, 77.1, 76.2, 76.2, 75.6, 75.1, 74.8, 74.7, 74.3, 73.9, 73.1, 72.0, 72.0, 71.2, 71.1, 70.3, 70.0, 68.9, 68.3, 68.2, 67.2, 67.1, 66.3, 63.3, 62.1, 61.4, 53.1, 51.5, 49.1, 35.8, 29.6, 23.2, 23.1, 21.0, 20.8, 20.7, 20.7, 20.5, 20.4, 20.3; MALDI MS: *m/z*: calcd for $C_{115}H_{128}N_2O_{38}Na$: 2,167.80; found: 2,167.91 [*M* + Na] $^+$.

Benzyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1→3)-2-acetamido-4,6-di-O-acetyl-2-deoxy-β-D-galactopyranosyl-(1→4)-{methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate-(2→3)}-2,6-di-O-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-benzyl-β-D-glucopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside (**24**) To a solution of compound **15** (105 mg, 64.5 μmol) and **21** (137 mg, 129 μmol) in CH_2Cl_2 (1.9 ml) was added molecular sieves 4 Å (300 mg). The suspension was stirred for 30 min and cooled to 0°C. To the mixture was added TMSOTf (1.2 μl, 6.5 μmol) and stirring was continued for 45 min. Completion of the reaction was confirmed by TLC (toluene/EtOAc=7:1). The reaction mixture was filtered through Celite. The combined filtrate and washings was extracted with $CHCl_3$, and the organic layer was washed with sat. Na_2CO_3 and brine, dried over Na_2SO_4 and concentrated. The residue was purified with column chromatography on silica gel ($CHCl_3$/MeOH= 200:3) to give **24** (110 mg, 69%).; $[\alpha]_D$=+ 0.0° (*c* 0.8, $CHCl_3$); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.18–7.09 (m, 45 H, 9 Ph), 5.88 (d, 1 H, $J_{5,NH}$=6.3 Hz, NH-c), 5.66 (m, 1 H, H-8b), 5.38–5.31 (m, 3 H, H-2a, 4c, 4d), 5.19 (dd, 1 H, $J_{6,7}$= 2.3 Hz, $J_{7,8}$=9.7 Hz, H-7b), 5.15 (d, 1 H, $J_{1,2}$=8.0 Hz, H-1c), 5.11–5.07 (m, 2 H, H-3a, 2d), 5.01–4.58 (m, 17 H, H-6a, 4b, 3c, 1d, 3d, 1f, NH-b, 10 CH*H*Ph), 4.48–4.37 (m, 5 H, $J_{1,2}$=7.4 Hz, H-1a, $J_{1,2}$=8.1 Hz, H-1e, 6e, 2 CH*H*Ph), 4.29–4.22 (m, 3 H, H-9b, 2 CH*H*Ph), 4.12–3.25 (m, 28 H, H-4a, 5a, 6a, 6'a, 5b, 6b, 9'b, 2c, 5c, 6c, 6'c, 5d, 6d, 6'd, 2e, 3e, 4e, 5e, 6'e, 2f, 3f, 4f, 5f, 6f, 6'f, -OMe), 2.73 (dd, 1 H, J_{gem}= 12.6 Hz, $J_{3eq,4}$=4.3 Hz, H-3b_{eq}), 2.19–1.49 (m, 37 H, H-3b_{ax}, 12 Ac); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 172.1, 170.9, 170.7, 170.5, 170.3, 170.2, 170.2, 170.0, 169.3, 168.4, 165.5, 165.1, 138.9, 138.6, 138.6, 138.5, 138.1, 137.6, 133.4, 133.1, 130.3, 130.1, 130.0, 129.6, 128.7, 128.4, 128.2, 128.0, 128.0, 127.9, 127.8, 127.7, 127.7, 127.6, 127.3, 127.3, 103.8, 102.7, 101.1, 100.7, 99.0, 97.9, 84.7, 83.2, 82.4, 81.8, 78.3, 75.7, 75.2, 75.0, 75.0, 74.8, 74.5, 73.9, 73.6, 72.9, 72.2, 71.9, 71.6, 71.3, 71.0, 70.6, 69.2, 69.1, 69.0, 68.6, 67.1, 66.9, 66.6, 63.2, 62.8, 62.6, 61.0, 55.3, 52.8, 49.3, 36.9, 29.8, 24.0, 23.2, 22.8, 21.4, 20.9, 20.8, 20.8, 20.8, 20.7, 20.7, 20.4, 20.3, MALDI MS: *m/z*: calcd for $C_{127}H_{144}N_2O_{46}Na$: 2,455.89; found: 2,455.52 [*M* + Na] $^+$.

β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-{5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid-(2→3)}-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranose (**1**) To a solution of compound **24** (95 mg, 39 μmol) in MeOH (1.6 ml) was added sodium methoxide (28% in MeOH; 14 mg). The mixture was stirred for 74 h under reflux condition, as the proceeding of the reaction was monitored by TLC ($CHCl_3$/MeOH/H_2O=3:2:0.3). H_2O (1.6 ml) was then added and stirring was continued for 14 h at ambient temperature. The reaction mixture was neutralized with Dowex (H^+) and filtered through cotton. The combined filtrate and washings was concentrated under diminished pressure to give a syrup compound. To a solution of the residue in H_2O (1.4 ml) was added palladium hydroxide [$Pd(OH)_2$] (20 wt% Pd on carbon; 345 mg). The mixture was vigorously stirred for 4 h at 40°C under hydrogen atmosphere, as the proceeding of the reaction was monitored by TLC (1-BuOH/MeOH/H_2O= 2:1:1). The reaction mixture was filtered through Celite, and the combined filtrate and washings was concentrated. The residue was purified with gel filtration column chromatography (Sephadex LH-20, H_2O as eluent) to give **1** (43 mg, 99%).; $[\alpha]_D$=+ 0.1° (*c* 1.0, H_2O); ^{1}H-NMR (600 MHz, CD_3OD): δ 5.16 (d, 1 H, $J_{1,2}$=3.7 Hz, H-1e), 4.79 (d, 1 H, H-1c), 4.57 (d, 1 H, $J_{1,2}$=8.0 Hz, H-1d), 4.49–4.45 (m, 3 H,H-1a, 1b, 1f), 4.15–3.19 (m, 39 H, ring H), 2.62 (dd, 1 H, H-3b_{eq}), 1.99 and 1.96 (2 s, 6 H, 2 Ac), 1.87 (m, 1 H, H-3b_{ax}), ^{13}C-NMR (150 MHz, CD_3OD) δ 175.0, 174.8, 174.1, 106.1, 105.7, 105.5, 104.1, 104.0, 103.4, 101.8, 97.0, 95.3, 94.2, 93.0, 91.5, 84.4, 81.2, 78.1, 77.6, 76.5, 75.1, 74.8, 74.5, 74.3, 73.9, 73.5, 73.4, 72.6, 72.1, 71.5, 70.8, 70.2, 68.9, 68.0, 67.1, 61.2, 61.0, 60.7, 59.7, 59.3, 58.8, 52.5, 51.6, 48.8, 47.5, 28.7, 25.9, 23.5; MALDI MS: *m/z*: calcd for $C_{43}H_{72}N_2O_{34}$: 1160.40; found: 1159.75 [*M*-H]$^-$.

2-Acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-{5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid-(2→3)}-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-D-glucopyranose (**2**) To a solution of compound **23** (38 mg, 18 μmol) in MeOH (2.0 ml) was added catalytic amounts of sodium methoxide (10 mg). The mixture was stirred for 96 h under reflux conditions, as

the proceeding of the reaction was monitored by TLC (1-BuOH/MeOH/H_2O=4:1:1). H_2O was then added and stirring was continued for 10 h at ambient temperature. The reaction mixture was neutralized with Dowex (H^+) and filtered through cotton. The combined filtrate and washings was concentrated under diminished pressure to give a syrupy compound. The residue was purified by gel filtration column chromatography on Sephadex LH-20 (MeOH) to give a white solid. To a solution of the solid in MeOH/H_2O (2.5/1 ml) was added palladium hydroxide [$Pd(OH)_2$] (20 wt% Pd on carbon; 40 mg). The mixture was vigorously stirred overnight at 40°C under hydrogen atmosphere, as the proceeding of the reaction was monitored by TLC (1-BuOH/MeOH/H_2O=2:1:1). The reaction mixture was filtered through Celite. The combined filtrate and washings was concentrated. The residue was purified with gel filtration column chromatography (Sephadex LH-20, MeOH/H_2O=1:1 as eluent) using MeOH as eluent, to give **2** (18 mg, 98%).; $[\alpha]_D$=+19.4° (*c* 1.7, MeOH:H_2O=1:1); ^{1}H-NMR (500 MHz, CD_3OD/D_2O=1:1): δ 2.69 (dd, 1 H, J_{gem}=11.4 Hz, $J_{3eq,4}$=4.6 Hz, H-3b$_{eq}$), 2.04 and 2.02 (2 s, 6 H, 2 NAc), 1.91 (t, 1 H, H-3b$_{ax}$); ^{13}C-NMR (125 MHz, CD_3OD/D_2O=1:1) δ 176.0, 175.4, 175.0, 103.9, 103.9, 103.7, 102.9, 97.3, 93.4, 80.0, 78.5, 77.0, 76.1, 75.8, 75.6, 75.4, 75.1, 71.0, 70.8, 69.8, 69.6, 69.5, 69.1, 64.2, 62.3, 61.5, 61.2, 53.5, 53.0, 49.5, 49.4, 48.4, 38.0, 23.6, 22.8; MALDI MS: *m/z*: calcd for $C_{37}H_{61}N_2O_{29}$: 997.33; found: 997.25 [*M*-H]$^-$.

{5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid-(2→3)}-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-D-glucopyranose (**3**) To a solution of compound **22** (45 mg, 24 μmol) in MeOH (3.0 ml) was added sodium methoxide (28% in MeOH; 11 mg). The mixture was stirred for 48 h at 45°C, as the proceeding of the reaction was monitored by TLC ($CHCl_3$/MeOH=5:1). H_2O (1.0 ml) was then added and stirring was continued for 18 h at 45°C. The reaction mixture was neutralized with Dowex (H^+) and filtered through cotton. The combined filtrate and washings was concentrated under diminished pressure to give a syrupy compound. To a solution of the residue in H_2O (2.0 ml) was added palladium hydroxide [$Pd(OH)_2$] (20 wt% Pd on carbon; 100 mg). The mixture was stirred for 8 h at ambient temperature under hydrogen atmosphere, as the proceeding of the reaction was monitored by TLC ($CHCl_3$/MeOH/H_2O=3:1:0.1). The reaction mixture was filtered through Celite. The combined filtrate and washings was concentrated. The residue was purified with gel filtration column chromatography (Sephadex LH-20, H_2O as eluent) to give **3** (14 mg, 76%).; $[\alpha]_D$=+ 8.3° (*c* 0.6, H_2O); ^{1}H-NMR (400 MHz, D_2O): δ 5.21 (d, 1 H, $J_{1,2}$=3.7 Hz, H-1e), 4.64–4.51 (m, 2 H, H-1a, 1f), 4.21–2.87 (m, 25 H, ring H), 2.75 (dd, 1 H, J_{gem}=12.0 Hz, $J_{3eq,4}$=4.6 Hz, H-3b$_{eq}$), 2.02 (s, 3 H, Ac), 1.77 (m, 1 H, H-3b$_{ax}$), ^{13}C-NMR (100 MHz, D_2O) δ 177.7, 176.6, 105.4, 105.2, 102.5, 98.7, 94.8, 80.9, 78.4, 77.9, 77.6, 77.5, 77.5, 77.0, 76.7, 75.6, 75.5, 75.4, 74.5, 74.1, 73.1, 72.2, 72.1, 71.5, 71.4, 71.1, 70.8, 70.2, 65.3, 65.2, 63.7, 62.7, 57.1, 54.4, 42.4, 24.8, 21.8, 17.7; MALDI MS: *m/z*: calcd for $C_{29}H_{24}NO_{24}$: 795.26; found: 794.24 [*M*-H]$^-$.

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A Chemical Synthesis of GlcNAcβ(1→4)GlcUA–UDP to Elucidate the Catalytic Mechanism of Hyaluronic Acid Synthases (HAS)

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Abstract: A first chemical synthesis of GlcNAcβ(1→4)GlcUA–UDP is described here. This compound can be an essential tool to elucidate the catalytic mechanism of hyaluronic acid synthases (HAS) and this synthetic strategy contributes to the synthesis of various UDP-sugars which include modified GlcUA moieties.

Key words: carbohydrates, enzymes, nucleotides, oligosaccharides, synthesis

Hyaluronic acid (HA), an important component of the extracellular matrix, is a linear polysaccharide composed of a repeating disaccharide unit of GlcNAcβ(1→4) and GlcUAβ(1→3), and plays an important role in a wide variety of biological processes.[2] Hyaluronic acid synthases (HAS), responsible for the synthesis of HA, have been cloned from bacteria and mammalian cells.[3] They have been even known as unique glycosyltransferases, which can catalyze two kinds of UDP-sugars (GlcNAc–UDP and GlcUA–UDP). They are classified into two families based on their primary structure as well as their enzyme characteristics.[4] However, the three-dimensional structure of HASs responsible for catalytic mechanism remains unclear.

We were interested in to obtaining the information about the catalytic mechanism, i.e. whether the HA elongating reaction occurs at the non-reducing end or at the reducing end of sugar chain, and also which substrate among GlcNAc–UDP and GlcUA–UDP serves as a primer of the elongation reaction. We believed that our target compound [GlcNAcβ(1→4)GlcUA–UDP] could be an essential tool for performing the above-mentioned tasks. Moreover, only a few reports about the enzymatic synthesis of GlcUA–UDP are available.[5,6] We describe herein the first chemical synthesis of GlcNAcβ(1→4)GlcUA–UDP which has the GlcUA–UDP moiety. Our synthetic strategy could be extended for the synthesis of various UDP-sugars including modified GlcUA structures.

For the synthesis of GlcNAcβ(1→4)GlcUA–UDP, the protected GlcNAcβ(1→4)GlcUA-1-phosphate derivative **10** (Figure 1) was prepared as a key compound. Benzyl group was added for protection of the sterically hindered 3-OH position of GlcUA, which could be removed under neutral conditions, since GlcUA is prone to undergo β-elimination under basic conditions.

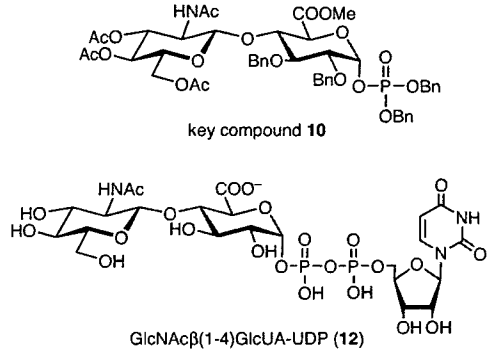

Figure 1 Structure of disaccharide-1-phosphate and UDP-disaccharide

Coupling of the donor **1**[7] and the suitably protected acceptor **3** prepared from **2**[8] in the presence of *N*-iodosuccinimide (NIS)–trifluoromethanesulfonic acid (TfOH) and 4 Å molecular sieves gave the disaccharide **4** in 86% yield. Deprotection of the Troc group and the subsequent selective acetylation of the amine gave **5** in 85% yield. Dess–Martin oxidation of 6-OH at the reducing end sugar, the further oxidation of the intermediate aldehyde to a carboxylic acid by the use of $NaClO_2$ in phosphate buffer,[9] and the following methyl esterification gave **6** with 88% overall yield in three steps. Selective removal of the 2-(trimethylsilyl)ethyl (SE) group in **6** with trifluoroacetic acid (TFA) afforded **7** in 92% yield (Scheme 1).

In order to obtain the α-monophosphate derivative of the disaccharide, coupling of the trichloroacetimidate derivative with dibenzylphosphate was carried out (Scheme 2).[10] Compound **7** was converted into the imidate **8** in a moderate yield,[11] which was directly coupled with dibenzylphosphate in CH_2Cl_2 for one day to give the unexpected β-oriented monophosphate derivative **9** as the major product. Treatment of **9** with TfOH in CH_2Cl_2 at 0 °C did not give the α compound. Alternatively, formation of lithium alkoxide of **7** by the action of lithium hexamethyldisilazide (LHMDS) followed by the coupling

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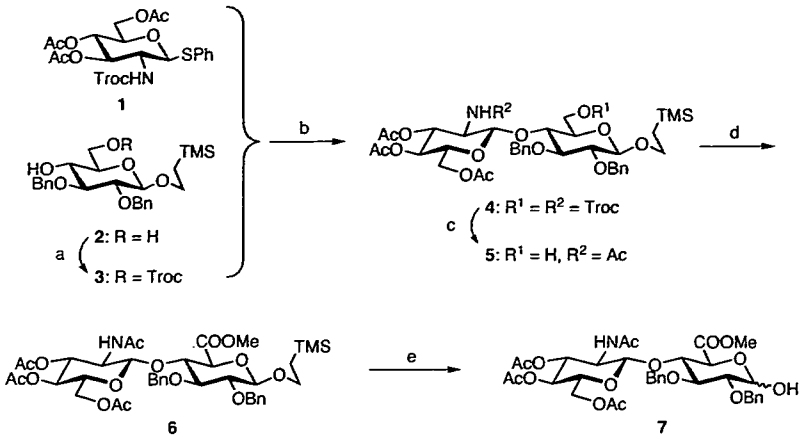
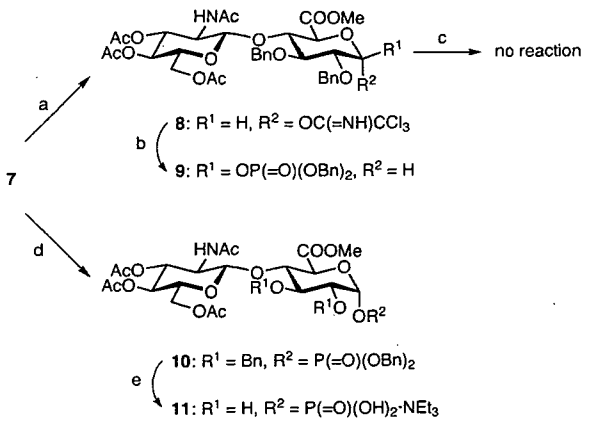


Scheme 1 Preparation of GlcNAcβ(1→4)GlcUA derivative. *Reagents and conditions*: (a) TrocCl, pyridine–CH_2Cl_2 (1:1), 0 °C, 2 h, 95%; (b) NIS, TfOH, CH_2Cl_2, MS 4A, –30 °C, 3.5 h, 86%; (c) Zn, Ac_2O, AcOH, THF, r.t., 2.5 h, 85%; (d) (i) Dess–Martin periodinane, $NaHCO_3$, CH_2Cl_2, r.t., 3 h; (ii) $NaClO_2$, 2-methyl-2-butene, NaH_2PO_4, *t*-BuOH, H_2O, r.t., 12 h; (iii) MeOH, WSC {water-soluble carbodiimide; 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride}, pyridine, CH_2Cl_2, r.t., 4 h, 88% (3 steps); (e) TFA, CH_2Cl_2, r.t., 2 h, 92% (α/β = 10:3).

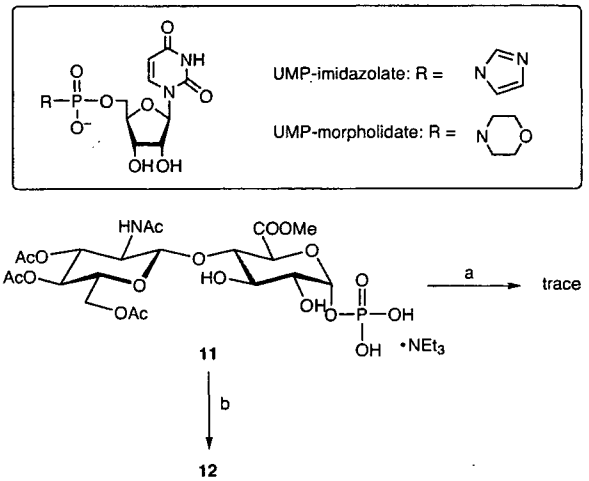
with tetrabenzylpyrophosphate gave the suitably protected GlcNAcβ(1→4)GlcUA-α-1-phosphate derivative **10**[12] in 67% yield. Significant signals in the ^{1}H NMR spectrum of **10** at δ = 5.88 (dd, 1 H, $J_{1,2}$ = 3.4 Hz, $^3J_{1,P}$ = 7.4 Hz, H-1 of GlcUA), and the signal in the ^{31}P NMR of **10** at δ = –1.83 (s, 1 P) were observed, confirming that the newly formed linkage between the disaccharide moiety and phosphoric acid moiety was α.

Removal of the benzyl groups in **10** by hydrogenation over 20% palladium hydroxide on carbon gave **11** in 94% yield. Several conditions to construct the UDP structure by the use of phosphoamidate did not give any desirable result. Condensation of **11** with UMP-imidazolate[5] in anhydrous DMF at room temperature gave many byproducts with β-elimination. This may be due to the basic conditions which were generated during the preparation of UMP-imidazolate. On the other hand, coupling of **11** with UMP-morpholidate[13] with 1*H*-tetrazole[12] in moderately basic condition (DMF–pyridine, 3:1) reduced side reactions. Subsequent deprotection of the acetyl groups and the methyl ester in MeOH–H_2O–Et_3N (7:3:1)[14] at 20 °C gave the desired GlcNAcβ(1→4)GlcUA–UDP (**12**)[15] in 52% yield in two steps after isolation and purification by reverse-phase column HPLC and gel-filtration column HPLC (Scheme 3).

In conclusion, we have succeeded in the first synthesis of GlcNAcβ(1→4)GlcUA–UDP by the chemical approach. This procedure could be extended to the synthesis of various UDP-sugars which contain the modified GlcUA-1-phosphate moiety. We also believe that the compound **12** will be an essential tool to gain information on the catalytic mechanism of hyaluronic acid syntheses.



Scheme 2 Preparation of disaccharide monophosphate derivative. *Reagents and conditions*: (a) trichloroacetonitrile, DBU, CH_2Cl_2, 0 °C, 2 h, 79%; (b) dibenzylphosphate, CH_2Cl_2, r.t., 24 h, (α form: 8%, β form: 77%); (c) dibenzylphosphate, TfOH, CH_2Cl_2, 0 °C, 12 h; (d) LHMDS, tetrabenzylpyrophosphate, THF, –78 °C → 0 °C, (α form: 67%, β form: 24%); (e) H_2 gas, $Pd(OH)_2$/C, MeOH, r.t., 18 h, 94%.



Scheme 3 Synthesis of GlcNAcβ(1→4)GlcUA–UDP. *Reagents and conditions*: (a) UMP-imidazolate, DMF, r.t., 1d; (b) (i) UMP-morpholidate, 1*H*-tetrazole, DMF–pyridine (3:1), r.t., 2 d; (ii) MeOH–H_2O–Et_3N (7:3:1), 20 °C, 2 d; (iii) RP column HPLC; (iv) gel-filtration column HPLC, 52% (from **11**).

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(15) The selected physical data of key compounds is listed. Compound **10**: $[\alpha]_D$ +17.6° (*c* = 1.7, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$): δ = 1.91 (s, 3 H, NAc), 1.94, 1.98, 2.01 (3 × s, 9 H, OAc), 3.49 (ddd, $J_{5',6'a}$ = 2.3 Hz, $J_{5',6'b}$ = 4.0 Hz, 1 H, H-5'), 3.57 (td, $J_{1,2}$ = 3.4 Hz, $J_{2,3}$ = 9.2 Hz, $^3J_{2,P}$ = 2.8 Hz, 1 H, H-2), 3.75 (s, 3 H, COOMe), 3.85 (t, $J_{2,3}$ = 9.2 Hz, $J_{3,4}$ = 9.2 Hz, 1 H, H-3), 3.86 (dd, $J_{5',6'a}$ = 2.3 Hz, $J_{6'a,6'b}$ = 12.6 Hz, 1 H, H-6'a), 3.97 (t, $J_{3,4}$ = 9.2 Hz, $J_{4,5}$ = 9.7 Hz, 1 H, H-4), 3.99 (q, $J_{1',2'}$ = 8.6 Hz, $J_{2',NH}$ = 9.2 Hz, 1 H, H-2'), 4.14 (dd, $J_{5',6'b}$ = 4.0 Hz, $J_{6'a,6'b}$ = 12.6 Hz, 1 H, H-6'b), 4.32 (d, $J_{4,5}$ = 9.7 Hz, 1 H, H-5), 4.61 (d, 1 H, CH_2Ph), 4.64 (d, $J_{1',2'}$ = 8.6 Hz, 1 H, H-1'), 4.67, 4.75, 4.98 (3 × d, CH_2Ph, 3 H), 5.02–5.11 (m, 6 H, H-3', H-4', CH_2Ph), 5.66 (d, $J_{2,NH}$ = 9.2 Hz, 1 H, NH), 5.88 (dd, $J_{1,2}$ = 3.4 Hz, $^3J_{1,P}$ = 7.4 Hz, 1 H, H-1), 7.20–7.33 (m, 20 H, CH_2Ph). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 20.5, 20.6, 20.6 (Me of OAc), 23.1 (Me of NAc), 53.0 (COOMe), 54.4 (C-2'), 61.6 (C-6'), 68.0 (C-4'), 69.2 ($J_{CH2Ph,P}$ = 4.8 Hz, $POCH_2Ph$), 69.5 ($J_{CH2Ph,P}$ = 4.8 Hz, $POCH_2Ph$), 71.4 (C-5), 71.9 (C-5'), 73.1 (C-3'), 73.3 (CH_2Ph), 75.3 (CH_2Ph), 77.7 ($J_{C-2,P}$ = 7.2 Hz, C-2), 78.4 (C-4), 78.7 (C-3), 94.9 ($J_{C-1,P}$ = 6.0 Hz, C-1), 100.0 (C-1'), 127.0, 127.0, 127.0, 127.0, 127.0, 128.0, 128.0, 128.0, 128.0, 128.0, 128.0, 135.0, 136.0, 136.0, 137.0, 139.0, 169.0 (C=O of OAc-4'), 170.0 (C=O of COOMe), 170.0 (C=O of NAc), 171.0 (C=O of OAc-6'), 171.0 (C=O of OAc-3'). ^{31}P NMR (202 MHz, $CDCl_3$): δ = –1.83 (s, 1 P). MS (MALDI–TOF, +ve): *m/z* $[M + Na]^+$ calcd for $C_{49}H_{56}NNaO_{18}P$: 1000.31; found: 1000.21. Compound **12**: $[\alpha]_D$ +8.75° (*c* = 0.4, H_2O). ^1H NMR (500 MHz, D_2O): δ = 1.95 (s, 3 H, NAc), 3.36–3.37 (m, 1 H, H-5'), 3.38–3.43 (m, 2 H, H-3', H-4'), 3.50 (td, $J_{1,2}$ = 4.0 Hz, $J_{2,3}$ = 9.2 Hz, $^3J_{2,P}$ = 2.9 Hz, 1 H, H-2), 3.59 (t, $J_{1',2'}$ = 8.6 Hz, 1 H, H-2'), 3.64 (t, $J_{2,3}$ = 9.2 Hz, 1 H, H-3), 3.64–3.68 (m, 2 H, H-4, H-6'a), 3.81 (d, 1 H, H-6'b), 4.01 (d, 1 H, H-5), 4.08 (ddd, $J_{Rib5a,Rib5b}$ = 12.0 Hz, $^3J_{Rib5a,P}$ = 2.3 Hz, 1 H, H-5a of Rib), 4.13 (ddd, $J_{Rib5a,Rib5b}$ = 12.0 Hz, $^3J_{Rib5b,P}$ = 2.3 Hz, 1 H, H-5b of Rib), 4.16–4.18 (m, 1 H, H-4 of Rib), 4.24–4.28 (m, 2 H, H-2 of Rib, H-3 of Rib), 4.42 (d, $J_{1',2'}$ = 8.6 Hz, 1 H, H-1'), 5.49 (dd, $J_{1,2}$ = 4.0 Hz, $^3J_{1,P}$ = 7.4 Hz, 1 H, H-1), 5.86 (d, $J_{Ura5,Ura6}$ = 8.6 Hz, 1 H, H-5 of Ura), 5.87 (d, 1 H, H-1 of Rib), 7.86 (d, $J_{Ura5,Ura6}$ = 8.6 Hz, 1 H, H-6 of Ura). ^{13}C NMR (126 MHz, D_2O): δ = 23.4 (C of Me), 56.4 (C-2'), 61.6 (C-6'), 65.8 ($J_{C-5,P}$ = 3.9 Hz, C-5 of Rib), 70.4 (C-3 of Rib), 70.7 (C-5'), 72.1 (C-3), 72.3 ($J_{C-2,P}$ = 9.6 Hz, C-2), 74.1 (C-5), 74.9 (C-4'), 75.2 (C-2 of Rib), 77.0 (C-3'), 81.0 (C-4), 84.3 ($J_{C-4,P}$ = 9.6 Hz, C-4 of Rib), 89.4 (C-1 of Rib), 96.3 ($J_{C-1,P}$ = 5.8 Hz, C-1), 101.0 (C-1'), 104.0 (C-5 of Ura), 142.0 (C-6 of Ura), 153.0 (C-4 of Ura), 167.0 (C-2 of Ura), 176.0 (COOH), 176.0 (C=O of NAc). ^{31}P NMR (202 MHz, D_2O): δ = –10.3 (d, *J* = 21.8 Hz, 1 P, P of Rib), –12.1 (d, *J* = 21.8 Hz, 1 P, P of GlcUA). MS (MALDI–TOF, –ve): *m/z* $[M - H]^-$ calcd for $C_{23}H_{34}N_3O_{23}P_2$: 782.10; found: 781.96.

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Evidence of Immunostimulating Lipoprotein Existing in the Natural Lipoteichoic Acid Fraction▽

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Lipoteichoic acid (LTA) is a cell surface glycoconjugate of gram-positive bacteria and is reported to activate the innate immune system. We previously reported that purified LTA obtained from *Enterococcus hirae* has no immunostimulating activity, but a subfraction (Eh-AF) in an LTA fraction possesses activity. In this study, we established a mouse monoclonal antibody neutralizing the activity of Eh-AF and investigated its inhibitory effects. Monoclonal antibody (MAbEh1) was established by the immunization of BALB/c mice with Eh-AF, followed by hybridoma screening based on its inhibitory effect for the production of interleukin-6 (IL-6) induced by Eh-AF. MAbEh1 neutralized the production of IL-6 by LTA fraction from not only *E. hirae* but also *Staphylococcus aureus*, while it failed to block that of lipopolysaccharide, suggesting that the antibody recognized a common active structure(s) in LTA fractions. Synthetic glycolipids in these LTAs did not induce cytokine production, at least in our system. Interestingly, the antibody was found to inhibit the activity of immunostimulating synthetic lipopeptides, Pam_3CSK_4 and FSL-1. These results suggest that MAbEh1 neutralizes the activity of lipoprotein-like compounds which is responsible for the activity of the LTA fraction of *E. hirae* and *S. aureus*.

Lipoteichoic acid (LTA) is a macroamphiphile distributing on the cell surfaces of gram-positive bacteria and is reported to exhibit immunostimulatory and inflammatory activities. LTA has been shown to have an antitumor effect (34, 36) and to induce inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and IL-6 (3, 31, 33). Recent research showed that such immunostimulatory activities of bacterial compounds were mediated by Toll-like receptor (TLR), a type I transmembrane receptor for innate immune activation (32). To date, more than 10 members of the TLR family have been discovered and most of their ligands were identified: TLR4 in combination with the adapter molecule MD-2 for lipopolysaccharide (LPS)/lipid A, an outer membrane component of gram-negative bacteria (21, 24); TLR9 for unmethylated CpG DNA (15); TLR3 and TLR7/8 for double- and single-stranded RNA (1, 14); TLR5 for bacterial flagellin (13); and TLR2 subfamily (TLR1, −2, and −6) for bacterial lipoprotein/lipopeptide (29, 30). LTA was also reported to be a ligand of TLR2 (22).

The structures of LTAs have been well studied and proposed as a glycoconjugates generally composed of a glycolipid anchor part, such as β-kojibiosyldiacylglycerol for *Enterococcus hirae* and *Streptococcus pyogenes* and β-gentiobiosyldiacylglycerol for *Staphylococcus aureus*, and a 1,3-linked poly(glycerophosphate) substituted by sugars and D-alanine at position 2 of the glycerol (4). Previously, we attempted to determine a structure of the LTA responsible for these activities. Fukase et al. prepared chemically synthetic glycoconjugates having fundamental structures of LTA from *E. hirae* and *S. pyogenes* and their glycolipid anchor parts (5, 6). However, these synthetic compounds exhibited no immunostimulating activities (28), suggesting that the proposed structures are not responsible for the activities. Thus, we reinvestigated the activity of LTA and found that an LTA fraction extracted from *E. hirae* by using a hot phenol (PhOH)-water method was able to be separated into two subfractions, a small amount of cytokine-inducing active fraction and an inactive major compound (27). Further, we determined that the structure of the inactive compound was identical to that of LTA (8). Those results suggested that the contaminating minor components in LTA fraction were responsible for the immunostimulation.

Recently, a structure-function relationship of LTA from *Staphylococcus aureus* has been reported. Morath et al. prepared a purified LTA by using a butanol (BuOH)-water extraction, followed by hydrophobic interaction chromatography, and showed that the LTA itself induces cytokine production (19). Further, those researchers synthesized an LTA counter-

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part and its glycolipid part and found that the counterpart displayed activity similar to that of natural LTA and even glycolipid possesses weak but distinct activity (20). From their observations, the researchers concluded that LTA itself was a potent immunostimulatory component in *S. aureus*. However, their conclusion for *S. aureus* disagreed with our data for *E. hirae*. One explanation for the contradictory results is an effect of the differences in the LTA structure, e.g., kojibiosyl is the backbone for glycolipid anchor in *E. hirae* LTA, whereas gentiobiose is the backbone in *S. aureus*, and partially alanylated oligoglucosyl is the substituent on glycerol at position 2 for *E. hirae*, but D-alanyl and glucosaminyl substitutions are made in *S. aureus*. This explanation may be supported by another report which showed that LTA exhibited from *Streptococcus pneumoniae* is 100-fold less potent than staphylococcal LTA (7). Pneumococcal LTA has been reported to be composed of a phosphocholine (PC)-linked tetraglycosylribitolphosphate polymer and a triglycosyldiacylglycerol anchor (2). Differences in extraction methods may be another possibility. Morath et al. also mentioned the critical role of D-alanine content in an LTA molecule from *S. aureus* (19), reporting that alkaline hydrolysis of the active LTA resulted in a loss of alanine substituent in LTA and reduced its activity. PhOH extraction of bacterial cells also decreased alanine, but BuOH extraction prevented alanine cleavage.

These interpretations might explain the inactivity of *E. hirae* LTA but do not clarify our minor active components. Therefore, we intended to reevaluate a principal compound responsible for the activity in *E. hirae*. In the present study, we established a mouse monoclonal antibody that neutralizes the activity of an LTA fraction from *E. hirae* and investigated its inhibitory effects for various bacterial stimuli.

MATERIALS AND METHODS

Bacterial compounds. *Enterococcus hirae* ATCC 9790 and *Staphylococcus aureus* DSM 20231 organisms were grown as previously described (27). The extraction of crude LTA fractions was performed using the BuOH-water method (19). The crude fractions were treated with DNase and RNase to digest contaminating nucleic acids and then subjected to hydrophobic interaction chromatography on octyl-Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden), with a batchwise elution using stepwise 1-propanol concentrations (15, 40, and 60%) as described previously (8). Since LTA was eluted mainly with the 40% 1-propanol fractions, these fractions were used as LTA fractions and designated Eh-Bu (for *E. hirae*) and Sa-Bu (for *S. aureus*). The immunostimulatory active fractions HGL-A, HGL-B1, and HGL-B2, previously prepared from the *E. hirae* LTA fraction (9), were combined, and the resulting fraction (designated Eh-AF) was used for immunization. Glycolipid anchors of LTA, β-kosibiosyldipalmytoylglycerol for *E. hirae* (5) and β-gentiobiosyldipalmytoylglycerol for *S. aureus* (data not shown), were synthesized. LPS from *Escherichia coli* O:111 was obtained from Sigma-Aldrich (St. Louis, MO) and subjected to phenol reextraction by using sodium deoxycholate (16). PC, phosphatidylethanolamine, and phosphatidylinositol were also obtained from Sigma-Aldrich. Synthetic lipopeptides, Pam_3CSK_4, O,O′-diacyl-type Pam_2CSK_4, FSL-1, *N*-monoacyl-type $PamCSK_4$, and deacyl-type $dhCSK_4$ were purchased from EMC Microcollections (Tübingen, Germany). Monoclonal antibody for LTA was purchased from Biogenesis (Oxford, United Kingdom).

Establishment of MAbEh1. A monoclonal antibody, MAbEh1, was established according to standard methods. Briefly, BALB/c mice were immunized with Eh-AF (0.25 mg/mouse) with Freund's complete adjuvant (Becton Dickinson, Franklin Lakes, NJ) on days 0 and 21 and spleen cells obtained on day 24 were fused with SP2/0-Ag14 myeloma cells. The hybridoma cells were cultured in hypoxanthine-aminopterin-thymidine medium and subcloned by limiting dilution. Hybridoma-secreting antibody neutralizing Eh-AF activity was screened on the basis of the inhibitory effect against the production of IL-6 in THP-1 cells stimulated with 300 ng/ml Eh-AF, and an antibody was designated MAbEh1. The hybridoma was cultured in CD hybridoma medium (Invitrogen, Carlsbad, CA), and the culture supernatant was used for the antibody stock solution. The stock solution was then subjected to gel filtration chromatography on Bio-Gel A5m (Bio-Rad, Hercules, CA) to give purified antibody. Isotyping of the antibody was performed with a mouse monoclonal isotyping kit (Serotec, Oxford, United Kingdom). Isotype control antibody was purchased from e-Bioscience (San Diego, CA).

Cytokine assays. Human monocytic leukemia cell line THP-1 was obtained from the Health Science Research Resources Bank (Osaka, Japan) and cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (FBS; MBL, Nagoya, Japan), 50 μg/ml penicillin, and 50 μg/ml streptomycin. THP-1 was differentiated with 10^{-7} M 1,25-dihydroxyvitamin D_3 for 3 days before use. Human peripheral blood mononuclear cells (PBMCs) were obtained from heparinized human peripheral blood collected from a healthy volunteer by density gradient centrifugation using Histopaque-1077 (Sigma).

The cells were plated onto 96-well microplates at 1×10^5 cells in 100 μl of RPMI 1640 with or without 10% FBS and stimulated with the indicated dose of the test specimens in the presence or absence of MAbEh1 for 24 h. Culture supernatants were collected and analyzed by using an enzyme-linked immunosorbent assay (ELISA) kit for secreted IL-6 (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction. The concentration of secreted IL-6 from cells was determined using a standard curve of recombinant IL-6 prepared in each assay and presented as the means ± standard deviations (SD). Inhibitory effects of FBS and MAbEh1 were statistically evaluated by using Welch's *t* test.

Luciferase assays. Ba/F3 cells stably expressing p55IgκLuc, an NF-κB/DNA binding activity-dependent luciferase reporter construct (Ba/κB), murine TLR2 and the p55IgκLuc reporter construct (Ba/mTLR2), and murine TLR4/MD-2 and the p55IgκLuc reporter construct (Ba/mTLR4/mMD-2) were kindly provided by K. Miyake (Institute of Medical Science, University of Tokyo, Tokyo, Japan). NF-κB-dependent luciferase activity in these cells was determined as follows. Cells were inoculated onto each well of a 96-well, flat-bottomed plate at 1×10^5 cells in 80 μl of RPMI 1640 supplemented with 10% FBS and stimulated with the indicated concentrations of the test specimens. After 4 h of incubation at 37°C in humidified air containing 5% CO_2, 80 μl of Bright-Glo luciferase assay reagent (Promega, Madison WI) was added to each well and luminescence was quantified with a luminometer ARVO SX multilabel counter (Perkin Elmer, Wellesley, MA). Results are shown as relative luciferase activity, which was the ratio of stimulated activity to nonstimulated activity in each cell line.

Immune blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) was performed by the Tris-glycine method using a mini-PAGE chamber AE-6530 and an AE-8450 power supply (ATTO, Tokyo, Japan) with a 15% gel. Materials in the gels were transferred to a nitrocellulose membrane (Bio-Rad) by using a semidry blotter AE-6677 (ATTO). For dot blot analysis, stimulus solution was placed on a nitrocellulose membrane and dried in the air. The membranes were incubated with blocking solution (3% nonfat milk in Tris-buffered saline containing 0.05% Tween 20) for 12 h at 4°C and then with 1/100 of MAbEh1 diluted in the diluent (1% nonfat milk in Tris-buffered saline containing 0.05% Tween 20) for 2 h at room temperature. The antibody was detected by incubation in peroxidase-labeled second antibody (KPL, Gaithersburg, MD; 1/2,000 in the diluent) for 2 h, followed by development using ECL (Amersham Bioscience). Luminescence was recorded with a LAS-1000 luminescence analyzer (Fuji Film, Kanagawa, Japan).

RESULTS

Preparation of LTA fractions. We previously separated small amounts of immunobiologically active fractions from *E. hirae* LTA fractions prepared by hot PhOH-water extraction (27). However, the structural elucidation of an essential compound(s) responsible for the activity was incomplete because of the difficulty of further purification based on its small amount. Recently, Morath et al. reported that an LTA fraction obtained from *S. aureus* by using BuOH-water extraction, followed by hydrophobic interaction chromatography, exhibited higher activity than that obtained by the PhOH method (19). Thus, we prepared LTA fractions by the BuOH method. *E. hirae* and *S. aureus* bacteria were subjected to BuOH extraction to give crude LTA fractions in yields of 1.5 to 2.1% and 1.6 to 2.6%, respectively. The crude extracts were digested with nu-

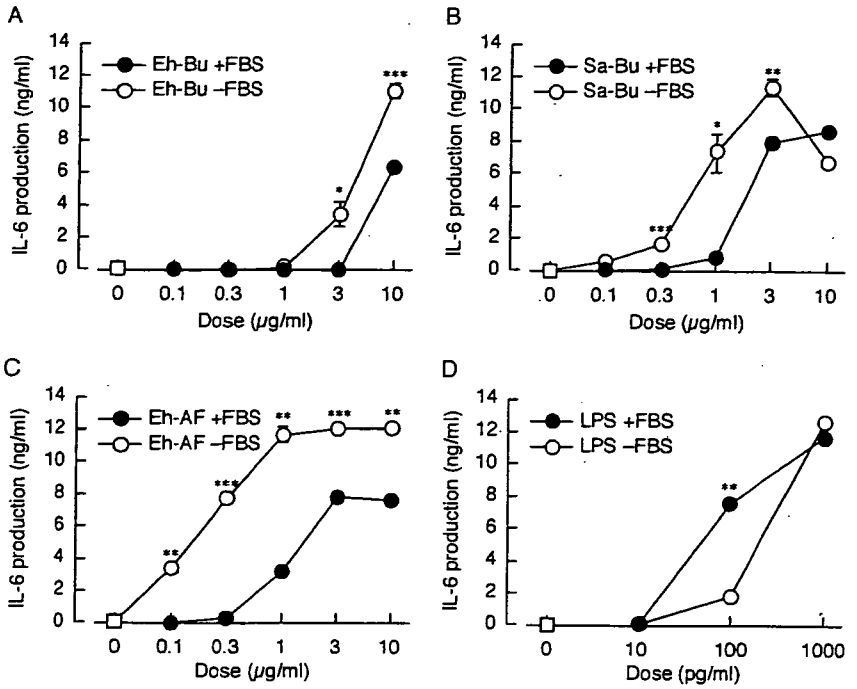

FIG. 1. IL-6 production in human peripheral blood mononuclear cells induced by (A) Eh-Bu, (B) Sa-Bu, (C) Eh-AF, or (D) LPS in the presence or absence of 10% FBS. Cells were stimulated with the indicated doses of stimuli for 24 h, and IL-6 production was determined by ELISA. The results represent the mean values (± SD [error bars]) obtained from three independent experiments. *P* values against stimuli without FBS are indicated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

clease and then subjected to hydrophobic interaction chromatography to obtain LTA fractions Eh-Bu (15 to 28%, yield from crude LTA fraction) and Sa-Bu (12 to 27%). Both fractions induced IL-6 production in PBMCs (Fig. 1). We previously demonstrated that IL-6 production in THP-1 cells stimulated with the active fraction was suppressed in the presence of FBS (9). Thus, the effect of serum was investigated, and the activities of Eh-Bu and Sa-Bu were found to decrease in the presence of FBS in a manner similar to that with Eh-AF, an active fraction previously prepared from *E. hirae* LTA fraction obtained by the PhOH method (Fig. 1). All of the fractions activated Ba/mTLR2 cells, but Ba/mTLR4/mMD-2 and negative control Ba/κB were not activated significantly (Fig. 2), indicating no endotoxin contamination.

Establishment of MAbEh1. We constructed an antibody that neutralized the activity of the fractions to evaluate a principal compound responsible for the activity. Mouse hybridoma cells were established by the immunization of BALB/c mice with Eh-AF. A hybridoma-secreting monoclonal antibody was screened for a neutralizing effect against the IL-6-inducing activity of Eh-AF in THP-1 cells. One hybridoma was found to secrete a neutralizing antibody named MAbEh1. The culture supernatant of the hybridoma cells in serum-free medium was subjected to gel filtration chromatography, and a fraction containing antibody was used for the solution of MAbEh1 (1.06 mg protein/ml). The isotype of the antibody was immunoglobulin M (IgM). MAbEh1 suppressed the activity of up to 1 μg/ml of Eh-AF dose dependently (Fig. 3). The antibody, in contrast, showed no inhibitory effect on the activity of LPS (Fig. 3). These results showed that MAbEh1 specifically suppresses the activity of the components in Eh-AF.

Neutralizing effects of MAbEh1. We next investigated the neutralizing effect of MAbEh1. The antibody also inhibited the activity of Eh-Bu and Sa-Bu (Fig. 4). These results suggest that structures of active components in Eh-Bu and Sa-Bu are common ones in LTA fractions and are similar to those in Eh-AF. Morath et al. reported that the glycolipid anchor in *S. aureus* LTA induced the production of TNF-α in human whole blood (19). Thus, we investigated the inhibitory effect on synthetic glycolipid anchors of *E. hirae* and *S. aureus*. However, in our assay system, neither glycolipid stimulated IL-6 production in human PBMCs (Fig. 5A and B). We recently showed that lipoproteins are predominant TLR2-activating ligands in *S. aureus* cell wall components (11). Thus, the inhibitory effects of MAbEh1 on the synthetic lipopeptides Pam_3CSK_4 and FSL-1 were studied. The activities of both synthetic counterparts were suppressed by the addition of the antibody dose dependently (Fig. 6A). The activities were also decreased in the presence of serum in a manner similar to those of Eh-AF, Eh-Bu, and Sa-Bu (Fig. 6B and C). Further, MAbEh1 bound lipopeptides Pam_3CSK_4, Pam_2CSK_4, $PamCSK_4$, and FSL-1 in dot blot analysis, but not other lipids (Fig. 7A). The antibody for LTA also failed to recognize these lipopeptides (Fig. 7B). These results suggested that MAbEh1 inhibits the activity of a compound containing lipopeptide structure.

DISCUSSION

LPS is a potent immunostimulatory compound in gram-negative bacteria. Although LPS is known to activate cells through TLR4, LPSs from some bacterial species have been reported to exhibit activity via TLR2 in addition to TLR4 (17,

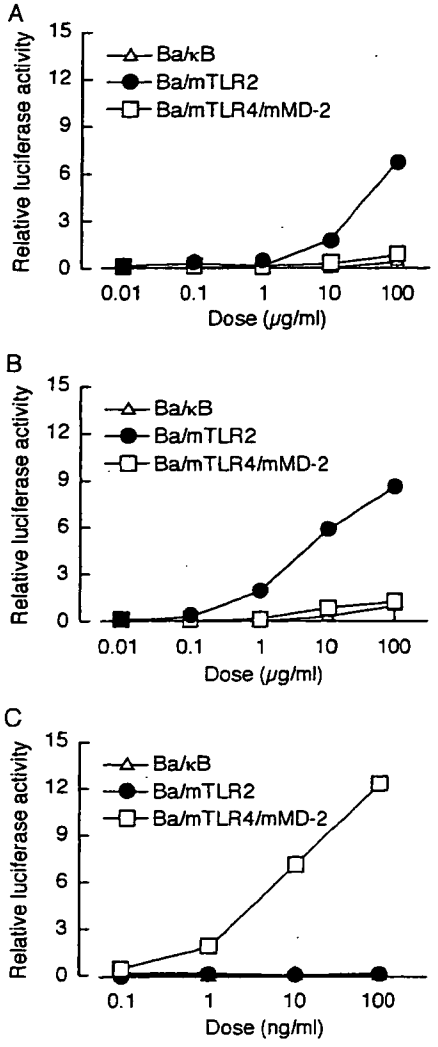

FIG. 2. NF-κB activation in Ba/κB, Ba/mTLR2, or Ba/mTLR4/mMD-2 cells induced by Eh-Bu (A), Sa-Bu (B), and LPS (C). Cells were incubated with the indicated doses of stimuli for 4 h. NF-κB activation was measured with a luciferase assay. Results are shown as relative luciferase activity, which was determined as the ratio of stimulated to nonstimulated activity.

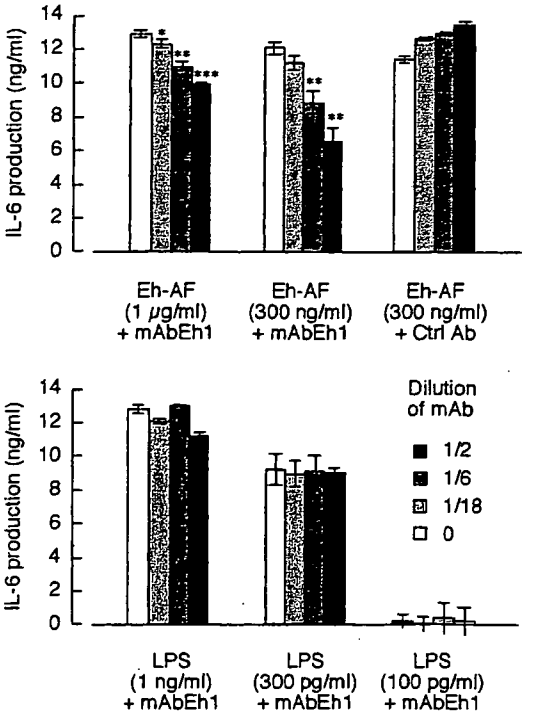

FIG. 3. Inhibitory effects of MAbEh1 or an isotype control antibody on IL-6 production in human peripheral blood mononuclear cells stimulated with Eh-AF or LPS. Cells were stimulated with the indicated doses of stimuli and antibody for 24 h in the absence of FBS, and IL-6 production was determined by ELISA. The results represent the mean values (± SD [error bars]) obtained from three independent experiments. *P* values against stimuli without antibody are indicated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

25, 35). Recent research proved that some of the TLR2-activating components were contaminated with small amounts of lipoproteins (10, 18). Lipoproteins are usually extracted from bacterial cells by surfactants such as Triton X-114 (23). LPS, which consists of a long hydrophilic polysaccharide and a hydrophobic lipid A anchor, may act as a surfactant. Since LTA is macroamphiphile, LTA may also work as a surfactant to extract lipoproteins from bacterial cells. In fact, we previously demonstrated that the activity of an LTA fraction, a BuOH extract, of *S. aureus* was not abrogated by hydrofluoric acid (HF) hydrolysis but by the following treatment with lipoprotein lipase, which cleaved acyl groups essential for the activity of lipoprotein and reduced the activity, indicating a possibility of the existence of lipoprotein in the LTA fraction (11). This indicated that lipoprotein but not the LTA molecule is responsible for the activity of LTA fractions. In the present study, we further confirmed the evidence of lipoprotein contamination in LTA fractions of *E. hirae* in addition to those of *S. aureus*. MAbEh1, which inhibited the immunostimulating activity of lipopeptides Pam_3CSK_4 and FSL-1, decreased the activity of the LTA fractions. This showed that lipoproteins were responsible for the activity of minor compounds previously separated from the LTA fraction of *E. hirae* (27).

We also characterized the binding affinity of MAbEh1. Dot blot analysis showed that the antibody binds to the lipopeptides triacylated Pam_3CSK_4, diacylated Pam_2CSK_4 and FSL-1, and

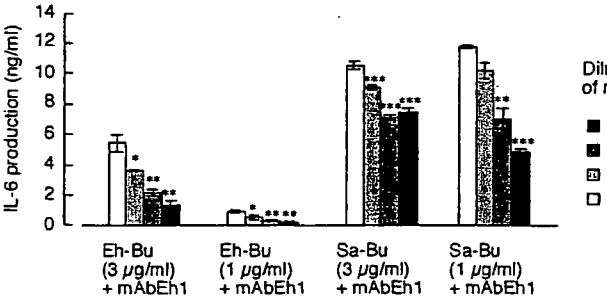

FIG. 4. Inhibitory effects of MAbEh1 on IL-6 production in human peripheral blood mononuclear cells stimulated with Eh-Bu or Sa-Bu. Cells were stimulated with the indicated doses of stimuli and antibody for 24 h in the absence of FBS, and IL-6 production was determined by ELISA. The results represent the mean values (± SD [error bars]) obtained from three independent experiments. *P* values against stimuli without antibody are indicated. *, $P < 0.05$; **, $P < 0.01$; or ***, $P < 0.001$.

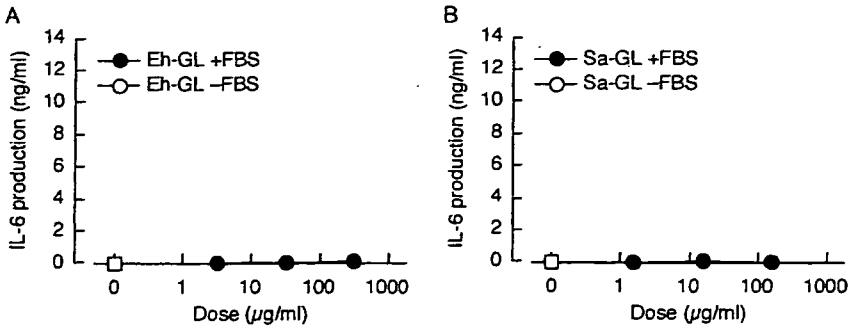

FIG. 5. IL-6 production in human peripheral blood mononuclear cells stimulated with (A) Eh-GL or (B) Sa-GL in the presence or absence of 10% FBS. Cells were stimulated with the indicated doses of stimuli for 24 h, and IL-6 production was determined by ELISA. The results are presented as the means ± SD.

monoacylated $PamCSK_4$ but not to deacylated $dhCSK_4$ (Fig. 7A). The antibody did not bind to LPS or diacylglyceryl lipids PC, phosphatidylethanolamine, and phosphatidylinositol (Fig. 7A). The lipopeptides were not visualized by an LTA antibody which bound to Eh-Bu (Fig. 7B). These results indicated that MAbEh1 recognized the N-terminal lipid moiety of lipopeptide. Unfortunately, both dot blot and Western blotting analysis of the lipoteichoic acid fraction using MAbEh1 failed to visualize any compound (data not shown), although the contamination of lipoprotein in the fraction was expected by the inhibition assay. This might be caused by its low concentration in the fraction as suggested in our previous work (11, 27) and/or low affinity of IgM antibody. In contrast to the specific binding, the inhibitory effect of MAbEh1 against not only lipoteichoic fraction but also synthetic lipopeptides was only partial (Fig. 3, 4, and 6). One interpretation for the partial effect may be the low affinity of IgM. We also assumed another possibility, which was that the inaccessibility of antibody to the N-terminal recognition center of lipoprotein was due to the incorporation into LTA micelles. Our previous observation, that lipoprotein lipase digestion of lipoproteins existing in the *S. aureus* LTA fraction (11) or the *Porphyromonas gingivalis* LPS fraction (10; our unpublished data) is unsuccessful, supported our second assumption.

Previously, we determined that LTA from *E. hirae* was inactive for the innate immune system (8). We also investigated the effect of HF degradation of the LTA fraction derived from *S. aureus* (11). Since HF cleaves the phosphodiester bonds in

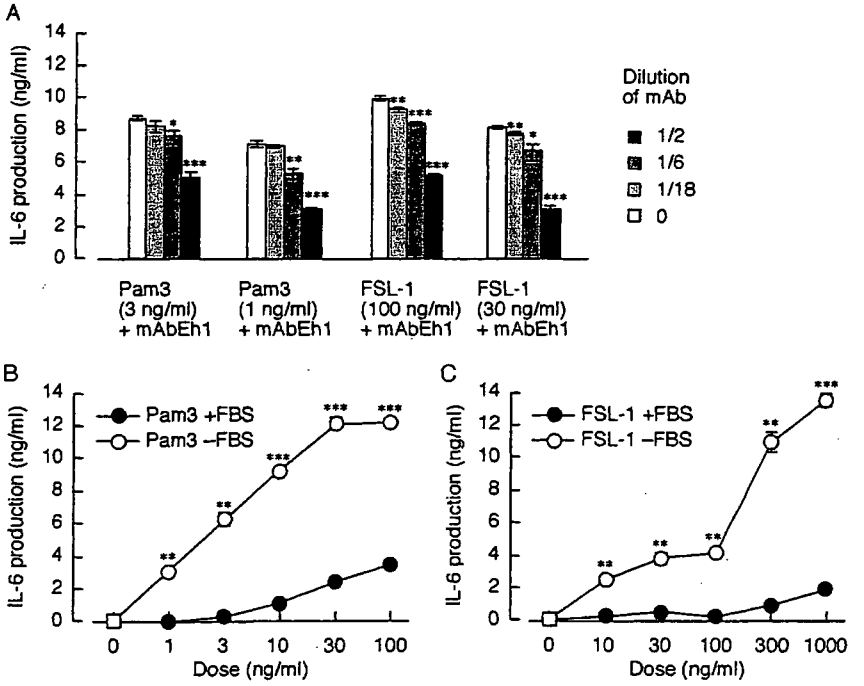

FIG. 6. (A) Inhibitory effects of MAbEh1 on IL-6 production in human peripheral blood mononuclear cells stimulated with synthetic lipopeptides Pam_3CSK_4 (Pam3) or FSL-1. Cells were stimulated with the indicated doses of stimuli and antibody for 24 h in the absence of FBS. (B and C) IL-6 production in human peripheral blood mononuclear cells stimulated with (B) Pam_3CSK_4 or (C) FSL-1 in the presence or absence of 10% FBS. IL-6 production was determined by ELISA. The results represent the mean values (± SD [error bars]) obtained from three independent experiments. *P* values against stimuli without antibody or FBS are indicated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

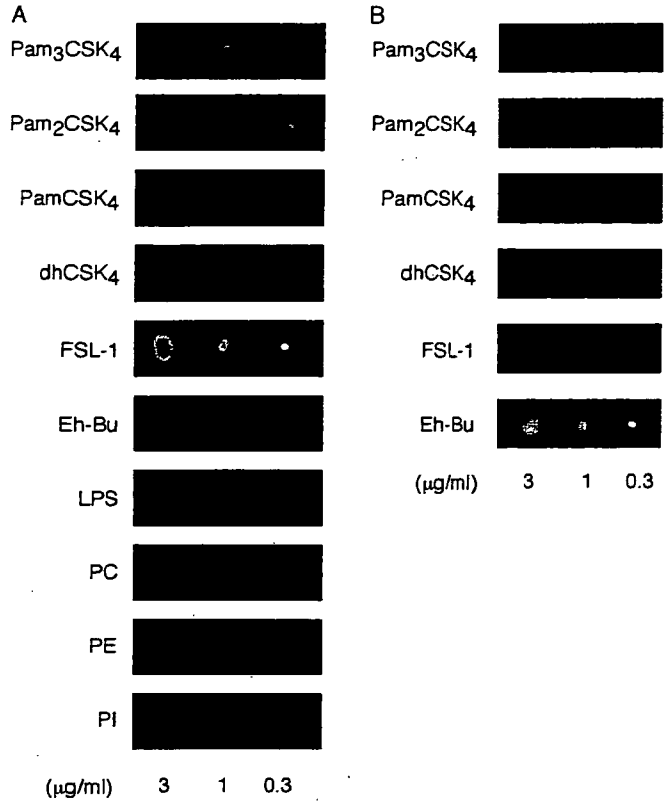

FIG. 7. Dot blot analysis against lipopeptide derivatives and other lipids with (A) MAbEh1 or (B) antibody for LTA. The indicated doses of stimuli were blotted onto a nitrocellulose membrane. The membranes were blocked with nonfat milk and incubated with MAbEh1. The bound antibody was detected with peroxidase-labeled second antibody using ECL reagents.

polyglycerophosphate, a hydrophilic part of LTA, most of the molecular mass of LTA is decomposed into small components, such as phosphate, glycerol, and phosphoglycerol (4). After HF degradation, no Alcian blue-stained band was found in the sodium dodecyl sulfate-PAGE gel, showing the complete decomposition of LTA. The treatment, however, did not abrogate the activity of the LTA fraction. Further, we showed that glycolipid parts of LTA for *E. hirae* and *S. aureus* were both inactive (Fig. 5). These results suggest that LTA itself was not an active molecule. However, we have not confirmed that natural LTA from *S. aureus* was immunobiologically inactive since a selective deletion of lipoprotein was not achieved. It was reported that *S. aureus* LTA was not separated into active and inactive fractions by the hydrophobic interaction and anion-exchange chromatographies which were used for the separation of *E. hirae* LTA (20). Direct lipoprotein lipase digestion of the LTA fraction was not successful (11), probably because contaminated lipoproteins may be incorporated into LTA micelles and the enzyme was not able to approach them. The reextraction of the natural LTA from *S. aureus* with PhOH containing deoxycholate, which was used for the extraction of contaminated lipoprotein from LPS (16), was also unsuccessful (data not shown). Since the reextraction method was also ineffective in some cases, such as for the extraction of lipoprotein from *Porphyromonas gingivalis* LPS (10, 16), it may be considered that the micellation of lipoprotein with LTA is very tight. Recently, we demonstrated that LTA from a lipoprotein diacylglycerol transferase deletion mutant of *S. aureus*, which contains no detectable lipoproteins (26), is 100-fold less active than that from the wild type (12). This result indicated that most of the activity of LTA fraction appears to be caused by lipoproteins. The identification of active lipoprotein species and the determination of chemical structure of compounds responsible for the residual activity in mutant LTA fraction are required for further understanding of biological activity of LTA molecule.

In conclusion, we established a monoclonal antibody that neutralizes the activity of natural LTA and demonstrated that the monoclonal antibody also blocked the activity of lipopeptides. These results strongly suggest that MAbEh1 neutralizes the activity of lipoprotein-like compounds existing in the natural LTA fraction from *E. hirae* and *S. aureus*.

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Human Herpesvirus 6 Infection of CD4$^+$ T-Cell Subsets

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Abstract: **The immune system includes CD4$^+$ regulatory T (T_{reg}) cells that play a role in self-tolerance and demonstrate functional variations that govern immune responses. HHV-6 is an important immunosuppressive virus that completely replicates *in vivo* and *in vitro* in only CD4$^+$ T cells. However, there have been no reports of the specific T-cell subpopulation that permits the replication of this virus. Here, we evaluated the infectivity of HHV-6 to specific T-cell populations such as CD4$^+$CD25^{high}, which includes the majority of T_{reg} cells, and CD4$^+$CD25$^-$. These cells were isolated from peripheral blood and then expanded. The expanded cell fractions were then infected with the HHV-6 variant B strain, and the spreads of infected cells were evaluated by immunofluorescence. Viral growth was also quantified by real-time PCR. The effects of virus infection on cytokine production from these T-cell subsets were examined using ELISA. Our results revealed that both these fractions permitted complete HHV-6 replication. Virus infection enhanced the production of both Th1- and Th2-type cytokines from CD4$^+$CD25$^-$ T cells; however, only Th2-type cytokine release was augmented from viral-infected CD4$^+$CD25^{high} T cells. Further, while virus-infected CD4$^+$CD25^{high} T cells shift their antiviral immunity toward Th2 dominance by producing IL-10, the role of virus-infected CD4$^+$CD25$^-$ T cells remains obscure.**

Key words: **HHV-6, Human CD4$^+$ T cell, Regulatory T cell, Cytokine production**

The immune system includes naturally occurring CD4$^+$ regulatory T (T_{reg}) cells, which maintain self-tolerance by suppressing the activation and expansion of self-reactive lymphocytes that may cause autoimmune diseases (32). T_{reg} cells develop in the thymus and its periphery after antigen (Ag) activation (10, 19, 37). T_{reg} cells perform regulatory functions in cells expressing high levels of the interleukin 2 (IL-2) alpha receptor CD25 (CD4$^+$CD25^{high}) (4). Additionally, the transcription factor forkhead box P3 (FoxP3) has recently been described as a key regulatory protein for T_{reg}-cell development in both mice and humans (18, 40). Ectopic FoxP3 expression confers peripheral CD4$^+$CD25$^-$ T cells with a suppressor function (13); these cells comprise 5%–10% of the peripheral CD4$^+$ T cells in humans. T_{reg} cells play a role in immune suppression by inhibiting CD4$^+$CD25$^-$ T-cell propagation in a cell contact-dependent manner (4). On the other hand, acquired regulatory T cells arise in the periphery. They suppress the activation of conventional T cells in a cytokine-dependent manner: TGF-β for Th3 cells, and IL-10 for T regulatory 1 (Tr1) cells (6, 8). Thus, these cells demonstrate functional variations that govern immune responses.

In 1986, a new herpesvirus was discovered in patients with acquired immune deficiency syndrome and hematological disorders (33); this virus has recently been designated as human herpesvirus 6 (HHV-6). Based on its biological, molecular, and antigenic features, this virus is classified into the following 2 variants: A and B (2, 9, 42). HHV-6 variant B has been identified as the causative agent of the childhood disease exanthem subitum (ES) (41), while no disease has been

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Abbreviations: Abs, antibodies; Ag, antigen; CD, cluster of differentiation; CD3/CD28 beads, anti-CD3 and anti-CD28-Ab-conjugated beads; ELISA, enzyme-linked immunosorbent assay; ES, exanthem subitum; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; FoxP3, forkhead box P3; gB, glycoprotein B; HHV-6, human herpesvirus 6; HIV, human immunodeficiency virus; HSV-1, herpes simplex virus type 1; HTLV-1, human T lymphotropic virus type 1; IFN, interferon; IL-2, interleukin 2; MOI, multiplicity of infection; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; $TCID_{50}$, 50% tissue culture infective dose; T_{reg}, regulatory T.

clearly associated with variant A. Most people experience primary HHV-6 infection by the age of 3 years (5, 29). Reactivation of or reinfection with the virus may cause opportunistic diseases in immunocompromised hosts (9, 27, 42). In fact, HHV-6 reactivation occurred in 40%–50% of bone marrow transplantation recipients (44), and occasionally resulted in serious outcomes (45). $CD4^+$ T cells are the only cells in which HHV-6 completely replicates *in vivo* (39) and *in vitro* (26); however, HHV-6 latently infects monocytes/macrophages (22). However, no additional work regarding the determination of the T-cell subpopulation that permits the replication of this virus has been reported.

Several reports have demonstrated that T_{reg} cells affected functional immunity to microbes (1, 25, 38, 43); this may facilitate microbial escape from the immune system and microbial propagation. If HHV-6 selectively infects a subpopulation of $CD4^+$ T cells, such as T_{reg} cells or $CD4^+CD25^-$ T cells, the host's immune responses may be modified. Indeed, although its mechanism is not completely understood, HHV-6 is known as an important immunosuppressive virus (9). In this study, we evaluated the infectivity of HHV-6 to specific cell populations such as $CD4^+CD25^{high}$, which includes the vast majority of T_{reg} cells, and $CD4^+CD25^-$ T cells. Our results revealed that both these T-cell fractions permitted complete HHV-6 replication, and cytokine productions by these cell subsets were modified by HHV-6 infection.

Materials and Methods

Cell isolation. Using Ficoll-Conray gradient centrifugation, human peripheral blood mononuclear cells (PBMCs) were isolated from healthy adults having HHV-6-positive serum antibodies (Abs). Informed consent was obtained from each individual before participation in the study. As shown in Fig. 1, by using a fluorescence-activated cell sorter (FACS) (FACSAria, Becton Dickinson, Los Angeles, Calif., U.S.A.), the PBMCs were further separated into 2 $CD4^+$ T-cell fractions—$CD4^+CD25^{high}$ and $CD4^+CD25^-$—that showed high and no expression of CD25, respectively. In this study, we used these $CD4^+$ T-cell fractions, but not the intermediate fraction expressing $CD25^{low}$, to obtain 2 distinct cell populations because one contained a majority of T_{reg} cells, while the other did not (4).

Propagation of $CD4^+$ T cells. The $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells were cultured in 96-well U-bottom plates by stimulating the cells with anti-CD3 and anti-CD28-Ab-conjugated beads (CD3/CD28 beads) (T-cell Expander, Dynal Biotech, Hamburg, Germany) at a ratio of 1 bead per cell in RPMI1640 medium supplemented with 10% fetal calf serum (complete RPMI) containing a high dose of IL-2 (500 units/ml).

HHV-6 infection of $CD4^+$ T cells. The expanded $CD4^+$ T cells were infected for 2 hr at 37 C with variant B HHV-6 (HST strain) at a multiplicity of infection (MOI) of 0.05, which was the 50% tissue culture infective dose ($TCID_{50}$) per cell. The cells were cultured in complete RPMI alone or with CD3/CD28 beads. The infected cells and culture supernatants were analyzed for the presence of viral Ags and viral DNAs, respectively.

Quantification of HHV-6-infected cells. The virus-infected cells were fixed on glass slides and treated with the anti-HHV-6 glycoprotein B (gB) mouse monoclonal Ab OHV1 (28). After washing, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgGs (Southern Biotechnology Associates, Inc., Birmingham, Ala., U.S.A.). The cells were washed, mounted with glycerol, and photographed using a fluorescence microscope. A total of 1,000 cells were counted, and the percentage of viral Ag-positive cells was calculated.

Detection of viral and cellular Ags at the single-cell level. The virus-infected cells were treated with a mixture of OHV1 and goat anti-human FoxP3 polyclonal Ab (Abcam, Cambridge, U.K.). After washing, rhodamine-conjugated anti-mouse IgGs (Chemicon Inter-

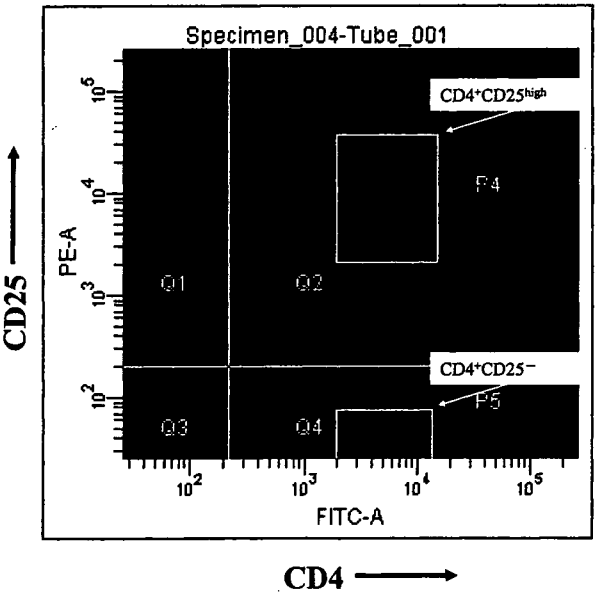

Fig. 1. Separation of PBMCs by FACS. Freshly drawn human PBMCs were stained with both PE-anti-CD25 and FITC-anti-CD4 Abs. The cells in this analysis were gated on lymphocytes via their forward and side scatter properties. $CD4^+CD25^{high}$ and $CD4^+CD25^-$ populations were sorted using the indicated sorting gates.

national, Inc., Temecula, Calif., U.S.A.) and FITC-conjugated anti-goat IgGs (Southern Biotech, Birmingham, Ala., U.S.A.) were added. The Ag-positive cells were detected by using a laser scanning confocal microscope (LSM510, Carl Zeiss Co., Ltd., Germany).

Quantification of HHV-6 DNAs by real-time PCR. The copy number of HHV-6 DNAs in the culture supernatants was assessed by real-time PCR, which targeted the HHV-6 U41 gene, based on *Taq*Man technology using an ABI PRISM 7900HT sequence detector (Applied Biosystems Japan, Ltd., Tokyo). Primers and a probe were selected by using Primer Express software (Applied Biosystems Japan, Ltd.). The sequence of the primers was as follows: forward primer, 5′-GGGAGCAAACTGTCAAATTAGCA-3′ and reverse primer, 5′-TTCTCGACCGCTGAAATTTTC-3′. The sequence of the probe was 5′-Fam CCCACAAAACTTATCTGGGTATCTCCGGAC Tamra-3′. The PCR protocol was as follows: 2 min at 50 C, 10 min at 95 C, and 40 cycles of 10 sec at 95 C, and 1 min at 64 C.

Cytokine assays. $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells that were freshly isolated from peripheral blood were mixed with the HST strain at a MOI of 1 $TCID_{50}$ per cell or with complete medium for 2 hr. After washing, the cells were cultured with complete medium in the presence or absence of CD3/CD28 beads. Culture supernatants were collected at 24 hr, replaced with new complete medium and the supernatants were picked up again at 48 hr and stored at −80 C until use. For evaluating the production of the human cytokines IL-2, IL-4, IL-10, and IFN-γ, commercially available ELISA kits (Human Th1/Th2 ELISA Panel, San Diego, Calif., U.S.A.) were used as indicated by the manufacturer.

Statistical analysis. Statistical analysis was performed using the Student's *t* test. Values were compared between groups with or without HHV-6 infection. *P* value<0.05 was considered statistically significant.

Results

Expansion of $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T Cells

Because the amounts of $CD4^+CD25^{high}$ T cells obtained from the PBMCs were usually too limited (5,000–10,000 cells/ml of blood) to be used in experiments, we first attempted to expand the cells. When the $CD4^+CD25^{high}$ T cells were cultured with CD3/CD28 beads tagged with IL-2 present in a high dose, the cells expanded approximately 20- fold at 9 days after cultivation (Fig. 2). On the other hand, the $CD4^+CD25^-$ T cells expanded more than 40-fold with the same treatment.

HHV-6 Infection to $CD4^+$ T Cells

It is well-known that HHV-6 is a $CD4^+$ T cell-tropic virus and that the activation of infected cells is required for its efficient replication (15). In order to clarify whether the virus can replicate completely in $CD4^+CD25^{high}$ cells, particularly T_{reg} cells, or in $CD4^+CD25^-$ T cells, HHV-6-infected cells were examined for the presence of gB, which is expressed in the late phase of viral replication, or FoxP3 by immunofluorescence testing. Most cells in both the T-cell populations showed typical cytopathic effects, expressed gB (Fig. 3A), and were finally destroyed when they were activated for propagation by a combination of IL-2-tagged CD3/CD28 beads. However, these findings were not

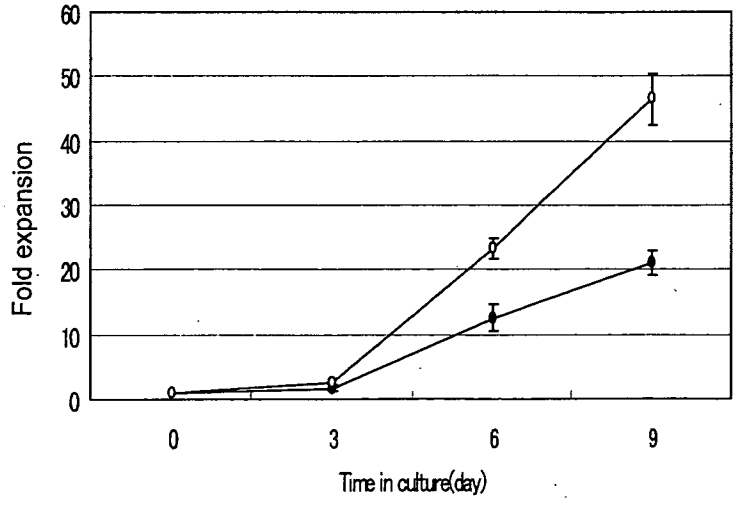

Fig. 2. $CD4^+$ T-cell expansion, and detection of T_{reg} cells. $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells were cultured with CD3/CD28 beads and IL-2 (500 units/ml); following this, the cell numbers were counted at intervals. Cell numbers were expressed as fold increase relative to those on day 0. (●): $CD4^+CD25^{high}$, (○): $CD4^+CD25^-$.

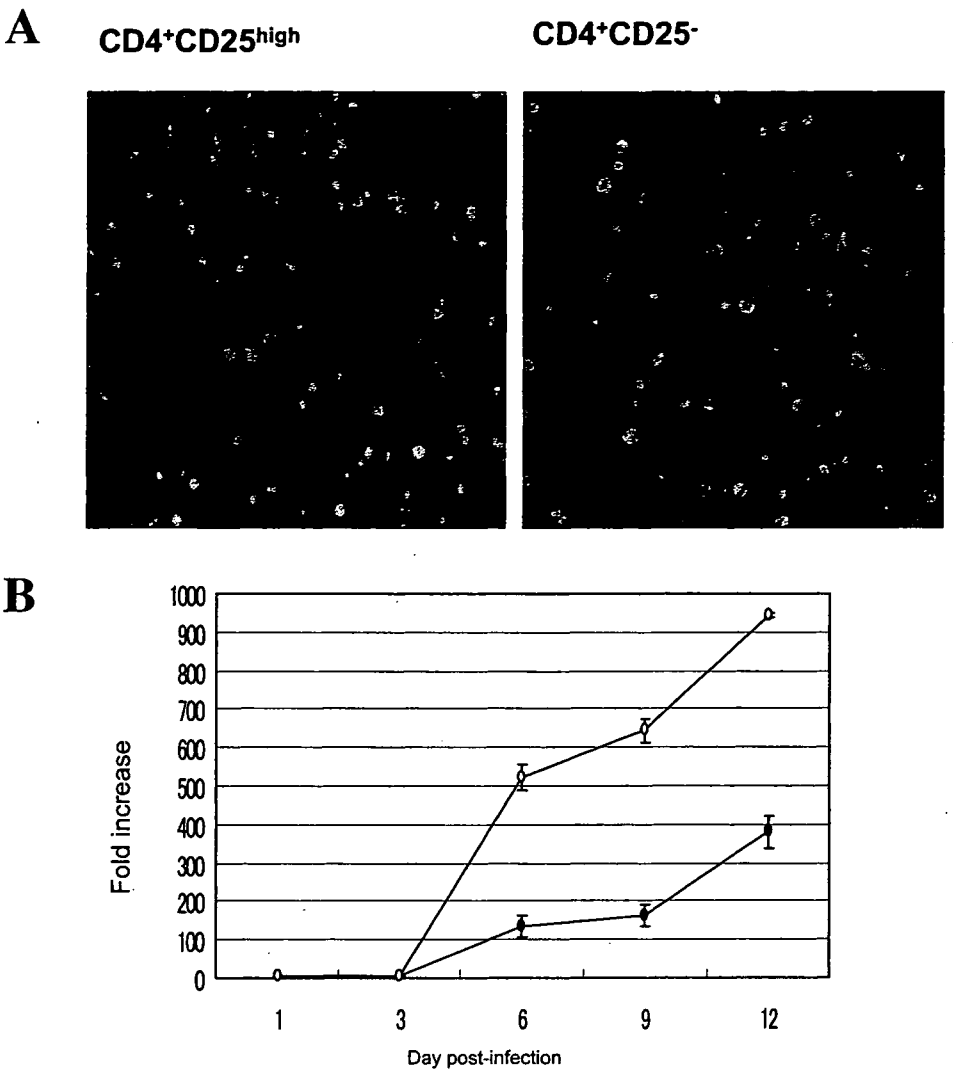

Fig. 3. HHV-6 replication in $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells. (A) Cells in each subset were cultured for 6 days after HHV-6 infection and examined for the presence of gB Ags. Green fluorescing cells represent gB Ag-positive cells. All cells were counterstained with Evans blue, and the red-stained cells were the uninfected cells. (B) HHV-6 DNA copy numbers in the culture supernatants were quantified by real-time PCR as described in "Materials and Methods." The values obtained were expressed as fold increase relative to that on day 1. (●): $CD4^+CD25^{high}$, (○): $CD4^+CD25^-$.

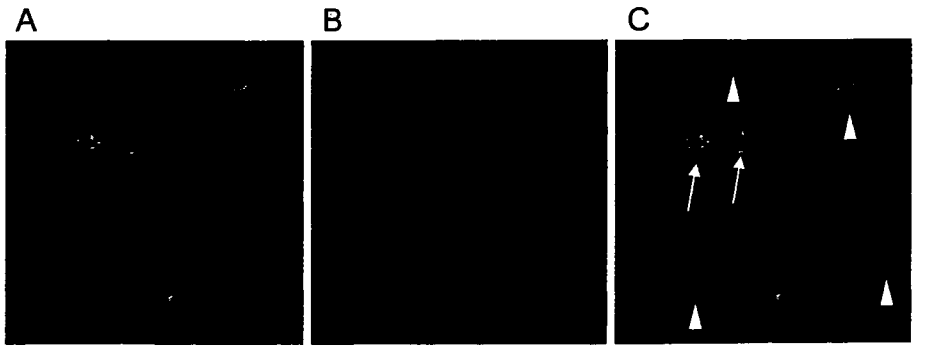

Fig. 4. Detection of FoxP3 and viral Ags in HHV-6-infected $CD4^+CD25^{high}$ T cells. HHV-6-infected cells were double labeled with anti-FoxP3 and anti-gB Abs as described in "Materials and Methods." (A) Green fluorescing cells indicate FoxP3 Ag-positive cells. (B) Red fluorescing cells indicate gB Ag-positive cells. (C) Merge. Arrows and arrowheads indicate Ag-positive cells and Ag-negative cells, respectively.

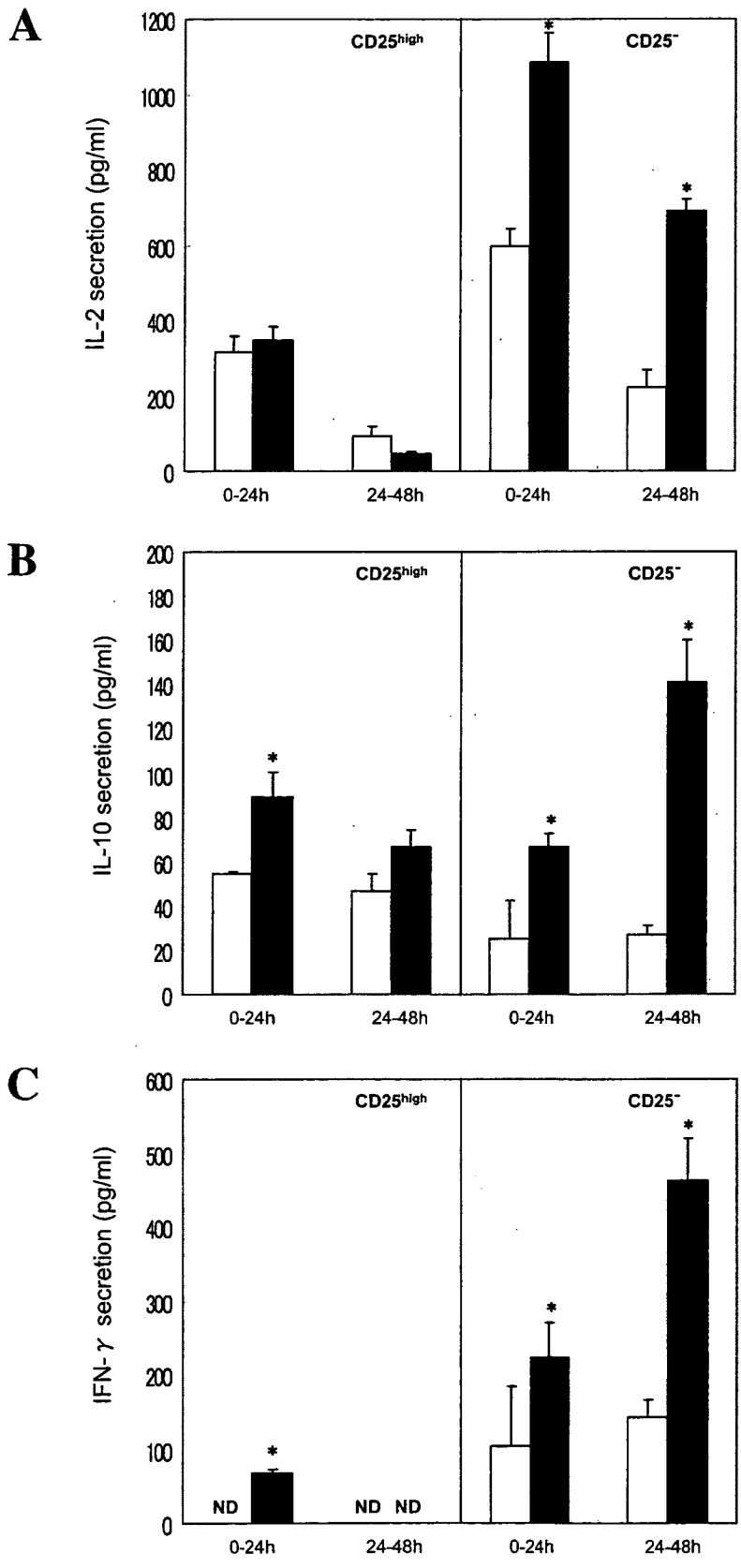

Fig. 5. Cytokine production from $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells with or without HHV-6 infection. Cytokine productions from T cells in both subsets, i.e., with (■) or without (□) HHV-6 infection, were assayed as described in "Materials and Methods." (A), (B) and (C) indicate IL-2, IL-10, and IFN-γ productions, respectively; ND: not detected. *$P<0.05$.