4.1.25. 4,5,6,7-Tetrachloro-*N*-{3-[(1*E*)-2-(3,4-dihydroxyphenyl) ethenyl]phenyl}phthalimide: PPT-97

Mp 257.0–260.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.51 (dt, 1H, J = 7.9, 1.2 Hz), 7.50 (m, 1H), 7.47 (t, 1H, J = 7.9 Hz), 7.26–7.25 (m, 1H), 7.05 (d, 1H, J = 1.8 Hz), 7.00 (d, 1H, J = 16.5 Hz), 6.95 (dd, 1H, J = 8.5, 1.8 Hz), 6.91 (d, 1H, J = 16.5 Hz), 6.85 (d, 1H, J = 8.5 Hz), 5.59 (br s, 1H), 5.54 (br s, 1H). FAB-MS m/z: 493 $[M]^+$, 494 $[M+H]^+$, 495 $[M+2]^+$, 496 $[M+3]^+$, 497 $[M+4]^+$, 498 $[M+5]^+$. HRMS (FAB) calcd for $C_{22}H_{11}Cl_4NO_4$ 492.9442; found: 492.9467 $(M)^+$.

4.1.26. *N*-{4-[(1*E*)-2-Phenylethenyl]phenyl}phthalimide: PPT-108

Mp 297.0–299.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.97 (dd, 2H, J = 5.5, 3.1 Hz), 7.81 (dd, 2H, J = 5.5, 3.1 Hz), 7.65 (d, 2H, J = 8.5 Hz), 7.54 (d, 2H, J = 7.9 Hz), 7.46 (d, 2H, J = 8.5 Hz), 7.38 (t, 2H, J = 7.9 Hz), 7.28 (tt, 1H, J = 7.9, 1.2 Hz), 7.15 (s, 2H). FAB-MS m/z: 325 $[M]^+$, 326 $[M+H]^+$. Anal. Calcd for $C_{22}H_{15}NO_2 \cdot 1/3H_2O$: C, 79.74; H, 4.77; N, 4.23. Found: C, 79.50; H, 4.69; N, 4.19.

4.1.27. 4,5,6,7-Tetrachloro-*N*-{4-[(1*E*)-2-phenylethenyl] phenyl}phthalimide: PPT-109

Mp 296.0–296.5 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.65 (d, 2H, J = 8.5 Hz), 7.54 (d, 2H, J = 7.3 Hz), 7.42 (d, 2H, J = 8.5 Hz), 7.38 (t, 2H, J = 7.3 Hz), 7.29 (tt, 1H, J = 7.3, 1.2 Hz), 7.17 (d, 1H, J = 16.5 Hz), 7.13 (d, 1H, J = 16.5 Hz). FAB-MS m/z: 461 $[M]^+$, 462 $[M+H]^+$, 463 $[M+2]^+$, 464 $[M+3]^+$, 465 $[M+4]^+$, 466 $[M+5]^+$. Anal. Calcd for $C_{22}H_{11}Cl_4NO_2$: C, 57.05; H, 2.39; N, 3.02. Found: C, 56.83; H, 2.69; N, 3.09.

4.1.28. 4,5,6,7-Tetrachloro-*N*-{4-[(1*E*)-2-(3,4-dimethoxyphenyl) ethenyl]phenyl}phthalimide: PPT-112

Mp 281.0–284.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.62 (d, 2H, J = 8.5 Hz), 7.40 (d, 2H, J = 8.5 Hz), 7.11 (d, 1H, J = 16.2 Hz), 7.09–7.05 (m, 2H), 7.00 (d, 1H, J = 16.2 Hz), 6.88 (d, 1H, J = 8.5 Hz), 3.96 (s, 3H), 3.91 (s, 3H). FAB-MS m/z: 521 $[M]^+$, 522 $[M+H]^+$, 523 $[M+2]^+$, 524 $[M+3]^+$, 525 $[M+4]^+$, 526 $[M+5]^+$. Anal. Calcd for $C_{24}H_{15}Cl_4NO_4 \cdot 1/2H_2O$: C, 54.16; H, 3.03; N, 2.63. Found: C, 54.24; H, 2.97; N, 2.55.

4.1.29. *N*-{4-[(1*E*)-2-(3,4-Dihydroxyphenyl)ethenyl]phenyl} phthalimide: PPT-113

Mp 278.0–281.0 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 7.97 (dd, 2H, J = 5.5, 3.1 Hz), 7.90 (dd, 2H, J = 5.5, 3.1 Hz), 7.67 (d, 2H, J = 8.5 Hz), 7.40 (d, 2H, J = 8.5 Hz), 7.14 (d, 1H, J = 16.2 Hz), 7.01 (d, 1H, J = 2.4 Hz), 6.97 (d, 1H, J = 16.2 Hz), 6.89 (dd, 1H, J = 8.5, 1.8 Hz), 6.73 (d, 1H, J = 7.9 Hz). FAB-MS m/z: 357 $[M]^+$, 358 $[M+H]^+$. Anal. Calcd for $C_{22}H_{15}NO_4 \cdot 1/4H_2O$: C, 73.02; H, 4.32; N, 3.87. Found: C, 73.21; H, 4.36; N, 3.91.

4.1.30. *N*-{4-[2-(3-Methoxyphenyl)ethyl]phenyl}phthalimide: PPT-121

Mp 127.0–127.5 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.96 (dd, 2H, J = 5.5, 3.1 Hz), 7.79 (dd, 2H, J = 5.5, 3.1 Hz), 7.36–7.32 (m, 4H), 7.22 (t, 1H, J = 7.9 Hz), 6.82 (d, 1H, J = 7.3 Hz), 6.76 (dd, 1H, J = 7.3, 1.8 Hz), 6.74 (d, 1H, J = 1.8 Hz), 3.79 (s, 3H), 3.00–2.96 (m, 2H), 2.95–2.91 (m, 2H). FAB-MS m/z: 357 $[M]^+$, 358 $[M+H]^+$. Anal. Calcd for $C_{23}H_{19}NO_3 \cdot 1/4H_2O$: C, 76.33; H, 5.43; N, 3.87. Found: C, 76.34; H, 5.40; N, 3.87.

4.1.31. 4,5,6,7-Tetrachloro-*N*-{4-[2-(3-methoxyphenyl) ethyl]phenyl}phthalimide: PPT-122

Mp 170.5–172.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.34–7.29 (m, 4H), 7.22 (t, 1H, J = 7.9 Hz), 6.80 (d, 1H, J = 7.3 Hz), 6.76 (dd, 1H, J = 7.9, 1.8 Hz), 6.73 (s, 1H), 3.79 (s, 3H), 3.00–2.97 (m, 2H), 2.95–2.91 (m, 2H). FAB-MS m/z: 493 $[M]^+$, 494 $[M+H]^+$, 495 $[M+2]^+$, 496 $[M+3]^+$, 497 $[M+4]^+$, 498 $[M+5]^+$. HRMS (FAB) calcd for $C_{23}H_{15}Cl_4NO_3$ 492.9806; found: 492.9834 $(M)^+$.

4.1.32. *N*-{4-[2-(4-Methoxyphenyl)ethyl]phenyl}phthalimide: PPT-123

Mp 196.5–199.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.96 (dd, 2H, J = 5.5, 3.1 Hz), 7.79 (dd, 2H, J = 5.5, 3.1 Hz), 7.35 (d, 2H, J = 9.2 Hz), 7.32 (d, 2H, J = 8.5 Hz), 7.13 (d, 2H, J = 8.5 Hz), 6.85 (d, 2H, J = 9.2 Hz), 3.80 (s, 3H). FAB-MS m/z: 357 $[M]^+$, 358 $[M+H]^+$. Anal. Calcd for $C_{23}H_{19}NO_3$: C, 77.29; H, 5.36; N, 3.92. Found: C, 77.21; H, 5.51; N, 3.83.

4.1.33. 4,5,6,7-Tetrachloro-*N*-{4-[2-(4-methoxyphenyl)ethyl] phenyl}phthalimide: PPT-124

Mp 209.5–211.5 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.30 (s, 4H), 7.10 (d, 2H, J = 8.5 Hz), 6.84 (d, 2H, J = 8.5 Hz), 3.80 (s, 3H), 2.96–2.93 (m, 2H), 2.91–2.90 (m, 2H). FAB-MS m/z: 494 $[M+H]^+$, 495 $[M+2]^+$, 496 $[M+3]^+$, 497 $[M+4]^+$, 498 $[M+5]^+$. Anal. Calcd for $C_{23}H_{15}Cl_4NO_3$: C, 55.79; H, 3.05; N, 2.83. Found: C, 55.43; H, 3.20; N, 2.76.

4.1.34. *N*-{4-[2-(3-Hydroxyphenyl)ethyl]phenyl}phthalimide: PPT-125

Mp 229.0–232.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.96 (dd, 2H, J = 5.5, 3.1 Hz), 7.80 (dd, 2H, J = 5.5, 3.1 Hz), 7.35–7.30 (m, 4H), 7.17 (t, 1H, J = 7.9 Hz), 6.80 (d, 1H, J = 7.3 Hz), 6.68 (dd, 1H, J = 7.9, 2.4 Hz), 6.60 (s, 1H), 2.98–2.95 (m, 2H), 2.92–2.89 (m, 2H). FAB-MS m/z: 343 $[M]^+$, 344 $[M+H]^+$. Anal. Calcd for $C_{22}H_{17}NO_3 \cdot 1/5H_2O$: C, 76.15; H, 5.05; N, 4.04. Found: C, 76.38; H, 5.24; N, 4.05.

4.1.35. 4,5,6,7-Tetrachloro-*N*-{4-[2-(3-hydroxyphenyl) ethyl]phenyl}phthalimide: PPT-136

Mp 267.0–268.0 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 9.25 (br s, 1H), 7.40 (d, 2H, J = 7.9 Hz), 7.31 (d, 2H, J = 7.9 Hz), 7.06 (t, 1H, J = 7.3 Hz), 6.67 (d, 1H, J = 7.3 Hz), 6.66 (s, 1H), 6.58 (d, 1H, J = 7.3 Hz), 2.92–2.90 (m, 2H), 2.84–2.81 (m, 2H). FAB-MS m/z: 479 $[M]^+$, 480 $[M+H]^+$, 481 $[M+2]^+$, 482 $[M+3]^+$, 483 $[M+4]^+$, 484 $[M+5]^+$. HRMS (FAB) calcd for $C_{22}H_{13}Cl_4NO_3$ 478.9650; found: 478.9694 $(M)^+$.

4.1.36. *N*-{4-[2-(4-Hydroxyphenyl)ethyl]phenyl}phthalimide: PPT-137

Mp 285.0–288.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.96 (dd, 2H, J = 5.5, 3.1 Hz), 7.80 (dd, 2H, J = 5.5, 3.1 Hz), 7.35–7.30 (m, 4H), 7.08 (d, 2H, J = 7.9 Hz), 6.77 (d, 2H, J = 8.5 Hz), 2.94–2.92 (m, 2H), 2.90–2.88 (m, 2H). FAB-MS m/z: 343 $[M]^+$, 344 $[M+H]^+$. Anal. Calcd for $C_{22}H_{17}NO_3 \cdot 1/2H_2O$: C, 74.99; H, 5.15; N, 3.97. Found: C, 74.97; H, 5.20; N, 3.89.

4.1.37. 4,5,6,7-Tetrachloro-*N*-{4-[2-(4-hydroxyphenyl) ethyl]phenyl}phthalimide: PPT-138

Mp 266.0–268.0 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 9.15 (br s, 1H), 7.37 (d, 2H, J = 8.5 Hz), 7.30 (d, 2H, J = 8.5 Hz), 7.03 (d, 2H, J = 8.5 Hz), 6.66 (d, 2H, J = 8.5 Hz), 2.89–2.86 (m, 2H), 2.81–2.78 (m, 2H). FAB-MS m/z: 479 $[M]^+$, 480 $[M+H]^+$, 481 $[M+2]^+$, 482 $[M+3]^+$, 483 $[M+4]^+$, 484 $[M+5]^+$. Anal. Calcd for $C_{22}H_{13}Cl_4NO_3$: C, 54.92; H, 2.72; N, 2.91. Found: C, 54.60; H, 3.00; N, 2.82.

4.2. Expression and purification of the PA endonuclease and PB2 627 domain proteins

The influenza (A/PR/8/34) H1N1 RNA polymerase PA and PB2 plasmids, pBMSA-PA and pBMSA-PB2, were sourced from the DNA Bank, Riken BioResource Center (Tsukuba, Japan; originally deposited by Dr. Susumu Nakada).[35] The cDNA fragment corresponding to the PA N-terminal endonuclease domain (residues

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1–220) was amplified by PCR[36] from pBMSA-PA using the primers PA endonuclease forward NdeI, GCCGTTCATATGGAAGATTTTG TGCGACAA and PA endonuclease reverse BamHI, GCCGTTGGATCCT ATTGGTCGGCAAGCTTGCG. A cDNA fragment of the PB2 627 domain (residues 535–759, previously denoted as the PB2 3/3 domain[28]) was amplified by PCR from pBMSA-PB2 using the following primers: PB2 Met 627, GCCGTTCATATGATGTGGGAGATTAA TGGT and PB2 stop BamHI, GCCGTTGGATCCTTAATTGATGGCCA TCCGAAT. These two amplified products were then subcloned into the pET28a(+) plasmid (Novagen, Madison, WI) at the NdeI and BamHI restriction sites. The two resulting constructs were then introduced into BL21-CodonPlus (Stratagene, La Jolla, CA) *Escherichia coli* cells. The induction of 6x his-tagged recombinant protein expression from these constructs were achieved by the addition of isopropyl β-D-thiogalactopyranoside (IPTG)[37] to TBG-M9 medium and this was followed by purification using Ni^{2+}-agarose.[38] The recombinant PA endonuclease domain protein was further purified to near homogeneity using a HiTrap™ Q FF column (GE Healthcare, Buckinghamshire, UK) with the Akta™ prime plus system (GE Healthcare). For further purification of the PB2 627 domain, the his-tagged proteins were cleaved by thrombin and purified using a HiTrap™ CM FF column (GE Healthcare) also with the Akta™ prime plus system.

4.3. PA endonuclease activity assay

Influenza A RNA polymerase PA endonuclease activity assays were performed essentially as described by Dias et al.[20–22] with some modifications. Briefly, we modified the pH conditions (from 8.0 to 7.3) and used 1 μg of M13mp18 single stranded circular phage DNA as the assay substrate. We added 0.35 μg of recombinant N-terminal endonuclease domain of the PA subunit to 100 μl of assay buffer in each reaction (the final concentration of the protein was about 0.1 μM). PPT analogs were then added to the reaction and products were analyzed by agarose electrophoresis and stained with ethidium bromide.

4.4. Electrospray ionization (ESI) mass spectrometry (MS)

Chemicals at a dose of 50 μM and recombinant PB2 627 domain protein at 1–3 μM were mixed in 200 μl of 10 mM ammonium acetate/methanol (1:1), to which 1% acetic acid was added. This mixture was then injected at 100 μl/h into an ESI-MS.[30,31] For binding analysis, recombinant PB2 627 domain protein was used.[28] Measurements were performed with a Fourier transform ion cyclotron resonance mass spectrometer (FT-ICRMS, Bruker Daltonics), equipped with an ESI ion source, using the following parameters: capillary −4.0 kV, spray shield −3.5 kV, dry temp 40 °C, resolution at m/z 2792 50 k, flow rate 100 μl/h, solvent 10 mM ammonium acetate buffer 49.5%, methanol 49.5%, and acetic acid 1% (v/v/v).

4.5. Inhibition of viral growth

Madin–Darby canine kidney (MDCK) cells[39] were cultured in MEM (Minimum Essential Medium; Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum in 5% CO_2 incubator at 37 °C. A confluent monolayer of MDCK cells was prepared in each well of a 96-well plate. Various concentrations (0.63–80 μM) of PPT analogs were mixed with or without 100 $TCID_{50}$ (50% of the infectious dose) of H1N1 influenza A virus (A/Puerto Rico/8/34 (PR8)) in the presence of trypsin and incubated at 37 °C for 30 min.[40] MDCK cells were washed with PBS(−) and the viral mixture was added to the cells. Treated cells were then incubated for four days at 34 °C under 5% CO_2. After incubation, the medium was removed and cells were fixed with a 10% formaldehyde solution. Viable cells were stained with NB solution (0.1% naphthol blue black, 0.1% sodium acetate, and 9% acetic acid) and the OD_{630} was measured.[40] Cell viability was calculated based on a calibration of the OD_{630} values observed in mock-infected and virus-only wells as 100% and 0%, respectively.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.05.035.

References and notes

1. Bartlett, J. B.; Dredge, K.; Dalgleish, A. G. *Nat. Rev. Cancer* **2004**, *4*, 314.
2. Hashimoto, Y. *Bioorg. Med. Chem.* **2002**, *10*, 461.
3. Hashimoto, Y. *Curr. Med. Chem.* **1998**, *5*, 163.
4. Hideshima, T.; Chauhan, D.; Shima, Y.; Raje, N.; Davies, F. E.; Tai, Y. T.; Treon, S. P.; Lin, B.; Schlossman, R. L.; Richardson, P.; Muller, G.; Stirling, D. I.; Anderson, K. C. *Blood* **2000**, *96*, 2943.
5. Hashimoto, Y. *Arch. Pharm. Weinheim* **2008**, *341*, 536.
6. Hashimoto, Y. *Mini-Rev. Med. Chem.* **2002**, *2*, 543.
7. Hashimoto, Y.; Tanatani, A.; Nagasawa, K.; Miyachi, H. *Drugs Future* **2004**, *29*, 383.
8. Taubenberger, J. K.; Reid, A. H.; Lourens, R. M.; Wang, R.; Jin, G.; Fanning, T. G. *Nature* **2005**, *437*, 889.
9. Morse, S. S. *Nat. Med.* **2007**, *13*, 681.
10. Horimoto, T.; Kawaoka, Y. *Nat. Rev. Microbiol.* **2005**, *3*, 591.
11. Hatta, M.; Gao, P.; Halfmann, P.; Kawaoka, Y. *Science* **2001**, *293*, 1840.
12. Neumann, G.; Noda, T.; Kawaoka, Y. *Nature* **2009**, *459*, 931.
13. De Clercq, E. *Nat. Rev. Drug Disc.* **2006**, *5*, 1015.
14. Hayden, F. G.; Atmar, R. L.; Schilling, M.; Johnson, C.; Poretz, D.; Paar, D.; Huson, L.; Ward, P.; Mills, R. G. N. *N. Eng. J. Med.* **1999**, *341*, 1336.
15. Reece, P. A. *J. Med. Virol.* **2007**, *79*, 1577.
16. Collins, P. J.; Haire, L. F.; Lin, Y. P.; Liu, J.; Russell, R. J.; Walker, P. A.; Skehel, J. J.; Martin, S. R.; Hay, A. J.; Gamblin, S. J. *Nature* **2008**, *453*, 1258.
17. Izumi, Y.; Tokuda, K.; O'dell, K. A.; Zorumski, C. F.; Narahashi, T. *Neurosci. Lett.* **2007**, *426*, 54.
18. Honda, A.; Ishihama, A. *Biol. Chem.* **1997**, *378*, 483.
19. Honda, A.; Mizumoto, K.; Ishihama, A. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 13166.
20. Yuan, P.; Bartlam, M.; Lou, Z.; Chen, S.; Zhou, J.; He, X.; Lv, Z.; Ge, R.; Li, X.; Deng, T.; Fodor, E.; Rao, Z.; Liu, Y. *Nature* **2009**, *458*, 909.
21. Dias, A.; Bouvier, D.; Crépin, T.; McCarthy, A. A.; Hart, D. J.; Baudin, F.; Cusack, S.; Ruigrok, R. W. *Nature* **2009**, *458*, 914.
22. Kuzuhara, T.; Iwai, Y.; Takahashi, H.; Hatakeyama, D.; Echigo, N. *PLoS Curr. Influenza* **2009**. RRN1052.
23. Subbarao, E. K.; London, W.; Murphy, B. R. *J. Virol.* **1993**, *67*, 1761.
24. Shinya, K.; Hamm, S.; Hatta, M.; Ito, H.; Ito, T.; Kawaoka, Y. *Virology* **2004**, *320*, 258.
25. Labadie, K.; Dos Santos Afonso, E.; Rameix-Welti, M. A.; van der Werf, S.; Naffakh, N. *Virology* **2007**, *362*, 271.
26. Gabriel, G.; Dauber, B.; Wolff, T.; Planz, O.; Klenk, H. D.; Stech, J. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 18590.
27. Tarendeau, F.; Crepin, T.; Guilligay, D.; Ruigrok, R. W.; Cusack, S.; Hart, D. J. *PLoS Pathog.* **2008**, *4*, e1000136.
28. Kuzuhara, T.; Kise, D.; Yoshida, H.; Horita, T.; Murazaki, Y.; Nishimura, A.; Echigo, N.; Utsunomiya, H.; Tsuge, H. *J. Biol. Chem.* **2009**, *284*, 6855.
29. Motoshima, K.; Ishikawa, M.; Sugita, K.; Hashimoto, Y. *Biol. Pharm. Bull.* **2009**, *32*, 1618.
30. Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, *246*, 64.
31. Baca, M.; Kent, S. B. H. *J. Am. Chem. Soc.* **1992**, *114*, 3992.
32. Yang, C. S.; Wang, X.; Lu, G.; Picinich, S. C. *Nat. Rev. Cancer* **2009**, *9*, 429.
33. Song, J. M.; Lee, K. H.; Seong, B. L. *Antiviral Res.* **2005**, *68*, 66.
34. Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J. *J. Comput. Chem.* **1998**, *19*, 1639.
35. Nakamura, Y.; Oda, K.; Nakada, S. *J. Biochem.* **1991**, *110*, 395.

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36. Mullis, K. B.; Faloona, F. A. *Methods Enzymol.* **1987**, *155*, 335.
37. Studier, F. W.; Moffatt, B. A. *J. Mol. Biol.* **1986**, *189*, 113.
38. Janknecht, R.; de Martynoff, G.; Lou, J.; Hipskind, R. A.; Nordheim, A.; Stunnenberg, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 8972.
39. Taub, M.; Saier, M. H., Jr. *Methods Enzymol.* **1979**, *58*, 552.
40. Hierholzer, J. C.; Killington, R. A. *Virology Methods Manual*; Mahy, B. W. J., Kangro, H. O., Eds.; Academic Press Limited: London, 1996; Chaper 2, pp 35–37.

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Review Article

A New Method for the Evaluation of Vaccine Safety Based on Comprehensive Gene Expression Analysis

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For the past 50 years, quality control and safety tests have been used to evaluate vaccine safety. However, conventional animal safety tests need to be improved in several aspects. For example, the number of test animals used needs to be reduced and the test period shortened. It is, therefore, necessary to develop a new vaccine evaluation system. In this review, we show that gene expression patterns are well correlated to biological responses in vaccinated rats. Our findings and methods using experimental biology and genome science provide an important means of assessment for vaccine toxicity.

1. Introduction

Vaccination effectively enables the control of many infectious diseases. However, we cannot always avoid the problem of adverse reactions accompanied by vaccination. While most adverse reactions are mild and local, some vaccines have been associated with very rare but severe systemic reactions. Therefore, all vaccines for public use are made in compliance with Good Manufacturing Practices (GMP) to prevent safety problems. Furthermore, manufacturers must submit samples and results of their in-house tests for each vaccine batch to the national control authorities before vaccines are released into the market. Among many quality control tests, conventional animal safety tests are performed to detect vaccine toxicity because residual vaccine toxicity has the potential to cause adverse reactions. For example, the animal body weight change test is the most commonly used test to evaluate the toxicity of vaccines [1]. Although a good correlation of the body weight loss with a vaccine's toxicity has been shown [2, 3], a greater understanding of the molecular mechanisms involved in the reaction to a vaccines' toxicity is needed. We, therefore, attempted to measure animals' responses to vaccines by determining changes in gene expression profiles.

Gene expression profiling is a unique way to characterize how cells or tissues are affected by abnormal conditions. The measurement of gene expression levels upon exposure to toxicants can be used to identify toxic products, and to provide information about the mechanism of toxicity [4]. DNA microarray technology has opened the way for the parallel detection and analysis of expression patterns of thousands of genes in a single experiment. Furthermore, the development of high-quality gene arrays has allowed DNA microarray technology to become a standard tool in molecular toxicology. Recently, the field of toxicogenomics has validated the concept of gene expression profiles as "signatures" of toxicant classes [5–7]. These signatures have effectively directed the analytical search for predictive toxicant biomarkers and they have contributed to the understanding of the dynamic responses of molecular mechanisms associated with toxic responses. In fact, many studies of gene-expression profiles have now been reported in the toxicology field. For example, Hamadeh et al. reported patterns of gene expression in liver tissue taken from rats exposed to different

chemicals [8]. DNA microarray assays have also been applied to the analysis of the side effects of medicines [9]. Recently, the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) have, either individually or together, started to review submissions for the qualification of biomarkers for medical products for specific purposes proposed by industry [10]. The introduction of pharmacogenomics, or pharmacogenetics, to the evaluation of medicines is a global trend.

For a better understanding of the molecular toxicology regarding vaccines, DNA microarray analysis promises to be an ideal method, as has been the case for pharmaceuticals. The FDA now encourages the voluntary submission of genomic data to the FDA outside of the regular review process [11]. However, no studies similar to those described above for pharmaceuticals have yet been conducted in the field of vaccines. At the beginning of this review, we summarized the current efforts used for the control of vaccine safety using conventional animal tests. We then referred to our recent efforts using DNA microarray analysis to identify "genetic signatures" for the toxicants remaining in vaccines. Since pertussis and influenza vaccines are among the most commonly used vaccines, we tried to develop a system to evaluate the "genetic signatures" of the toxicity of these vaccines.

2. Current Vaccine Safety Test

2.1. Body Weight Change in Vaccinated Animals. To screen for general toxicity of vaccines, the body weight of vaccine-treated animals can be analyzed as the general safety test [12]. Five mL of the vaccine are injected into the peritoneum of guinea pigs weighing 300–400 g, and the weight loss experienced by the animals is analyzed at days 1, 2, 3, 4, and 7 after administration. None of the animals should show any abnormal signs; no statistically significant ($P = .01$) difference in weight loss should be observed between the treated animals and the control group on any observation day. This test has been applied to a wide variety of vaccines in a unified way, and plays an important role in ensuring the safety and consistency of vaccine batches [12]. For pertussis vaccine (inactivated whole cell formulation), the effects of vaccine treatment were also measured using test for toxicity to mouse weight gain, in addition to the general safety test. All mice were weighed on days 0, 1, 2, 3, 4, and 7 after vaccine administration. The criterion for judgment is that mean body weight 3 days after injection should be no less than that at the time of injection upon statistical analysis, and no mice showed any abnormal sign during the observation periods [12]. When the reference vaccine (RE: the inactivated whole cell pertussis vaccine) was administrated, weight loss was observed on day 1 after administration (**Figure 1(a)**).

2.2. Leukocytosis-Promoting Toxicity in Vaccinated Animals. To detect the toxin present in pertussis vaccines, the number of peripheral leukocytes can also be analyzed. Pertussis vaccine is injected into the peritoneum of mice at a dose of 0.5 mL. Leukocytes present in peripheral blood are then counted 3 days after injection [12]. The white blood cell (WBC) counts in peripheral blood of reference vaccine-treated mice reach approximately 2,500 cells/μL (**Figure 1(b)**). The standard criterion of safety for pertussis vaccine (inactivated whole cell formulation) is that the mean count of leukocytes in peripheral blood, 3 days after injection, should not exceed 10 times that before injection [12].

2.3. Leukopenic Toxicity Test in Vaccinated Animals. Quality control of influenza vaccines is performed using the general safety test and the leukopenic toxicity test (LTT), which is based on peripheral WBC counts in mice 12–18 hours after intraperitoneal injection of a vaccine. The criterion for judgment is that the leukopenic toxicity of the test sample relative to that of the toxicity reference sample should be no higher than the value corresponding to 80% of the leukocyte count of the control relative to that of the toxicity reference sample [12–14].

3. DNA Microarray-Based Safety Test

The currently used quality control and safety tests, such as the LTT and the general safety test, have been used to evaluate vaccine safety for over 50 years [3]. We are now developing a new quality control method for vaccines using DNA microarray analysis as a substitute for the conventional animal tests [15–17]. The principle of this method is to translate vaccine quality, immunogenicity, and reactogenicity, into gene expression profile data. This method is expected to be informative, rapid, and highly sensitive.

For DNA microarray analysis using vaccines, 8 week-old male rats, weighing 180–220 g, were intraperitoneally administered with 5 mL of vaccine or physiological saline (SA). Three to 6 rats were used for each group. Vaccinated rats were sacrificed to obtain whole lung, kidney, brain, and the lateral left lobe of the liver on day 1, 2, 3, and 4 postadministration (**Figure 2**). Tissues were immediately frozen in liquid nitrogen for storage. Thawed tissue was homogenized and poly(A)$^+$ RNA was purified from the lysate. Cyanine 5-labeled poly(A)$^+$ RNA was subjected to DNA microarray analysis. Blood was also collected, however, this could not be analyzed due to the low quality of purified RNA.

For DNA microarray analysis, a set of synthetic polynucleotides (80-mers) representing 11,468 rat transcripts and including most of the RefSeq genes deposited in the NCBI database (MicroDiagnostic, Tokyo, Japan) was arrayed on aminosilane-coated glass slides [18, 19]. Cyanine 5-labeled poly(A)$^+$ RNA was competitively hybridized on the slide with cyanine 3-labeled common reference RNA. Hybridization signals were measured, processed into primary expression ratios ([Cyanine 5-intensity obtained from each sample]/[Cyanine 3-intensity obtained from common reference RNA]), and then normalized by multiplying normalization factors calculated for each microarray feature.

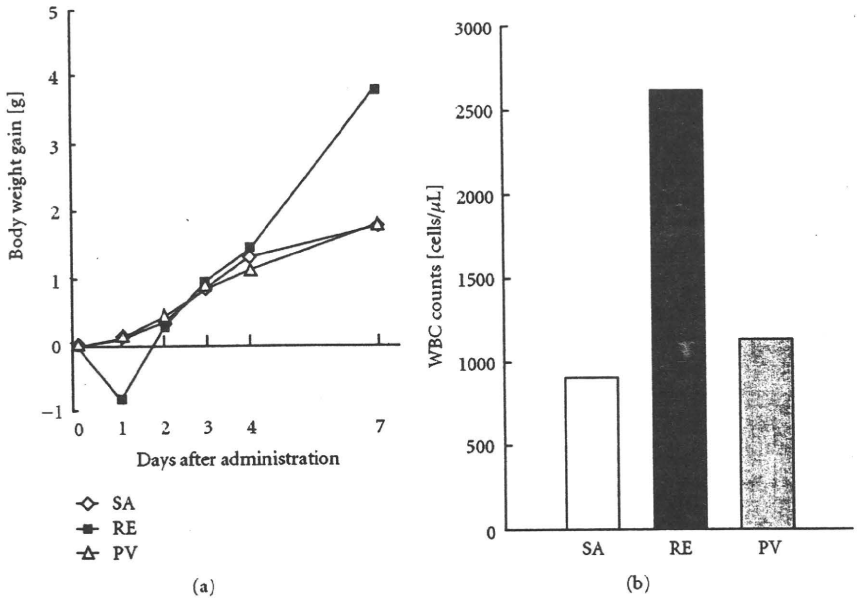

FIGURE 1: Safety control tests for pertussis vaccines. (a) Test for toxicity to mouse weight gain. Physiological saline (SA), an inactivated whole-cell pertussis vaccine (RE), or an acellular pertussis vaccine (PV)-administered mice were weighed on 0, 1, 2, 3, 4, and 7 days postadministration. Ten mice in each group were used, and the mean changes in body weight are indicated. (b) Leukocytosis promoting activity of various pertussis vaccines. White blood cell (WBC) counts in peripheral blood were measured 3 days after vaccine administration. Ten mice in each group were used and the mean WBC counts are indicated.

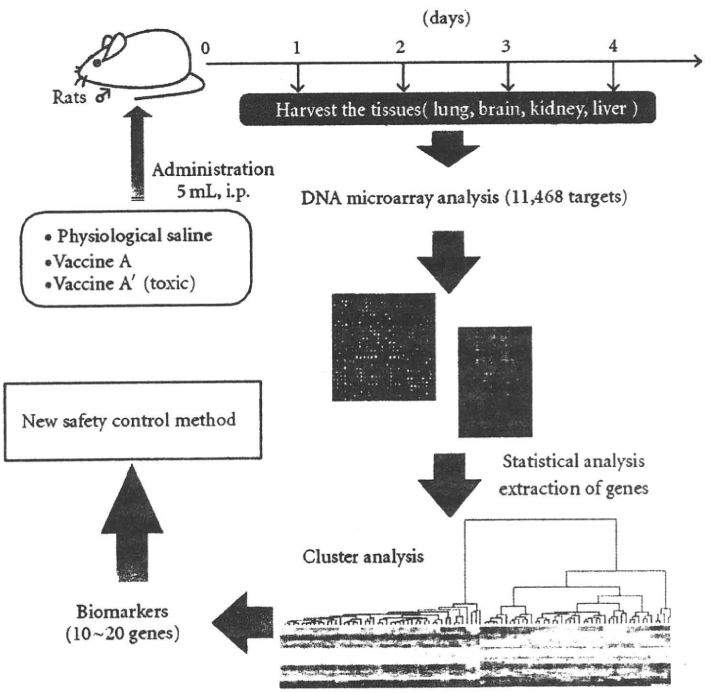

FIGURE 2: The gene expression analysis procedure. The detail of the procedure is described in the text.

For data processing and hierarchical cluster analysis, the primary expression ratios were converted into $\log_2$ ratios ($\log_2$ Cyanine-5 intensity/Cyanine-3 intensity). The genes with $\log_2$ ratios over 1 or under −1 in at least one sample were extracted from the primary data matrix, then subjected to two-dimensional hierarchical cluster analysis for samples and genes.

For the identification of biomarker genes for pertussis vaccines, we extracted differentially expressed genes from physiological saline and pertussis toxin-treated lung samples using the t-test ($P < .01$). Among the extracted genes, we further selected genes that exhibited mean average $\log_2$ ratio differences greater than 0.75 between the two sample groups [17]. For influenza vaccines, we extracted differentially expressed genes from physiological saline and inactivated whole-virion vaccine-treated lung samples using the t-test ($P < .005$) [16].

4. Pertussis Vaccines

Pertussis, or whooping cough, is an infectious respiratory disease caused by a Gram-negative bacillus, *Bordetella pertussis*. *Bordetella pertussis* possesses several pathogenic components, including pertussis toxin (PT) [20]. PT is known as a leukocytosis promoting factor, a major contributor to the pathogenesis of pertussis, and an antigen in immunity to pertussis [21]. At present, whole-cell pertussis vaccines and acellular pertussis vaccines containing inactivated PT are in commercial use [20].

Although pertussis vaccines are effective in the prevention of whooping cough, they have occasionally caused local reactions such as redness, swelling, and pain at the injection site. However, little is known about the overall responses to these vaccines. To address this problem, we applied DNA microarray analysis and quantification of specific genes to analyze the toxicants in pertussis vaccines [15, 17]. Three preparations, an acellular vaccine containing inactivated pertussis toxin (PV), an inactivated whole-cell vaccine (RE), and a purified pertussis toxin (PT) were prepared. RE is a reference vaccine for National Quality Control Tests of pertussis vaccines in Japan and is made from formaldehyde-inactivated *Bordetella pertussis* preparations. Physiological saline (SA) was used as a control. For comprehensive gene expression analysis, 5 mL of SA, PV, PT, and RE were each injected into 3 rats and the vaccinated tissues, lung, brain, kidney, and liver, were harvested at 1, 2, 3, and 4 days after vaccine administration. The experiments were performed twice and purified poly(A)$^+$ RNA from a total of 384 samples was subjected to DNA microarray analysis.

Of the 4 organs tested, the lung expressed genes that were extracted by DNA microarray analysis were classified sharply into clusters depending on sample treatment. From the DNA microarray analysis of vaccinated rat lungs at day 1, 13 genes for which expression levels were dynamically changed in response to PT treatment were [17] (accession numbers were updated in **Table 1**). Interestingly, the DNA microarray-based gene expression data correlated well with the body weight change of vaccine-treated mice (**Figure 1(a)**) and rats [17]. The real-time PCR quantification results of the expression levels of the 13 genes were comparable to the relative expression ratios from the DNA microarray analysis. Furthermore, cluster analysis using the 13 genes could distinguish SA- and PV-treated groups from PT- and RE-treated groups. These 13 genes are likely to be closely involved in the toxicity of pertussis vaccines. To quantify these genes in a convenient way, the QuantiGene Plex assay was applied. The QuantiGene Plex assay enabled the simultaneous analysis of the 13 genes. We evaluated the expression levels of the 13 genes in the lungs of rats vaccinated with various doses of RE. Nine genes, *S100A9*, *S100A8*, *IRF7*, *MX2*, *IFI27L*, *BEST5*, *MMP9*, *MMP8*, and *CYP2E1* (indicated in bold letters in **Table 1**) showed dose-dependent up-or down-regulation in response to the various doses of RE treatment. RE vaccine toxicity could be measured by the expression level in lung lysate of these 9 genes. The quantification of these 9 genes using the QuantiGene Plex assay is, we believe, a promising candidate for a new control test for pertussis vaccines.

5. Influenza Vaccines

Influenza virus triggers a highly contagious acute respiratory disease and has caused epidemics and global pandemics, partly because it possesses the capacity for gradual antigenic change in two surface antigens, hemagglutinin (HA) and neuraminidase (NA) [22]. To combat influenza, split vaccines consisting of subvirion preparations and whole-virus vaccines are manufactured using strains recommended annually by the WHO, based on the antigenic characteristics of HAs and NAs. Furthermore, the recent circulation of the highly pathogenic avian influenza A (H5N1) virus has raised concerns about the preparations for a coming influenza pandemic [23]. Many efforts are underway to develop vaccines against influenza A (H5N1).

To identify biomarkers for influenza vaccine toxicity, 3 vaccines were used: trivalent influenza HA vaccine (HAv, a split vaccine), trivalent influenza vaccine (WPv, an inactivated whole-virion vaccine), and prepandemic influenza vaccine (PDv, inactivated whole-virion (A/H5N1) absorbed onto an aluminum salt). All were produced by Kaketsuken, The Chemo-Sero-Therapeutic Research Institute, Japan. Physiological saline (SA) was used as a control. For comprehensive gene expression analysis, SA, HAv, WPv, and PDv were each injected into 5 rats, and the vaccinated tissues, lung, liver, brain, and peripheral blood, were harvested at 1, 2, 3, and 4 days after vaccine administration. Purified poly(A)$^+$ RNA from a total of 320 samples was subjected to DNA microarray analysis [16]. Based on the analysis of pertussis vaccines, described above, the gene expression profiles from lung samples were subjected to two-dimensional hierarchical cluster analysis. PDv- and WPv-treated samples at day 1 formed an independent cluster from other samples, indicating distinct profiles in gene expression of these groups. As was the case with pertussis vaccines, we tried to identify several biomarkers from the analysis of lung gene expression. The analysis of lungs from vaccinated rats at day 1 resulted in the extraction of 76 genes, whose expression levels were statistically different between SA- and

Table 1: Biomarkers for pertussis vaccine toxicity.

Category	Accession no.	Symbol	Brief description
Inflammation	NM_053587	***S100A9***	A calcium binding protein that may be associated with acute inflammatory processes, coupled with S100a8
	NM_053822	***S100A8***	May play a role in inflammatory responses such as cell motility, coupled with S100a9
	NM_019323	*MCPT9*	A serine protease expressed in mast cells, but the precise function has not yet been determined
	NM_031530	*CCL2*	A ligand for CCR2 that acts as a chemoattractant of monocytes
IFN inducible, immune response	NM_001033691	***IRF7***	Unknown
	NM_134350	***MX2***	Involved in inhibiting vesicular stomatitis virus
	NM_203410	***IFI27***	Induced by steroid hormone, IFN, and LPS in endometrium at implantation, dendric cells, and macrophases
	NM_001007694	*IFIT3*	May induced by IFN or virus infection
	Y07704	***BEST5***	Induced by IFN and involved in bone formation
Peptidoglycan metabolism	NM_031055	***MMP9***	Metalloproteinase involved in extracellular matrix remodeling, bone resorption, and immune responses
	NM_022221	***MMP8***	May play a role in appositional bone formation and regulation of the extracellular matrix
Xenobiotic metabolism	J02627	***CYP2E1***	Protects hepatocytes from stress-induced cell death
Others	NM_001106862	*NGP*	Unknown

Table 2: Biomarkers for influenza vaccine toxicity.

Category	Accession No.	Symbol	Brief description
IFN inducible gene	NM_172019	*IFI47*	Mouse homolog may be a guanine nucleotide-binding protein induced by IFN-gamma
	AF329825	*TRAFD1*	Putative TRAF-interacting zinc finger protein
	NM_019242	*IFRD1*	May be involved in proliferation of neuronal and glial precursors
IFN inducible, immune response	NM_001033691	*IRF7*	Unknown
	NM_134350	*MX2*	Involved in inhibiting vesicular stomatitis virus
Immune response	NM_172222	*C2*	Likely component of the classical pathway of the complement cascade
	NM_012708	*PSMB9*	Subunit of the proteasome complex, which may play a role in protein catabolism
	NM_032056	*TAP2*	Transports peptides into the ER lumen for binding with MHC class I molecules; plays a role in antigen processing and presentation
	NM_033098	*TAPBP*	Facilitates the binding of MHC class I molecules to the transporter associated with antigen processing (TAP) in MHC class I assembly
	NM_017264	*PSME1*	May play a role in proteasome activation
Chemokine and Cytokine function	AF065438	*LGALS3BP*	Displays differential expression in a fibroblast cell line transformed by human T-cell leukemia virus type 1 Tax protein
	NM_012977	*LGALS9*	A highly selective urate transporter/channel
	NM_053819	*TIMP1*	Acts as an inhibitor of metalloprotease activity; may play a role in vascular tissue remodeling
	NM_023981	*CSF1*	Plays a role in macrophage formation
	NM_145672	*CXCL9*	Chemokine which plays a role in the recruitment of mononuclear cells and in allograft rejection
	XM_223236	*CXCL11*	Mouse homolog is a chemokine and is involved in the immune response
Transcription activity	AJ302054	*ZBP1*	DNA binding protein; thought to bind Z-DNA, which is largely controlled by the amount of supercoiling

WPv-treated samples ($P < .005$) [16]. The cluster analysis using these 76 genes successfully distinguished WPv- and PDv-treated groups at day 1 from other groups, indicating the suitability of the 76 genes as biomarkers for influenza vaccines.

The extracted 76 genes were categorized according to function, such as interferon-inducible, chemokine and cytokine function, immune response, transcriptional activity, and so on. Among the 76 genes, 17 genes met the requirement for high expression levels and were chosen as representatives for each functional category (Table 2). Among the 17 genes, *IRF7* and *MX2* were also nominated for biomarkers of pertussis vaccine toxicity. Real-time PCR quantification results of the expression levels of the 17 genes were comparable to the relative expression ratios determined by DNA microarray analysis. We are now working to establish a rapid quantification system for these 17 biomarkers using the QuantiGene Plex assay.

6. Japanese Encephalitis Vaccines

Japanese encephalitis (JE) is a seasonal and sporadic encephalitis in East Asia caused by the JE virus. Vaccination is very important to prevent JE infection, because palliative care is the only treatment available for JE patients. Recently, a Vero cell-derived JE vaccine had been licensed in Japan as an alternative to the long-used mouse brain-derived JE vaccines. The newly developed Vero cell-derived vaccine should be at least equivalent to the mouse brain-derived vaccines, because the mouse brain-derived vaccines were considered generally safe and succeeded in the near elimination of JE in certain endemic regions. In this context, we performed DNA microarray analysis of tissues from rats administered with mouse brain-derived or Vero cell-derived JE vaccine and compared the gene expression profiles. As expected, the gene expression patterns in brain and liver were comparable between mouse brain-derived and Vero cell-derived vaccines, indicating that both vaccines possessed equivalent reactivity characteristics in rats [24].

7. Conclusions

Over recent decades, the safety control of vaccines has been assessed using several animal tests, including the body weight change test and white blood cell counts. However, conventional animal safety tests need to be improved in many aspects. For example, the number of test animals used needs to be reduced and the test period needs to be shortened. This requires the development of a new vaccine evaluation system. In this review, we showed that gene expression patterns were well correlated to the biological responsiveness of vaccinated animals. From the DNA microarray analysis of lungs from vaccinated rats, we identified 13 and 17 biomarkers to detect the toxicity of pertussis and influenza vaccines, respectively.

Furthermore, the QuantiGene Plex assay for gene expression analysis is being introduced. The QuantiGene Plex assay was revealed to be as accurate as real-time PCR and has the great benefit of being able to evaluate all biomarkers simultaneously. Using the QuantiGene Plex assay, we could rapidly and sensitively detect the gene expression changes that accompany biological reactivity in vaccinated rats.

Thus, it may be concluded that DNA microarray technology is an informative, rapid, and highly sensitive method with which to evaluate vaccine quality. Our data suggest that this new method has the potential to shorten the time for safety tests and can reduce the number of animals used. In addition, our test may contribute to the development of urgently required vaccines. Further analyses are required to confirm that gene expression changes correlate with vaccine quality.

In this review, we referred to our recent efforts of exploring new safety control methods using gene expression pattern indexes, focusing on pertussis and influenza vaccines. In the future, for the evaluation of all kinds of vaccines, microarray analysis is expected to play an important role in the new safety control test, especially for checking toxin-reactive transcripts.

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References

[1] Y. Horiuchi, M. Takahashi, T. Konda, et al., "Quality control of diphtheria tetanus acellular pertussis combined (DTaP) vaccines in Japan," *Japanese Journal of Infectious Diseases*, vol. 54, no. 5, pp. 167–180, 2001.

[2] M. Kurokawa, "Toxicity and toxicity testing of pertussis vaccine," *Japanese Journal of Medical Science and Biology*, vol. 37, no. 2, pp. 41–81, 1984.

[3] T. Mizukami, A. Masumi, H. Momose, et al., "An improved abnormal toxicity test by using reference vaccine-specific body weight curves and histopathological data for monitoring vaccine quality and safety in Japan," *Biologicals*, vol. 37, no. 1, pp. 8–17, 2009.

[4] T. Lettieri, "Recent applications of DNA microarray technology to toxicology and ecotoxicology," *Environmental Health Perspectives*, vol. 114, no. 1, pp. 4–9, 2006.

[5] E. K. Lobenhofer, P. R. Bushel, C. A. Afshari, and H. K. Hamadeh, "Progress in the application of DNA microarrays," *Environmental Health Perspectives*, vol. 109, no. 9, pp. 881–891, 2001.

[6] W. Pennie, S. D. Pettit, and P. G. Lord, "Toxicogenomics in risk assessment: an overview of an HESI collaborative research program," *Environmental Health Perspectives*, vol. 112, no. 4, pp. 417–419, 2004.

[7] A. H. Harrill and I. Rusyn, "Systems biology and functional genomics approaches for the identification of cellular responses to drug toxicity," *Expert Opinion on Drug Metabolism & Toxicology*, vol. 4, no. 11, pp. 1379–1389, 2008.

[8] H. K. Hamadeh, P. R. Bushel, S. Jayadev, et al., "Gene expression analysis reveals chemical-specific profiles," *Toxicological Sciences*, vol. 67, no. 2, pp. 219–231, 2002.

[9] N. Ejiri, K.-I. Katayama, N. Kiyosawa, Y. Baba, and K. Doi, "Microarray analysis on phase II drug metabolizing enzymes expression in pregnant rats after treatment with

pregnenolone-16α-carbonitrile or phenobarbital," *Experimental and Molecular Pathology*, vol. 79, no. 3, pp. 272–277, 2005.

[10] European Medicines Agency Concept Paper, "Pharmacogenomics (PG) biomarker qualification: format and data standards," in *Proceedings of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use*, June 2008, EMEA/CHMP/190395/2008, **http://www.emea.europa.eu/pdfs/human/pharmacogenetics/19039508en.pdf**.

[11] F. W. Frueh, "Impact of microarray data quality on genomic data submissions to the FDA," *Nature Biotechnology*, vol. 24, no. 9, pp. 1105–1107, 2006.

[12] *Minimum Requirements for Biological Products*, National Institute of Infectious Diseases, Tokyo, Japan, 2006, **http://www.nih.go.jp/niid/MRBP/files/seibutsuki_english.pdf**.

[13] M. Kurokawa, S. Ishida, S. Asakawa, S. Iwasa, and N. Goto, "Toxicities of influenza vaccine: peripheral leukocytic response to live and inactivated influenza viruses in mice," *Japanese Journal of Medical Science and Biology*, vol. 28, no. 1, pp. 37–52, 1975.

[14] F. Chino, "The views and policy of the Japanese control authorities on the three Rs," *Developments in Biological Standardization*, vol. 86, pp. 53–62, 1996.

[15] I. Hamaguchi, J.-I. Imai, H. Momose, et al., "Two vaccine toxicity-related genes Agp and Hpx could prove useful for pertussis vaccine safety control," *Vaccine*, vol. 25, no. 17, pp. 3355–3364, 2007.

[16] T. Mizukami, J.-I. Imai, I. Hamaguchi, et al., "Application of DNA microarray technology to influenza A/Vietnam/1194/2004 (H5N1) vaccine safety evaluation," *Vaccine*, vol. 26, no. 18, pp. 2270–2283, 2008.

[17] I. Hamaguchi, J.-I. Imai, H. Momose, et al., "Application of quantitative gene expression analysis for pertussis vaccine safety control," *Vaccine*, vol. 26, no. 36, pp. 4686–4696, 2008.

[18] E. Ito, R. Honma, J.-I. Imai, et al., "A tetraspanin-family protein, T-cell acute lymphoblastic leukemia-associated antigen 1, is induced by the Ewing's sarcoma-Wilms' tumor 1 fusion protein of desmoplastic small round-cell tumor," *American Journal of Pathology*, vol. 163, no. 6, pp. 2165–2172, 2003.

[19] S. Kobayashi, E. Ito, R. Honma, et al., "Dynamic regulation of gene expression by the Flt-1 kinase and Matrigel in endothelial tubulogenesis," *Genomics*, vol. 84, no. 1, pp. 185–192, 2004.

[20] K. M. Edwards and M. D. Decker, "Pertussis vaccines," in *Vaccines*, S. A. Plotkin, W. A. Orenstein, and P. A. Offit, Eds., pp. 467–517, Elsevier, New York, NY, USA, 5th edition, 2008.

[21] H. Sato and Y. Sato, "Bordetella pertussis infection in mice: correlation of specific antibodies against two antigens, pertussis toxin, and filamentous hemagglutinin with mouse protectivity in an intracerebral or aerosol challenge system," *Infection and Immunity*, vol. 46, no. 2, pp. 415–421, 1984.

[22] C. B. Bridges, J. M. Katz, R. A. Levandowski, and N. J. Cox, "Inactivated influenza vaccines," in *Vaccines*, S. A. Plotkin, W. A. Orenstein, and P. A. Offit, Eds., pp. 259–290, Elsevier, New York, NY, USA, 5th edition, 2008.

[23] K. Ungchusak, P. Auewarakul, S. F. Dowell, et al., "Probable person-to-person transmission of avian influenza A (H5N1)," *The New England Journal of Medicine*, vol. 352, no. 4, pp. 333–340, 2005.

[24] H. Momose, J.-I. Imai, I. Hamaguchi, et al., "Induction of indistinguishable gene expression patterns in rats by Vero cell-derived and mouse brain-derived Japanese encephalitis vaccines," *Japanese Journal of Infectious Diseases*, vol. 63, no. 1, pp. 25–30, 2010.

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ORIGINAL ARTICLE

Immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1, NIBRG-14) vaccine administered by intramuscular or subcutaneous injection

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ABSTRACT

The immunogenicity and safety profile of an inactivated whole-virion influenza A (H5N1, NIBRG-14) vaccine with alum adjuvant that was administered by IM or SC injection in a phase I clinical study involving 120 healthy Japanese men aged 20–40 years is described. The serological response of the IM group was stronger than that of the SC group. Local adverse events were less severe with IM injection than with SC injection, while similar systemic adverse events were seen in both groups. These results indicate that, when administering an inactivated whole virion vaccine with alum adjuvant for pandemic influenza, IM injection may achieve better immunogenicity and safety than SC injection.

Key words influenza, intramuscular injection, pandemic, subcutaneous injection.

According to a WHO survey, the H5N1 virus has killed more than 260 people since 2003 (1). Although the WHO has not yet declared phase 4 in response to the H5N1 influenza, cases of human infection in Egypt, China, and Vietnam have continued to be reported in 2009 (1). Therefore, it is important to develop vaccines for H5N1 viruses as part of the preparation for a possible pandemic. The Japanese pandemic influenza preparedness action plan stipulates that, when the Minister of Health, Labor and Welfare declares stage one (corresponding to phase 4), health care workers and public servants can be vaccinated with prototype vaccines from the national stockpile as an emergency measure. There are several reference vaccine strains that have been recommended by the WHO in consideration of the epidemiological situation in humans, poultry, and wild birds (2). However, it is extremely hard to predict a pandemic strain in advance, and it takes some time to distribute an appropriate vaccine after declaration of a pandemic. Thus, prior to the declaration of stage one, the possibility of using a pre-pandemic vaccine to vaccinate people who are involved with critical infrastructure has been discussed in Japan.

Influenza pandemic vaccines are being developed and assessed clinically by several vaccine manufacturers (3). In our previous investigation of vaccination for the A/Hong Kong/156/97(H5N1) strain, the immunogenicity of the

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List of Abbreviations: CC, cell control containing no serum or virus; CHMP, The Committee for Medicinal Products for Human Use; GMT, geometric mean titer; HA, hemagglutinin; HI, hemagglutination-inhibition; ID, intradermal; IM, intramuscular; NIBSC, National Institute for Biological Standards and Control; NT, neutralization titer; PT, preferred term; RDE, receptor destroying enzyme; SC, subcutaneous; TCID50, 50% tissue culture infectious dose; VC, virus control.

vaccine in humans was very low, even when an inactivated whole virion vaccine was employed (4), suggesting that an adjuvant was needed in order to elicit a more robust immune response (5, 6). Therefore, we developed a whole-virion vaccine with alum adjuvant to enhance the immune response. Alum (aluminum hydroxide) was selected as the adjuvant because of its excellent safety record, proven ability to enhance the immune response to various antigens, and low cost (7).

The route of administration is an important factor in relation to both the immunogenicity and safety of a vaccine. A seasonal influenza vaccine is routinely immunized subcutaneously in Japan, but it is still unclear what route is suitable for the aluminum-adjuvanted whole influenza vaccine. One report states that the influenza vaccine is more immunogenic and less reactogenic after IM injection (8). In addition, in the USA, it is recommended that vaccines with adjuvant such as aluminum gel be administered intramuscularly because the SC or ID route might induce more local reactions (9). We therefore compare two routes, IM and SC injection, in this clinical trial.

This report describes the results of a phase I study of an inactivated adjuvanted whole-virion influenza A (H5N1, NIBRG-14) vaccine, with a detailed comparison between the results obtained after administration via IM and SC routes.

METHODS

Vaccine

The vaccine used in this study, an inactivated monovalent A/H5N1 whole-virion influenza vaccine with 0.3 mg/ml aluminum hydroxide as the adjuvant, was manufactured by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan) according to the Good Manufacturing Practice regulations under Biosafety Level 2 Enhanced conditions. The vaccine was produced in embryonated hens eggs using an H5N1 pandemic influenza reference strain (NIBRG-14) established by the UK NIBSC (Potters Bar, UK). NIBRG-14, a reassortant that was generated by reverse genetics, has a modified HA gene and an original NA gene from A/Vietnam/1194/2004, as well as other internal genes from A/PR/8/34 (H1N1).

The original virus obtained from the NIBSC was grown once in specific pathogen-free embryonated hen's eggs, and was then used for vaccine production as an inoculum. Allantoic fluid containing virus particles was concentrated, purified by ultrafiltration, and inactivated with formalin. The vaccine was then formulated by adding aluminum hydroxide (final concentration: 0.3 mg/ml) as the adjuvant. Vaccines of two different concentrations, containing either 10 μg or 30 μg of HA per 1.0 ml, were prepared. The HA concentration was determined by SDS-PAGE/densitometry analysis. Purified viruses were separated by 12.5% of SDS-PAGE and the proportion of HA in the total viral proteins was analyzed by densitometry of the stained gel. The HA concentration was calculated by multiplying the proportion of HA by the protein concentration. As a preservative, 0.001 w/v% thimerosal was added and the vaccines were stored at 2–8°C before use.

Pre-clinical studies

Before conducting the phase I clinical study, we performed several toxicity studies according to Good Laboratory Practice standards, including single-dose toxicity, repeated-dose toxicity, local irritation, reproductive toxicity, and general pharmacology tests. Because no data suggesting toxicity of the vaccine was obtained by these tests, we decided to perform a phase I clinical study.

Clinical study

The Phase I clinical study was conducted from January 2007 to April 2007 at the Medical Corporation Kouryokai CPC Clinic. We obtained permission from the Institutional Review Board of the clinic before initiating the study and the study was conducted in full compliance with the Good Clinical Practice regulations. It was performed as an open-label study.

Subjects

Healthy Japanese men aged 20–40 years were carefully screened, including assessment for any history of drug allergy and anaphylactic shock. Then 120 eligible volunteers who gave written informed consent were enrolled.

Vaccination and examination

Three different antigen doses were evaluated in this study, namely 15 μg, 5 μg, and 1.7 μg per dose of vaccine. For the 15 μg and 5 μg doses, 0.5 ml of the vaccine containing 30 μg/ml or 10 μg/ml of antigen was administered, respectively. For the 1.7 μg dose, 0.17 ml of the vaccine containing 10 μg/ml of antigen was administered. Administration was conducted either intramuscularly or subcutaneously, and two doses were given to each volunteer 21 days apart. On days 2, 8, and 21, volunteers were examined by an investigator. Volunteers also recorded local symptoms (such as pain, erythema, swelling, and pruritus), systemic symptoms (such as malaise, headaches, and chills), and their axillary temperature every day for 21 days after each vaccination. Adverse events were classified from 1 to 4 (grade 1 was mild and grade 4 was severe). Serum samples for assessment of the antibody response were obtained on day 0 (pre-vaccination), day 21, and day 42.

Neutralizing antibody test and HI test

Neutralizing antibody tests were performed by the method of the National Institute of Infectious Diseases in Japan. Serum was treated with RDE (II) (Denka Seiken, Tokyo, Japan) overnight at 37°C, and incubated for 30 min at 56°C. Then the serum samples were diluted 2-fold from 1:10 and placed in 96-well microplates. To each well of the plate, an equal volume of diluent containing 1×10^2 $TCID_{50}/50$ μl of NIBRG-14 virus was added. There were eight control wells in each plate, including a VC and a CC. After 1 hr of incubation at 37°C in a humidified atmosphere with 5% CO_2, 100 μl of the mixture of diluted serum and the virus solution was added to Madin-Darby canine kidney cells and subsequently incubated for 4 days. Then the cells were fixed with 10% formalin for 10 min or more, and subsequently stained with 0.1% Neutral Blue solution (Sigma, St Louis, MO, USA). After dissolving the stain the absorbance of each well was read at 630 nm. The NT was expressed as the reciprocal of the highest serum dilution where the absorbance of the well was equal to or more than the half absorbance of non-infected well calculated by (VC absorbance + CC absorbance)/2.

The immune response elicited after vaccination was evaluated by the HI test (10). In brief, serum samples were treated with RDE (II) overnight at 37°C, incubated for 1 hr at 56°C, and then incubated with 50% horse erythrocytes for 1 hr at 4°C. The samples were diluted 2-fold from 1:10 in V-shaped 96-well plates and the HI titer was defined as the highest dilution that completely inhibited hemagglutination. In both assays, negative samples were assigned a titer of 5 for calculation purposes.

Statistical analysis

The HI and NT values were transformed into $\log_{10}$ titers for calculation of the GMT at every time of assessment (day 0, day 21, and day 42). The HI results were evaluated according to the European Union CHMP criteria for annual licensing of seasonal influenza vaccines (11). All data manipulations and statistical computations were done with SAS/Base/Stat (version 9.1) and Microsoft excel (version 2002).

Role of the funding source

The Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan) was involved in the study design, laboratory assays, data collection, management, analysis, and writing of this report. The corresponding author had full access to all the data in the study and has final responsibility for the decision to submit this report for publication.

RESULTS

Subjects

A total of 120 healthy men were selected according to the inclusion criteria and randomized. The mean ages in different subgroups ranged from 25.3 to 26.6 years. There were no significant age differences between the subgroups (Table 1). One man in the 15 μg group did not receive the second dose of vaccine for family reasons. The remaining 119 individuals received both of the planned doses, attended all visits, completed clinical observation, and provided three blood samples (days 0, 21, and 42).

Adverse events

All doses of the vaccine were well tolerated, with no serious adverse events being reported between day 0 and day 42 (no grade 4 events). In general, there was no marked difference in adverse events between the first and second doses (Table 2).

The major local adverse events were injection site erythema (63.3%, 76/120), pain (41.7%, 50/120), injection site swelling (26.7%, 32/120) and injection site pruritus (15.0%, 18/120). The SC group had more local adverse events than the IM group, especially in relation to erythema, swelling, and pruritus. Although most adverse

Table 1. Trial profile

	SC			IM		
	1.7 ug ($n = 20$)	5 ug ($n = 20$)	15 ug ($n = 19$)	1.7 ug ($n = 20$)	5 ug ($n = 20$)	15 ug ($n = 20$)
Age						
Mean (SD)	26.2 (4.3)	26.6 (4.2)	26.5 (3.8)	26.1 (5.3)	26.0 (5.4)	25.3 (4.3)
Median	26.0	26.5	27.0	25.0	23.5	24.0
BMI						
Mean (SD)	22.1 (2.5)	22.4 (2.4)	21.3 (2.0)	22.3 (2.4)	22.2 (2.3)	21.8 (2.3)
Median	21.7	22.8	20.6	21.8	21.0	21.9

BMI, body mass index; SD, standard deviation.

Table 2. Rates of adverse events to vaccination

(a) SC

	PT	Total ($n = 60^{\dagger}$)	1.7 ug ($n = 20$)	5 ug ($n = 20$)	15 ug ($n = 20^{\ddagger}$)
After first dose					
Local adverse events	Pain	22 (37%; 25, 50)	5 (25%; 9, 49)	5 (25%; 9, 49)	12 (60%; 36, 81)
	Erythema	40 (67%; 53, 78)	12 (60%; 36, 81)	15 (75%; 51, 91)	13 (65%; 41, 85)
	Swelling	14 (23%; 13, 36)	2 (10%; 1, 32)	7 (35%; 15, 59)	5 (25%; 9, 49)
	Induration	2 (3%; 0, 12)	1 (5%; 0, 25)	0 (0%; 0, 17)	1 (5%; 0, 25)
	Pruritus	11 (18%; 10, 30)	2 (10%; 1, 32)	6 (30%; 12, 54)	3 (15%; 3, 38)
Systemic adverse events	Pyrexia	3 (5%; 1, 14)	0 (0%; 0, 17)	2 (10%; 1, 32)	1 (5%; 0, 25)
	Headache	10 (17%; 8, 29)	5 (25%; 9, 49)	2 (10%; 1, 32)	3 (15%; 3, 38)
	Malaise	18 (30%; 19, 43)	5 (25%; 9, 49)	6 (30%; 12, 54)	7 (35%; 15, 59)
	Chill	6 (10%; 4, 21)	1 (5%; 0, 25)	2 (10%; 1, 32)	3 (15%; 3, 38)
	Diarrhea	4 (7%; 2, 16)	1 (5%; 0, 25)	1 (5%; 0, 25)	2 (10%; 1, 32)
After second dose					
Local adverse events	Pain	18 (31%; 19, 44)	3 (15%; 3, 38)	8 (40%; 19, 64)	7 (37%; 16, 62)
	Erythema	44 (75%; 62, 85)	13 (65%; 41, 85)	14 (70%; 46, 88)	17 (89%; 67, 99)
	Swelling	16 (27%; 16, 40)	3 (15%; 3, 38)	4 (20%; 6, 44)	9 (47%; 24, 71)
	Induration	1 (2%; 0, 9)	1 (5%; 0, 25)	0 (0%; 0, 17)	0 (0%; 0, 18)
	Pruritus	6 (10%; 4, 21)	0 (0%; 0, 17)	0 (0%; 0, 17)	6 (32%; 13, 57)
Systemic adverse events	Pyrexia	4 (7%; 2, 16)	1 (5%; 0, 25)	1 (5%; 0, 25)	2 (11%; 1, 33)
	Headache	6 (10%; 4, 21)	3 (15%; 3, 38)	1 (5%; 0, 25)	2 (11%; 1, 33)
	Malaise	10 (17%; 8, 29)	2 (10%; 1, 32)	4 (20%; 6, 44)	4 (21%; 6, 46)
	Chill	3 (5%; 1, 14)	1 (5%; 0, 25)	0 (0%; 0, 17)	2 (11%; 1, 33)
	Diarrhea	7 (12%; 5, 23)	2 (10%; 1, 32)	2 (10%; 1, 32)	3 (16%; 3, 40)

(b) IM

	PT	Total ($n = 60$)	1.7 ug ($n = 20$)	5 ug ($n = 20$)	15 ug ($n = 20$)
After first dose					
Local adverse events	Pain	21 (35%; 23, 48)	3 (15%; 3, 38)	9 (45%; 23, 68)	9 (45%; 23, 68)
	Erythema	15 (25%; 15, 38)	6 (30%; 12, 54)	6 (30%; 12, 54)	3 (15%; 3, 38)
	Swelling	4 (7%; 2, 16)	0 (0%, 0, 17)	2 (10%; 1, 32)	2 (10%; 1, 32)
	Induration	1 (2%; 0, 9)	1 (5%; 0, 25)	0 (0%; 0, 17)	0 (0%; 0, 17)
	Pruritus	1 (2%; 0, 9)	0 (0%; 0, 17)	1 (5%; 0, 25)	0 (0%; 0, 17)
Systemic adverse events	Pyrexia	5 (8%; 3, 18)	0 (0%; 0, 17)	2 (10%; 1, 32)	3 (15%; 3, 38)
	Headache	11 (18%; 10, 30)	3 (15%; 3, 38)	3 (15%; 3, 38)	5 (25%; 9, 49)
	Malaise	12 (20%; 11, 32)	1 (5%; 0, 25)	4 (20%; 6, 44)	7 (35%; 15, 59)
	Chill	4 (7%; 2, 16)	1 (5%; 0, 25)	2 (10%; 1, 32)	1 (5%; 0, 25)
	Diarrhea	4 (7%; 2, 16)	0 (0%; 0, 17)	2 (10%; 1, 32)	2 (10%; 1, 32)
After second dose					
Local adverse events	Pain	13 (22%; 12, 34)	2 (10%; 1, 32)	4 (20%; 6, 44)	7 (35%; 15, 59)
	Erythema	9 (15%; 7, 27)	2 (10%; 1, 32)	6 (30%; 12, 54)	1 (5%; 0, 25)
	Swelling	4 (7%; 2, 16)	1 (5%, 0, 25)	2 (10%; 1, 32)	1 (5%; 0, 25)
	Induration	0 (0%; 0, 6)	0 (0%; 0, 17)	0 (0%; 0, 17)	0 (0%; 0, 17)
	Pruritus	1 (2%; 0, 9)	0 (0%; 0, 17)	0 (0%; 0, 17)	1 (5%; 0, 25)
Systemic adverse events	Pyrexia	5 (8%; 3, 18)	4 (20%; 6, 44)	1 (5%; 0, 25)	0 (0%; 0, 17)
	Headache	5 (8%; 3, 18)	2 (10%; 1, 32)	2 (10%; 1, 32)	1 (5%; 0, 25)
	Malaise	5 (8%; 3, 18)	3 (15%; 3, 38)	2 (10%; 1, 32)	0 (0%; 0, 17)
	Chill	4 (7%; 2, 16)	2 (10%; 1, 32)	2 (10%; 1, 32)	0 (0%; 0, 17)
	Diarrhea	2 (3%; 0, 12)	1 (5%; 0, 25)	1 (5%; 0, 25)	0 (0%; 0, 17)

†After second dose: $n = 59$. ‡After second dose: $n = 19$. Data presented as n (%, 95% confidence limits).

events were grade 1 or grade 2, a total of 11 grade 3 events occurred in the SC group. Most of the grade 3 events were erythema (larger than 5 centimeters in diameter), of which 9 were reported in the 15 μg SC group and 1 in the 5 μg SC group. One case of grade 3 swelling (larger than 5 centimeter in diameter) was reported in the 15 μg SC group. In contrast, no grade 3 events were reported in the IM group.

The major systemic adverse events were malaise (30%, 36/120), headache (23%, 27/120), pyrexia (13%, 16/120), chills (13%, 15/120), and diarrhea (13%, 15/120). There was no marked difference in systemic adverse events between SC and IM injection. One case of grade 3 pyrexia (≥39.0°C for less than one day) was reported in the 1.7 μg IM group, and it was shown by a rapid diagnostic test that this subject had influenza due to H1N1 virus.

Immune responses

Before vaccination, 12.5% (15/120) and 1.7% (2/120) of the participants had detectable antibodies against H5N1 in the neutralization and HI tests, respectively. However, only 3.3% (two for both the NT and HI titer and two for the NT only) of the participants had antibody titers over 20. Similar antibody titers have been reported in other H5N1 vaccine trials (12–14).

With regard to the neutralizing antibody response to NIBRG-14 virus (Table 3, Fig. 1), more than 60% of the 5 μg and 15 μg groups achieved an NT ≥ 40 after two doses of vaccine via the IM route. After SC injection, however, the ratio was 53% in the 15 μg group and only 20% in the 5 μg group. After a single dose of vaccine, a similar superiority for the IM route was noted.

The results of the HI test using horse erythrocytes showed a similar pattern to those obtained by measurement of NT (Table 3, Fig. 1). After both IM and SC injection, the 15 μg group complied with two of the three CHMP criteria for seasonal vaccines (seroconversion factor over 2.5 and seroconversion ratio over 40%) at 21 days after the first dose. After the second dose, both the 5 μg and 15 μg IM groups and the 15 μg SC group met these two criteria. Unfortunately, none of the groups achieved the seroprotection criterion (>70%). HI antibody titers were also measured using chicken erythrocytes, but these titers were generally lower than the titers obtained using horse erythrocytes (data not shown).

Discussion

We investigated the immunogenicity and safety profile of an inactivated whole-virion influenza A (H5N1, NIBRG-14) vaccine with alum adjuvant after injection by the IM or SC routes in a phase I clinical study. Both administration routes were well tolerated, but local adverse events were less severe after IM than after SC injection. Similar results were obtained in relation to systemic adverse events.

Several inactivated whole-virion vaccines for H5N1 virus have already been evaluated in clinical studies. A Sinovac vaccine (a whole-virion vaccine grown in eggs with alum adjuvant) met all the CHMP criteria after

Table 3. NT and HI response to the homologous recombinant A/Vietnam/1194/2004 NIBRG-14 vaccine strain after the first and second doses of vaccine

		SC			IM		
	CHMP criteria	1.7 ug (n = 20)	5 ug (n = 20)	15 ug (n = 20†)	1.7 ug (n = 20)	5 ug (n = 20)	15 ug (n = 20)
After first dose							
GMT (NT)	No standard	10.4 (6.4–16.7)	9.0 (6.4–12.6)	20.7 (10.6–40.6)	10.7 (6.1–18.9)	20.7 (12.7–33.7)	28.3 (14.4–55.7)
GMT (HI)	No standard	7.1 (5.4–9.3)	6.2 (5.0–7.6)	14.4 (8.8–23.5)	7.3 (5.0–10.8)	10.4 (7.1–15.0)	20.7 (11.9–36.0)
Seroconversion factor	>2.5	1.23 (1.06–1.43)	1.23 (0.99–1.52)	**2.88 (1.76–4.70)**	1.46 (0.99–2.15)	2.07 (1.43–3.00)	**4.14 (2.39–7.19)**
Seropositivity	>70%	5.0% (0.1–24.9)	0.0% (0.0–16.8)	26.3% (9.1–51.2)	5.0% (0.1–24.9)	15.0% (3.2–37.9)	40.0% (19.1–63.9)
Seroconversion	>40%	0.0% (0.0–16.8)	10.0% (1.2–31.7)	**42.1% (20.3–66.5)**	10% (5.4–9.3)	35% (15.4–59.2)	**65% (40.8–84.6)**
After second dose							
GMT (NT)	No standard	13.2 (7.5–23.3)	17.4 (11.8–25.7)	34.6 (19.7–60.7)	13.2 (7.5–23.3)	41.4 (28.6–60.1)	51.0 (31.0–83.8)
GMT (HI)	No standard	8.7 (5.9–12.8)	8.1 (6.0–11.0)	24.0 (14.3–40.4)	9.3 (6.3–13.8)	14.6 (10.1–21.2)	31.4 (18.9–52.2)
Seroconversion factor	>2.5	1.52 (1.16–1.98)	1.62 (1.20–2.19)	**4.80 (2.85–8.08)**	1.87 (1.26–2.76)	**2.93 (2.02–4.25)**	**6.28 (3.78–10.43)**
Seropositivity	>70%	10.0% (1.2–31.7)	5.0% (0.1–24.9)	36.8% (16.3–61.6)	5.0% (0.1–24.9)	20.0% (5.7–43.7)	45.0% (23.1–68.5)
Seroconversion	>40%	20.0% (5.7–43.7)	20.0% (5.7–43.7)	**68.4% (43.5–87.4)**	20.0% (5.7–43.7)	**50.0% (27.2–72.8)**	**75.0% (50.9–91.3)**

†After second dose: n = 19. Data presented as value (95% confidence limits).
Seroconversion, proportion of individuals with at least four-fold increase in titers; seroconversion factor, the ratio of post-vaccination GMT to prevaccination GMT; seropositivity, proportion of individuals achieving HI titer of >40.

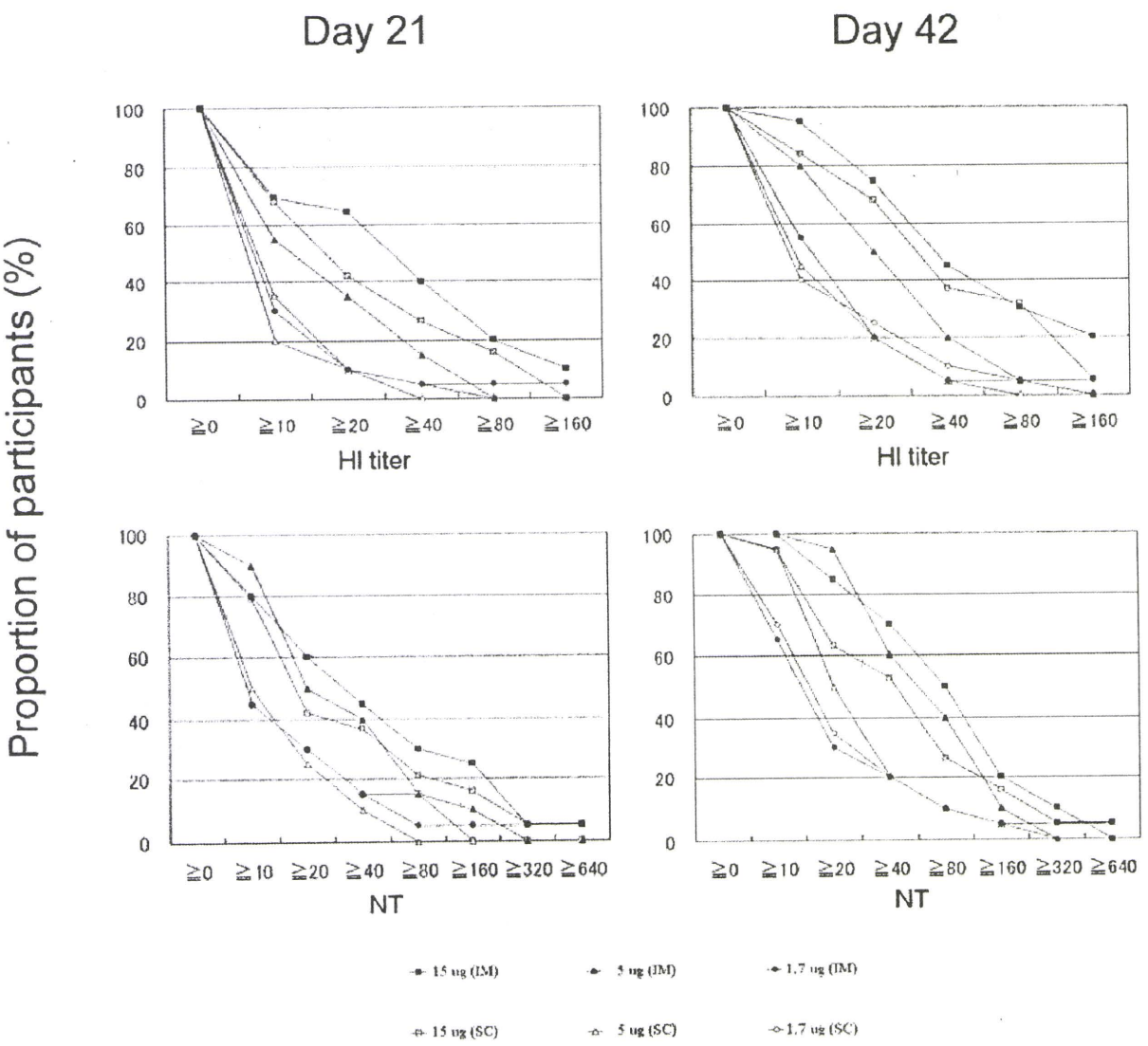

Fig. 1. Reverse cumulative distribution of NT and HI at days 21 and 42. These graphs show the proportion of study participants for neutralizing titer (bottom panels) and the HI antibody titer (upper panels) at day 21 (left panels) and day 42 (right panels) in this Phase I clinical study.

administration at 10 μg HA (12). A Baxter vaccine (a cell-culture derived whole-virion vaccine without any adjuvant) complied with both the seroprotection factor and seroconversion factor criteria after doses of 7.5 or 15 μg HA in the microneutralization test (15). These vaccines revealed cross-reactivity against heterologous H5N1 strains (15, 16). The immunogenicity of these two whole-virion vaccines did not seem to be significantly different, because there is considerable variation among institutions with respect to influenza serological assays (17). Regarding safety, both vaccines were well tolerated and no severe reactions were reported (12, 15, 16). Therefore, whole-virion vaccines would seem to be a reasonable choice for an H5N1 vaccine (18).

The present clinical study did not include a non-adjuvant group because alum adjuvant enhanced the NT and HI titer in BALB/c mice [data not shown], and an inactivated whole-virion H5N1 vaccine elicited low NT and HI titers in the human clinical trial previously described (4). In addition, a 90 μg HA/dose injection of baculovirus-expressed H5 HA is needed to induce an immune response in most recipients without adjuvant

(13). Therefore, we could not find reasons to incorporate a non-adjuvant group in this clinical study, which was aimed at improving immune responses and achieving dose-sparing. In fact, our alum-adjuvanted H5N1 vaccine elicited NT and HI antibody titer, while non-adjuvanted whole-virion H5N1 vaccine did not (4). In contrast, it has been reported that the immunogenicity of a whole-virion H5N1 vaccine grown in Vero cells was not enhanced by alum adjuvant in either CD1 mice (19) or humans (15). This difference in the enhancement effect of the alum-adjuvant may be due to the following differences: i) physicochemical form of the aluminum gels; ii) alum concentrations; iii) seed viruses of the inactivated antigens (RG or wild type); and iv) cell substrates.

Serological responses were stronger in the IM group than in the SC group, although the 15 μg HA dose delivered via both administration routes complied with two out of three European Union CHMP criteria according to the HI test. Some previous clinical reports have also indicated that IM injection achieves much better immunogenicity and safety than SC injection in the case of both influenza vaccines (8, 20) and other vaccines (21–23). As an alternative route of injection, ID injection might be expected to reduce the dose of antigen needed, as has been shown for H1N1 and H3N2 influenza vaccines (24). However, ID injection of an A/VN/1203/04 (H5N1) subvirion vaccine did not actually elicit a stronger immune response than IM injection (25). Therefore, the IM route seems to be the most effective route for delivery of an inactivated whole-virion vaccine with alum adjuvant in terms of both immunogenicity and safety. The mechanisms which lead to differences in immune response and safety between the IM and SC routes are unclear.

Although whole-virion influenza vaccines are generally thought to be more immunogenic and reactogenic than split-virion vaccines, our IM group results were actually comparable with those reported for split-virion vaccines (13, 14, 26). The incidence of fever was also comparable among these vaccines, although the reporting systems differed slightly for each clinical trial.

An alternative adjuvant to alum is an oil emulsion (AS03 [27] or MF59 [28]). Although H5N1 influenza vaccines with oil emulsion adjuvant may be more immunogenic than vaccines with no adjuvant or alum adjuvant, such emulsions have to be licensed by the patent holders and thus are not easy for some vaccine manufacturers to use.

In conclusion, IM administration is better than SC administration from the viewpoint of immunogenicity and reactogenicity in a whole-virion H5N1 vaccine with alum. Furthermore, such a vaccine may be one of the choices for adults. The suitability of this vaccine for elderly and pediatric populations should also be investigated.

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REFERENCES

1. WHO. (2009) Cumulative number of confirmed human cases of avian influenza A (H5N1) reported to WHO. Accessed July 1, 2009, at: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_07_01/en/index.html.
2. WHO. (2009) Antigenic and genetic characteristics of H5N1 viruses and candidate H5N1 vaccine viruses developed for potential use as pre-pandemic Vaccines. Accessed July 18, 2009, at: http://www.who.int/csr/disease/avian_influenza/guidelines/200902_H5VaccineVirusUpdate.pdf.
3. WHO. (2007) Tables on the Clinical trials of pandemic influenza prototype vaccines. WHO meeting Available at: http://www.who.int/vaccine_research/diseases/influenza/flu_trials_tables/en/index3.html.
4. Tashiro M. (2003) Safety and efficacy of an inactivated whole-virion influenza A (H5N1) vaccine. *MHLW Res*: 200201019A (Japanese language only).
5. Hehme N., Engelmann H., Kuenzel W., Neumeier E., Saenger R. (2004) Immunogenicity of a monovalent, aluminum-adjuvanted influenza whole virus vaccine for pandemic use. *Virus Res* **103**: 163–71.
6. Hehme N., Engelmann H., Kunzel W., Neumeier E., Sanger R. (2002) Pandemic preparedness: lessons learnt from H2N2 and H9N2 candidate vaccines. *Med Microbiol Immunol* **191**: 203–8.
7. Ninomiya A., Imai M., Tashiro M., Odagiri T. (2007) Inactivated influenza H5N1 whole-virus vaccine with aluminum adjuvant induces homologous and heterologous protective immunities against lethal challenge with highly pathogenic H5N1 avian influenza viruses in a mouse model. *Vaccine* **25**: 3554–60.
8. Cook I.F., Barr I., Hartel G., Pond D., Hampson A.W. (2006) Reactogenicity and immunogenicity of an inactivated influenza vaccine administered by intramuscular or subcutaneous injection in elderly adults. *Vaccine* **24**: 2395–402.
9. American Academy of Pediatrics. (1997) RED BOOK, 24th Edition (ISBN: 0-910761-85-x). p14.
10. Stephenson I., Wood J.M., Nicholson K.G., Charlett A., Zambon M.C. (2004) Detection of anti-H5 responses in human sera by HI using horse erythrocytes following MF59-adjuvanted influenza A/Duck/Singapore/97 vaccine. *Virus Res* **103**: 91–5.
11. European Medicine Agency. (1997) Committee for Proprietary Medicinal Products (CPMP). Note for guidance on harmonization of requirements for influenza vaccines (CPMP/BWP/214/96). Available at: http://www.emea.europa.eu/pdfs/human/bwp/021496en.pdf.
12. Lin J., Zhang J., Dong X., Fang H., Chen J., Su N., Gao Q., Zhang Z., Liu Y., Wang Z., Yang M., Sun R., Li C., Lin S., Ji M., Liu Y., Wang X., Wood J., Feng Z., Wang Y., Yin W. (2006) Safety and

immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. *Lancet* **368**: 991–7.
13. Treanor J.J., Campbell J.D., Zangwill K.M., Rowe T., Wolff M. (2006) Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. *N Engl J Med* **354**: 1343–51.
14. Bresson J.L., Perronne C., Launay O., Gerdil C., Saville M., Wood J., Hoschler K., Zambon M.C. (2006) Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomised trial. *Lancet* **367**: 1657–64.
15. Ehrlich H.J., Muller M., Oh H.M., Tambyah P.A., Joukhadar C., Montomoli E., Fisher D., Berezuk G., Fritsch S., Low-Baselli A., Vartian N., Bobrovsky R., Pavlova B.G., Pollabauer E.M., Kistner O., Barrett P.N. (2008) A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. *N Engl J Med* **358**: 2573–84.
16. Wu J., Fang H.H., Chen J.T., Zhou J.C., Feng Z.J., Li C.G., Qiu Y.Z., Liu Y., Lu M., Liu L.Y., Dong S.S., Gao Q., Zhang X.M., Wang N., Yin W.D., Dong X.P. (2009) Immunogenicity, safety, and cross-reactivity of an inactivated, adjuvanted, prototype pandemic influenza (H5N1) vaccine: a phase II, double-blind, randomized trial. *Clin Infect Dis* **48**: 1087–95.
17. Stephenson I., Das R.G., Wood J.M., Katz J.M. (2007) Comparison of neutralising antibody assays for detection of antibody to influenza A/H3N2 viruses: an international collaborative study. *Vaccine* **25**: 4056–63.
18. Tada Y. (2008) Characterization of a whole, inactivated influenza (H5N1) vaccine. *Influenza Other Respi Viruses* **2**: 261–6.
19. Kistner O., Howard M.K., Spruth M., Wodal W., Bruhl P., Gerencer M., Crowe B.A., Savidis-Dacho H., Livey I., Reiter M., Mayerhofer I., Tauer C., Grillberger L., Mundt W., Falkner F.G., Barrett P.N. (2007) Cell culture (Vero) derived whole virus (H5N1) vaccine based on wild-type virus strain induces cross-protective immune responses. *Vaccine* **25**: 6028–36.
20. Ruben F.L., Jackson G.G. (1972) A new subunit influenza vaccine: acceptability compared with standard vaccines and effect of dose on antigenicity. *J Infect Dis* **125**: 656–64.
21. Fisch A., Cadilhac P., Vidor E., Prazuck T., Dublanchet A., Lafaix C. (1996) Immunogenicity and safety of a new inactivated hepatitis A vaccine: a clinical trial with comparison of administration route. *Vaccine* **14**: 1132–6.
22. Carlsson R.M., Claesson B.A., Kayhty H., Selstam U., Iwarson S. (1999) Studies on a Hib-tetanus toxoid conjugate vaccine: effects of co-administered tetanus toxoid vaccine, of administration route and of combined administration with an inactivated polio vaccine. *Vaccine* **18**: 468–78.
23. Mark A., Carlsson R.M., Granstrom M. (1999) Subcutaneous versus intramuscular injection for booster DT vaccination of adolescents. *Vaccine* **17**: 2067–72.
24. Belshe R.B., Newman F.K., Cannon J., Duane C., Treanor J., Van Hoecke C., Howe B.J., Dubin G. (2004) Serum antibody responses after intradermal vaccination against influenza. *N Engl J Med* **351**: 2286–94.
25. Shital Patel R.A., El-Sahly H., Cate T., Keitel W. (2007) A Randomized, Open-Label, Phase I Clinical Trial Comparing the Safety, Reactogenicity, and Immunogenicity of Immunization with Inactivated Influenza A/H5N1 Vaccine Administered by the Intradermal (ID) or the Intramuscular (IM) Route Among Healthy Adults. Available at: http://www.who.int/vaccine_research/diseases/influenza/160207_keitel.pdf.
26. Nolan T.M., Richmond P.C., Skeljo M.V., Pearce G., Hartel G., Formica N.T., Hoschler K., Bennet J., Ryan D., Papanaoum K., Basser R.L., Zambon M.C. (2008) Phase I and II randomised trials of the safety and immunogenicity of a prototype adjuvanted inactivated split-virus influenza A (H5N1) vaccine in healthy adults. *Vaccine* **26**: 4160–7.
27. Leroux-Roels I., Borkowski A., Vanwolleghem T., Drame M., Clement F., Hons E., Devaster J.M., Leroux-Roels G. (2007) Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial. *Lancet* **370**: 580–9.
28. Nicholson K.G., Colegate A.E., Podda A., Stephenson I., Wood J., Ypma E., Zambon M.C. (2001) Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. *Lancet* **357**: 1937–43.