

# Significant neutralizing activities against H2N2 influenza A viruses in human intravenous immunoglobulin lots manufactured from 1993 to 2010

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**Abstract:** Influenza A H2N2 virus, also known as the Asian flu, spread worldwide from 1957 to 1967, although there have been no cases reported in humans in the past 40 years. A vaccination program was introduced in Japan in the 1960s. Older Japanese donors could have been naturally infected with the H2N2 virus or vaccinated in the early 1960s. Human intravenous immunoglobulin (IVIG) reflects the epidemiological status of the donating population in a given time period. Here, the possible viral neutralizing (VN) activities of IVIG against the H2N2 virus were examined. Hemagglutination inhibition (HI) and VN activities of IVIG lots manufactured from 1993 to 2010 in Japan and the United States were evaluated against H2N2 viruses. High HI and VN activities against H2N2 viruses were found in all the IVIG lots investigated. HI titers were 32–64 against the isolate in 1957 and 64–128 against the isolates in 1965. VN titers were 80–320 against the isolate in 1957 and 1280–5120 against the isolates in 1965. Both the HI and VN titers were higher against the isolate in 1965 than in 1957. Thus, antibody titers of IVIG against influenza viruses are well correlated with the history of infection and the vaccine program in Japan. Therefore, evaluation of antibody titers provides valuable information about IVIGs, which could be used for immune stimulation when a new influenza virus emerges in the human population.

**Keywords:** IVIG, influenza, H2N2, neutralization

## Introduction

The highly pathogenic avian influenza A H5N1 virus has spread among wild birds worldwide. As of April 2012, there have been 602 cases of human infections, with an extremely high mortality rate of about 59%.<sup>1</sup> Therefore, there are public health concerns regarding the possible global emergence of an H5N1 pandemic virus. However, a swine-origin pandemic influenza A H1N1 virus suddenly emerged in 2009. This novel virus spread among human populations within a short period of time because of the low level of immune responses against the virus, especially among young people. Thus, a pandemic influenza virus may be transmitted to humans because of limited immune responses against the virus in humans. The H2N2 virus could be considered one such virus, because it was prevalent in humans between 1957 and 1967, and a vaccination program was introduced in Japan in the 1960s.<sup>2</sup> However, although H2N2 continues to circulate among birds and pigs, this virus has not infected humans for the last several decades.<sup>3</sup> Based on the above background, it might be worthwhile examining the neutralizing activity of human intravenous immunoglobulin (IVIG) against the H2N2 virus.

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Generally, IVIGs are manufactured in individual lots with serum donations from over 10,000 healthy donors. Therefore, IVIG contains various antibodies against numerous human pathogens including seasonal influenza viruses. In fact, not only current (manufactured in 2008) but also previous (manufactured in 1999) IVIGs contain antibodies with significant neutralizing titers against seasonal and pandemic 2009 influenza viruses.<sup>4</sup> The antibodies comprising IVIG therefore reflect the epidemiological status of the donating population, in a particular time period and geographical area.

In this study, IVIG lots manufactured from 1993 to 2010 were evaluated for hemagglutination inhibition (HI) and virus neutralizing (VN) activities against the H2N2 virus.

## Material and methods

Clinical isolates of H2N2 (A/Okuda/1957, A/Izumi/5/1965, A/Kaizuka/2/1965), IVIGs manufactured from 1993 to 2010 from healthy donors in Japan (currently Kenketsu Venoglobulin®-IH; Benesis Corporation, Osaka, Japan), and IVIGs manufactured in 1993 and 1999 from healthy donors in the United States were used in this study (Table 1).

The viruses were propagated in Madin–Darby canine kidney (MDCK) cells or in the allantoic cavity of 11-day-old embryonated chicken eggs. The culture media and allantoic fluids were stored at  $-80^{\circ}\text{C}$  prior to use. Viral infectivity (FFU/mL) was titrated in MDCK cells using the peroxidase-antiperoxidase (PAP) staining technique.<sup>5</sup> The HI test using 0.7% guinea pig erythrocytes was carried

out as described previously.<sup>5</sup> HI titers are expressed as the reciprocal of the highest dilution of the IVIG preparation showing inhibition. The VN test was also carried out as described previously.<sup>5</sup> Briefly, each IVIG was serially diluted two-fold with serum-free minimal essential medium. The IVIG dilutions (30  $\mu\text{L}$  of each) were mixed with 100 FFU (30  $\mu\text{L}$ ) of virus. After incubation for 30 minutes at  $37^{\circ}\text{C}$ , the mixture (30  $\mu\text{L}$ ) was applied to MDCK cells in a 96-well microplate. After incubation for 16 hours, the cells were fixed with ethanol and stained using PAP technique. The results are expressed as the reciprocal of the dilution resulting in 50% neutralization ( $\text{VN}_{50}$ ).

## Results

As summarized in Table 1, the titers were significant against all three isolates of H2N2: HI titers of 32–64 and  $\text{VN}_{50}$  titers of 80–320 against Okuda/1957, HI titers of 64–128 and  $\text{VN}_{50}$  titers of 1280–5120 against Izumi/5/1965, and HI titers of 64–128 and  $\text{VN}_{50}$  titers of 1280–2560 against Kaizuka/2/1965. Both the HI and  $\text{VN}_{50}$  titers were higher against the isolate in 1965 than in 1957 (Table 1). There were no apparent differences in the HI and  $\text{VN}_{50}$  titers against H2N2 virus isolates among the IVIG products prepared in the United States and Japan in different years.

## Discussion

Currently, most of the donor population has not been exposed to the H2N2 virus. IVIG is manufactured from pooled plasma. In a previous study, HI and VN activities against seasonal influenza H1N1 and H1N1-pdm2009 were examined in IVIG lots manufactured from 1999 to 2008. The IVIGs indicated high and stable HI and VN activity against H1N1. It was taken into account that the donor population may have been immunized through native infections and/or vaccine programs. Interestingly, the IVIGs also showed low but significant HI and VN activities against H1N1-pdm2009 despite most of the donor population never having been exposed to swine H1N1 or the Spanish flu.<sup>4</sup>

In this study, HI and VN activities against the H2N2 virus, which has not been included in vaccine programs for the past 45 years, were measured in IVIGs manufactured from 1993 to 2010 in Japan. Both the HI and  $\text{VN}_{50}$  titers were higher against H2N2 isolates in 1965 than in 1957. It has been reported that people under the age of 50 years have little or no immunity to H2N2 and that people older than 50 years who have been exposed to the virus show a higher rate of resistance.<sup>6</sup> Therefore, older Japanese donors could have been naturally infected with the H2N2 virus or vaccinated in the

**Table 1** Cross-reactivity of intravenous immunoglobulins to the influenza H2N2 virus

IVIG	H2N2 virus					
	Okuda/ 1957		Izumi/5/ 1965		Kaizuka/2/ 1965	
	HI	$\text{VN}_{50}$	HI	$\text{VN}_{50}$	HI	$\text{VN}_{50}$
1993JP, Lot A	32	160	64	1280	64	1280
1993US, Lot B	64	320	128	5120	128	2560
1999JP-A, Lot C	32	160	64	1280	64	1280
1999JP-B, Lot D	32	80	64	1280	64	1280
1999JP-C, Lot E	32	160	64	1280	64	1280
1999JP-D, Lot F	32	160	64	1280	64	1280
1999US, Lot G	64	320	128	5120	128	2560
2000JP, Lot H	32	160	64	1280	64	1280
2003JP, Lot I	32	80	64	1280	64	1280
2006JP, Lot J	32	160	64	1280	64	1280
2007JP, Lot K	32	160	64	1280	64	1280
2008JP, Lot L	32	160	64	1280	64	1280
2009JP, Lot M	32	80	64	1280	64	1280
2010JP, Lot N	32	80	64	1280	64	1280

**Abbreviations:** HI, hemagglutination inhibition titer; IVIG, intravenous immunoglobulin; JP, Japan; US, United States;  $\text{VN}_{50}$ , virus neutralization titer giving 50% inhibition of infectivity.

early 1960s. Consequently, antibody titers against the H2N2 virus in the general population will decrease in the future, which could lead to an H2N2 pandemic. It is not clear why such differences of HI and VN activities of IVIG preparations were observed between the United States and Japan. One possible explanation may be derived from the difference in environmental factors and population for blood donation between the two countries.

## Conclusion

The results suggest that the antibody titers against influenza viruses in IVIGs correlate with the history of infection and with vaccination programs. Therefore, the evaluation of antibody titers provides valuable information about IVIGs, which could be used for immune stimulation when a new influenza virus emerges in the human population.

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## Disclosures

Yoshinobu Okuno and Ritsuko Kubota-Koketsu are employed by The Research Foundation for Microbial Diseases of Osaka University. Mikihiro Yunoki is employed by the Benesis Corporation. All other authors report no conflicts of interest in this work.

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