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Significant neutralizing activity of human immunoglobulin preparations against pandemic 2009 H1N1

The influenza-like illness that began in the Unites States and Mexico was first reported by the World Health Organization (WHO) on 24 April, 2009, and declared a phase 6 pandemic on 11 June. As of 6 July 2009, over 90 000 cases and more than 400 deaths in some 120 countries had been confirmed (WHO, 2009). Importantly, on July 8th, the WHO announced that oseltamivir (Tamiflu)-resistant viruses had been identified in Denmark, Japan and Hong Kong (WHO, 2009).

The pandemic virus 2009 H1N1 was a triple reassortant of human-, swine- and avian-derived influenza A virus segments and the HA gene was classified as being of swine-origin (Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, (2009). Evidence is accumulating that specific IgG antibodies against this virus are present in certain populations, especially the elderly (Itoh *et al*, 2009). However, Katz *et al* (2009) reported that cross reactive IgG against a pandemic influenza virus (A/California/04/2009) was found in no serum specimens of children aged 6 months–9 years old, 8% of samples from 5- to 9-year olds, 9% of samples from 18- to 64-year olds, 6% of samples of 18- to 40-year olds and 33% of samples of those over 60 years old, suggesting that immunoglobulin preparations derived from pooled plasma from over 10 000 healthy donors could contain such cross reactive IgG. The present study evaluated haemagglutinin-inhibition (HI) and virus neutralization (VN) activities against 2009 H1N1 and seasonal H1N1 as a positive control in intravenous human immunoglobulin (IVIG) preparations manufactured in 1999 and 2008.

An influenza A/H1N1 vaccine strain (A/New Caledonia/20/99), a clinical isolate of A/H1N1 (A/Osaka/16/2008), a classical swine isolate of A/H1N1 (A/Swine/Hokkaido/2/1981) and a pandemic influenza isolate of A/H1N1 (A/Osaka/168/2009 H1N1 pdm) were used in this study. Three lots (Lot. A, B and C) of IVIG derived from pooled plasma collected in Japan and manufactured in 2008 (IVIG2008JP, 'Kenketsu Venoglobulin®-IH Yoshitomi; Benesis Corp., Osaka, Japan') were also used. In addition, two lots of IVIG that were manufactured in 1999, derived from plasma pooled collected in Japan and the USA (IVIG1999JP 'Kenketsu Venoglobulin®-IH', IVIG1999US 'Venoglobulin®-IH; Yoshitomi Pharmaceutical Industries, Ltd. at the time, currently Benesis Corp.'), were used.

The viruses were propagated in Madin-Darby canine kidney (MDCK) cells or in the allantoic cavity of chicken embryonated eggs. The culture media and the allantoic fluids

Table I. Cross reactivity of several lots of IVIG against pandemic 2009, classical swine and seasonal H1N1 viruses.

	Pandemic 2009 H1N1 A/Osaka/168/2009(H1N1pdm)		Classical swine H1N1 A/Swine/Hokkaido/2/1981		Vaccine strain H1N1 A/NC/20/99		Clinical isolate H1N1 A/Osaka/16/2008	
IVIG	HI	VN	HI	VN	HI	VN	HI	VN
2008JP, Lot. A	8	64	8	64	160	640	20	160
2008JP, Lot. B	8	64	8	64	160	640	20	160
2008JP, Lot. C	8	64	8	64	320	1280	40	160
1999US, Lot. D	16	64	16	64	40	128	16	32
1999JP, Lot. E	8	32	4	64	10	32	4	8

JP, Japan; US, United States; HI, haemagglutinin-inhibition; VN, virus neutralization.

were stored at −80°C prior to use. Infectivity, as infectious focus-forming units (FFU) per ml, was titrated in MDCK cells using peroxidase and an anti-peroxidase (PAP) staining technique (Okuno *et al*, 1990). The haemagglutinin-inhibition (HI) test using 0·75% guinea pig red blood cells was carried out as described previously (Okuno *et al*, 1993). The results were expressed as the reciprocal of the highest dilution of the culture medium to show inhibition. The virus neutralization (VN) test was carried out as described (Okuno *et al*, 1990). Briefly, IVIG was diluted twofold with serum-free medium. The diluted IVIG (50 μl) was mixed with 100 FFU (50 μl) of virus, then applied to MDCK cells in a 96-well microplate. After culturing, the cells were fixed with ethanol and stained by PAP as above. The results were expressed as the reciprocal of the dilution giving 50% neutralization.

Intravenous human immunoglobulins were manufactured using plasma pooled from over 10 000 healthy donors. The HI and VN activities of IVIGs were titrated against pandemic, seasonal human and swine influenza A viruses (Table I). Of note, both the 1999 and 2008 IVIGs were shown to have anti pandemic and classical swine influenza A/H1N1 virus titres with HI (×4–×8) and VN (×32–×64). The 2008 IVIGs showed titres against the vaccine strain A/New Caledonia/20/99, which was isolated in 1999, with HI (×160–×320) and VN (×640–×1280), while the 1999 IVIGs showed titres with HI (×10–×40) and VN (×32–×128). These results suggested that the IVIG derived from the pooled plasma contained a certain amount of functional IgG, including IgG against pandemic or classical swine influenza A/H1N1. Of note, such IgG titres were slightly higher in the IVIG2008JP products compared with IVIG1999JP. However, the titres were slightly higher in IVIG1999US than in IVIG1999JP. Higher titres against the vaccine and clinical strains were observed in IVIG1999US than IVIG1999JP. Interestingly, the difference in the increase in titres against the vaccine strain was much greater between the products manufactured in 2008 and 1999 than between the others. This difference seems to be an outcome of vaccination. Our preliminary results showed a HI titre >×40 in 1·2% (7/580), ×20 in 3·1% (18/580) and ×10 in 4·3% (25/580), indicating the possible production of hyperimmune globulin with these sources of plasma collected in 2008, Japan.

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VKORC1 mutations in patients with partial resistance to phenprocoumon

Coumarin derivatives, such as warfarin, phenprocoumon and acenocoumarol, are used for long-term prevention of thromboembolic events. The management of oral anticoagulation with coumarin derivatives is complicated by a large variability in the dose-response relationship, which is partly determined by genetic constitution (Rost *et al*, 2004; Bodin *et al*, 2005; Sconce *et al*, 2005). Coumarins act by inhibiting the vitamin K epoxide reductase (VKOR), encoded for by the *VKORC1* (VKOR complex, subunit 1) gene. This enzyme recycles vitamin K epoxide to the reduced form of vitamin K, an essential cofactor in the formation of the active clotting factors II, VII, IX, and X and the inhibitors protein C and S through γ-glutamyl carboxylation. While the most common *VKORC1* genetic variants result in the need for lower doses of warfarin during long-term therapy (Rieder *et al*, 2005; Sconce *et al*, 2005), some genetic variants confer coumarin resistance (Bodin *et al*, 2005). International Normalized Ratio (INR) values in combination with a plasma concentration of the coumarin in use give a good indication of possible resistance (Harrington *et al*, 2008). The known *VKORC1* sequence variants associated with coumarin resistance were recently summarised (Peoc'h *et al*, 2009).

We report three patients presented with confirmed (partial) coumarin resistance. Patient 1 was initially treated with acenocoumarol 12 mg/day, and subsequently with phenprocoumon, up to 9 mg/day. At this dose the phenprocoumon serum concentration, determined by non-stereospecific reversed phase high performance liquid chromatography and diode array detector detection, was 4·3 mg/l (therapeutic range 1–3 mg/l). The INR did not rise above 1·4. Patient 2 was treated with up to 9 mg phenprocoumon, which resulted in a serum phenprocoumon concentration of 7·6 mg/l, while INRs remained below 2·0. Patient 3 was initially treated with acenocoumarol 8 mg/day, subsequently with phenprocoumon 9 mg/day. Serum phenprocoumon concentration was 6·6 mg/l, while the INR was 1·3.

To investigate whether a genetic predisposition of coumarin resistance was present in these three patients the *VKORC1* 5′ UTR and coding sequence were analysed [AY587020 (Rieder *et al*, 2005) annotates the wild type *VKORC1* genomic sequence]. Both Patients 1 and 3 were heterozygous for a previously described nucleotide variation *g.1310T>C* (= *g.6621T>C* in AY587020) in exon 2, leading to p.Trp59Arg (Wilms *et al*, 2008). Patient 2 was heterozygous for a new nucleotide variation, also a missense mutation *g.155C>T* in exon 1 leading to p.Ser52Leu. None of the other previously reported sequence variations associated with coumarin resistance were detected in the three patients. In addition, no other genetic alterations were found in the 5′UTR (positions *g.*−226–1 analysed), in exon 1 (positions *g.1–173*), in exon 2 (positions *g.1309–1418*) and exon 3 (positions *g.3388–3596*). Both Patients 1 and 2 were heterozygous for *g.1173C>T*, while Patient 3 carried wild type. Individuals carrying *g.1173T* allele in general require less phenprocoumon or acenocoumarol than individuals carrying *g.1173C* alleles (Bodin *et al*, 2005; Rieder *et al*, 2005; Sconce *et al*, 2005). Thus, the putative increased