Virus	Origin of gene segment								Virus titer		Virus recovery			
	PB2	PB1	PA	HA	NP	NA	M	NS	Brain	Lung	Trachea	Kidney	Liver	Colon
HK483 PB2/MON3									3.8, 2.5, 2.5	4.8, 3.8, 4.3	++	+++	++	++
HK483 PB1/MON3									4.8, 3.5, 2.8	2.0, 5.5, 3.5	+	+++	++	+++
HK483 PA/MON3									5.3, 5.3, -	7.3, 2.8, 3.5	+++	+++	+++	+++
HK483 HA/MON3									6.3, 5.3, 1.8	6.8, 5.3, 5.8	+++	+++	+++	+++
HK483 NP/MON3									4.8, 2.5, ≤ 1.8	5.5, 4.8, 3.3	+++	+++	++	++
HK483 NA/MON3									7.5, 6.5, 3.5	3.8, 6.3, 3.5	+++	+++	+++	+++
HK483 M/MON3									9.3, 7.8, 5.8	5.3, 6.3, 6.3	+++	+++	+++	+++
HK483 NS/MON3									4.3, -, -	5.3, 3.5, 2.5	+	+++	+	++
MON3 PB2/HK483									-, -, -	2.4, 2.0, 2.0	-	+	-	-
MON3 PB1/HK483									-, -, -	-, -, -	-	-	-	+
MON3 PA/HK483									-, -, -	3.5, 3.3, 2.0	+	+	+	+++
MON3 HA/HK483									-, -, -	3.0, 2.4, -	+	++	+	+
MON3 NP/HK483									-, -, -	4.0, 3.5, 3.5	++	+++	+	+
MON3 NA/HK483									-, -, -	5.7, 4.3, -	+	+	-	++
MON3 M/HK483									-, -, -	2.5, 2.0, -	+	+	+	+
MON3 NS/HK483									-, -, -	3.8, 3.5, -	-	-	+	-

Figure 4 Replication of single-gene reassortants in 2-week-old ducks. A series of single-gene reassortants between HK483 and MON3 was generated by reverse genetics. Each of the viruses was inoculated into 2-week-old ducks and tissue samples were collected on 3 dpi. Suspension of each tissue sample was inoculated into 10-day-old embryonated eggs for virus titration or recovery. White and gray boxes indicate the derivation of virus gene segments from HK483 and MON3, respectively. Virus titers in tissues were expressed as log EID_{50}/g and the lower limit of detection was $10^{1.5}$ EID_{50}/g. +, ++, and +++: viruses were recovered from one, two, and three of three birds tested, respectively. -: viruses were not detected from tissue samples.

PA were involved in the virulence of H5N1 HPAIVs in domestic ducks. Here, we showed that the PB2, PA, HA, NP, and NS genes of MON3 were required to show full pathogenicity in ducks. The two strains utilized in the present study, HK483 and MON3, have comparably distant evolutionary relationship amongst H5N1 HPAIVs [17]. HA genes of HK483 and MON3 were classified into clade 0 and 2.2, respectively [17], and each gene segment of them possesses only 86.3–95.1% homology (Table 2). However, MON3 and HK483 share the same amino acid residues at all positions reported to be involved in the pathogenicity of H5N1 HPAIVs in ducks,

Virus	Origin of gene segment								Virus titer		Virus recovery			
	PB2	PB1	PA	HA	NP	NA	M	NS	Brain	Lung	Trachea	Kidney	Liver	Colon
MON3 PB2-NP-NS/HK 483									2.5, -, -	4.3, 3.3, 2.7	-	+	-	+
MON3 PB2-PB1-NP -NS/HK483									-, -, -	4.5, 4.3, 3.7	++	+++	+	++
MON3 PB2-PA-NP -NS/HK483									-, -, -	5.0, 3.3, 2.7	+	+++	++	+++
MON3 PB2-HA-NP -NS/HK483									-, -, -	4.7, 3.3, -	+	++	+++	++
MON3 PB2-PB1-PA -NP-NS/HK483									5.7, 2.5, -	4.5, 5.7, 5.7	+++	+++	++	+++
MON3 PB2-PB1-HA -NP-NS/HK483									-, -, -	3.3, 2.0, ≤1.7	++	+	+++	++
MON3 PB2-PA-HA -NP-NS/HK483									5.0, -, -	5.3, 6.0, 3.0	+	++	++	+++
MON3 PB2-PB1-PA -HA-NP-NS/HK483									3.0, 3.0, 2.0	6.5, 4.0, 2.4	++	+++	+	++

Figure 5 Replication of multi-gene reassortants in 2-week-old ducks. A series of multi-gene reassortants between HK483 and MON3 was generated by reverse genetics. Each of the viruses was inoculated into 2-week-old ducks and tissue samples were collected on 3 dpi. Suspension of each tissue sample was inoculated into 10-day-old embryonated eggs for virus titration or recovery. White and gray boxes indicate the derivation of virus gene segments from HK483 and MON3, respectively. Virus titers in tissues were expressed as log EID_{50}/g and the lower limit of detection was $10^{1.5}$ EID_{50}/g. +, ++, and +++: viruses were recovered from one, two, and three of three birds tested, respectively. -: viruses were not detected from tissue samples.

Table 2 Homology of viral genes and proteins between HK483 and MON3

	Nucleotide (%)	Amino acid (%)	No. of different amino acids
PB2	86.3	96.4	27
PB1	90.9		
PB1		97.2	21
PB1-F2		80.0	18
PA	88.5	95.3	34
HA	95.1	94.9	39
NP	92.1	97.6	12
NA	88.0	90.4	43
M	92.5		
M1		95.2	12
M2		95.9	4
NS	89.8		
NS1		85.2	34
NS2		90.1	12

suggesting other molecular determinants for the high pathogenicity of MON3 in ducks.

The present study demonstrated that the PB2, NP, and NS genes of MON3 were prerequisite for high pathogenicity in ducks (Figures 1, 2, 4, and 5). Some explorations to clarify the relationship between the constellation of gene segments and pathogenicity of influenza A viruses were hitherto performed mainly using mammals, such as mice, ferrets, and monkeys [26-29]. These reports showed that the growth potential of reassortants differed depending on the constellation of the polymerase genes, suggesting the major contribution of the polymerases to efficient replication in mammals. NP was also shown to play an important role in the efficient replication and expansion of tissue tropism of H5N1 HPAIVs in chickens [30,31]. Recently, it was demonstrated that the interaction of PB2 and NP with importin-α, a host factor mediating trafficking into the nucleus, where transcription and replication of the viral genome occur, was correlated with host adaptation of influenza A viruses [32]. Amino acid differences of the PB2 and NP between HK483 and MON3 are 27 and 12 positions, respectively (Table 2). These differences are probably responsible for the incompatibility of the PB2 and NP of HK483 with viral proteins of MON3 to show high pathogenicity in ducks. Taken together, it is likely that the polymerase and the NP of MON3 may play a principal role in efficient replication in systemic organs of ducks coordinately with employing host factors involved in virus replication.

Sarmento *et al.* [33] investigated the contribution of the NS gene to the pathogenicity of H5N1 HPAIVs in ducks and discussed that the NS gene products had a minimal influence on the viral pathogenicity in ducks. Conversely, the present data showed that the NS gene segment of MON3 was also prerequisite for high pathogenicity in ducks (Figures 1 and 4). NS1, encoded in the NS gene, is well known to serve multiple functions in the life cycle of influenza A viruses [34]. The major role of NS1 is considered to be the inhibition of host innate immune responses by limiting interferon production and the subsequent antiviral effects of interferon-induced proteins [34]; therefore, NS1 has been extensively studied as a molecular determinant of viral pathogenicity mainly in mice and chickens. It was recently reported that deletion of amino acid residues 80–84 in NS1, which is a very common characteristic among H5N1 HPAIVs isolated after 2000, enhanced the pathogenicity of H5N1 viruses in mice and chickens [35]. It is noteworthy that MON3 also has the deletion of amino acid residues 80–84 in NS1 but HK483 does not; however, a plausible explanation of how NS1 with the deletion contributes to high pathogenicity is lacking and it is also unclear whether this finding is applicable to the pathogenicity of H5N1 viruses in ducks. Recently, Barber *et al.* [36] demonstrated that the cytoplasmic pathogen sensor RIG-I, triggering antiviral effects by recognition of viral RNA, might be associated with the natural resistance of ducks to influenza A virus infection, while RIG-I homologue of chickens has not been identified. It is known that the NS1 of some strains interacted with RIG-I and blocked its signaling pathway and subsequent interferon-β induction [34]; therefore, it is of interest whether the NS1 of MON3 suppresses innate immune responses of ducks more strongly than that of HK483.

Consistent with the data of Pantin-Jackwood *et al.* [37], the present study showed that the age of ducks is one of the factors which influenced their susceptibility to H5N1 HPAIVs. MON3 killed all of the 1-day- and 2-week-old ducks, but two of the three 4-week-old ducks infected with MON3 survived. While none of the ducks infected with HK483 died, this virus was recovered from the brains of 1-day-old ducks but not from the brains of 2- and 4-week-old ducks. As previously described by Kishida *et al.* [12], virus replication in the brain might be a cause of death upon HPAIV infection. Neurological symptoms, such as torticollis, blindness, and convulsion, were distinct characteristics of MON3 infection of ducks. MON3 showed considerably strong tropism for the central nervous system of 2-week-old ducks but HK483 were not detected in their brains (Figure 3), suggesting that efficient virus replication in the brain might correlate with neurological dysfunction and death. It is also known that chickens and humans die of multiple organ failure upon H5N1 virus infection [38,39]. In 2-week-old ducks, titers of MON3 were significantly higher than those of HK483 in all of the tissue tested (Figure 3). Furthermore, reassortants lethal to ducks showed wide tissue tropism and

efficient replication in the lungs and brains (Figures 4 and 5); therefore, multiple organ failure also appeared to be critical for the high pathogenicity of MON3 in ducks.

The present study demonstrated that polygenic factors are involved in the high pathogenicity of MON3. In particular, the PB2, NP, and NS genes of MON3 were prerequisite. Emergence of H5N1 HPAIVs, which caused the death of the water birds in 2002, changed the idea that natural reservoir hosts (i.e., ducks) generally do not die due to influenza A virus infection. H5N1 HPAIVs that emerged in 1997 are still expanding their geographical distribution and the epidemiological study of HPAI in the 2010 migration season suggested the strong possibility that H5N1 HPAIVs might be perpetuated in a migratory bird population in the eastern Eurasian region [40]. These trends underscore the increasing importance of further study on avian influenza in migratory birds. To control HPAI not only in poultry but also in wild birds, the molecular basis of the high pathogenicity of H5N1 viruses in ducks should be clarified, in addition to continued global monitoring of HPAIVs in a migratory bird population.

Conclusions

Experimental infection studies in ducks revealed that the PB2, NP, and NS gene segments of MON3 were prerequisite for the high pathogenicity of MON3 in ducks. A set of the PB2, PA, HA, NP, and NS gene segments of MON3 was required to show full pathogenicity in ducks. These data indicate that multigenic factors are responsible for the pathogenicity of MON3 in ducks. MON3 and reassortants that were lethal to ducks efficiently replicated in the tissues tested, especially in the brain, suggesting a possible correlation between virus growth in the brain and the death of ducks accompanying neurological dysfunction.

Methods

Viruses and cells

HK483 was isolated from throat aspirates of the third case of a 13-year-old girl. MON3 was isolated from the brain of a dead whooper swan found at Lake Khunt, Mongolia [17]. Viruses were propagated in 10-day-old embryonated chicken eggs for 48 hours at 35°C. Madin-Darby canine kidney (MDCK) cells and human embryonic kidney 293 T (293 T) cells were cultured according to Tsuda *et al.* [41].

Generation of reassortants by reverse genetics

Viral RNAs of HK483 and MON3 were extracted as described previously [41]. Full-length genomes of the eight gene segments were cloned into a dual-promoter plasmid, pHW2000 [42]. HK483, MON3, and 24 reassortants between HK483 and MON3 were generated by reverse genetics (Figures 1 and 2). Briefly, MDCK and 293 T cells were cocultured in Opti-MEM 1 (Invitrogen) and transfected with 1 μg of each of the eight plasmids using TransIT-293 (Mirus Bio) according to the manufacturer's protocol. After six hours of incubation at 37°C, transfection mixture was replaced with Opti-MEM 1. At 72 hours post-transfection, culture supernatant was collected and injected into the allantoic cavity of 10-day-old chicken embryonated eggs. Virus titers in harvested allantoic fluids were determined by the method of Reed and Muench [43] using embryonated eggs and expressed as EID_{50} per milliliter.

Experimental infection of ducks

One-day-, 2-week-, and 4-week-old domestic ducks (Cherry Valley strain) were used to assess the pathogenicity of viruses. Viruses ($10^{6.0}$ EID_{50} in 100 μl of allantoic fluid) were inoculated intranasally into three to nine ducks and clinical signs were monitored at 24-hour intervals over 14 dpi. Undiluted virus stocks of the following seven reassortants were inoculated into ducks, because their titers in 100 μl allantoic fluids did not reach $10^{6.0}$ EID_{50} despite attempts to increase their titers through several passages in embryonated eggs (i.e., $10^{4.7}$, $10^{5.5}$, $10^{5.4}$, $10^{4.5}$, $10^{4.8}$, $10^{5.0}$, and $10^{4.5}$ EID_{50} were used for MON3 HA/HK483, MON3 NS/HK483, MON3 PB2-PA-NP-NS/HK483, MON3 PB2-HA-NP-NS/HK483, MON3 PB2-PB1-HA-NP-NS/HK483, MON3 PB2-PA-HA-NP-NS/HK483, and MON3 PB2-PB1-PA-HA-NP-NS/HK483, respectively). Ducks exhibiting severe disease signs were euthanized by intravenous injection of pentobarbital (Dainippon Sumitomo Pharma) and recorded as having died the next day. To examine virus replication, each of the reassortants was inoculated into two or three ducks as described above. On 3 dpi, ducks were euthanized and the brains and lungs of 1-day-old ducks and the brains, tracheas, lungs, kidneys, livers, and colons of 2- and 4-week-old ducks were collected aseptically. To make 10% suspensions in minimum essential medium (Nissui Pharmaceutical), the tissue samples were homogenized by a Multi-Beads Shocker (Yasui Kikai). Virus titers in these suspensions were determined by the same method as described above and expressed as EID_{50} per gram of tissue.

Experimental infection of ducks was carried out in the biosafety level 3 facilities at the Graduate School of Veterinary Medicine, Hokkaido University, Japan according to the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan (http://www.scj.go.jp/ja/info/kohyo/pdf/kohyo-20-k16-2e.pdf). The protocol was approved by the Hokkaido University Animal Care and Use Committee (Permit Number: 08-0157).

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
MK drafted the manuscript. MK, KS, and KM carried out generation of reassortants by reverse genetics and experimental infection of ducks. YS, MO, AT, and HK participated in the coordination of the study. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

The nucleoprotein is responsible for intracerebral pathogenicity of A/duck/Mongolia/47/2001 (H7N1) in chicks

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Abstract Avian influenza viruses A/duck/Mongolia/47/2001 (H7N1) (47/01) and A/duck/Mongolia/867/2002 (H7N1) (867/02) were defined as low-pathogenic avian influenza viruses (LPAIVs) using an intravenous pathogenicity test in chickens. On the other hand, the intracerebral pathogenicity indices of 47/01 and 867/02 were 1.30 and 0.00, respectively. A series of reassortant viruses were generated between 47/01 and 867/02, and their intracerebral pathogenicity was compared in one-day-old chicks to identify the protein(s) responsible for the intracerebral pathogenicity of 47/01. The results indicate that the amino acids at positions 50 and 98 of the nucleoprotein are related to the pathogenicity of 47/01 in chicks by intracerebral inoculation. A significant association was found between mortality of the chicks inoculated intracerebrally with 47/01 and virus replication in the lungs and/or brain. These results indicate that the NP of avian influenza viruses may be responsible for intracerebral pathogenicity in the host.

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Introduction

Highly pathogenic avian influenza viruses (HPAIVs) cause severe systemic infection with high mortality in chickens. It is known that low-pathogenic avian influenza viruses (LPAIVs) of the H5 or H7 subtype acquire pathogenicity during repeated passages in chicken populations [9].

The pathogenicity of avian influenza viruses is defined by an intravenous pathogenicity test using 4- to 8-week-old chickens. Insertion of multiple basic amino acids at the cleavage site of the haemagglutinin (HA) is well established as an indicator of potential pathogenicity for H5 and H7 influenza viruses in chickens [4, 18]. In 1992, the criteria for the pathogenicity of avian influenza viruses were defined by the European Union (EU) on the basis of an intravenous pathogenicity index (IVPI), growth in cultured cells in the absence of trypsin, and amino acid residues at the cleavage site of the HA protein, and World Organization for Animal Health (OIE) has also adopted these criteria [4, 18]. It is generally recognized that the HA subtypes of HPAIVs are restricted to H5 or H7, although it has been reported that the IVPIs of two H10 subtypes are over 1.20 [29]. These H10 viruses do not have multiple basic amino acids at the cleavage site of the HA and do not cause severe disease when inoculated intranasally. On the other hand, four H5 avian influenza viruses have multiple basic amino acids at the cleavage site of the HA but show some pathogenicity (IVPI < 1.2) when the viruses are inoculated intravenously into chickens [13].

H5N1 HPAIVs have been isolated from aquatic migratory birds as well as terrestrial poultry since 2005 [3, 12]. Ducks experimentally infected with viruses isolated during outbreaks of highly pathogenic avian influenza in Japan in 2004, the HA gene of which belongs to clade 2.5, did not show any disease signs [8, 10, 23], whereas ducks

inoculated with isolates from dead migratory birds in Mongolia, the HA genes of which belong to clade 2.2 or 2.3.2, showed severe neurological signs, such as depression, blindness, and intermittent head-shaking [23]. Chickens infected with H5 HPAIVs die due to systemic infection. On the other hand, ducks infected with H5 HPAIVs show neurological signs resulting in death [23]. Histopathological studies indicated that neurological signs of the birds infected with HPAIVs, the HA genes of which belong to clades 1, 2.5, and 8, were associated with inflammation and necrosis in the brain [21, 26, 30]. Nevertheless, the viral factors responsible for intracerebral pathogenicity of HPAIVs have not been identified.

A/duck/Mongolia/47/2001 (H7N1) (47/01), isolated from a fecal sample from a duck that migrated from nesting lakes in Siberia in autumn, was defined as an LPAIV by IVPI [25]; however, eight of the 10 one-day-old chicks that were inoculated intracerebrally with 47/01 died, and the intracerebral pathogenicity index (ICPI) was 1.30. Taking into account that the highest ICPI of 91 LPAIVs isolated from wild birds by Otsuki et al. was 0.83 [20], the ICPI of 47/01 was apparently high. It has been reported that an H7N1 LPAIV spread systemically in chickens after a combined intranasal and intratracheal inoculation [22]. Introduction of intracerebral pathogenicity factors found in 47/01 into LPAIVs that can spread systemically in a host might result in severe intracerebral pathogenicity via the natural infection route. In the present study, the pathogenicity of a series of reassortant viruses generated between 47/01 and an intracerebrally low-pathogenic virus, A/duck/Mongolia/867/2002 (H7N1) (867/02), was compared in one-day-old chicks. The results indicate that the amino acids at positions 50 and 98 of the nucleoprotein (NP) of 47/01 are related to the pathogenicity in chicks when tested by intracerebral inoculation.

Materials and methods

Viruses and animals

A/duck/Mongolia/47/2001 (H7N1) and A/duck/Mongolia/867/2002 (H7N1) were isolated from fecal samples from migratory ducks in Mongolia in 2001 and 2002, respectively. These two viruses were propagated in 10-day-old embryonated chicken eggs at 35 °C for 48 hours, and the infectious allantoic fluids were used as viral stocks.

Boris brown chickens and their fertile eggs were purchased from a hatchery vendor (Hokuren Chuou Syukeijo). Chicks were hatched from fertile eggs in our laboratory. These animals were housed in self-contained isolator units (Tokiwa Kagaku) at a BSL 3 biosafety facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan.

Sequencing

Virus RNAs were extracted using TRIzol LS Reagent (Invitrogen) and reverse-transcribed with the Uni12 primer [6] and M-MLV reverse transcriptase (Invitrogen). The full-length genome of each gene segment was amplified by polymerase chain reaction (PCR) with gene-specific primer sets [6]. Direct sequencing of each gene segment was carried out using an automated sequencer, CEQ 2000XL (Beckman Coulter). The nucleotide sequences of these two viruses in the present study were deposited in the DDBJ/EMBL/GenBank database (GenBank ID: AB473548, AB302788, AB268552-AB268557, and AB473540-AB473547, respectively)

Generation of recombinant viruses

Two recombinant viruses, rg-A/duck/Mongolia/47/2001 (H7N1) (rg-47) and rg-A/duck/Mongolia/867/2002 (H7N1) (rg-867), were generated according to Hoffmann et al. [5, 7]. Each of the PCR products of the eight gene segments of 47/01 and 867/02 was cloned into pGEM®-T Easy Vector (Promega). Each of the eight segments of 47/01 and 867/02 was cloned into the dual-promoter plasmid pHW2000 [5]. The plasmids carrying the NP gene of either 47/01 or 867/02 were manipulated using a QuikChange® II Site-Directed Mutagenesis Kit (Stratagene) in order to generate variant plasmids introducing single or double amino acid substitutions into the NP of each virus. Each of the resulting 14 plasmids that had single or double amino acid substitutions in the NP of either 47/01 or 867/02 was obtained and designated as pHW-47-NP-G34S, pHW-47-NP-N50S, pHW-47-NP-K98F, pHW-47-NP-N319K, pHW-47-NP-A350T, pHW-47-NP-M352V, pHW-47-NP-R384Q, pHW-867-NP-S34G, pHW-867-NP-S50N, pHW-867-NP-F98K, pHW-867-NP-K319N, pHW-867-NP-T350A, pHW-867-NP-V352M, pHW-867-NP-Q384R, pHW-47-NP-N50S-K98F, and pHW-867-NP-S50N-F98K.

Reverse-genetic viruses were generated by DNA transfection according to Tsuda et al. [27]. Briefly, 293T cells and Mardin-Darby canine kidney (MDCK) cells were co-cultured and transfected with 1 μg of each of the eight plasmids and 16 μl of TransIT-293 (Mirus Bio) in a total volume of 1 ml of Opti-MEM (Gibco). After 30 hours, 1 ml of Opti-MEM with 5 μg/ml of acetylated trypsin (Sigma) was added and incubated at 35 °C for 48 hours. One hundred microliters of the supernatant was inoculated into 10-day-old embryonated chicken eggs and incubated at 35 °C for 48 hours.

Pathogenicity tests in chicken embryos and chickens

The mean death time (MDT) of chicken embryos was determined according to Waterson et al. [28]. Viruses that were serially diluted tenfold with phosphate-buffered saline (pH 7.2) (PBS) were inoculated into 10 nine-day-old embryonated chicken eggs. The eggs were examined every eight hours by candling; the integrity of the circulatory system and movement of the embryo were criteria for viability until 120 hours post-inoculation (p. i.). The MDT was calculated as the mean time in hours required for the minimum lethal dose (MLD) to kill all 10 embryos.

An intravenous pathogenicity test for influenza viruses was carried out according to the OIE manual [18]. Two hundred microliters of virus that had been diluted tenfold with PBS was inoculated intravenously into eight six-week-old chickens. The birds were observed for signs of disease at intervals of 24 hours until 10 days p. i., and each bird was scored 0 if normal, 1 if sick, 2 if severely sick (including paralysis), and 3 if dead, at each observation. The IVPI was the mean score per bird per observation until 10 days p. i.

An intracerebral pathogenicity test was carried out basically according to the OIE manual of Newcastle Disease and Alexander et al. [1, 19]. One hundred microliters of virus that had been diluted tenfold with PBS or $10^{6.0}$ EID_{50} of wild-type or recombinant virus generated by the reverse genetics method was inoculated into 10 one-day-old chicks by intracerebral injection. The birds were observed for disease signs at intervals of 24 hours until 8 days p. i., and each bird was scored 0 if normal, 1 if sick, and 2 if dead, at each observation. The ICPI was the mean score per bird per observation until 8 days p. i. Birds were housed in self-contaminated isolator units (Tokiwa Kagaku, Tokyo) at a BSL 3 biosafety facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan. All animal experiments were authorized by the committee of Animal Experiments at the Graduate School of Veterinary Medicine, Hokkaido University.

Plaque assay

Viruses that had been serially diluted tenfold with PBS were inoculated onto confluent monolayers of MDCK cells and incubated at 35 °C for 1 hour. The cells were washed with minimal essential medium (MEM). Cells were then overlaid with MEM containing 0.7 % Bact-Agar (Gibco) and 5 μg/ml of acetylated trypsin (Sigma). After a 48-hour incubation at 35 °C, cells were stained with 0.005 % neutral red.

Virus growth potential in MDCK cells

Viruses were inoculated onto MDCK cell monolayers at a multiplicity of infection of 0.01 and incubated at 35 °C for 1 hour. Each virus was inoculated into three wells of MDCK cells. Cells were then washed with MEM. MEM with or without 5 μg/ml of acetylated trypsin was added to the cells, and they were incubated at 35 °C for 72 hours. Culture supernatants were collected, and each of the supernatants was titrated for its infectivity by plaque assay.

Virus recovery from chicks challenged intracerebrally with H7N1 viruses

In order to assess the virus growth potential in one-day-old chicks, $10^{6.0}$ EID_{50} of either rg-47 or rg-867 was inoculated into 60 one-day-old chicks by intracerebral injection. Three chicks in each challenged group were sacrificed every 12 hours from 12 hours p. i. If more than three birds died in an observation, all of the dead birds were sampled and no chicks were sacrificed at that time. When the birds were sacrificed or died, the brain and lung tissues were collected aseptically. In order to make a 10 % suspension with MEM, the collected tissue samples were homogenized using a Multi-Beads Shocker (Yasui Kikai). Suspensions of each sample were titrated in triplicate by plaque assay, and titers were expressed as the common logarithm of plaque-forming units (PFU) per gram of tissue.

Statistical analysis

To examine whether virus replication in the host is responsible for pathogenicity, one-tailed Fisher's exact tests were conducted at the 5 % confidence level.

Results

Pathogenicity of two wild-type H7N1 avian influenza viruses

Pathogenicity of wild-type 47/01 and 867/02 in nine-day-old chicken embryos, one-day-old chicks, and six-week-old chickens was assessed. The MLD of the chicken embryos inoculated with 47/01 and those inoculated with 867/02 were both 10 EID_{50} (data not shown). The MDTs of the chicken embryos inoculated with 47/01 and 867/02 were 93.0 and 94.7 hours, respectively, indicating that both viruses had low pathogenicity in chicken embryos (Table 1). Neither 47/01 nor 867/02 replicated in MDCK cells in the absence of trypsin. All eight six-week-old chickens that were inoculated intravenously with 867/02 survived for 10 days without showing any signs of disease, indicating that the IVPI of the virus was 0.00. Two out of the eight chickens inoculated with 47/01 died, indicating that the IVPI of the virus was 0.58. In addition, insertion of multiple basic amino acids at the cleavage site of HA was

not found in either H7N1 virus. The above results indicate that both 47/01 and 867/02 can be categorized as LPAIVs according to the OIE criteria.

None of the one-day-old chicks inoculated intracerebrally with 867/02 showed disease signs, and all survived for 8 days after inoculation. On the other hand, eight of the 10 one-day-old chicks inoculated intracerebrally with 47/01 died within 6 days p. i. The ICPI of 47/01 was thus scored as 1.30. Insertion of multiple basic amino acids at the cleavage site of HA was not found in the viruses recovered from the brain tissues of the dead chicks after intracerebral inoculation with rg-47 (data not shown).

Virus recovery from the chicks inoculated intracerebrally with recombinant H7N1 viruses

In order to clarify whether the intracerebral pathogenicity of the recombinant viruses is associated with virus growth potential in the brain, virus growth in the brain and lungs of the chicks that were inoculated intracerebrally with either rg-47 or rg-867 was assessed. Each virus, at a dose of $10^{6.0}$ EID_{50}, was inoculated intracerebrally into 30 one-day-old chicks.

The viruses were recovered from the brains of 19 chicks inoculated with rg-47 (Table 2). Since a high titer of viruses was directly inoculated intracerebrally into chicks and virus titers of the brain from 12 to 36 hours p. i. were gradually getting low, high virus titers from the brains as well as lungs of the chicks inoculated with the viruses from 12 to 36 hours p. i. should not be attributed to virus replication but instead to residual virus remaining after challenge. Of the 21 chicks sampled after 48 hours p.i., 15 were dead, and the other six were sacrificed at each time point. Of the 15 dead chicks, challenge virus was recovered from 14 of them as well as from three out of the six sacrificed birds. Mortality of the chicks after intracerebral inoculation with rg-47 was significantly associated with virus replication either in the lungs or in the brain ($P < 0.05$). Virus replication in the brain was confirmed in 10 out of the 21 chicks inoculated with rg-47 between 48 and 108 hours p. i. Virus replication in the lungs was confirmed in 16 out of the 21 chicks inoculated with rg-47 between 48 and 108 hours p. i. One of the birds inoculated with rg-47 died at 48 hours p. i. Seven of the birds inoculated with rg-47 died at 60 hours p. i., and seven other birds died between 72 and 96 hours p. i. Virus replication in the lungs was confirmed in 13 of the 15 dead chicks and three of the six chicks that were sacrificed, and virus replication in the brain was confirmed in eight of the 15 dead chicks and two of the six that were sacrificed.

Between 48 and 120 hours p. i., virus replication in the brain and lungs was confirmed in three and four, respectively, of the 21 chicks that were inoculated with rg-867. One of the birds inoculated with rg-867 died at 72 hours p. i., and the virus was not recovered from the brain.

Table 2 Virus recovery from the dead chicks inoculated either with rg-47 or rg-867

Virus	Hours post inoculation	No. of birds[a]	Virus recovery from the following tissues	
			Brain	Lungs
rg-47	12	3	3[b] (5.8)[c]	2 (2.5)
	24	3	3 (3.8)	3 (3.7)
	36	3	3 (3.1)	1 (2.5)
	48	1^+	1 (5.3)	1 (6.2)
	48	2	0	0
	60–96	7^+	7 (3.7)	6 (5.0)
	60–84	6^+	0	6 (3.5)
	60	1^+	0	0
	72–108	4	2 (2.8)	3 (3.6)
rg-867	12	3	3 (5.3)	2 (3.0)
	24	3	3 (4.2)	1 (2.5)
	36	3	3 (2.7)	0
	48–120	17	0	0
	72	1^+	0	1 (6.1)
	72–108	3	3 (3.4)	3 (3.4)

[a] Crosses indicate that all of the birds died

[b] Number of birds from which challenge virus was recovered. Zero indicates that no virus was recovered from any of the bird tissues

[c] The average virus titer is shown in parentheses (log PFU/g)

Table 1 Pathogenicity indices of two H7N1 influenza virus strains

Virus	MDT (h)	Growth potential in MDCK cell in the absence of trypsin	IVPI test		Amino acid sequence at cleavage site of the HA	ICPI test	
			IVPI	Dead/Total		ICPI	Dead/Total
A/duck/Mongolia/47/2001 (H7N1)	93.0	-	0.58	2/8	IPKGR/G	1.30	8/10
A/duck/Mongolia/867/2002 (H7N1)	94.7	-	0.00	0/8	IPKGR/G	0.00	0/10

Abbreviation name of pathogenicity indices, IVPI; intravenous pathogenicity index, MDT; mean death time of embryonated chicken eggs, ICPI; intracerebral pathogenicity index, Dead/Total; number of dead one-day-old chicks per ten chicks in the ICPI test

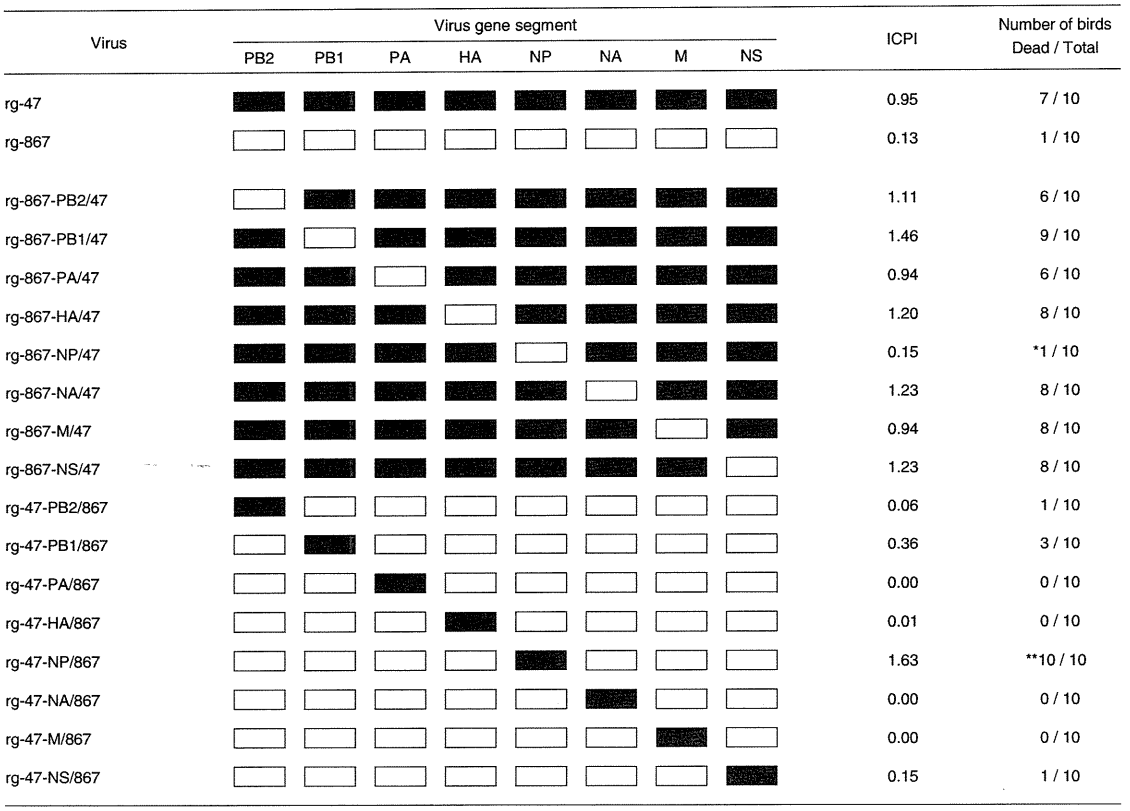

Fig. 1 Pathogenicity of recombinant virus in chicks after intracerebral inoculation. A series of viruses that were recombinants between 867/02 and 47/01 were generated by the reverse genetics method. Each of the recombinant viruses was injected intracerebrally into eight one-day-old chicks, and the ICPI was calculated according to the OIE manual of Newcastle Disease. White and black boxes indicate the virus gene segments derived from 867/02 and 47/01, respectively. Single and double stars indicate significant differences between the number of dead birds inoculated with rg-47 and with rg-867-NP/47, respectively, and there was a significant difference between the number of dead birds inoculated with rg-867 and with rg-47-NP/867

Intracerebral pathogenicity of recombinant viruses in chicks

In order to determine the virus protein(s) of 47/01 responsible for pathogenicity in chicks, a series of viruses that were recombinants between 47/01 and 867/02 was generated. Two recombinant viruses, rg-A/duck/Mongolia/47/2001 (H7N1) (rg-47) and rg-A/duck/Mongolia/867/2002 (H7N1) (rg-867), showed pathogenicity in chicks similar to 47/01 and 867/02, respectively (Table 1 and Fig. 1). All of the 10 chicks inoculated with rg-47-NP/867 (H7N1), which had the NP gene segment from 47/01 and the others from 867/02, died, and the ICPI of rg-47-NP/867 (H7N1) was 1.63 (Fig. 1). A significant difference between the number of dead chicks inoculated with rg-867 and rg-47-NP/867 was found ($P < 0.05$), indicating that the intracerebral pathogenicity of rg-47-NP/867 (H7N1) was apparently higher than that of rg-867. In contrast, none of the other single-gene reassortant viruses, which had the other seven genes of 867/02, showed severe intracerebral pathogenicity in chicks, and no significant differences between the number of dead chicks inoculated with each of them and with rg-867 were found. Nine out of the 10 chicks inoculated with rg-867-NP/47 (H7N1), which had the NP gene segment from 867/02 and the others from 47/01, survived for 8 days p. i. and the ICPI of rg-867-NP/47 (H7N1) was 0.15. A significant difference was found between the number of dead chicks inoculated with rg-47 and with rg-867-NP/47 ($P < 0.05$). In contrast, all of the other single-gene reassortant viruses, which had the other seven genes of 47/01, were highly pathogenic in chicks, and no significant differences between the number of dead chicks inoculated with each of them and with rg-47 were found. The present results indicate that the NP is responsible for pathogenicity after intracerebral inoculation of 47/01 in chicks.

Between the NPs of 47/01 and 867/02, seven amino acid differences were found at positions 34, 50, 98, 319, 350, 352, and 384 (Fig. 2). In order to identify the amino acid positions in the NP that are responsible for the pathogenicity of 47/1 by intracerebral inoculation in chicks, fourteen clones of recombinant 47/01 or 867/02, each of which had a single amino acid substitution in the NP, were generated. Three recombinant viruses, rg-867-NP-S50 N/867

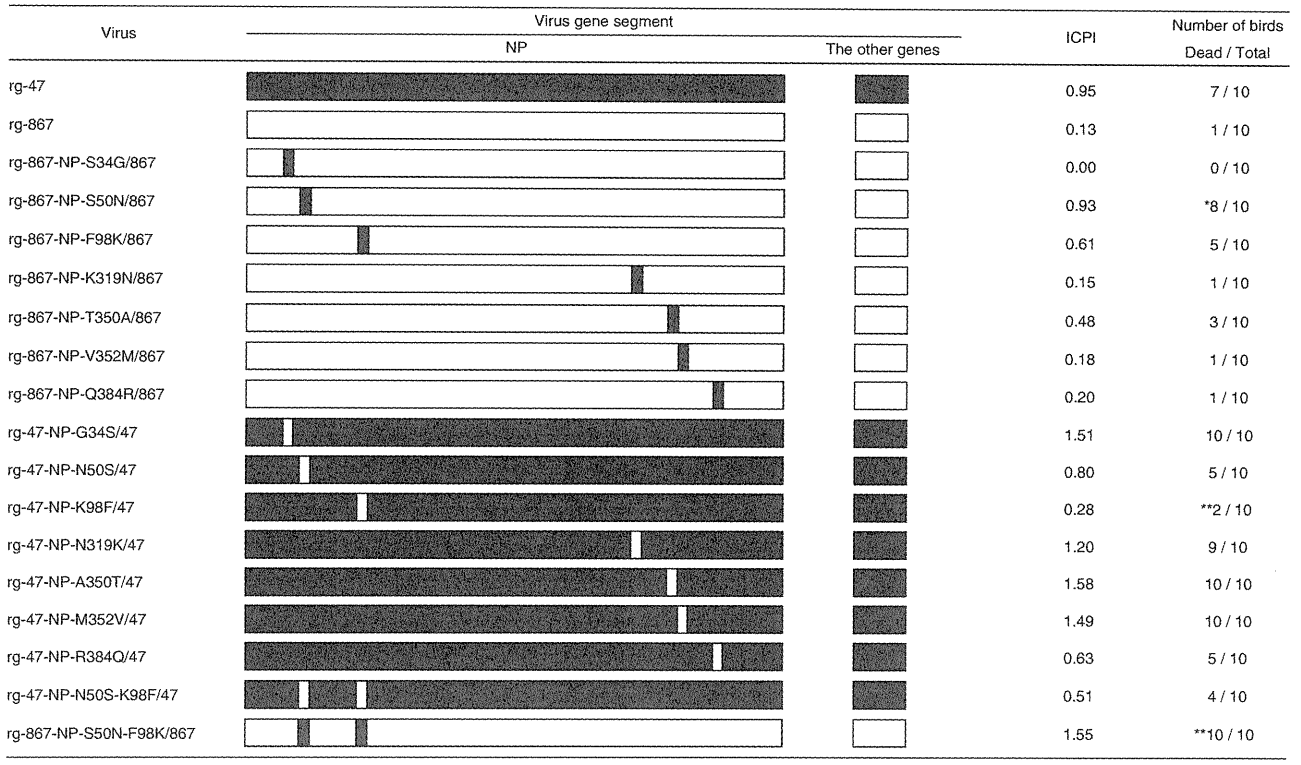

Fig. 2 Pathogenicity of recombinant virus containing a single amino acid substitution in the NP after intracerebral inoculation. Fourteen clones of recombinant 47/01 or 867/02, each of which had a single amino acid substitution in the NP, were generated. Each of the recombinant viruses was inoculated intracerebrally into eight one-day-old chicks, and the ICPI was calculated according to the OIE manual of Newcastle Disease. White and black boxes or lines indicate the virus gene segments derived from 867/02 and 47/01, respectively. The numbers indicate the positions of the amino acid differences. A significant difference between the number of dead birds inoculated with rg-867 and those inoculated with the single-point mutant is indicated by a single star. A significant difference between the number of dead birds inoculated with rg-47 and those inoculated with the single-point mutant are indicated by a double star

(H7N1), which had an amino acid substitution from serine to asparagine at position 50 in the NP of 867/02, rg-867-F98K/867 (H7N1), which had an amino acid substitution from phenylalanine to lysine at position 98 in the NP of 867/02, and rg-867-NP-T350A/867 (H7N1), which had an amino acid substitution from threonine to alanine at position 350 in the NP of 867/02, were more pathogenic than rg-867. Amino acid substitutions at positions 50, 98, or 350 were responsible for the pathogenicity of rg-867 by intracerebral inoculation, although a significant difference was only found between the number of dead chicks inoculated with rg-867-NP-S50N/867 (H7N1) and rg-867 ($P < 0.05$). Three mutant viruses, rg-47-NP-N50S/47 (H7N1), which had an amino acid substitution from asparagine to serine at position 50 in the NP of 47/01, rg-47NP-K98F/47 (H7N1), which had an amino acid substitution from lysine to phenylalanine at position 98 in the NP of 47/01, and rg-47-NP-R384Q/47 (H7N1), of which an amino acid substitution from arginine to glutamine was introduced at position 384 in the NP of47/01, were less pathogenic than rg-47. Amino acid substitutions at positions 50, 98, or 384 were responsible for the pathogenicity of rg-47 by intracerebral inoculation, although a significant difference was only found between the number of dead chicks inoculated with rg-47-NP-K98F/47 (H7N1) and rg-47 ($P < 0.05$). Taken together, we conclude that the pathogenicity of 47/01 by intracerebral inoculation in chicks was influenced by the amino acid at position 50 or 98 of the NP, although no significant difference was found between the number of dead chicks that had been inoculated with rg-867-F98K/867 (H7N1) and those inoculated with rg-867 or between the number of dead chicks that had been inoculated with rg-47-N50S/47 (H7N1) and those inoculated with rg-47.

Since the results described above suggested that amino acid substitutions at positions 50 and 98 of the NP of rg-47 are related to pathogenicity in chicks, two recombinant viruses with double amino acid substitutions were generated. The recombinant virus rg-867-NP-S50N-K98F/867 (H7N1) showed higher pathogenicity in chicks than either rg-867-NP-S50N/867 (H7N1) or rg-867-NP-F98K/47 (H7N1), whereas the pathogenicity of rg-47-NP-N50S-K98F/47 (H7N1) was not obviously different from those of rg-47-NP-N50S/47 (H7N1) and rg-47-K98F/47 (H7N1).

Discussion

Avian influenza virus 47/01, which possesses the typical monobasic motif of LPAIVs at the cleavage site of the HA, showed high pathogenicity in chicks when injected intracerebrally. In the present study, the amino acid sequence at the cleavage site of the HA of the viruses recovered from the dead birds was the same as that of the inoculum virus (data not shown), indicating that the intracerebral pathogenicity of 47/01 in chicks was not attributed to the fusion activity of the HA. These results are in agreement with an earlier report in which an H5N1 influenza virus strain that met the criteria according to the cleavage site of the HA but whose IVPI and ICPI values were both 0.0 became more pathogenic after serial intracerebral passages in 1- or 2-day-old chicks [14].

In the present study, a significant association was found between the pathogenicity of the virus and its replication in the host, although specific organs associated with virus pathogenicity were not determined. Interestingly, although many chicks died following inoculation with rg-47 via the intracerebral route, viruses were recovered from the lungs of 13 out of 15 dead chicks inoculated with rg-47, whereas no viruses were recovered from the brains of 7 out of 15 dead birds. Virus replication in the lungs is therefore responsible for the pathogenicity of 47/01 in the host, although a significant difference was not observed ($P = 0.11$). The probability that the severe pathogenicity in the host can be attributed to organ failure of the lungs subsequent to virus replication there is high. However, taking into consideration that no virus was recovered from one of the 15 dead chicks, there may be other causes of death in these birds after intracerebral inoculation with 47/01, such as organ failure in the brain or another organ, which was not investigated in the present study, or systemic failure subsequent to virus replication in a given organ, which is shown as systemic inflammatory response syndrome caused by HPAIV infection.

In the animal experiments using the recombinant viruses, it was demonstrated that the NP of 47/01 is responsible for pathogenicity in chicks after intracerebral injection. Furthermore, it was found that amino acids at positions 50 and 98 of the NP of 47/01 are related to the pathogenicity in chicks after intracerebral inoculation. The NP is known as a protein that encapsidates the virus genome, forming the nucleocapsid, which packages of the viral RNA. Structural analysis of the NP indicates that amino acids at positions 50 and 98 are located in the body domain of the NP [31]. Amino acids at positions 50 and 98 of the NP are in one of the three regions where the NP binds to the PB2 protein [2]. It has been suggested that the efficiency with which a given nucleocapsid ensures transcription and replication of virus RNA in 293T cells is related to the efficiency of the binding of NP to the PB1-PB2-PA complex [11]. On the basis of virus RNA replication, the NP is believed to play a major role in the switch from mRNA transcription to cRNA synthesis by interacting with one or more polymerase proteins through a conformational change in the polymerase complex [15–17, 24]. These reports indicate that the NP is indirectly responsible for virus replication and for determining cell/host tropism by controlling viral polymerase activity

In the present study, an LPAIV defined by the IVPI test caused severe pathogenicity in one-day-old chicks after intracerebral inoculation. The definition of the pathogenicity of avian influenza viruses by the IVPI or amino acid sequence at the cleavage site of the HA is the result of interactions between pathogens and hosts, and this may conceal other important interactions or factors. Although the HA should play a major role in pathogenicity of avian influenza virus, virus pathogenicity associated with viral proteins other than HA has been observed in many studies. We believe that the present results will be helpful for investigating the intracerebral pathogenicity or neuropathology of avian influenza viruses.

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Memory Immune Responses against Pandemic (H1N1) 2009 Influenza Virus Induced by a Whole Particle Vaccine in Cynomolgus Monkeys Carrying Mafa-A1*052:02

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Abstract

We made an H1N1 vaccine candidate from a virus library consisting of 144 (=16 HA×9 NA) non-pathogenic influenza A viruses and examined its protective effects against a pandemic (2009) H1N1 strain using immunologically naïve cynomolgus macaques to exclude preexisting immunity and to employ a preclinical study since preexisting immunity in humans previously vaccinated or infected with influenza virus might make comparison of vaccine efficacy difficult. Furthermore, macaques carrying a major histocompatibility complex class I molecule, Mafa-A1*052:02, were used to analyze peptide-specific $CD8^+$ T cell responses. Sera of macaques immunized with an inactivated whole particle formulation without addition of an adjuvant showed higher neutralization titers against the vaccine strain A/Hokkaido/2/1981 (H1N1) than did sera of macaques immunized with a split formulation. Neutralization activities against the pandemic strain A/Narita/1/2009 (H1N1) in sera of macaques immunized twice with the split vaccine reached levels similar to those in sera of macaques immunized once with the whole particle vaccine. After inoculation with the pandemic virus, the virus was detected in nasal samples of unvaccinated macaques for 6 days after infection and for 2.67 days and 5.33 days on average in macaques vaccinated with the whole particle vaccine and the split vaccine, respectively. After the challenge infection, recall neutralizing antibody responses against the pandemic virus and $CD8^+$ T cell responses specific for nucleoprotein peptide NP262-270 bound to Mafa-A1*052:02 in macaques vaccinated with the whole particle vaccine were observed more promptly or more vigorously than those in macaques vaccinated with the split vaccine. These findings demonstrated that the vaccine derived from our virus library was effective for pandemic virus infection in macaques and that the whole particle vaccine conferred more effective memory and broader cross-reactive immune responses to macaques against pandemic influenza virus infection than did the split vaccine.

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Introduction

A pandemic (2009) H1N1 influenza A virus has been transmitted among humans since April 2009 [1]. We revealed that the pandemic (2009) H1N1 virus replicated efficiently in non-human primates and caused more severe pathological changes in the lungs of infected macaques than did a circulated human H1N1 (Russian flu) virus [2]. A substantial number of hospitalized individuals did not have underlying health issues during the pandemic [3,4], and their symptoms were as severe as those seen in cynomolgus macaques [2,5,6]. In addition, cynomolgus macaques are susceptible to other unadapted human influenza viruses after minimal passages in cell culture for isolation of the virus [7]. Since the clinical symptoms seen in cynomolgus macaques infected with influenza viruses closely reflect the signs of disease observed in humans, cynomolgus macaque models of influenza virus infection are useful for predicting symptoms and extrapolating pathogenesis in humans. Therefore, we examined the efficacy of vaccines against pandemic (H1N1) 2009 influenza virus using macaques.

In the present study, we selected a vaccine strain from a non-pathogenic influenza A virus library that contains 144 different

combinations of 16 hemagglutinins (HA) and 9 neuraminidases (NA) subtypes, and we examined the efficacy of the vaccine [8–11], and then compared differences in formulations of vaccines, whole particle vaccines and split vaccines. Although the efficacy of whole particle vaccines has been described previously in humans [12], it is difficult to exclude disturbance of pre-existing immunity due to previous infection with influenza viruses [2,13,14]. We used immunologically naïve non-human primates to test the vaccine efficacy with focus on induction of memory cytotoxic T lymphocyte (CTL) responses. In addition, animal models enable examination of the time lag between infection with a virus and initiation of immune responses, which is shorter in recall memory responses than in primary responses. Thus, non-human primates would be excellent tools to examine memory responses after vaccination.

A problem in studies using non-human primates is the difficulty in searching for epitopic peptides in individual animals to analyze peptide-specific T cell responses since major histocompatibility complex (MHC) genes are polymorphic and most of the macaques used for biomedical research are not inbred strains [15–18]. To solve this problem and to precisely analyze CTL responses specific for influenza virus peptides in macaques, we used macaques expressing Mafa-A1*052:02, which was observed at a frequency of 17% in the Mafa-A1 allele of cynomolgus macaques originating from the Philippines (Shiina et al., unpublished data).

To examine peptide-specific memory CTL responses, a Mafa-A1*052:02- binding motif and epitopes of nucleoprotein (NP) of the pandemic virus were determined using two approaches. Firstly, we used a peptide-binding assay with overlap peptides. These peptides were mixed with cells lacking transporter associated with antigen processing (TAP) proteins, which do not present endogenous cytosolic peptides on MHC class I molecules or do not allow stable expression of MHC class I molecules on the cell surface unless appropriate exogenous peptides are added [19,20]. Therefore, binding of peptides to MHC class I is detected as stable expression of MHC class I molecules. Secondly, we identified naturally processed peptides eluted from MHC molecules using liquid chromatography with tandem mass spectrometry (LC-MS/MS) and a genetic information database as previously described [21]. Consequently, we determined NP peptides that bound to Mafa-A1*052:02.

Recall memory CTL responses specific for NP peptides in lymph node cells from macaques immunized with the whole particle vaccine were more vigorous than those in lymph node cells from macaques immunized with the split vaccine. Furthermore, recall neutralization antibody responses against the pandemic challenge strain in macaques vaccinated with the whole particle vaccine were observed more promptly than those in macaques vaccinated with the split vaccine. Therefore, the results suggest that the inactivated whole particle vaccine is more effective against pandemic influenza virus infection than is the split vaccine, inducing cross-reactive memory CTL and antibody responses.

Results

Antibody Responses After Inoculation of an Inactivated Whole Virus Particle Vaccine or a Split Vaccine Prepared from a Non-pathogenic H1N1 Strain in a Virus Library

To compare immune responses in different vaccine formulations, we prepared an inactivated whole viral particle vaccine and a split vaccine using the A/swine/Hokkaido/2/1981 (H1N1) (Hokkaido2) strain that was selected from our virus library previously described [8]. The vaccine was subcutaneously inoculated twice into each macaque, and antibody responses in the vaccinated macaques were examined during a 7-week period between the second vaccination and challenge with an H1N1 pandemic virus. The macaques showed no systemic symptoms after the vaccination. No skin reaction at the site of injection was observed after inoculation with either the whole particle vaccine or split vaccine.

Serum IgM specific for Hokkaido2 antigen was clearly detected in 2 of the 3 macaques vaccinated with the whole particle vaccine one week after the first vaccination and in all macaques vaccinated with the whole particle vaccine 2 weeks after the first vaccination (before the second vaccination that was performed 2 weeks after the first vaccination) (Fig. 1A). In the 3 macaques vaccinated with the whole particle vaccine, serum total IgG, IgA, and IgG1 specific for Hokkaido2 were detected 2 weeks after the first vaccination and production of IgG1 antibody was enhanced after the second vaccination (Fig. 1B–D). Antigen-specific antibody levels gradually declined from 2 weeks after the second vaccination. On the other hand, serum IgM, IgG, IgA and IgG1 responses specific for Hokkaido2 in macaques vaccinated with the split vaccine were lower than those in macaques vaccinated with the whole particle vaccine (Fig. 1A–D). Antigen-specific IgG antibodies in nasal swab samples from all of the macaques vaccinated with the whole particle vaccine and 2 of the 3 macaques vaccinated with the split vaccine (except #750) were detected 2 weeks after the first vaccination (Fig. 1E). Antigen-specific IgA was detected in swab samples from 2 macaques vaccinated with the whole particle vaccine (#312 and #784) and in swab samples from all 3 macaques vaccinated with the split vaccine (Fig. 1F).

Next, we examined neutralization activity of serum antibody from macaques vaccinated with the whole particle or the split vaccine. Sera from macaques vaccinated with the whole particle vaccine showed significantly higher neutralization activity against Hokkaido2 than did sera from macaques inoculated with the split vaccine from week 2 to week 8 ($P<0.05$) (Fig. 2A) and less potent neutralization activity against the pandemic strain A/Narita/1/2009 (H1N1) pdm (Narita1) [22,23] than against Hokkaido2 (Fig. 2B). Sera from two macaques vaccinated with the split vaccine 2 weeks after the first vaccination showed no detectable neutralization activity against Narita1. Sera from two macaques (#729, #742) vaccinated with the split vaccine 2 weeks after the second vaccination (week 4) showed neutralization activity against Narita1 at a level comparable to that in sera from macaques vaccinated once with the whole particle vaccine (week 2). The average of 50% neutralization titers against Narita1 in sera from macaques vaccinated with the whole particle vaccine was significantly higher than that in sera from macaques vaccinated with the split vaccine in week 8 ($P<0.05$) (Fig. 2B). These results suggested that a single vaccination with a whole particle vaccine has potency similar to that of two inoculations with split vaccines in serum neutralization activity.

T lymphocyte Responses Specific for Vaccine Antigens After the Second Vaccination in Cynomolgus Macaques

Two weeks after the second vaccination (week 4), cytokine production by T lymphocytes specific for the vaccine antigen was analyzed. $CD4^+$ and $CD8^+$ T cells from all macaques vaccinated with the whole particle vaccine produced interferon (IFN)-γ and interleukin (IL)-2 (Fig. 3). In contrast, T cells from macaques vaccinated with the split vaccine produced smaller amounts of IFN-γ and IL-2 than did those from macaques vaccinated with the

Figure 1. Antibody responses specific for H1N1 vaccine antigens in cynomolgus macaques immunized with vaccines

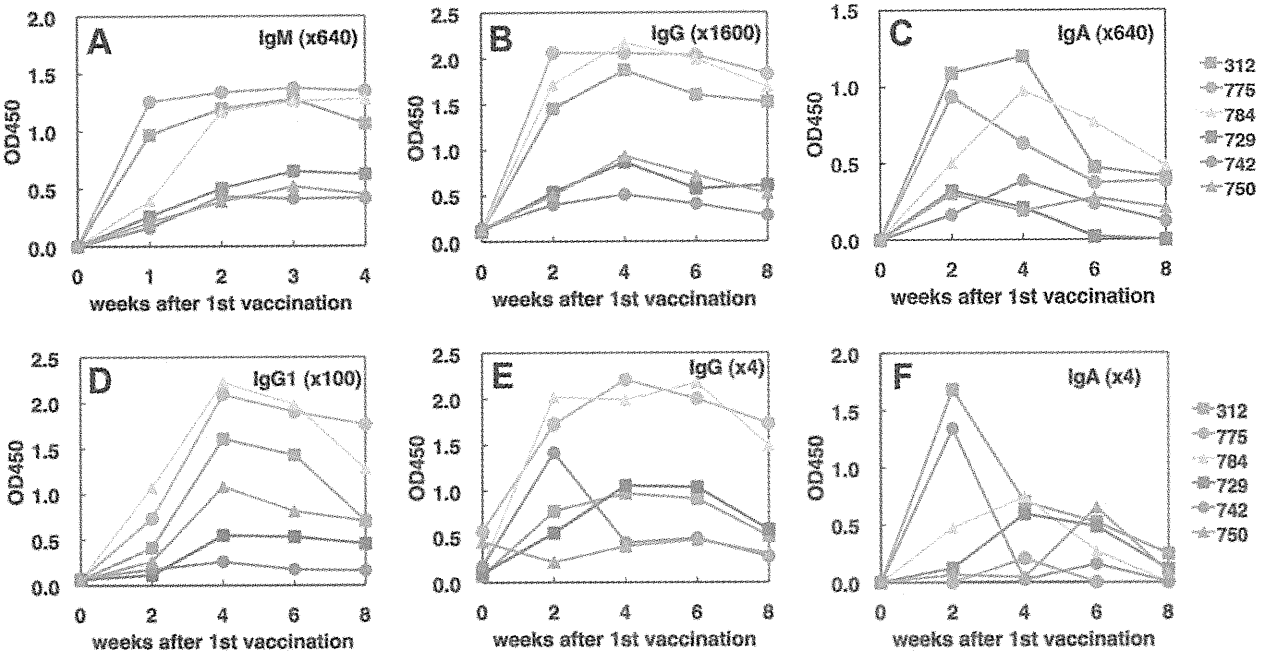

Figure 1. Antibody responses specific for H1N1 vaccine antigens in vaccinated cynomolgus macaques. Cynomolgus macaques were subcutaneously immunized twice (in weeks 0 and 2) with a whole virus particle vaccine (#312, #775 and #784, orange symbols) or with a split vaccine (#729, #742 and #750, blue symbols) derived from Hokkaido2. Sera and swab samples were collected in indicated weeks after the first vaccination. IgM (A), IgG (B, E), IgA (C, F), and IgG1 (D) antibodies specific for Hokkaido2 antigens in sera (A-D) and nasal swab samples (E, F) were analyzed using ELISA. Optical densities at 450 nm are shown. In IgM and IgA measurements (A, C, F), the OD450 values before vaccination as non-specific background responses were subtracted from those after vaccination.
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Figure 2. Neutralization activity of sera in the vaccinated cynomolgus macaques.

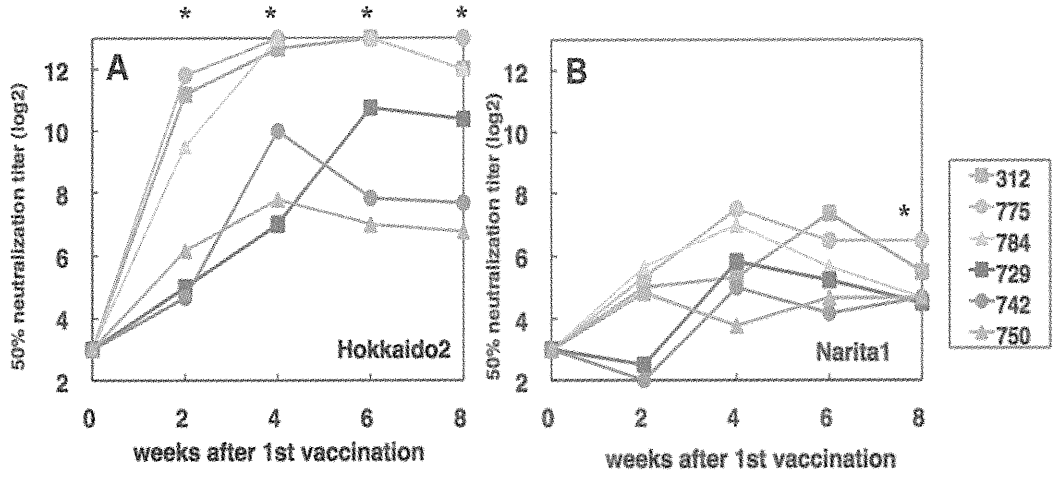

Figure 2. Neutralization activity of sera in the vaccinated cynomolgus macaques. Sera were collected in the indicated weeks after the first vaccination. Sera of week 2 were obtained before the second vaccination. The neutralization titers against Hokkaido2 (A) and Narita1 (B) were expressed as reciprocals of dilution of the serum samples that showed CPE in 50% of the wells. Detection limits were 1:8 in week 0 and 1:4 in the other weeks. P values calculated with Student's *t*-test are less than 0.05 in comparison between the whole particle vaccine group and split vaccine group against Hokkaido2 and Narita1 (*) when the titers below 1:4 are calculated as 1:4.
doi:10.1371/journal.pone.0037220.g002

Figure 3. Cytokine production by T lymphocytes from the vaccinated macaques.

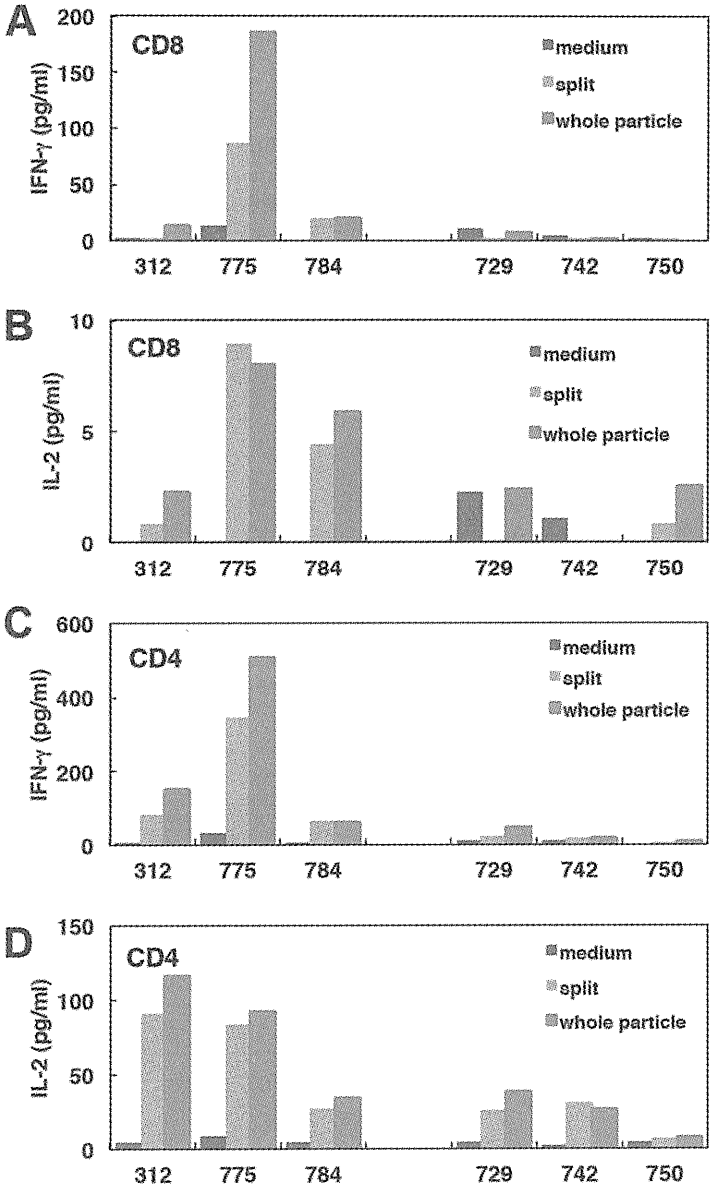

Figure 3. Cytokine production by T lymphocytes from the vaccinated macaques. $CD8^+$ cells (A, B) and $CD4^+$ cells (C, D) were separated from the blood cells 2 weeks after the second vaccination (week 4). T lymphocytes were cultured with irradiated APC and the whole particle or split vaccine antigen (10 μg/ml) for 48 h. Culture without antigen is indicated as medium. Cytokines (IFN-γ; A, C, IL-2; B, D) in the supernatants were measured with a multiple cytokine array. IL-4 production was under the detection limit.
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whole particle vaccine even when cells were stimulated with the whole particle antigen, though the differences were not statistically significant. These results indicated that subcutaneous vaccinations with the whole particle vaccine induced greater T cell responses, especially Th1 type responses in both $CD4^+$ and $CD8^+$ T cells, than did the split vaccine, as well as IgG1 antibody responses (Fig. 1), which are assisted by Th1 cells in humans.

Protective Effects of Two Formulations of the Inactivated Vaccine Against a Pandemic (H1N1) 2009 Strain in Cynomolgus Macaques

Next, to examine efficacy of the vaccines, the pandemic (H1N1) 2009 virus strain, Narita1, was inoculated into nasal cavities of macaques 7 weeks after the second vaccination. Body temperature was expressed by calculating the average of the highest and lowest

temperatures in one day, and body temperature after the virus challenge was compared with that before the virus challenge. When we focused on temperature changes until day 7 after infection, higher body temperature than that before the challenge was observed for 7 to 8 days after infection in the unvaccinated macaques (7.67 days on average, Fig. 4, left panels), while raised body temperatures in the macaques vaccinated with the whole particle vaccine and in macaques vaccinated with the split vaccine were observed for 2 to 8 days (5 days on average, Fig. 4, middle panels) and 6 to 7 days (6.33 days on average, Fig. 4, right panels), respectively, though differences among the three groups were not statistically significant. Therefore, these results indicated a tendency for vaccination with inactivated Hokkaido2 to accelerate recovery of body temperature and reduce morbidity caused by infection with the pandemic influenza virus. No macaques either with or without vaccination lost weight or appetite after inoculation with Narita1 (data not shown).

We examined the virus titers in swab samples after challenge with the pandemic virus Narita1. The virus was detected in swab samples from nasal cavities of the unvaccinated macaques for 6 days (6 days on average) and in swab samples from tracheas for 3 to 5 days (4.33 days on average) after inoculation with Narita1 (Fig. 5A, D). On the other hand, in the macaques vaccinated with the whole particle vaccine, the virus was detected until day 5 (3.67 days on average) in nasal samples and until day 4 (3.67 days on average) in tracheal samples (Fig. 5B, E). The virus in nasal and tracheal samples of macaques vaccinated with the split vaccine was detected until day 6 (5.33 days on average) and until day 3 (3 days on average), respectively (Fig. 5C, F). The average areas under the curves (AUC) of virus titers were 16.6, 4.37 and 13.4 $\log_{10}$TCID$_{50}$/ml•day in nasal samples and 4.50, 2.30 and 3.95 $\log_{10}$TCID$_{50}$/ml•day in tracheal samples of macaques inoculated with saline, the whole particle vaccine and the split vaccine, respectively. Significant differences of virus titer AUC in nasal samples were observed between the saline group and whole particle vaccine group ($P = 0.016$) and between the whole particle vaccine group and split vaccine group ($P = 0.03$). Therefore, inoculation with the whole particle vaccine of Hokkaido2 derived from the non-pathogenic virus library interfered with propagation of the pandemic strain Narita1 in the upper respiratory tracts more effectively than did inoculation with the split vaccine.

Memory Antibody Responses after Challenge Infection with the Pandemic Influenza Virus

We examined recall antibody responses against the vaccine strain Hokkaido2 and the pandemic strain Narita1 after challenge infection. In the sera from macaques without vaccination, neutralization activity against Hokkaido2 and Narita1 was detected 6 to 10 days after challenge infection with Narita1 (Fig. 6A, D). Neutralization activity against Hokkaido2 and Narita1 was observed in the sera from macaques vaccinated with the whole particle vaccine before the challenge infection (day 0) (Fig. 6B, E), and neutralization titers against Narita1 were increased 2 days after the challenge, suggesting that the challenge infection induced immediate recall antibody responses against Narita1.

Figure 4. Body temperatures of macaques after inoculation of the pandemic (H1N1) 2009 influenza virus.

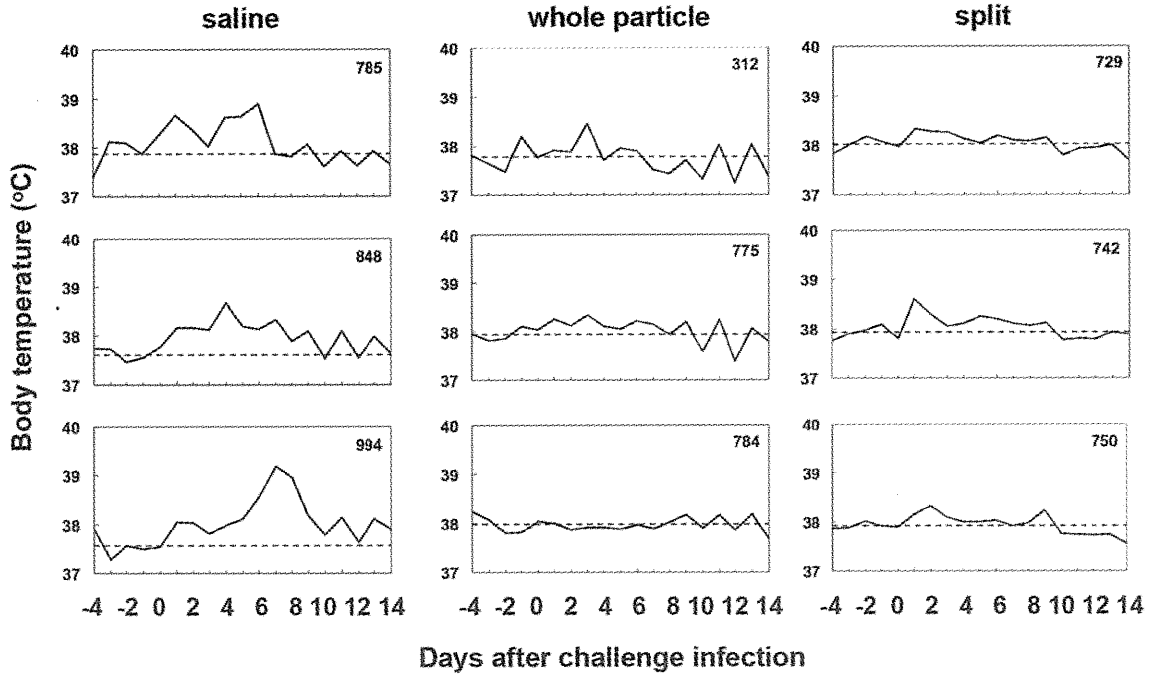

Figure 4. Body temperatures of macaques after inoculation of the pandemic (H1N1) 2009 influenza virus. Cynomolgus macaques were subcutaneously immunized with the whole particle vaccine (middle column) or with the split vaccine (right column). Cynomolgus macaques injected with saline were used as unvaccinated controls (left column). On day 0, 7 weeks after the second vaccination, Narita1 (2×10^5 TCID$_{50}$) was inoculated into nasal cavities of the macaques. Lines horizontally drawn indicate average temperature levels at pre-infection.
doi:10.1371/journal.pone.0037220.g004

Figure 5. Virus recovery from macaques inoculated with Narita1.

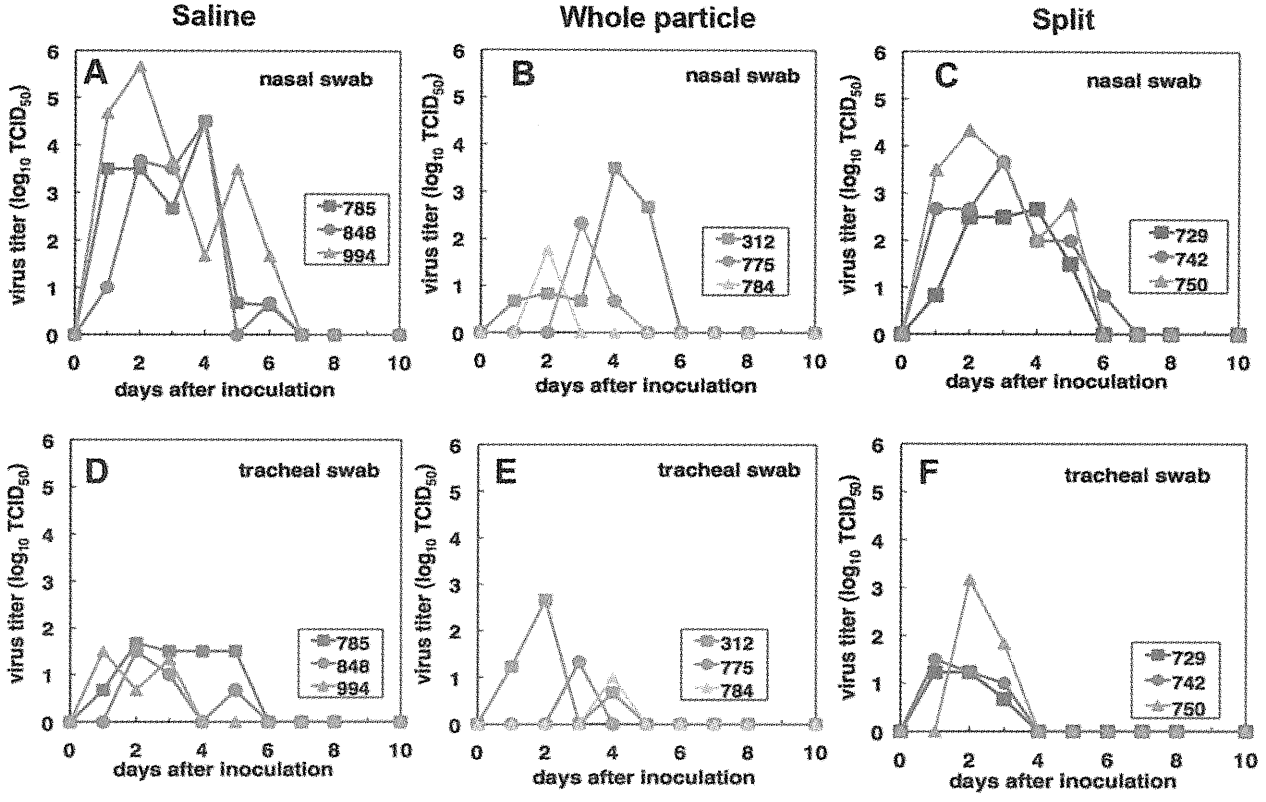

Figure 5. Virus recovery from macaques inoculated with Narita1. Each macaque was inoculated with Narita1 on day 0 (saline: A, D; whole particle vaccine, B, E; split vaccine, C, F). Virus titers in nasal (A-C) and tracheal (D-F) swab samples were determined using MDCK cells. Virus titers were under detection limit after day 7.
doi:10.1371/journal.pone.0037220.g005

Low neutralization activity in sera from macaques vaccinated with the split vaccine was observed against Hokkaido2 but not against Narita1 before the challenge infection, and neutralization activity against Hokkaido2 was increased on days 4 to 6 after the challenge (Fig. 6C, F). On the other hand, neutralization activity against Narita1 was increased on days 8 to 10 in sera from macaques vaccinated with the split vaccine and was significantly lower on day 4 and day 6 than that with the whole particle vaccine ($P<0.05$). These responses were similar to responses observed in the unvaccinated macaques. These findings indicate that vaccination with the whole particle vaccine generated immunological memory in B cells that produced antibodies against not only the vaccine strain but also the challenge strain (i.e., crossreaction), whereas vaccination with the split vaccine induced memory B cell responses against the vaccine antigens but not against the antigens of the challenge strain.

Identification of Nucleoprotein Peptides that Induced Mafa-A1*052:02-restricted CD8$^+$ T cell Responses After Challenge with the Pandemic (H1N1) 2009 Strain

Next, we examined memory CTL responses in macaques vaccinated with the whole particle vaccine and the split vaccine. To measure antigen-specific CTL responses, we used macaques carrying an MHC class I molecule, Mafa-A1*052:02 (Table 1). Furthermore, we prepared 10-mer influenza virus peptides derived from NP, which overlapped 5 amino acids in consecutive peptides and showed amino acid sequences of some peptides since NP was shown in mice to be a nuclear/cytosolic target antigen for CTL (Fig. 7A). Firstly, we determined peptides bound to Mafa-A1*052:02. To perform a peptide binding assay, we established an RMA-S cell line that lacked TAP-2 protein for presentation of endogenous peptides and expressed Mafa-A1*052:02 and human β2-microglobulin for presentation of exogenous peptides. Using the RMA-S transfectant, we found that 3 NP peptides (#16, #53 and #74) bound to Mafa-A1*052:02 (Fig. 7B). NP#44 showed a weak binding capacity to Mafa-A1*052:02 on the surface of the RMA-S transfectant.

We cultured CD8$^+$ T cells from Narita1-infected macaques with the NP peptides and measured IFN-γ responses. IFN-γ production was detected in the culture of CD8$^+$ T cells from macaques vaccinated with the whole particle vaccine with NP#44, #45, #53 and #74 and from macaques vaccinated with the split vaccine with NP#12, #16, #37, #44, #53 and #74 (Fig. 7C).

Since IFN-γ production by stimulation with NP#53 was observed in the culture of cells from all vaccinated macaques (Fig. 7C), we performed further analyses of the NP#53 peptide. A peptide lacking C-terminal residue 270 (NP261-269) lost binding capacity to Mafa-A1*052:02 (Fig. 8A, B), suggesting that the residue 270 V was one of the anchor residues. Furthermore, deletion of residue 261 enhanced binding of the peptide (NP262-270) to Mafa-A1*052:02, and NP263-270 showed binding to Mafa-A1*052:02 similar to that of NP261-270, suggesting that

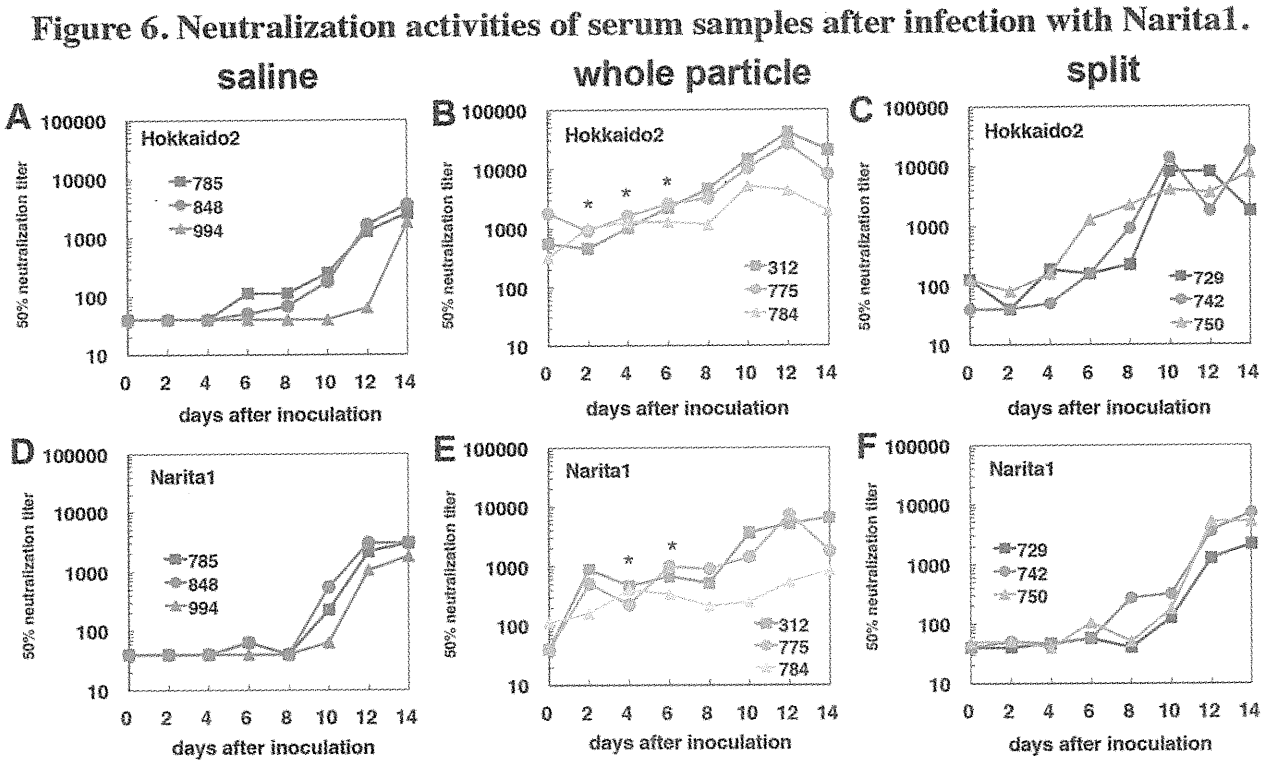

Figure 6. Neutralization activities of serum samples after infection with Narita1. Cynomolgus macaques were vaccinated with saline (A, D), whole particle vaccine (B, E), or split vaccine (C, F) as described in the legend to Fig. 1. Macaques were inoculated with Narita1 as described in the legend to Fig. 4. Sera were collected on indicated days after the challenge infection. Fifty percent neutralization titers against Hokkaido2 (A-C) and Narita1 (D-F) were determined. Statistically significant differences of average neutralization titers between the whole particle vaccine group and split vaccine group are indicated with asterisks ($P<0.05$ with Student's *t*-test).
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NP262-270 was an optimal sequence bound to Mafa-A1*052:02. Henceforth, we used NP262-270 as a basic peptide bound to Mafa-A1*052:02. Deletion of residue 263 impaired binding to the Mafa-A1*052:02 transfectant, indicating that position 263 was one of the anchor residues or that it affected binding capacity.

To determine the precise anchor residues, we used a series of peptides of which amino acids were substituted (Fig. 8C). Substitutions at residues 264, 266 and 270 diminished binding of peptides to Mafa-A1*052:02 (Fig. 8D), suggesting that residues 264 (position 3 from the N-terminus), 266 (position 5) and 270 (position 9) were anchor residues that were essential for presentation on Mafa-A1*052:02 and that L at position 3, L at position 5 and V at position 9 comprised a binding motif for Mafa-A1*052:02. Substitutions at residues 263, 265 and 267 had weak effects on the binding of peptides to Mafa-A1*052:02.

We examined sequences of naturally processed peptides bound to Mafa-A1*052:02 using a 721.221 transfectant established by introduction of a Mafa-A1*052:02 gene into an MHC null human B cell line and LC-MS/MS [21]. In analyses of peptides eluted from Mafa-A1*052:02 with 9 to 12 amino acids in length, hydrophobic residues were observed at position 9 when C-terminal residues were aligned as position 9 (Table 2). In addition, leucine or isoleucine was detected at position 5 in 8 of the 15 peptides, and leucine, isoleucine or valine was detected at position 3 in 4 of the 15 peptides. This motif was compatible with the sequence of NP262-270 (SALILRGSV).

Memory T Cell Responses After Challenge with the Pandemic (H1N1) Strain in Vaccinated Macaques

Finally, we examined $CD8^+$ T cell responses against NP262-270 using cervical lymph node cells of the infected macaques. IFN-γ production in $CD8^+$ T cells was observed after culture with NP262-270 and restimulation (Fig. 9A). The percentage of IFN-γ-producing cells in $CD8^+$ T cells stimulated *in vitro* with NP262-270 from macaques vaccinated with the whole particle vaccine (48.3% on average) was significantly higher than the percentage of those from macaques vaccinated with saline (24.6% on average, $P = 0.034$ in saline vs. whole particle vaccine) and with the split vaccine (21.7% on average, $P = 0.049$ in whole particle vaccine vs. split vaccine) (Fig. 9B). Since IFN-γ production in $CD8^+$ T cells from the macaques inoculated with saline was the primary response with Narita1 infection, the higher percentage of IFN-γ-producing $CD8^+$ T cells in the whole particle group than that in the saline group seemed to indicate memory responses induced by vaccination. These responses were likely to be memory recall responses since the recall responses in macaques inoculated with the whole particle vaccine were examined 7 weeks after the vaccination. In contrast, there was no significant difference in the percentage of IFN-γ-producing $CD8^+$ cells between the saline group and split vaccine group (Fig. 9B), though IFN-γ production level per cell (mean fluorescense intensity) in the split vaccine group was higher than that in the saline group (Fig. 9A). These results indicated that the whole particle vaccine more effectively generated memory T cells than did the split vaccine.