MSM-*Mx*, and B6.MSM-*Oas* mice were injected intraperitoneally with 200 μg of the synthetic double-stranded RNA (dsRNA) analog, poly (I:C) (GE Healthcare, UAS). Animals were sacrificed at 24 hr after the injection and the spleens were dissected. Total RNAs were isolated from the spleens using TRIzol reagent (Invitrogen, USA), and cDNAs were generated with oligo (dT) primers using ReverTra Ace (TOYOBO, Japan). PCR was performed using the following primers: ACGATGGATTCTGTGAATAATCTGT (nt 211–235) and TCTAGATAGCCTGGTTAATCG GAGAATTT (nt 2,095–2,121) for *Mx1*; GGCTG CAGAGGTATTAGCTGGACCT (nt 36–60) and CAGGAGGATGGCAATATCCAAGACA (nt 1202–1226) for *Oas1b*; and TGATGGTGGGAAT GGGTCAG (nt 207–226) and GAAGGCTG GAAAAGAGCCTC (nt 854–873) for mouse *Actb* (NM_007393). PCR products were electrophoresed in 1% agarose gels and visualized by ethidium bromide staining. Sequences of the *Oas1b* PCR products were determined with an ABI Prism 377 DNA Sequencer and an ABI Prism Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

Experimental infection of mice with influenza virus and West Nile virus: To assess the lethality of viruses in the congenic mice, 8-week-old male mice were infected intranasally with 30 μl inoculum containing 10^2–$10^4 \times 50\%$ of lethal dose (LD_{50}) of a highly pathogenic avian influenza strain, A/whooper swan/Mongolia/3/2005 (H5N1), diluted with phosphate-buffered saline (PBS) supplemented with 100 U/ml penicillin G and 100 μg streptomycin under anesthesia with 50 mg/kg sodium pentobarbital (Somnopentyl, Schering-Plough Animal Health U. S. A). The LD_{50} value was calibrated using 8-week-old B6 mice. After infection, mice were monitored daily for 21 days. For West Nile virus infection, 6-week-old male mice were infected intraperitoneally with 200 μl inoculum containing 1 or 10 plaque forming units (PFUs) of West Nile virus 6LP strain diluted with PBS under anesthesia with diethyl ether inhalation. After infection, mice were followed up for 4 weeks.

Results

Generation of Mx1 and Oas1b congenic mice

Congenic strains were generated using a marker-assisted speed congenic strategy as reported previously[17]. To generate congenic mice in which the *Mx* or *Oas* locus from MSM mice was introduced into the genetic background of B6 mice, the B6 and MSM alleles of *Mx1* and *Oas1b* were determined to select candidate mice. In the *Mx1* locus of B6 mice, a large deletion from exon 9 to 11 was reported previously[2,33]. Therefore, primers were designed for amplifying the region from intron 10 to exon 11 to distinguish the B6 allele from the MSM allele as reported previously[2]. As shown in Fig. 1A, these primers can detect MSM allele. On the other hand, since a single nucleotide polymorphism has been found in the *Hin*fI site of the *Oas1b* gene in the MSM allele (Fig. 1B), genotyping was performed by digestion of the PCR products of the *Oas1b* gene with *Hin*fI (Fig. 1C). The 'best' male mice, those carrying the most homozygous B6 alleles in 134 microsatellite markers with heterozygosity in the *Mx1* or *Oas1b* gene, were selected for breeding next generation. Backcrossing was performed six and seven times for exchanging to the B6 genetic background in the B6.MSM-*Oas* and B6.MSM-*Mx* mice, respectively. Finally, heterozygous sibling pairs were mated and homozygous mice were selected. To estimate the length of the chromosomal regions derived from MSM mice, the genotype and position of microsatellite markers surrounding the *Mx* or *Oas* locus were confirmed (Fig. 2). As shown in Fig. 2A, the genotypes of *D16Mit71* and *D16Mit106* in the B6.MSM-*Mx* mice were homozygous MSM (M/M), whereas that of *D16Mit20* was homozygous B6 (B/B), suggesting that the region between *D16Mit71* and *D16Mit106* was derived from MSM and recombination occurred at two points between *D16Mit20*

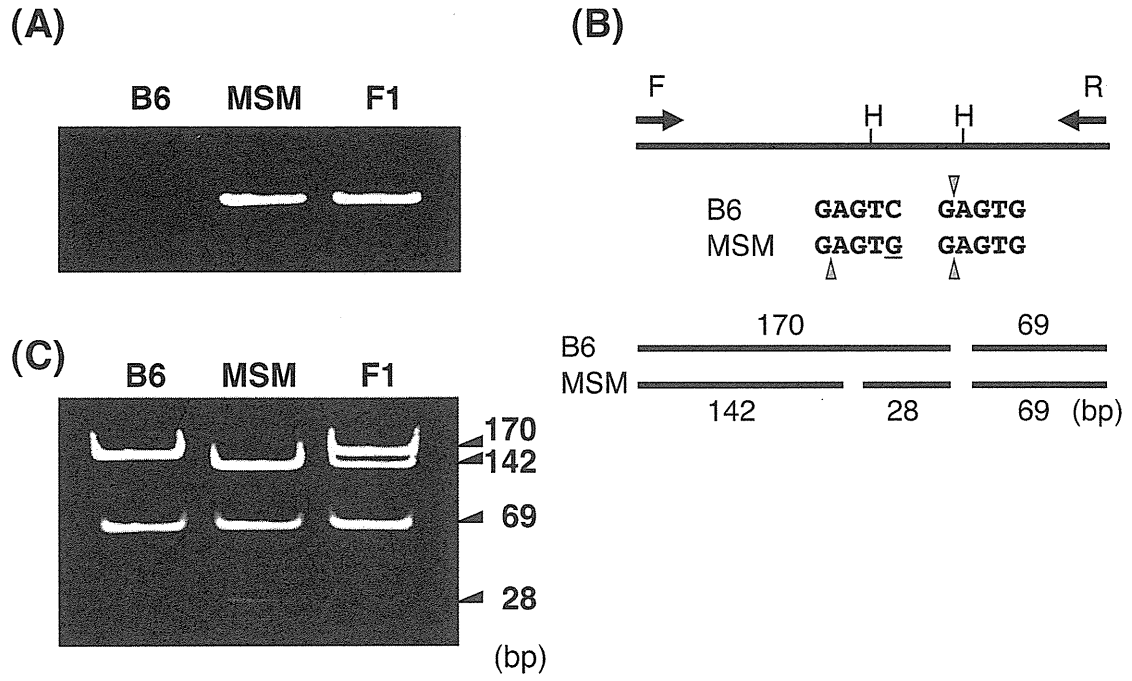

Fig. 1. Detection of the B6 and MSM alleles of the *Mx1* and *Oas1b* genes. (A) The results of PCR amplification of the Mx1 gene. The alleles of MSM but not of B6 mice show the PCR product. (B) Schematic diagram of the *Oas1b* gene in B6 and MSM mice. The arrows show PCR primers used for *Oas1b* genotyping. The lower diagram shows the expected results of *Hin*fI digestion after PCR amplification. H; *Hin*fI site. (C) The result of genotyping of the *Oas1b* gene. The 28-bp and 142-bp bands are derived from the MSM allele.

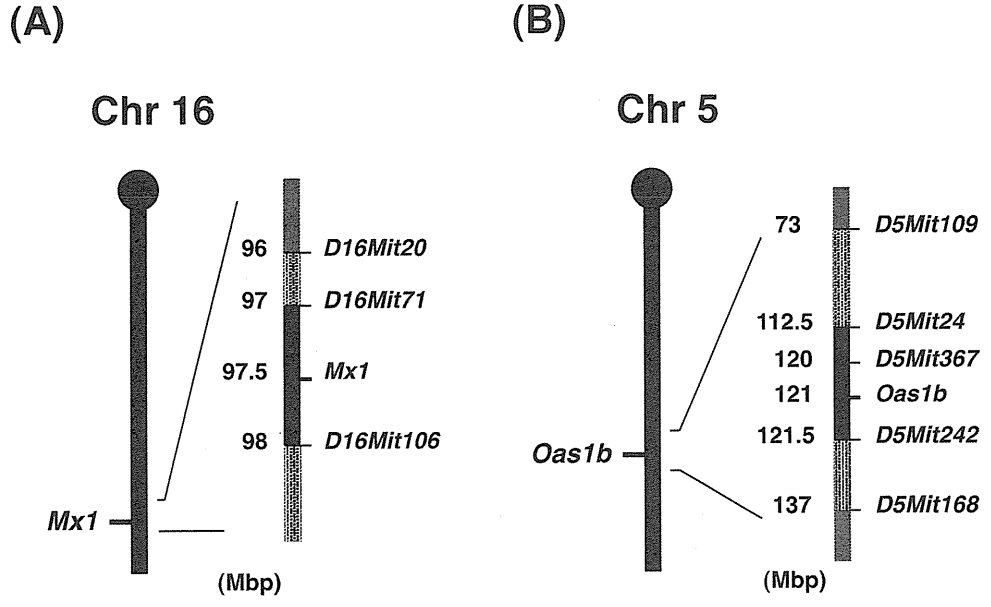

Fig. 2. Schematic diagrams of the genomic structure surrounding *Mx1* and *Oas1b* genes in the B6.MSM-*Mx* (A) and B6.MSM-*Oas* (B) congenic strains, respectively. Black and gray bars represent the MSM-derived and B6-derived genomes, respectively. Dotted bars represent recombined regions between the MSM and B6 genomes. The numbers to the left of the bars represent physical locations based on the NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview).

and *D16Mit71,* and between *D16Mit106* and telomere. On the other hand, the genotypes of *D5Mit24*, *D5Mit367*, and *D5Mit242* in B6.MSM-*Oas* mice were M/M, whereas those of *D5Mit109* and *D5Mit168* were B/B (Fig. 2B), suggesting that the region between *D5Mit24* and *D5Mit242* was derived from MSM mice and recombination occurred at two points between *D5Mit109* and *D5Mit24*, and between *D5Mit242* and *D5Mit168*.

Next we confirmed by RT-PCR that *Mx* or *Oas1b* mRNA was transcribed from both MSM alleles in congenic mice. After induction of IFN using poly (I:C) injection, expression of the *Mx1* gene in the spleens of B6.MSM-*Mx*, MSM, and B6 mice was analyzed. Intact *Mx1* could be distinguished from the mutant type due to the large deletion in the *Mx1* gene of the B6 mouse (Fig. 3A). In addition, no *Mx2* gene expression was detected in the spleens of B6.MSM-*Mx* and MSM mice (Fig. 3A). On the other hand, since *Oas1b* genes expressed from the B6 and MSM mice could not be distinguished by the length of RT-PCR products (Fig. 3B), we determined the origin of the *Oas1b* gene by DNA sequencing. A nonsense mutation was observed in the B6 *Oas1b* cDNA, whereas the sequence of the *Oas1b* cDNA in the B6.MSM-*Oas* mice was identical to that of MSM mice (data not shown). Interestingly, both genes are basally expressed in B6 background and are more induced by dsRNA stimulation than those of original MSM mice (Fig. 3). This result is in agreement with previous report showing that MSM strain is hyporesponsive to poly (I:C) due to a mutation in toll-like receptor 3 activated by dsRNA[35]. Thus, we have confirmed that these congenic strains are able to express the intact *Mx1* and *Oas1b* genes by the stimulation of dsRNA.

Experimental infection of congenic strain with orthomyxovirus and flavivirus

We performed viral injection to confirm whether these congenic mice were resistant to the infection of orthomyxovirus and flavivirus. A highly pathogenic avian influenza virus and West Nile virus were selected as the representative orthomyxo- and flaviviruses, respectively. When

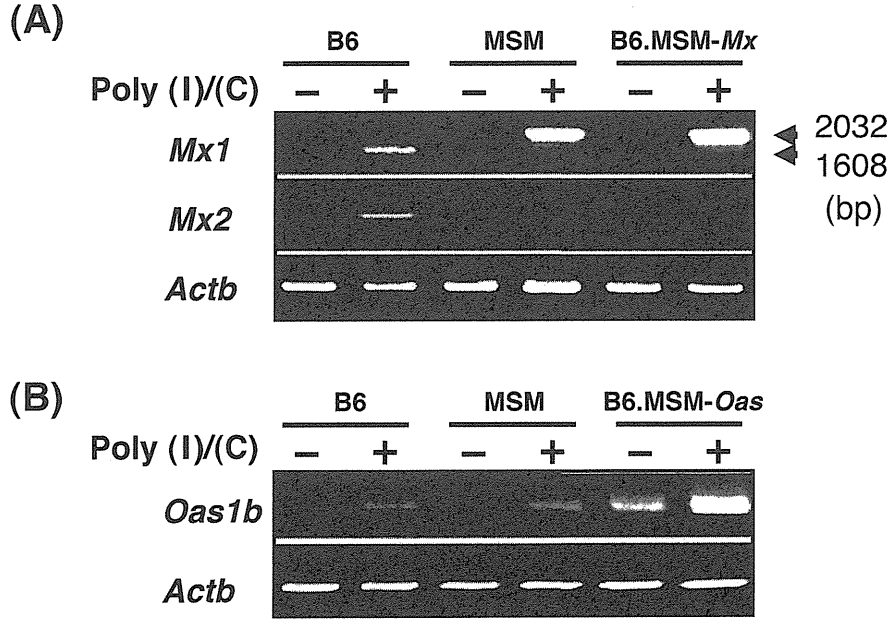

Fig. 3. Expression of intact *Mx1* and *Oas1b* genes in congenic mice. (A) Expression of *Mx1* and *Mx2* genes in the spleen tissues of B6, MSM, and B6.MSM-*Mx* mice in response to poly (I)/(C). (B) Expression of *Oas1b* gene in the spleen tissues of B6, MSM, and B6.MSM-*Oas* mice in response to poly (I)/(C).

infected with a 10^2 LD_{50} dose of influenza A virus, all B6 and B6.MSM-*Oas* mice died within 14 days after infection, whereas 5 of 7 (71%) B6.MSM-*Mx* mice survived. When B6.MSM-*Mx* mice were infected with 2 higher doses, 10^3 LD_{50} and 10^4 LD_{50}, 6 of 7 (86%) and 7 of 7 (100%) B6.MSM-*Mx* mice survived, respectively (Fig. 4A). On the other hand, all B6.MSM-*Oas* mice infected with 1 and 10 PFUs of West Nile virus survived, whereas most of B6 and B6.MSM-*Mx* mice died within 14 days after infection. The survival rate in each experiment was as follows; B6 mice infected with 1 PFU, B6 with 10 PFU, B6.MSM-*Mx* with 1 PFU, and B6.MSM-*Mx* with 10 PFU were 20% (3 out of 15), 7% (1 out of 14), 10% (1 out of 10), and 30% (3 out of 10), respectively (Fig. 4B). These results indicate that Mx1 and Oas1b have specific anti-virus activity against influenza- and West Nile viruses, respectively, in mice with the same B6 genetic background.

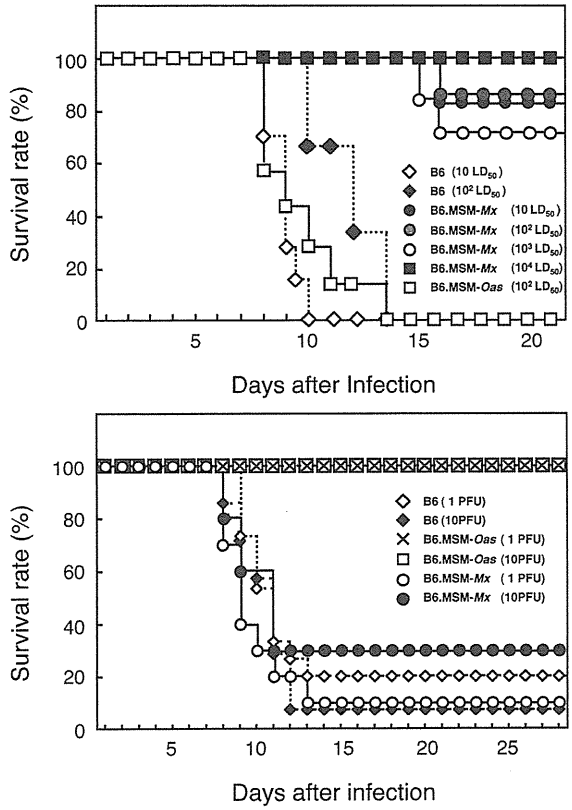

Fig. 4. Experimental infection with orthomyxovirus and flavivirus using congenic mouse strains. Survival rates of B6 mice are shown by dotted lines in each experiment. (A) Black and white diamonds represent B6 mice infected with 10 LD_{50} and 10^2 LD_{50} doses of influenza virus, respectively, whereas white squares represent B6.MSM-*Oas* mice infected with 10^2 LD_{50}. B6.MSM-*Mx* mice infected with 10 LD_{50}, 10^2 LD_{50}, 10^3 LD_{50}, and 10^4 LD_{50} inoculum doses are shown by black circles, white circles, gray circles, and black squares, respectively. (B) Survival rates of B6 (diamonds) and B6.MSM-*Mx* (circles) mice after infection with 1 PFU (white symbols) and 10 PFU (black symbols) of West Nile virus, respectively. Crosses and white squares represent B6.MSM-*Oas* mice infected with 1 PFU and 10 PFU, respectively.

Discussion

Orthomyxovirus and flavivirus are considered to be important viruses from both the medical and sanitary position. Influenza A, B, and C viruses, classified as orthomyxoviruses, cause the epidemic respiratory disease known as 'flu' in humans that spreads worldwide. Last century, pandemic influenza emerged several times. Further, a new emerging pandemic influenza virus is much concerned[13,15,21]. On the other hand, 20–30 members of the flavivirus family are known to be involved in human diseases. Some of these viruses cause severe diseases such as fatal encephalitis and haemorrhagic fevers in humans[10,31]. Last century, yellow fever virus, Dengue virus, Japanese encephalitis virus, tick-borne encephalitis virus, and West Nile virus caused large outbreaks worldwide[10]. Taken together, infectious diseases associated with orthomyxoviruses and flaviviruses are the subject of much important study. In mice, both Mx1 and Mx2 proteins have been identified, and Mx1 localizes in the nucleus and inhibits virus polymerase activity[5,14,22,25,39]. Therefore, mice carrying intact Mx1 show resistance to orthomyxoviruses such as influenzavirus[1,33]. B6.MSM-*Mx* and B6 mice showed notably different survival rates after a challenge with a highly pathogenic avian influenza virus. Although it remains to be determined whether Mx in all vertebrates possesses anti-viral

activity against orthomyxoviruses, anti-viral activity of the Mx protein has been shown in rodents, human, and other animals, suggesting that laboratory mice lacking these genes may not reflect the normal infectious conditions in humans and animals[39]. In addition, these data indicate that studies performed using mice lacking Mx would lead to incorrect evaluations of viral virulence, effects of vaccine, drug and therapy and so on. MSM mice do not express Mx2, and B6.MSM-*Mx* mice do not recover Mx2 expression, suggesting that the absence of Mx2 expression in MSM mice is not due to the MSM genetic background but due to the presence of a putative cis-acting element in the Mx locus. Mouse Mx2 protein localizes in the cytoplasm in the same manner as the Mx proteins in humans and some animals[2,25,39]. Mouse Mx2 can inhibit the replication of negative-stranded RNA viruses that replicate in the cytoplasm such as vesicular stomatitis virus and hanta virus, but not those that replicate in the nucleus such as influenza virus. On the other hand, Mx proteins in humans and some animals localize in cytoplasm and can inhibit influenza virus replication[2,25,39]. The reason for the differential anti-viral activity between mouse Mx2 and Mx proteins of other species is unknown.

Oas genes are induced by type 1 IFN, and synthesize 2-5 A, and consequently RNA degradation, by activating latent RNase L[9,28,41]. In mice, a gene cluster has been identified on Chr 5 that is comprised of ten *Oas* family genes[19,27]. Although mouse *Oas1b* is one of these genes, Oas1b protein is not regarded as a typical Oas protein, because it lacks enzymatic activity[7]. Although mice carrying intact *Oas1b* show resistant in flavivirus infection, the mechanism by which Oas1b confers resistance to the infection of flaviviruses on mice remains unclear[18,26,30]. In recent reports, the *Oas1b* gene of wild-derived mice was shown to confer differential resistance to the infection of flaviviruses due to the polymorphisms[8,29,38]. In our study, B6.MSM-*Mx* but not B6.MSM-*Oas* mice showed resistance to the infection of influenza virus. On the other hand, B6.MSM-*Oas* but not B6.MSM-*Mx* mice showed resistance to the infection of West Nile virus, suggesting that the Oas1b protein of MSM mice possesses anti-West Nile virus characteristics but murine Mx1 protein does not. In addition, these results indicate that Mx1 and Oas1b specifically inhibit influenza virus and West Nile virus replications, respectively.

As there is only limited information on the mechanisms of the pathogenesis of virus infection and the role of host innate immune response in humans, animal models are necessary to identify relationship between virus and host defense in infectious diseases. Mice are frequently used as an animal model to study the viral virulence and vaccine efficacy before using other larger animals[3,6]. However, standard laboratory mice do not possess certain key components of the innate immune system that mediates protection against the infection of these viruses. It has been reported that the proinflammatory cytokines, such as interleukin 1, interleukin 6, and macrophage inflammatory protein-1, are significantly increased in highly pathogenic influenza-infected mice; however, the course of the disease and the extent of virus replication and spread in these knockout mice were not different from those observed in wild-type mice[36]. On the other hand, IFN α/β receptor-deficient mice are highly susceptible to pathogenic influenza virus[37]. These results suggest a role for IFN signaling, including Mx1, which is essential for protection of the host in the early stages of infection in mice. Thus, the congenic mice generated in this study are useful for the further investigation of orthomyxovirus and flavivirus infectious diseases, particularly, the precise mechanism and timing of the interplay between components of pro- and anti-inflammatory signaling pathways, and may allow the eventual identification of an effective target on these viruses.

Acknowledgements

We thank Professor T. Shiroishi, National Institute of Genetics, Japan, for providing MSM/Ms mice. We wish to thank our lab members for their helpful discussions. K. M. is a research assistant of the Global Center Of Excellence (GCOE) program. This work was supported in part by the GCOE Program, 'Establishment of International Collaboration Centers for Zoonosis Control' from Japan Society for the Promotion of Science and Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

1) Arnheiter, H., Skuntz, S., Noteborn, M., Chang, S. and Meier, E. 1990. Transgenic mice with intracellular immunity to influenza virus. *Cell*, **62**: 51–61.
2) Asano, A., Jin, H. K. and Watanabe, T. 2003. Mouse *Mx2* gene: organization, mRNA expression and the role of the interferon-response promoter in its regulation. *Gene*, **306**: 105–113.
3) Charlier, N., Leyssen, P., De Dlercq. and E. Neyts, J. 2004. Rodent models for the study of therapy against flavivirus infections. *Antiviral Res.*, **63**: 67–77.
4) De Zoyza, M., Kang, H. S., Song, Y. B., Jee, Y., and Lee, Y. D. and Lee, J. 2007. First report of invertebrate Mx: cloning, characterization and expression analysis of Mx cDNA in disk abalone (*Haliotis discus discus*). *Fish Shellfish Immunol.*, **23**: 86–96.
5) Dittmann, J., Stertz, S., Grimm, D., Steel, J., García-Sastre, A., Haller, O. and Kochs, G. 2008 Influenza A virus strains differ in sensitivity to the antiviral action of Mx-GTPase. *J. Virol.*, **82**: 3624–3631.
6) Eichelberger, M. C. 2007. The cotton rat as a model to study influenza pathogenesis and immunity. *Viral Immunol.*, **20**: 243–249.
7) Elbahesh, H. 2005. Functional analysis of the murine oligoadenylate synthetase 1b (Oas1b). Georgia State University Master's Thesis.
8) Ferguson, W., Dvora, S., Gallo, J., Orth, A. and Boissinot. S. 2008. Long-term balancing selection at the west nile virus resistance gene, Oas1b, maintains transspecific polymorphisms in the house mouse. *J. Virol.*, **82**: 8978–8985.
9) Ghosh, A., Sarkar, S. N., Guo, W., Bandpadhyay, S. and Sen, G. C. 1997. Enzymatic activity of 2'-5'-oligoadenylate synthetase is impaired by specific mutations that affect oligomerization of the protein. *J. Biol. Chem.*, **272**: 33220–33226.
10) Gould, E. A. and Solomon, T. 2008. Pathogenic flaviviruses. *Lancet.* **371**: 500–509.
11) Jin, H. K., Yamashita, T., Ochiai, K., Haller, O. and Watanabe, T. 1998. Characterization and expression of the *Mx1* gene in wild mouse species. *Biochem. Genet.*, **36**: 311–322.
12) Kajaste-Rudnitski, A., Mashimo, T., Frenkiel, M. P., Guenet J. L., Lucas, M. and Despres, P. 2006. The 2', 5'-oligoadenylate synthetase 1b is a potent inhibitor of West Nile virus replication inside infected cells. *J. Biol. Chem.*, **281**: 4624–4637.
13) Khanna, M., Kumar, P., Choudhary, K., Kumar, B. and Vijayan, V. K. 2008. Emerging influenza virus: a global threat. *J. Biosci.*, **33**: 475–482.
14) Krug, R. M., Shaw, M., Broni, B., Shapiro, G. and Haller, O. 1985. Inhibition of influenza viral mRNA synthesis in cells expressing the interferon-induced Mx gene product. *J. Virol.*, **66**: 2564–2569.
15) Maines, T. R., Szretter, K. J., Perrone, L., Belser, J. A., Bright, R. A., Zeng, H., Tumpey, T. M. and Katz, J. M. 2008. Pathogenesis of emerging avian influenza viruses in mammals and the host innate immune response. *Immunol. Rev.*, **225**: 68–84.
16) Malathi, K., Dong, B., Gale, M. Jr. and Silverman, R. H. 2007. Small self-RNA generated by RNase L amplifies antiviral innate immunity. *Nature*, **448**: 816–819.
17) Markel, P., Shu, P., Ebeling, C., Carlson, G. A.,

Nagle D. L., Smutko, J. S. and Moore, K. J. 1997. Theoretical and empirical issues for marker-assisted breeding of congenic mouse strains. *Nat. Genet.*, **17**: 280–284.

18) Mashimo, T., Lucas, M., Simon-Chazottes, D., Frenkiel, M. P., Montagutelli, X., Ceccaldi, PE., Deubel, V., Guénet, JL. and Desprès, P. 2002. A nonsense mutation in the gene encoding 2'-5' oligoadenylate synthetase/L1 isoform is associated with West Nile virus susceptibility in laboratory mice. *Proc. Natl. Acad. Sci. U. S. A.*, **99**: 11311–11316.
19) Mashimo, T., Glaser, P., Lucas, M., Ceccaldi, P. E., Montagutelli, X., Desprès, P. and Guénet, J. L. 2003. Structural and functional genomics and evolutionary relationships in the cluster of genes encoding murine 2', 5'-oligoadenylate synthetases. *Genomics*, **82**: 537–552.
20) Mouse Genome Sequencing Consortium. 2002. Initial sequencing and comparative analysis of the mouse genome. *Nature*, **420**: 520–562.
21) McFee, R. B. 2007. Avian Influenza: The next pandemic? 2007. *Dis. Mon.*, **57**: 348–387.
22) Nakayama, M., Nagata, K., Kato, A. and Ishihama, A. 1991. Interferon-inducible mouse Mx1 protein that confers resistance to influenza virus is GTPase. *J. Biol. Chem.*, **266**: 21404–21408.
23) Nakayama, M., Yazaki, K., Kusano, A., Nagata, K., Hanai, N. and Ishihama, A. 1993. Structure of mouse Mx1 protein. molecular assembly and GTP-dependent conformational change. *J. Biol. Chem.*, **268**: 15033–15038.
24) Nygaard, R., Husgard, S., Sommer, A. I., Leong, J. A. and Robertsen, B. 2000. Induction of Mx protein by interferon and double-stranded RNA in salmonid cells. *Fish Shellfish Immunol.*, **10**: 435–450.
25) Pavlovic, J., Haller, O. and Steaheli, P. 1992. Human and mouse Mx proteins inhibit different steps of the influenza virus multiplication cycle. *J. Virol.*, **66**: 2564–2569.
26) Perelygin, A. A., Scherbik, S. V., Zhulin, I. B., Stockuman, B. M., Li, Y. and Brunton, M. A. 2002. Positional cloning of the mureine flavivirus resistance gene *Proc. Natl. Acad. Sci. U. S. A.*, **99**: 9322–9327.
27) Perelygin, A. A., Zharkikh, A. A., Scherbik, S. V. and Brinton, M. A. 2006. The mammalian 2'-5'oligoadenyrate synthetase gene family: Evudence for concerted evolution of paralogous Oas1 genes in rodentia and artiodactyla. *J. Mol. Evol.*, **63**: 562–576.
28) Samuel, C. E. 2001. Antiviral actions of interferons. *Clin. Microbiol. Rev.*, **14**: 778–809.
29) Sangster M. Y., Heliams, D. B., MacKenzie, J. S. and Shellam, G. R. 1993. Genetic studies of flavivirus resistance in inbred strains derived from wild mice: evidence for a new resistance allele at the flavivirus resistance locus (Flv). *J. Virol.*, **67**: 340–347.
30) Scherbik, S. V., Kluetzman, K., Perelygin, A. A. and Brinton, M. A. 2007. Knock-in of the Oas1b(r) allele into a flavivirus-induced disease susceptible mouse generates the resistant phenotype. *Virology*, **368**: 232–237.
31) Silvia, O. J., Pantelic, L., Mackenzie, J. S., Shellam, G. R., Papadimitriou, J. and Urosevic, N. 2004. Virus spread, tissue inflammation and antiviral response in brains of flavivirus susceptible and resistant mice acutely infected with Murray Valley encephalitis virus. *Arch. Virol.*, **149**: 447–464.
32) Staeheli, P. and Haller, O. 1985. Interferon-induced human protein with homology to protein Mx of influenza virus-resistant mice. *Mol. Cell. Biol.*, **5**: 2150–2153.
33) Staeheli, P., Grob, R., Meier, E., Sutcliffe, J. G. and Haller, O. 1988. Influenza virus-susceptible mice carry Mx genes with a large deletion or a nonsense mutation. *Mol. Cell Biol.*, **8**: 4518–4523.
34) Staeheli, P., Yu, Y-X., Grob, R. and Haller, O. 1989. A double-stranded RNA-inducible fish gene homologous to the murine influenza virus resistance gene Mx. *Mol. Cell. Biol.*, **9**: 3177–3121.

35) Stephan, K., Smirnova, I., Jacque, B. and Poltorak, A. 2007. Genetic analysis of the innate immune responses in wild-derived inbred strains of mice. *Eur. J. Immunol.*, **37**: 212–223.

36) Szretter, K. J., Gangappa, S., Lu, X., Smith, C., Shieh, W. J., Zaki, S. R., Sambhara, S., Tumpey, T. M. and Katz, J. M. 2007. Role of host cytokine responses in the pathogenesis of avian H5N1 influenza viruses in mice. *J. Virol.*, **81**: 2736–2744.

37) Szretter, K. J., Gangappa, S., Belser, J. A., Zeng, H., Chen, H., Matsuoka, Y., Sambhara, S., Swayne, D. E., Tumpey, T. M. and Katz, J. M. 2009. Early control of H5N1 influenza virus replication by the type I interferon response in mice. *J. Virol.*, **83**: 5825–5834.

38) Urosevic, N., Silvia, O. J., Sangster, M. Y., Mansfield, J. P., Hodgetts, S. I. and Shellam, G. R. 1999. Development and characterization of new flavivirus-resistant mouse strains bearing Flv(r)-like and Flv(mr) alleles from wild or wild-derived mice. *Gen. Virol.*, **80**: 897–906.

39) Watanabe, T. 2007. Polymorphisms of the chicken antiviral MX gene. *Cytogenet Genome Res.*, **117**: 370–375.

40) Weins, M., Kuusksal, A., Kelve, M. and Muller, W. E. 1999. Origin of the interferon-inducible (2'-5') oligoadenylate synthetases: cloning of the (2'-5') oligoadenylate synthetase from the marine sponge *Geodia cydonium*. *FEBS Lett.*, **462**: 12–18.

41) Wreschner, D. H., McCauley, J. W., Skehel, J. J. and Kerr, I. M. 1981. Interferon action-sequence specificity of the ppp $(A2'p)_n$ A-dependent ribonuclease. *Nature*, **289**: 414–417.

Japanese Journal of Veterinary Research 56(4): 189-198, 2009

FULL PAPER

Evaluation of the potency, optimal antigen level and lasting immunity of inactivated avian influenza vaccine prepared from H5N1 virus

Takashi Sasaki[1,*], Norikazu Isoda[2], Kosuke Soda[2], Ryuichi Sakamoto[3], Kazue Saijo[4], Junko Hagiwara[5], Norihide Kokumai[1], Toshiaki Ohgitani[1], Takashi Imamura[3], Akira Sawata[4], Zhifeng Lin[5], Yoshihiro Sakoda[2] and Hiroshi Kida[2,6]

[1]Avian Biologics Department, Kyoto Biken Laboratories, Inc., Uji 611-0041, Japan
[2]Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan
[3]Division 2, Second Research Department, The Chemo-Sero-Therapeutic Research Institute, Kikuchi 869-1298, Japan
[4]Research Center for Biologicals, The Kitasato Institute, Kitamoto 364-0026, Japan
[5]Research Department, Nippon Institute for Biological Science, Ome 198-0024, Japan
[6]Research Center for Zoonosis Control, Hokkaido University, Sapporo 060-0818, Japan

Received for publication, November 18, 2008; accepted, February 4, 2009

Abstract

Test vaccines comprised of inactivated water-in-oil emulsions containing various antigen levels were prepared using a non-pathogenic H5N1 avian influenza (AI) virus, A/duck/Hokkaido/Vac-1/04 (H5N1). The potencies of these test vaccines were evaluated by two experiments. In the first experiment, the triangular relationship among the antigen levels of test vaccines, the hemagglutination inhibition (HI) antibody response, and the protective effect against challenge with a highly pathogenic avian influenza (HPAI) virus, A/chicken/Yamaguchi/7/04 (H5N1), was confirmed. Then lasting immunity of chickens after a single-shot vaccination was confirmed in the second experiment. As a result, complete protection after the challenge was observed in chickens immunized by test vaccines with an antigen level of 160 HA units/dose or higher. Thus, it was ascertained that the minimum antigen level in the AI vaccine was 160 HA units/dose, and the minimum HI antibody titer that could protect chickens from HPAI virus infection-related death was considered to be 1:16. Dose-dependent HI antibody responses were observed in chickens after the vaccination. Thus, 640 HA units/dose was thought to be similar to the optimal antigen level. Alternatively, the HI antibody titers of chickens, injected with the vaccine containing 640 HA units/dose, were maintained at 1:181 or higher for 100 weeks after the single-shot vaccination.

Key words: avian influenza vaccine, minimum antigen, minimum HI antibody, lasting immunity, optimal antigen level

*Corresponding author: Takashi Sasaki, Avian Biologics Department, Kyoto Biken Laboratories, Inc., 24-16 Makishima-cho, Uji 611-0041, Japan
Phone:+81-774-22-4518. Fax:+81-0774-24-1407. E-mail: keibyou_one@kyotobiken.co.jp

Introduction

In 2004, an outbreak of highly pathogenic avian influenza (HPAI) occurred in Yamaguchi Prefecture. It was the first outbreak for 79 years in Japan. Subsequent outbreaks then occurred in Oita and Kyoto prefectures at a total of four poultry farms, and about 275,000 fowls were eventually culled. The outbreak in Kyoto Prefecture caused the largest damage because the emergent condition at the poultry farm was intentionally hidden[3,8,15].

The H5N1 viruses isolated from birds in Yamaguchi, Oita, and Kyoto prefectures showed close genetic homology with each other[7]. A/chicken/Yamaguchi/7/04 (H5N1) also showed marked homology with a virus isolated from fowls in Korea in 2003[6]. Thus, these viruses isolated in Japan were strongly suspected to have originated from the Korean Peninsula, possibly being brought by migrating birds[5].

At the beginning of 2007, HPAI virus infection in chickens was confirmed on three poultry farms in Miyazaki prefecture and one in Okayama prefecture[4].

Under the present regulations in Japan, the practical application of avian influenza (AI) vaccine on actual poultry farms is permitted on limited occasions when the spread of the virus is impossible to curb by ordinary actions such as banning the movement of fowls or culling. However, it is very important to prepare a reliable vaccine against any AI outbreak in the future[1,2,10,13,14]. Thus, we tried to develop a vaccine that would induce a good immunological response to protect chickens from HPAI virus infection-related death by single-shot vaccination.

This study investigated the potency of our vaccine by two experiments. In the first experiment, the triangular relationship among the antigen levels of test vaccines, the hemagglutination inhibition (HI) antibody response, and the protective effect against challenge with a highly pathogenic avian influenza (HPAI) virus, A/chicken/Yamaguchi/7/04 (H5N1), was confirmed. Lasting immunity of the chickens after the single-shot vaccination was confirmed in the second experiment.

Materials and Methods

Viruses: A/duck/Hokkaido/Vac-1/04 (H5N1) (hereinafter referred to as Dk/Vac-1/04), a Eurasian lineage of a non-pathogenic AI virus produced as a reassortant virus of A/duck/Mongolia/54/01 (H5N2) and A/duck/Mongolia/47/01 (H7N1) in 2004 at Hokkaido University, was used for vaccine preparation[12].

A/chicken/Yamaguchi/7/04 (H5N1) (hereinafter referred to as Ck/Yamaguchi/04) was isolated from a dead chicken following an AI outbreak in 2004 in Yamaguchi Prefecture by the National Institute of Animal Health of Japan and was used as the challenge virus.

Each AI virus strain, Dk/Vac-1/04 and Ck/Yamaguchi/04, was inoculated into the allantoic cavity in 10- to 12-day-old embryonated chicken eggs, and incubated for 48 hr at 34℃ for the former, and 35℃ for the latter to prepare viral suspensions.

Vaccine preparation: A viral suspension of Dk/Vac-1/04 was inactivated by incubation with formalin at a final concentration of 0.2% for 3 days at 4℃. Inactivation of the virus was confirmed by the inoculation of embryonated chicken eggs.

The inactivated viral suspension was diluted with phosphate-buffered saline (PBS) to appropriate concentrations based on the hemagglutination (HA) titer. A 2.5 volume of each viral suspension with HA titers of 1:256, 1:64, and 1:32 was mixed with a 7.5 volume of oil adjuvant containing 3.9% anhydromannitol-octadecenoate-ether and light mineral oil to comprise the remaining volume. These mixtures were then homogenized using an ultra-homomixer to produce water-in-oil type Vaccines A, B, and C, respectively.

The antigen levels of Vaccines A, B, and C were calculated as 640, 160, and 80 HA units/dose, respectively, using the formula shown below:

$$\text{HA units/dose} = \frac{\text{Volume (μl) of viral suspension/dose} \times \text{HA titer of viral suspension}}{50\ \mu l}$$

Animals: Four-week-old specific pathogen-free (SPF) white leghorn chickens were used in this study. The chickens were hatched and fed in Kyoto Biken Laboratories, Inc. The chickens prepared for challenge test were transported to a biosafety level 3 facility at Hokkaido University 7 weeks after the vaccination. All procedures were performed according to the animal experiment guidelines of Hokkaido University.

HA antigen: The viral suspension of Dk/Vac-1/04 was inactivated by incubation with formalin at a final concentration of 0.2% for 3 days at 4℃, followed by dilution of the antigen with PBS to adjust the HA titer to 1:8.

HI test protocols: One volume of each serum was respectively mixed with 3 volumes of 10% chicken red blood cells (RBCs), and stored overnight at 4℃. The mixtures were centrifuged at 1,000 g for 5 min, and the supernatants were then collected as fourfold-diluted sera.

One-hundred μl of each of the supernatants described above was dispensed into several wells of the first lane of a plastic V-bottomed microtitration plate. Fifty μl of PBS was dispensed into all other wells, after which 50 μl of twofold serial dilutions of the supernatants were then added to the wells. Fifty μl of each HA antigen (1:8 HA titer) was then dispensed into all wells of the plates, and they were incubated for 30 min at room temperature. Finally, 100 μl of 0.5% chicken RBCs was dispensed into all wells, and they were incubated again for 60 min at room temperature.

HI antibody titers were expressed as the highest dilution of the serum sample that showed complete hemagglutination inhibition.

Experiment 1 (Confirmation of HI antibody responses of immunized chickens and the minimum vaccinal antigen level required for protection against HPAI virus challenge): Seven, ten, and eleven chickens were vaccinated intramuscularly in the lower thigh with 0.5 ml of Vaccines A, B, and C, respectively, and 13 other chickens were prepared as non-vaccinated controls. Sera of all chickens were collected every week after the vaccination, and the geometric mean of the HI antibody titer against Dk/Vac-1/04 was calculated by the method described above. Chicken groups injected with Vaccines A, B, and C were designated Groups A, B, and C, respectively. The chicken group without vaccination was designated the Control group.

All chickens were challenged intranasally with a 100-fold 50% chicken lethal dose ($10^{5.3}$ 50% egg infectious dose) of Ck/Yamaguchi/04 at 7 weeks after the vaccination. Clinical signs were monitored for 14 days post-challenge (p.c.).

Cloacal swabs on day 4 p.c. were individually collected from all surviving chickens to detect viral shedding. Both tracheal and cloacal swabs were also collected individually at the time of death and euthanasia on day 14 p.c. As primary screening for viral shedding, swabs were individually suspended in 1.0 ml of Eagle's minimum essential medium (MEM) containing a moderate amount of antibiotics. A 0.1-ml aliquot of each suspension was then inoculated into the allantoic cavity in 10-day-old embryonated chicken eggs and incubated at 35℃ for 48 hr, followed by refrigeration at 4℃. The allantoic fluids showing typical HA activity were judged as an indication of positive virus growth.

For the quantification of viral shedding, suspensions that were positive in the primary screening test were serially diluted tenfold with MEM, and inoculated into the allantoic cavity in 10-day-old embryonated chicken eggs to calculate the recovered viral titers by the same method and conditions described above. The recovered viral titers from swabs were judged by typical HA activity of the allantoic fluids. These were calculated by the method of Reed and Muench and expressed as the 50% egg infectious dose per ml (EID_{50}/ml)[11].

Experiment 2 (Immunization of chickens and se-

rum sampling until 100 weeks after the vaccination to confirm lasting immunity): Vaccine A was considered similar to the optimal antigen level based on the results of Experiment 1. We designed another experiment to determine if there was lasting immunity. Eight chickens were vaccinated intramuscularly with 0.5ml of Vaccine A in the lower thigh, and the other 3 chickens were used as non-vaccinated controls. Sera from all chickens were collected every week after the vaccination until 7 weeks, and then collected regularly at 9- to 12-week intervals until 100 weeks. The HI antibody titer against Dk/Vac-1/04 was calculated by the method described above. The chicken group injected with Vaccine A was designated the Test group, and the chicken group without vaccination was designated the Control group.

Results

Experiment 1 (Confirmation of HI antibody responses of immunized chickens and the minimum vaccinal antigen level required for protection against HPAI virus challenge)

HI antibody titers of chickens after vaccination with test vaccines are presented in Table 1. The geometric means of HI antibody titers every week after vaccination were <1:4, 1:58, 1:1,131, 1:2,497, 1:1,522, 1:1,248, and 1:1,248 in Group A, <1:4, 1:9, 1:294, 1:832, 1:724, 1:724, and 1:832 in Group B, and <1:4, <1:4, 1:9, 1:41, 1:56, 1:106, and 1:73 in Group C. Dose-dependent HI antibody responses were observed in chickens after the vaccination.

Clinical signs observed for 14 days after the challenge in all chickens are presented in Table 2. There were no clinical signs in any chickens in Groups A and B with HI antibody titers of 1:1,024 to 1:4,096 and 1:512 to 1:2,048, respectively, at the time of challenge. However, the HI antibody titers of Group C showed variable results ranging from 1:4 to 1:512. Chicken No. 148, which had an HI antibody titer of 1:4 at the time of challenge showed typical clinical signs (gloom, anorexia and nervous symptoms) from day 3 p.c. and died on day 8 p.c. Moreover, chicken No. 143, which had an HI antibody titer of 1:8 at the time of challenge also showed typical clinical signs from day 3 p.c. and died on day 4 p.c. Chickens with HI antibody titers of 1:16 or higher did not show any clinical signs for 14 days after the challenge. All chickens in the Control group showed HI antibody titers of <1:4 at the time of challenge and died on day 2 or 3 p.c.

Viral shedding in each group is presented in Table 3. None of the chickens in Groups A and B showed any virus shedding at any time investigated after the challenge. The HI antibody titers of vaccinated chickens in these groups were 1:512 or higher at the time of challenge. In Group C, $10^{1.0}$ EID_{50}/ml of the virus was recovered on day 4 p.c., and subsequently, $10^{4.3}$ and $10^{3.7}$ EID_{50}/ml of the virus were recovered from cloacal and tracheal swabs, respectively, at the time of death on day 8 p.c. in chicken No. 148, which showed an HI antibody titer of 1:4 at the time of challenge. Moreover, $10^{2.3}$ and $10^{3.5}$ EID_{50}/ml of the virus were recovered from cloacal and tracheal swabs, respectively, at the time of death on day 4 p.c. in chicken No. 143, which had an HI antibody titer of 1:8 at the time of challenge. None of the chickens with an HI antibody titer of 1:16 or higher showed any viral shedding at any time point examined. All chickens in the Control group demonstrated viral shedding from the cloaca and trachea at the time of death, and the maximum titer of recovered virus from the swabs was $10^{7.3}$ EID_{50}/ml.

Experiment 2 (Immunization of chickens and serum sampling until 100 weeks after the vaccination to confirm lasting immunity)

HI antibody titers of chickens until 100 weeks after vaccination are presented in Table 4. All chickens exceeded an HI antibody titer of 1:64 within 2 weeks after vaccination. The geometric mean of HI antibody titers reached a maximal value of 1:1,722 at 4 weeks after vaccination, and then slowly decreased. However, the geometric mean of HI antibody titers in the Test group remained 1:181 or higher for 100 weeks after vacci-

Table 1. HI antibody titers of chickens after the vaccination with test vaccines

Group	HA units/dose	Chicken No.	HI antibody titers at the following weeks after vaccination						
			1	2	3	4	5	6	7
A	640	105	<4	64	1,024	2,048	1,024	1,024	1,024
		109	<4	8	1,024	2,048	1,024	1,024	1,024
		115	<4	128	1,024	4,096	2,048	1,024	1,024
		116	<4	128	1,024	2,048	2,048	1,024	1,024
		117	<4	128	1,024	2,048	1,024	1,024	1,024
		120	<4	64	2,048	2,048	1,024	1,024	1,024
		114	<4	32	1,024	4,096	4,096	4,096	4,096
	GM[a)]		<4	58	1,131	2,497	1,522	1,248	1,248
B	160	123	<4	8	256	512	512	256	512
		128	<4	128	2,048	2,048	1,024	512	512
		131	<4	32	1,024	1,024	1,024	512	512
		133	<4	<4	128	256	256	512	512
		138	<4	<4	16	128	128	512	512
		122	<4	<4	256	1,024	1,024	1,024	1,024
		125	<4	16	128	1,024	1,024	1,024	1,024
		127	<4	—[b)]	128	2,048	1,024	1,024	1,024
		134	<4	4	256	1,024	1,024	1,024	2,048
		135	<4	32	4,096	2,048	2,048	2,048	2,048
	GM		<4	9	294	832	724	724	832
C	80	148	<4	<4	<4	<4	4	4	4
		143	<4	<4	<4	4	8	8	8
		154	<4	<4	<4	4	8	32	16
		141	<4	<4	<4	16	32	32	32
		151	<4	<4	<4	32	32	128	128
		153	<4	<4	128	512	256	256	128
		158	<4	<4	16	64	128	256	128
		147	<4	<4	16	128	128	256	256
		155	<4	<4	32	128	128	512	256
		159	<4	<4	64	512	512	1,024	256
		146	<4	<4	16	256	256	512	512
	GM		<4	<4	9	41	56	106	73
Control		161	<4	<4	<4	<4	<4	<4	<4
		164	<4	<4	<4	<4	<4	<4	<4
		165	<4	<4	<4	<4	<4	<4	<4
		166	<4	<4	<4	<4	<4	<4	<4
		167	<4	<4	<4	<4	<4	<4	<4
		170	<4	<4	<4	<4	<4	<4	<4
		172	<4	<4	<4	<4	<4	<4	<4
		173	<4	<4	<4	<4	<4	<4	<4
		162	<4	<4	<4	<4	<4	<4	<4
		163	<4	<4	<4	<4	<4	<4	<4
		168	<4	<4	<4	<4	<4	<4	<4
		169	<4	<4	<4	<4	<4	<4	<4
		175	<4	<4	<4	<4	<4	<4	<4
	GM		<4	<4	<4	<4	<4	<4	<4

a) GM: geometric mean, b) not tested

Table 2. Clinical signs of chickens after challenge with a highly pathogenic avian influenza virus

Group	HA units/dose	Chicken No.	HI antibody titer[a]	Clinical signs on days after challenge													
				1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	640	105	1,024	-[b]	-	-	-	-	-	-	-	-	-	-	-	-	-
		109	1,024	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		115	1,024	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		116	1,024	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		117	1,024	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		120	1,024	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		114	4,096	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	160	123	512	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		128	512	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		131	512	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		133	512	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		138	512	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		122	1,024	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		125	1,024	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		127	1,024	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		134	2,048	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		135	2,048	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C	80	148	4	-	-	+	+	+	+	+	D						
		143	8	-	-	+	D										
		154	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		141	32	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		151	128	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		153	128	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		158	128	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		147	256	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		155	256	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		159	256	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		146	512	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Control		161	<4	-	D												
		164	<4	-	D												
		165	<4	-	D												
		166	<4	-	D												
		167	<4	-	D												
		170	<4	-	D												
		172	<4	-	D												
		173	<4	-	D												
		162	<4	-	+	D											
		163	<4	-	+	D											
		168	<4	-	-	D											
		169	<4	-	-	D											
		175	<4	-	-	D											

a) HI antibody titer at the time of challenge, b) -: No abnormal signs, +: typical clinical signs (gloom, anorexia and nervous symptoms), D: Death

Table 3. Virus isolation from cloacal and tracheal samples of chickens after challenge with a highly pathogenic avian influenza virus

Group	HA units/dose	Chicken No.	HI antibody titer[a]	Virus titers on days after challenge									
				2		3		4		8		14	
				C[b]	T	C	T	C	T	C	T	C	T
A	640	105	1,024					-[c]				-	-
		109	1,024					-				-	-
		115	1,024					-				-	-
		116	1,024					-				-	-
		117	1,024					-				-	-
		120	1,024					-				-	-
		114	4,096					-				-	-
B	160	123	512					-				-	-
		128	512					-				-	-
		131	512					-				-	-
		133	512					-				-	-
		138	512					-				-	-
		122	1,024					-				-	-
		125	1,024					-				-	-
		127	1,024					-				-	-
		134	2,048					-				-	-
		135	2,048					-				-	-
C	80	148	4					1.0[d]		4.3	3.7		
		143	8					2.3	3.5				
		154	16					-				-	-
		141	32					-				-	-
		151	128					-				-	-
		153	128					-				-	-
		158	128					-				-	-
		147	256					-				-	-
		155	256					-				-	-
		159	256					-				-	-
		146	512					-				-	-
Control		161	<4	5.0	6.8								
		164	<4	7.0	6.0								
		165	<4	6.3	5.5								
		166	<4	4.3	5.5								
		167	<4	5.3	7.3								
		170	<4	5.3	5.5								
		172	<4	5.5	6.6								
		173	<4	7.3	6.3								
		162	<4			1.5	4.8						
		163	<4			3.8	5.8						
		168	<4			4.5	3.3						
		169	<4			4.7	3.5						
		175	<4			5.8	3.5						

a) HI antibody titer at the time of challenge, b) C: Cloaca, T: Trachea, c) -: Not detected,
d) virus titer ($\log_{10}$ EID_{50}/ml)

Table 4. HI antibody titers of chickens until 100 weeks after the vaccination

Group	HA units/dose	Chicken No.	HI antibody titers at the following weeks after vaccination														
			1	2	3	4	5	6	7	16	28	40	52	64	76	88	100
Test	640	1	<4	512	1,024	1,024	2,048	2,048	1,024	1,024	256	256	128	128	128	128	128
		2	<4	128	2,048	4,096	2,048	2,048	2,048	1,024	512	512	512	256	256	256	256
		3	<4	256	1,024	2,048	2,048	2,048	2,048	2,048	1,024	1,024	1,024	512	512	512	512
		4	<4	64	512	1,024	1,024	1,024	1,024	1,024	512	256	256	256	256	256	128
		5	<4	128	2,048	2,048	2,048	1,024	1,024	1,024	256	256	128	128	128	128	128
		6	<4	128	512	1,024	1,024	1,024	1,024	1,024	256	256	256	128	128	128	128
		7	<4	128	512	2,048	2,048	1,024	1,024	256	128	128	128	128	128	128	128
		8	<4	512	1,024	2,048	2,048	2,048	1,024	1,024	512	512	512	512	512	512	256
	GM[a)]		<4	181	939	1,722	1,722	1,448	1,218	939	362	332	279	215	215	215	181
Control		9	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
		10	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
		11	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
	GM		<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4

a) GM: geometric mean

nation.

Discussion

Based on the results of Experiment 1, it was demonstrated that the HI antibody responses of immunized chickens were correlated with the antigen levels of test vaccines. The minimum HI antibody titer that could protect chickens from HPAI virus infection-related symptoms and death was considered to be 1:16, because chickens with HI antibody titers of 1:4 and 1:8 at the time of challenge died on days 8 and 4 p.c., respectively, and distinct viral shedding was observed. In contrast, chickens with an HI antibody titer of 1:16 or higher survived without symptoms of AI and there was no viral shedding after the challenge.

Vaccine A, containing 640 HA units/dose, provided excellent protection for chickens by single-shot vaccination without viral shedding. Transit of the HI antibodies and the presence of anti-NS-1 antibodies (considered an indicator of infection by AI virus[9)]) after the challenge were not monitored in this study. We could not clearly assess the presence or absence of AI virus infection in the surviving chickens. However, Vaccine A might be able to protect chickens from HPAI virus infection. Vaccine B, containing 160 HA units/dose, induced an antibody response at 1:16 or higher in all chickens at 3 weeks after the vaccination. This was sufficient to protect the chickens from HPAI virus infection-related symptoms and death. Vaccine C, containing 80 HA units/dose, was inadequate to protect the chickens from HPAI virus infection-related death by single-shot vaccination, because it did not induce an adequate HI antibody response in any of them 2 weeks after vaccination. Moreover, the geometric mean 3 weeks after the vaccination was only 1:9. Two chickens died on days 4 and 8 after challenge. It would be possible to employ an emergency vaccination against severe outbreaks of HPAI if the vaccine contains an amount of antigen similar to that in Vaccine A. In this experiment, challenge was performed 7 weeks after the vaccination. Though it seems desirable to confirm the potency of an AI vaccine for emergency use via a challenge test as early as possible after vaccination, observation of the antibody response several weeks after the vaccination was also an important objective of the experiment. Thus, the challenge was performed at a relatively late time after vaccination, when the antibody titer had reached a plateau.

Based on the results of Experiment 2, Vaccine A, containing 640 HA units/dose, showed a good

immunological response. It showed rapid as well as long-lasting high-level immunity for 100 weeks after a single-shot vaccination. We are currently planning to continue the long-term observation further to determine the duration of the HI antibody titer, and to conduct an eventual challenge with HPAI at an appropriate time around 200 weeks after the vaccination. However, we speculate that the chickens of the vaccinated group could be protected from death even at that time because the HI antibody titers of all the chickens in the Test group remained 1:128 or higher for 100 weeks after single-shot vaccination, and the decrease of the HI antibody level to date has been extremely slow. Based on the results of Experiment 1, those HI antibody titers were considered sufficient to protect chickens from challenge with HPAI virus.

References

1) Ellis, T.M., Leung, C.Y., Chow, M.K., Bissett, L. A., Wong, W., Guan, Y. and Malik, Peiris, J.S. 2004. Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission. *Avian Pathol.,* **33**: 405-412.
2) Garcia, A., Johnson, H., Srivastava, D.K., Jayawardene, D.A., Wehr, D.R. and Webster, R. G. 1998. Efficacy of inactivated H5N2 influenza vaccines against lethal A/Chicken/Queretaro/19/95 infection. *Avian Dis.,* **42**: 248-256.
3) Highly Pathogenic Avian Influenza Infection Route Elucidation Team. 2004. Routes of infection of highly pathogenic avian influenza in Japan. www.maff.go.jp/tori/20040630report.pdf.
4) Highly Pathogenic Avian Influenza Infection Route Elucidation Team. 2007. Routes of infection of highly pathogenic avian influenza in Japan. www.maff.go.jp/j/syouan/douei/tori/pdf/report2007.pdf.
5) Liu, J., Xiao, H., Lei, F., Zhu, Q., Qin, K., Zhang, X.W., Zhang, X.L., Zhao, D., Wang, G., Feng, Y., Ma, J., Liu, W., Wang, J. and Gao, G.F. 2005. Highly pathogenic H5N1 influenza virus infection in migratory birds. *Science,* **309**: 1206.
6) Mase, M., Kim, J.H., Lee, Y.J., Tsukamoto, K., Imada, T., Imai, K. and Yamaguchi, S. 2005. Genetic comparison of H5N1 influenza A viruses isolated from chickens in Japan and Korea. *Microbiol. Immunol.,* **49**: 871-874.
7) Mase, M., Tsukamoto, K., Imada, T., Imai, K., Tanimura, N., Nakamura, K., Yamamoto, Y., Hitomi, T., Kira, T., Nakai, T., Kiso, M., Horimoto, T., Kawaoka, Y. and Yamaguchi, S. 2005. Characterization of H5N1 influenza A viruses isolated during the 2003-2004 influenza outbreaks in Japan. *Virology,* **332**: 167-176.
8) Nishiguchi, A., Yamamoto, T., Tsutsui, T., Sugizaki, T., Mase, M., Tsukamoto, K., Ito, T. and Terakado, N. 2005. Control of an outbreak of highly pathogenic avian influenza, caused by the virus sub-type H5N1, in Japan in 2004. *Rev. Sci. Tech.,* **24**: 933-944.
9) Ozaki, H., Sugiura, T., Sugita, S., Imagawa, H. and Kida, H. 2001. Detection of antibodies to the nonstructural protein (NS1) of influenza A virus allows distinction between vaccinated and infected horses. *Vet. Microbiol.,* **82**: 111-119.
10) Qiao, C., Yu, K., Jiang, Y., Li, C., Tian, G., Wang, X. and Chen, H. 2006. Development of a recombinant fowlpox virus vector-based vaccine of H5N1 subtype avian influenza. *Dev. Biol. (Basel),* **124**: 127-132.
11) Reed, L. J. and Muench, H. 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.,* **27**: 493-497.
12) Soda, K., Sakoda, Y., Isoda, N., Kajihara, M., Haraguchi, Y., Shibuya, H., Yoshida, H., Sasaki, T., Sakamoto, R., Saijo, K., Hagiwara, J. and Kida, H. 2008. Development of vaccine strains of H5 and H7 influenza viruses. *Jpn. J. Vet. Res.,* **55**: 93-98.
13) Swayne, D.E., Beck, J.R. and Mickle, T.R. 1997. Efficacy of recombinant fowl poxvirus vaccine

in protecting chickens against a highly pathogenic Mexican-origin H5N2 avian influenza virus. *Avian Dis.,* **41**: 910-922.

14) Swayne, D.E., Lee, C.W. and Spackman, E. 2006. Inactivated North American and European H5N2 avian influenza virus vaccines protect chickens from Asian H5N1 high pathogenicity avian influenza virus. *Avian Pathol.,* **35**: 141-146.

15) Webster, R.G., Guan, Y., Poon, L., Krauss, S., Webby, R., Govorkovai, E. and Peiris, M. 2005. The spread of the H5N1 bird flu epidemic in Asia in 2004. *Arch. Virol. Suppl.,* **19**: 117-129.

Vaccine 27 (2009) 5174–5177

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Short communication

Long lasting immunity in chickens induced by a single shot of influenza vaccine prepared from inactivated non-pathogenic H5N1 virus particles against challenge with a highly pathogenic avian influenza virus

Takashi Sasaki[a,*], Norihide Kokumai[a], Toshiaki Ohgitani[a], Ryuichi Sakamoto[b], Noriyasu Takikawa[c], Zhifeng Lin[d], Masatoshi Okamatsu[e], Yoshihiro Sakoda[e], Hiroshi Kida[e,f]

[a] Avian Biologics Department, Kyoto Biken Laboratories, Inc., 24-16 Makishima-cho, Uji, Kyoto 611-0041, Japan
[b] Division 2, Second Research Department, The Chemo-Sero-Therapeutic Research Institute, Kikuchi, Kumamoto 869-1298, Japan
[c] Research Center for Biologicals, The Kitasato Institute, Kitamoto, Saitama 364-0026, Japan
[d] Research Department, Nippon Institute for Biological Science, Ome, Tokyo 198-0024, Japan
[e] Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan
[f] Research Center for Zoonosis Control, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan

ARTICLE INFO

Article history:
Received 12 May 2009
Received in revised form 12 June 2009
Accepted 22 June 2009
Available online 9 July 2009

Keywords:
Avian influenza vaccine
Lasting protective immunity
H5 virus

ABSTRACT

An influenza vaccine was prepared from inactivated whole particles of the non-pathogenic strain A/duck/Hokkaido/Vac-1/04 (H5N1) virus using an oil adjuvant containing anhydromannitol-octadecenoate-ether (AMOE). The vaccine was injected intramuscularly into five 4-week-old chickens, and 138 weeks after vaccination, they were challenged intranasally with 100 times 50% chicken lethal dose of the highly pathogenic avian influenza (HPAI) virus A/chicken/Yamaguchi/7/04 (H5N1). All 5 chickens survived without exhibiting clinical signs of influenza, although 2 days post-challenge, 3 vaccinated chickens shed limited titres of viruses in laryngopharyngeal swabs.

1. Introduction

Influenza A viruses are divided into H1–H16 and N1–N9 subtypes on the basis of antigenic specificity of two glycoproteins [1,2]. Each of the subtypes has been isolated from migrating waterfowls, which play a role in the spread of the influenza A viruses [3,4]. During repeated passage through a chicken population, the viruses acquire transmissibility and pathogenicity against chickens, resulting in highly pathogenic avian influenza (HPAI) outbreaks in domestic poultry [5–9].

HPAI caused by H5 and H7 subtype viruses have occurred in many parts of the world, and such outbreaks have resulted in huge economic losses in poultry industries. A recent outbreak of H5N1 virus infection emerged in South Asia and spread through Eurasia and Africa. In addition, direct transmission of H5N1 viruses from birds to humans with high mortality occurred. Since 2003, more than 400 human cases with 60% mortality have been reported as of 11 May 2009 [10].

The standard measure undertaken for the control of HPAI in poultry is stamping out. Vaccination is allowed as an optional-tool to decrease the amount of viruses shed from infected chickens when standard measure cannot enough to control the outbreak in the field [11]. Although commercial vaccines prepared from viruses of the North American lineage are available, they may be less effective for the control of HPAI outbreaks caused by infection by viruses of the Eurasian lineage [12]. Thus, a vaccine prepared from a Eurasian lineage virus may provide better protection against Asian HPAI virus infections [13–16].

We have developed an H5N1 reassortant virus of the Eurasian lineage that is non-pathogenic for chickens and chicken embryos, and exhibits good growth in embryonated chicken eggs [12]. Subsequently, we prepared test vaccines using this reassortant virus, and confirmed the optimal antigen concentration and its protective potency against a currently prevalent Eurasian lineage HPAI virus [17]. We demonstrated that the test vaccine is able to induce protective immunity against HPAI virus starting 8 days post-vaccination and that chickens challenged with a HPAI virus, A/whooperswan/Mongolia/3/2005 (H5N1) strain did not show clinical signs of HPAI [14]. In the present study, we examined whether the vaccine induces long lasting immunity (138 weeks) by challenging vaccinated chickens with HPAI virus.

* Corresponding author. Tel.: +81 774 22 4518; fax: +81 774 24 1407.
E-mail address: keibyou_one@kyotobiken.co.jp (T. Sasaki).

0264-410X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2009.06.070