Comparison of the antiviral potentials among the pseudorabies-resistant transgenes encoding different soluble forms of porcine nectin-1 in transgenic mice

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Nectin-1 is an alphaherpesvirus receptor that binds to virion glycoprotein D by the first immunoglobulin (Ig)-like domain. The possibility of making animals resistant to pseudorabies virus (PRV) infection has been investigated by generating transgenic mice expressing soluble forms of porcine nectin-1. Previously, transgenic mice were generated that expressed a fusion protein made of the entire ectodomain of nectin-1 fused to the Fc portion of human IgG, or the first Ig-like domain fused to the Fc portion of porcine IgG. Here, the contribution of the second and third Ig-like domains of nectin-1 was analysed by generating transgenic mice expressing the entire ectodomain of nectin-1 fused to the porcine Fc portion. Transgenic mice expressing each of three different fusion proteins were challenged with PRV for comparison of their resistance. Altogether, mice transgenic for a chimera that carried the entire ectodomain were more resistant than those transgenic for a chimera that carried the first Ig-like domain.

Pseudorabies virus (PRV), a representative member of the subfamily Alphaherpesvirinae, causes lethal encephalitis in piglets, acute respiratory syndrome in growing pigs and abortion and infertility in breeding sows, and leads to latent infection in surviving pigs. PRV infection is a major economic risk in the swine industry worldwide. As an alternative to the vaccination strategies, we proposed a novel approach for generating farm animals resistant to pseudorabies. We set our sights on inhibition of viral entry to induce protection of the animals against PRV infection. The entry of herpes simplex virus (HSV) into cells takes place in three steps. In the first, two viral glycoproteins, gC and gB, bind to heparan sulfate proteoglycans (Herold et al., 1994; Mettenleiter et al., 1990; Shieh et al., 1992; WuDunn & Spear, 1989). In the second step, another glycoprotein, gD, interacts with specific cellular receptors that are required for virus entry into mammalian cells (Campadelli-Fiume et al., 2000; Spear et al., 2000). In the third step, gH, gL and gB execute the fusion of the viral envelope with the cell membranes (Cai et al., 1988; Forrester et al., 1992; Ligas & Johnson, 1988; Roop et al., 1993). Nectin-1, a gD receptor, has the broadest specificity for mediating alphaherpesvirus entry and is present in a broad range of tissues and cells (Cocchi et al., 1998a; Geraghty et al., 1998). Nectin-1 has an extracellular region containing three immunoglobulin (Ig)-like domains, a single transmembrane region and a cytoplasmic region. The three Ig-like domains consist of the first or N-terminal variable (V)-like domain and two constant (C)-like domains. The first Ig-like domain of nectin-1 contains the gD-binding site. In addition to the regions that are critical for binding to gD (Cocchi et al., 1998a; Krummenacher et al., 1999, 2000) and HSV entry (Cocchi et al., 1998b, 2001), it is known that the first Ig-like domain contains the regions for homotypic and heterotypic trans-interactions (Fabre et al., 2002; Krummenacher et al., 2002; Miyahara et al., 2000), but little is known about the functions of the second and third domains. Recent studies have shown that the second domain is concerned with cis-interactions (Momose et al., 2002).
Recently, we reported that transgenic mouse lines expressing a soluble form of the first Ig-like domain of porcine nectin-1 (PHveC-VpIg; VpIg), consisting of the first Ig-like domain and the Fc portion of porcine IgG, showed significant resistance to PRV infection (Ono et al., 2006). However, these animals were less resistant overall than transgenic mice expressing the entire ectodomain of porcine nectin-1 fused to the Fc portion of human IgG1, indicating that the two C domains of nectin-1 both have roles in antiviral potential. However, different Fc portions may affect the in vivo effects. There is a possibility that the primary effect of the fusion protein is to bind to the virus and promote its clearance. We cannot exclude the possibility that there may be functional differences between whole human and porcine Fc portions, when placed in the context of a murine immune system. Based on these findings, we aimed to investigate the role of the second and third domains and the effects of Fc portions from different species in antiviral potential against PRV infection. Here, we generated transgenic mouse lines expressing a soluble form of porcine nectin-1 (PHveCpIg; VCCpIg), consisting of the entire ectodomain and the Fc portion of porcine IgG.

The cDNA of the entire domain of porcine nectin-1 was amplified by PCR under conditions described previously (Ono et al., 2004a), in which pCXN2/PHveC Ig (Ono et al., 2004a) was used as a template. The PCR primers used to amplify the cDNA were 5′-GGACCCCTCGAGGCGGC-ATGGCT-3′ and 5′-CTGAGCGGATCCGTGTTCGGGAG-GAGACGGGGTGTA-3′. The cDNA was inserted into the plasmid carrying the porcine IgG–Fc cDNA (Ono et al., 2006). Then, the fragment containing the chimeric gene, encoding a fusion protein (PHveCpIg) consisting of the entire ectodomain of porcine nectin-1 and the Fc portion of porcine IgG, was inserted into the pCXN2 vector (Niwa et al., 1991). The transgene fragment containing the CAG promoter (cytomegalovirus immediate-early enhancer and chicken β-actin promoter), the PHveCpIg gene and the rabbit β-globin poly(A) signal was microinjected into C57BL/6 mouse eggs to generate transgenic mice. Three founder mice (PHveCpIg150, 151 and 185) carrying the transgene were identified by PCR analysis of their tail DNA. All founders gave rise to offspring in crosses with wild-type C57BL/6 mice and transmitted the introduced gene in a Mendelian fashion. The transgenic mouse lines PHveCpIg22, 32 and 37 (Ono et al., 2004a), expressing a soluble form of the entire ectodomain of porcine nectin-1 fused to the human Fc portion, and the transgenic mouse lines PHveC-Vplg21, 30 and 72 (Ono et al., 2006), expressing a soluble form of the first Ig-like domain fused to the porcine Fc portion, were used for comparison of the resistance to PRV infection. A schematic representation of each fusion protein is shown in Fig. 1(a). All mice were maintained in the animal facility at our institute and treated according to the Laboratory Animal Control Guidelines of our institute, which conform to those of the US National Institutes of Health.

Expression of VCCpIg was confirmed by Western blot analysis as described previously (Ono et al., 2006). A rabbit anti-pig IgG-specific band, the molecular mass of which is comparable to that of VCCpIg, was detected in all three transgenic mouse lines, but not in non-transgenic littermates, although non-specific bands were also detected in transgenic and non-transgenic mice (Fig. 2a). To confirm expression of VCCpIg in the nasal cavities, paraffin sections from the transgenic mice, prepared as described previously (Ono et al., 2004a), were examined by immunofluorescence staining using anti-pig IgG antibodies. Nasal respiratory epithelia of nasal cavities were stained specifically with the antibodies, although there were quantitative and/or cell type-specific differences among the transgenic mice (Fig. 2b). No staining was observed in sections from non-transgenic littermates (Fig. 2b). We observed no gross abnormalities in the transgenic mice.

To measure concentrations of fusion proteins in sera of the transgenic mice, a competitive ELISA system with a mAb against nectin-1 (CK6; Santa Cruz Biotechnology) was used as described previously (Ono et al., 2006). As shown in Fig. 1(b), PHveCpIg (VCCpIg) concentrations in serum samples ranged in each line from 6.2 ± 1.9 to 112.5 ± 42.4 μg ml⁻¹. On the other hand, PHveCpIg (VCChIg) or PHveC-Vplg (VpIg) concentrations were 20.0 ± 10.2–24.7 ± 8.3 μg ml⁻¹ in PHveCpIg lines and 108.0 ± 28.7–333.9 ± 105.2 μg ml⁻¹ in PHveC-Vplg lines. Expression seemed to be sufficient for comparison of the resistance to PRV infection among the three transgenic mouse lines expressing each different type of fusion protein, because the transgenic mice expressing only 5 μg VCChIg ml⁻¹ in their sera showed significant resistance against PRV infection via both intraperitoneal and intranasal routes (Ono et al., 2004a). Discrepancies from previous results in VCChIg or VpIg concentration may be due to the usage of different antibodies in the competitive ELISA. In the present study, a mAb against nectin-1 was used as the first antibody. On the other hand, polyclonal antibodies against nectin-1 for VCChIg or polyclonal antibodies against pig IgG for VpIg were used in previous studies (Ono et al., 2004a, 2006).

To find out whether the transgenic mice expressing VCCpIg were protected from PRV infection, transgenic and non-transgenic offspring were infected intraperitoneally with 20, 100 or 1000 LD₅₀ PRV strain YS-81. The LD₅₀ was determined as described previously (Ono et al., 2004b). The survival data are summarized in Fig. 3. Significant protection was observed in all of the lines: 82 % (nine of 11), 100 % (12 of 12), and 90 % (nine of ten) of the animals from lines 150, 151 and 185 survived, respectively. In contrast, approximately 90 % (111 of 122) of all control littermates died within 14 days. This resistance was confirmed to a slightly lower extent in more severe intraperitoneal challenges. Intraperitoneal inoculation with
100 LD$_{50}$ was lethal for 95% of all control mice (113 of 119 non-transgenic littermates died), whereas 73% (16 of 22), 83% (10 of 12) and 64% (nine of 14) of the animals from lines 150, 151 and 185 survived, respectively. In addition, the challenge with 1000 LD$_{50}$ was lethal for $>$98% of all control mice (119 of 121 non-transgenic littermates died),

**Fig. 1.** Schematic representation of fusion proteins. (a) PHveCpIg, consisting of the entire ectodomain (aa 1–345) of porcine nectin-1 and the Fc portion (aa 346–578) of porcine IgG. PHveC-VpIg is a fusion protein made of the first Ig-like (V) domain (aa 1–143) and the porcine Fc portion (aa 144–376). PHveC-lg is made of the entire ectodomain (aa 1–345) and the human Fc portion (aa 346–581). (b) Concentration of fusion proteins in transgenic mouse serum. Values represent the mean concentration of fusion protein, as determined by competitive ELISA with three transgenic offspring.

**Fig. 2.** Detection of PHveCpIg expressed in transgenic mice. (a) Western blot analysis of sera from the transgenic mice. The positions of molecular mass markers are shown on the left (in kDa). The positions of detected PHveCpIg and a non-specific band are indicated by the arrow and asterisk, respectively. (b) Immunofluorescence of nasal mucosa with anti-pig IgG antibody of transgenic mice of lines 150, 151 and 185, and a wild-type mouse (Non-Tg) as a control.
whereas 57% (16 of 28), 75% (nine of 12) and 71% (10 of 14) of the animals from lines 150, 151 and 185 survived, respectively. As PRV usually enters the body in pigs via infection of mucosal epithelium, intranasal challenges with PRV were performed. PRV (10 LD$_{50}$) was inoculated intranasally into the transgenic mice and their non-transgenic littermates. This challenge was lethal for 90% of all control mice (132 of 145 non-transgenic littermates died), whereas 70% (seven of ten), 57% (12 of 21) and 50% (five of ten) of the animals from lines 150, 151 and 185 survived, respectively (approx. 60% mean survival rate across lines). The data demonstrate that all transgenic mouse lines showed significant resistance to PRV infection.

To compare the effects of human and porcine Fc portions on the antiviral potential of the soluble form of the entire ectodomain, three transgenic mouse lines (PHveCIG22, 32 and 37) expressing VCChIg were used for experimental infection. Consistent with the earlier study, significant protection was observed after intraperitoneal inoculation with 20 LD$_{50}$ PRV: 100% (21 of 21), 100% (11 of 11) and 95% (19 of 20) of the animals from lines 22, 32 and 37 survived, respectively. In the challenge with 100 LD$_{50}$, 89% (17 of 19), 100% (nine of nine) and 70% (14 of 20) of the animals from lines 22, 32 and 37 survived, respectively. In addition, 86% (12 of 14), 69% (11 of 16) and 69% (11 of 16) of the animals from lines 22, 32 and 37 survived, respectively, in the challenge with 1000 LD$_{50}$. Significant resistance to the intranasal challenge was also observed: approximately 72% on average across lines [survival rates of 70% (19 of 27) in line 22, 71% (15 of 21) in line 32 and 75% (15 of 20) in line 37]. Direct comparison of the effects of Fc portions from human IgG and porcine IgG demonstrates that there is not much difference between them in antiviral potential. However, it seems that the antiviral potential of VCChIg is slightly stronger than that of VCCpIg (mean survival rates: 98% in VCChIg versus 90%
in VCCpIg for the intraperitoneal challenge with 20 LD₅₀; 83 versus 73 % for 100 LD₅₀; 74 versus 68 % for 1000 LD₅₀; 72 versus 60 % for the intranasal challenge with 10 LD₅₀).

To compare the effects of the entire ectodomain and the first Ig-like domain fused to the porcine Fc portion on antiviral potential, three transgenic mouse lines (PHveC-VpIg 21, 30 and 72) expressing VpIg were used for experimental infection. The survival data demonstrate that they showed lower resistance to PRV challenge with 100 LD₅₀ survival rates of approximately 40 % for each line, although these were statistically significantly different from the results after challenge with 20 LD₅₀ [survival rates of 86 % (12 of 14), 60 % (nine of 15) and 100 % (nine of nine) for lines 21, 30 and 72, respectively], consistent with a previous study (Ono et al., 2006). Furthermore, it was especially noteworthy that almost no protection was observed in the challenge with 1000 LD₅₀; only two of 27 mice survived the challenge. Consistent with earlier studies, they showed lower resistance to the intranasal challenge: approximately 40 % on average across lines [survival rates of 56 % (nine of 16), 55 % (six of 11) and 18 % (three of 17) for lines 21, 30 and 72, respectively]. Taken together, the antiviral potential of VpIg is weaker than that of VCCpIg (mean survival rates: 79 % in VpIg versus 90 % in VCCpIg for intraperitoneal challenge with 20 LD₅₀; 40 versus 73 % for 100 LD₅₀; 7 versus 68 % for 1000 LD₅₀; 40 versus 60 % for the intranasal challenge with 10 LD₅₀). Direct comparison of the effects of the entire ectodomain and the first Ig-like domain fused to the porcine Fc portion demonstrates that the second and third Ig-like domains enhance the antiviral effect against PRV infection.

There may be several explanations for the roles of the second and third Ig-like domains in antiviral potential in the transgenic mice. Firstly, the two C-like domains provide a certain degree of flexibility to the first Ig-like domain for suitable interaction with gD, and/or enhance the binding affinity to gD. It is known that the second and third C-like domains of nectin-1 increase the efficiency of HSV entry and cell fusion with the human Fc portion rendered the mice slightly more resistant to PRV infection than those transgenic for a chimera that carried the first Ig-like domain. Furthermore, the human Fc portion rendered the mice slightly more resistant to challenge than those that carried the porcine IgG. These findings indicate that the PHveC-Vg gene (VCChlg) is the best among the pseudorabies-resistant transgenes encoding different soluble forms of porcine nectin-1 in transgenic mice.

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