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Induction of protective immunity in chickens vaccinated with infectious bronchitis virus S1 glycoprotein expressed by a recombinant baculovirus

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A recombinant baculovirus containing the S1 glycoprotein gene of the virulent nephropathogenic KM91 strain of infectious bronchitis virus (IBV) was constructed in order to investigate protective immunity in vaccinated chickens. Results from the protection test were evaluated by re-isolation of virus from the kidneys and tracheas of vaccinated chickens after challenge with strain KM91. After three immunizations, the recombinant S1 (rS1) glycoprotein induced 50% protection of the kidney, whilst inactivated KM91 induced 88% and 50% protection of the kidney and trachea, respectively. In chickens primed with the attenuated H120 vaccine strain, which is heterologous to KM91, the rS1 glycoprotein induced 83% protection of the kidney after two immunizations. Haemagglutination-inhibition titres were also increased in chickens immunized with the rS1 glycoprotein after three immunizations, and significantly higher titres were detected after challenge. These data indicate that the expressed rS1 glycoprotein alone can induce a protective immune response as well as an antibody response.

Introduction

Infectious bronchitis virus (IBV) is the causative agent of infectious bronchitis (IB), a respiratory and urogenital track disease in chickens (King & Cavanagh, 1991; Picault *et al.*, 1986). IB is responsible for severe financial losses to the poultry industry and despite the wide use of live attenuated and inactivated vaccines, IB still remains one of the most important poultry diseases in Korea and many other countries of the world. A number of IBV serotypes have been identified worldwide, and some of these serotypes cannot be controlled by heterologous serotype vaccines (Endo-Munoz & Faragher, 1989; Lambrechts *et al.*, 1993; Muneer *et al.*, 1988). Antigenically different serotypes and newly emerged variants from field chicken flocks sometimes cause 'vaccine breaks'. The generation of genetic variants is thought to result from a few amino acid changes in the spike (S) glycoprotein of IBV (Cavanagh *et al.*, 1992; Kant *et al.*, 1992). The widespread use of live attenuated vaccine may play an important role in increasing the number of new genetic variants (Kusters *et al.*, 1987; Wang *et al.*, 1993).

IBV, a member of the family *Coronaviridae*, has a single-stranded RNA genome, approximately 27 kb in length, of positive polarity which specifies the production of three major structural proteins; the phosphorylated nucleocapsid protein, the membrane glycoprotein and the surface (S) glycoprotein, which is post-translationally cleaved into two subunits, S1 (92 kDa) and S2 (84 kDa). The S glycoprotein extends from the viral membrane and the S1 glycoprotein is anchored to the viral membrane by the S2 glycoprotein (Binns *et al.*, 1985; Cavanagh, 1983*a*, *b*, *c*). The S1 glycoprotein induces virus neutralization (VN) and haemagglutination-inhibition (HI) antibody and it has also been identified as a major inducer of protective immune responses (Cavanagh *et al.*, 1984; Koch *et al.*, 1990; Mockett *et al.*, 1984). Chickens vaccinated with inactivated urea-treated IBV, which lacked the S1 glycoprotein, failed to induce protection in the trachea of chickens, whereas intact inactivated IBV induced some protection following one intramuscular inoculation (Cavanagh *et al.*, 1986). Furthermore, four immunizations of purified S1 glycoprotein induced an antibody response that resulted in 80% and 71% protection at

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the kidney and trachea level, respectively, when challenged with virulent nephropathogenic IBV (Ignjatovic & Galli, 1994). These observations indicate that the S1 glycoprotein is a major determinant for the induction of protective immune responses and that for an effective immune response and protection, multiple immunizations are needed. As an alternative approach, it was reported that the IBV S glycoprotein, expressed by a recombinant vaccinia virus, could potentially be used as a live IBV vaccine (Tomley *et al*., 1987). In this study, the entire S glycoprotein gene was recombined into vaccinia virus and mice vaccinated with this recombinant vaccinia virus were shown to have VN antibodies to IBV.

In the present study, we demonstrate that the S1 gene can be expressed as an IBV S1 glycoprotein in a baculovirus expression system and the recombinant S1 (rS1) glycoprotein could induce protective immunity in chickens against challenge with virulent nephropathogenic IBV.

Methods

■ **Viruses and cells.** The 6th embryo passage of the virulent nephropathogenic KM91 strain (KM91p6) was propagated in 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs (Hyvac) at 37 °C for 48 h. The allantoic fluid from eggs infected with KM91p6 [10^7 egg infectious dose $(EID)_{50}$/ml] was harvested and clarified by low-speed centrifugation. The clarified allantoic fluid was inactivated with 0·2% β-propiolactone (Sigma) at 25 °C for 3 h. To assay for inactivation, this material was inoculated into the chorioallantoic cavity of 10-day-old SPF embryonated chicken eggs and passaged into eggs at least twice. *Autographa californica* nucleopolyhedrovirus (AcNPV) and recombinant AcNPV were grown and assayed in *Spodoptera frugiperda* clone 21 (Sf21) cells in Grace's insect cell culture medium (Gibco BRL) supplemented with 10% foetal bovine serum and antibiotics.

■ **Chickens.** All the chickens were derived from an SPF flock of White Leghorn parents and were housed in positive-pressure isolators.

■ **Amplification of the S1 gene of IBV.** Two primers, which included 18 bp complementary to a region at the 5′ end (KMS1F1; 5′ CGGGATCCATGTCGGGGAAGTTACTG 3′) and 3′ end (KMS1R1; 5′ CGGGATCCTTAACGTCTAAAACGACGTGT 3′) of the S1 gene of the nephropathogenic KM91 IBV strain, were used to amplify the complete S1 gene. *Bam*HI sites were added to the 5′ ends of each primer to facilitate cloning, and start and stop codons were added next to the *Bam*HI sites of the two primers, respectively. These primers were made with reference to the sequence of the KM91 strain (our unpublished data). Viral RNA extraction and PCR were performed according to the methods previously described by Kwon *et al*. (1993).

■ **Cloning of the S1 gene of IBV and construction of recombinant viruses.** The amplified PCR product was ligated into plasmid pCRII using a TA Cloning kit (Invitrogen) according to the manufacturer's recommendations. *Escherichia coli* INVαF′ was infected, and ampicillin-resistant colonies were screened by restriction enzyme analysis. A plasmid including the complete S1 gene of the KM91 strain was designated pCRKMS1. To construct a recombinant baculovirus expressing the KM91 S1 glycoprotein, the S1 gene was obtained by digestion of pCRKMS1 with *Bam*HI. The excised fragment containing the KM91 S1 gene was ligated into the unique *Bam*HI site of the pVL1393 vector (Invitrogen) to produce the transfer vector pVLKMS1. The orientation of the S1 gene was confirmed by restriction enzyme analysis. To transfer the S1 gene into the AcNPV DNA, Sf21 cells were co-transfected with BaculoGold-linearized baculovirus DNA (Pharmingen) and pVLKMS1 transfer DNA as previously described (Kitts *et al*., 1990). After three successive plaque purifications, recombinant AcNPV stocks (above 10^7 p.f.u./ml) were obtained using Sf21 cells and designated rAcKMS1.

■ **Immunofluorescence assay and HI test.** Sf21 cells infected with rAcKMS1 or wild-type AcNPV (wAcNPV) at an m.o.i. of 5 were incubated at 27 °C for 48 h, and then washed three times with PBS. The fixed and unfixed cells were reacted with IBV-specific hyperimmune sera as first antibody and with a fluorescence isothiocyanate-conjugated goat anti-chicken immunoglobulin (Cappel) as second antibody. Haemagglutination antigen of the nephropathogenic KM91 strain and HI test were done as described previously (King & Hopkins, 1983). The HI titre were read as the reciprocal of the highest dilution showing complete inhibition and the results were recorded as the geometric mean of $\log_2$ titres.

■ **Immunization of chickens.** The sonicated Sf21 cells and the inactivated KM91 were mixed with an equal volume of Montanide Incomplete Sepic Adjuvant (ISA-50). For vaccination, all the chickens were inoculated intramuscularly with oil-emulsion inoculum which contained 1×10^7 Sf21 cells infected with rAcKMS1 or wild-type AcNPV (wAcNPV), and $1 \times 10^{6·0}$ EID_{50} of inactivated KM91 (0·5 ml per chicken). Chickens primed with live IBV were inoculated intraocularly with $1 \times 10^{3·0}$ EID_{50} of attenuated H120 commercial vaccine (Intervet) and 120th embryo passage of KM91 strain (KM91p120), respectively; chickens for challenge were inoculated intraocularly with $1 \times 10^{4·5}$ EID_{50} of KM91p6.

■ **Protection assay.** The protective efficacy of the rS1 glycoprotein expressed by rAcKMS1 in Sf21 cells was examined and compared with inactivated KM91 using 6-week-old chickens (Table 1, Experiment 1) and 9-week-old chickens pre-immunized at 6 weeks old with live IBVs (Table 1, Experiment 2). In Experiment 1, 64 6-week-old chickens were divided into four groups. Sixteen chickens from each group were housed in separated isolators. Chickens in groups 1 and 3 were immunized with rAcKMS1- and wAcNPV-infected cell lysates, respectively. Chickens in group 2 were immunized with inactivated KM91. Chickens in group 4 were kept as uninoculated controls. For booster vaccination, the 16 chickens in groups 1, 2 and 3 were equally divided into two subgroups. Each of the eight chickens was immunized twice and the other eight chickens were immunized three times at 3 week intervals with the same antigen used for the primary immunization. Three weeks after the second and third immunization, eight chickens from each group were challenged with virulent KM91p6.

In Experiment 2, 72 6-week-old chickens were divided into six groups. Twelve chickens from each group were housed in separated isolators. All the chickens in groups 1, 2 and 3 and groups 4, 5 and 6 were pre-immunized with attenuated H120 and live KM91p120, respectively. Three weeks after priming, chickens in groups 1 and 4 were immunized with rAcKMS1-infected cell lysates and chickens in groups 3 and 6 were immunized with wAcNPV-infected cell lysates. Chickens in groups 2 and 5 were immunized with inactivated KM91. Three weeks after booster immunization, six chickens from each group were re-immunized with the same antigen used for the first booster immunization. Three weeks after the first and second booster immunization with rAcKMS1 or inactivated KM91, six chickens from each group were challenged with virulent KM91p6. Five days after challenge, the chickens in Experiments 1 and 2 were sacrificed and kidney and trachea tissues were collected for re-isolation of the challenge virus. Chickens were bled 3 weeks after each immunization and 5 days after challenge.

Re-identification of the challenge virus was performed using a dot-immunoblotting assay (Song *et al*., 1998), and tissue samples collected

from Experiments 1 and 2 were passaged into egg at least twice before virus re-isolation attempts were considered to be negative.

■ **Statistical analyses.** For statistical analyses, we employed the one-tailed Fisher's exact test and the Student's *t*-test for protective immunity and antibody response. Results were considered to be statistically significant only if the comparison to each of wAcNPV-immunized group gave a P value of $< 0{\cdot}05$.

Results and Discussion

By immunofluorescence assay using a polyclonal antibody specific to the nephropathogenic KM91 strain, the rS1 glycoprotein was found to transport to the cell surface (data not shown). As shown in Fig. 1, the rS1 glycoprotein had an M_r similar to that of the authentic KM91 strain.

Two immunizations of chickens with rAcKMS1 provided no statistically significant protection at the kidney or trachea level against virulent KM91p6 challenge ($P > 0{\cdot}05$ by Fisher's exact test), respectively. In contrast, three immunizations of chickens with rAcKMS1 induced 50% protection at the kidney level ($P < 0{\cdot}05$; Table 1, Experiment 1, group 1). Chickens immunized twice with inactivated KM91 showed 50% protection of kidneys ($P < 0{\cdot}05$; Table 1, Experiment 1, group 2) and three immunizations of chickens with inactivated KM91 provided high levels of protection, significantly greater than that seen for the wAcNPV-immunized group at the kidney ($P < 0{\cdot}001$) and trachea ($P < 0{\cdot}05$) level, respectively. These data indicated that although rAcKMS1 was less effective for protection than whole inactivated IBV, multiple immunizations with rAcKMS1 did induce some protective immunity against challenge with virulent KM91p6. Also, these results clearly showed that both rAcKMS1 and inactivated KM91 provided better protection for kidney than trachea. Ignjatovic & Galli (1994) reported that at least three immunizations with the S1 glycoprotein obtained by immunoaffinity purification were needed to induce a significant level of protection in vaccinated chickens. In the present study, we did not immunize more than three times with rAcKMS1. To induce a significant level of protection in vaccinated chickens at the level of trachea, further immunizations with rAcKMS1 seem to be necessary.

Using chickens primed with attenuated H120, rAcKMS1 provided no statistically significant protection against virulent KM91p6 challenge following the first booster immunization ($P < 0{\cdot}1$), but it did provide 83% protection at the kidney level after the second booster immunization ($P < 0{\cdot}05$; Table 1, Experiment 2, group 1). On the other hand, inactivated KM91 showed 100% protective immunity only at the kidney level after the first booster immunization ($P < 0{\cdot}05$). However, chickens boosted twice with inactivated KM91 developed a stronger protective immunity than that seen for rAcKMS1 at the kidney ($P < 0{\cdot}01$) and trachea ($P < 0{\cdot}05$) level, respectively (Table 1, Experiment 2, group 2). These results indicated that although H120 is a heterologous strain, rAcKMS1 and inactivated KM91 could induce enhanced protection compared with the control chickens after priming with attenuated H120.

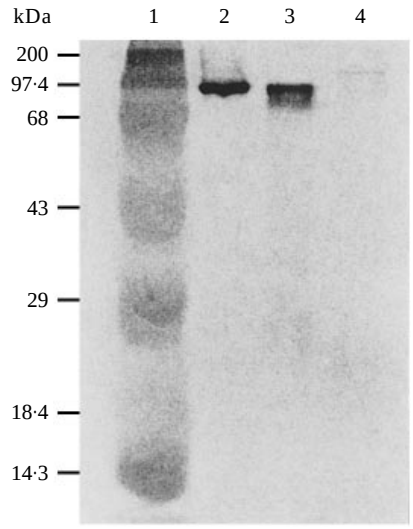

Fig. 1. Western blot analysis of the rS1 glycoprotein expressed by rAcKMS1. Sf21 cells, infected with rAcKMS1 or wAcNPV, and allantoic fluid from eggs infected with the nephropathogenic KM91 strain, were separated by SDS–PAGE and transferred to nitrocellulose membrane. The transferred cellular proteins were reacted with monoclonal antibody specific to strain KM91 S1 glycoprotein. Lane 1, M_r marker; lane 2, KM91-infected allantoic fluid; lane 3, rAcKMS1-infected cells; lane 4, wAcNPV-infected cells. A band with an M_r of about 92000 was found in lanes 2 and 3. The positions of M_r markers are indicated on the left.

In contrast to the results comparing groups primed with attenuated H120, all the chickens primed with live KM91p120 were completely protected against challenge, even in the control group of chickens immunized with wAcNPV-infected cell lysates (Table 1, Experiment 2, groups 4, 5 and 6). We tried to evaluate the booster effects of rAcKMS1 on homologous strain-primed chickens but we could not obtain clear results, because live KM91p120 induced a strong immunity after priming to all the chicken groups.

In the present study, we also investigated the possibility that chickens immunized with rAcKMS1 could induce IBV HI antibody. The HI titres induced by rAcKMS1 after the second immunization were slightly higher than those in the group inoculated with wAcNPV (Table 2, Experiment 1, groups 1 and 3). However, this difference was not statistically significant ($P > 0{\cdot}25$ by Student's *t*-test). The level of HI titres was increased in chickens immunized with rAcKMS1 after the third immunization ($P < 0{\cdot}05$), and significantly higher than that of wAcNPV-immunized control after challenge ($P < 0{\cdot}005$; Table 2, Experiment 1, groups 1 and 3). The titres induced by inactivated KM91 after the second and third immunizations were significantly higher than that of the rAcKMS1- or wAcNPV-immunized groups ($P < 0{\cdot}005$; Table 2, Experiment 1, groups 1, 2 and 3). Furthermore, using chickens primed with attenuated H120, all of the chickens immunized with rAcKMS1 showed similar HI titres compared with the chickens immunized with wAcNPV-infected cells ($P > 0{\cdot}5$; Table 2, Experiment 2, groups 1 and 3). Chickens primed with live KM91p120, then immunized twice with rAcKMS1, showed a

Table 1. Protective effect in chickens immunized with rSl glycoprotein against challenge with the nephropathogenic KM91 strain of IBV using SPF chickens or live-IBV-primed chickens

Expt	Group	Live IBV pre-immunized	Antigen immunized	No. of chickens from which the challenge virus was re-isolated/ no. of chickens challenged (% protected)					
				1st immunization		2nd immunization		3rd immunization	
				Kidney	Trachea	Kidney	Trachea	Kidney	Trachea
1	1	ND	rAcKMSI	ND	ND	4/7 (43)	7/7 (0)	4/8 (50)*	6/8 (25)
	2	ND	Inactivated KM91	ND	ND	4/8 (50)*	7/8 (13)	1/8 (88)‡	4/8 (50)*
	3	ND	wAcNPV	ND	ND	7/7 (0)	7/7 (0)	8/8 (0)	8/8 (0)
	4	ND	Control	ND	ND	8/8 (0)	8/8 (0)	8/8 (0)	8/8 (0)
2	1	H120	rAcKMSI	2/6 (67)	4/6 (33)	1/6 (83)*	2/6 (67)	ND	ND
	2	H120	Inactivated KM91	0/6 (100)*	2/6 (67)	0/6 (100)†	1/6 (83)*	ND	ND
	3	H120	wAcNPV	4/6 (33)	4/6 (33)	5/6 (17)	5/6 (17)	ND	ND
	4	KM91p120	rAcKMSI	0/6 (100)	0/6 (100)	0/6 (100)	0/6 (100)	ND	ND
	5	KM91p120	Inactivated KM91	0/6 (100)	0/6 (100)	0/6 (100)	0/6 (100)	ND	ND
	6	KM91p120	wAcNPV	0/6 (100)	0/6 (100)	0/6 (100)	0/6 (100)	ND	ND

* $P < 0{\cdot}05$ by Fisher's exact test.
† $P < 0{\cdot}01$ by Fisher's exact test.
‡ $P < 0{\cdot}001$ by Fisher's exact test.
ND, Not done.

Table 2. HI antibody response in chickens after immunization with rSl glycoprotein and post-challenge with the nephropathogenic KM91 strain of IBV using SPF chickens or live-IBV-primed chickens

Expt	Group	Live IBV pre-immunized	Antigen immunized	Geometric mean HI titre ($\log_2$) ± SD							
				Pre-immunization		1st immunization		2nd immunization		3rd immunization	
				Pre-challenge	Post-challenge	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge
1	1	ND	rAcKMSI	ND	ND	3·0 ± 1·40	ND	3·1 ± 2·19	3·7 ± 2·14*	4·1 ± 1·89*	6·9 ± 2·03†
	2	ND	Inactivated KM91	ND	ND	5·9 ± 1·64	ND	9·0 ± 1·77†	8·4 ± 1·30†	9·5 ± 1·41†	9·9 ± 1·55†
	3	ND	wAcNPV	ND	ND	2·3 ± 0·46	ND	2·4 ± 0·74	1·4 ± 0·98	2·1 ± 1·13	3·0 ± 0·58
	4	ND	Control	ND	ND	0·6 ± 0·74	ND	1·6 ± 1·06	1·9 ± 1·25	1·8 ± 0·71	2·1 ± 0·99
2	1	H120	rAcKMSI	5·9 ± 1·37	ND	5·1 ± 0·83	6·3 ± 0·82	5·3 ± 2·25	7·3 ± 1·97	ND	ND
	2	H120	Inactivated KM91	5·9 ± 1·37	ND	7·3 ± 1·23	8·8 ± 1·17	10·5 ± 1·38*	11·2 ± 1·17†	ND	ND
	3	H120	wAcNPV	5·9 ± 1·37	ND	4·4 ± 1·08	6·8 ± 1·17	3·7 ± 0·82	5·7 ± 1·10	ND	ND
	4	KM91p120	rAcKMSI	7·8 ± 1·06	ND	8·9 ± 0·90	9·3 ± 1·37	8·8 ± 2·04	9·8 ± 0·96	ND	ND
	5	KM91p120	Inactivated KM91	7·8 ± 1·06	ND	9·9 ± 1·51	9·5 ± 1·52	10·5 ± 0·55	10·7 ± 1·03	ND	ND
	6	KM91p120	wAcNpv	7·8 ± 1·06	ND	8·8 ± 1·76	10·0 ± 1·22	6·5 ± 0·55	7·7 ± 0·52	ND	ND

* $P < 0{\cdot}05$ by Student's *t*-test.
† $P < 0{\cdot}005$ by Student's *t*-test.
ND, Not done.

significantly higher HI titre compared with chickens immunized with wAcNPV-infected cells ($P < 0{\cdot}05$ or better; Table 2, Experiment 2, groups 4 and 6). These data indicated that although rAcKMS1 was less effective in inducing HI antibodies than inactivated KM91, multiple immunizations with rAcKMS1 could induce HI antibody. In the present study, the HI titres did not correlate with protection. These results are in accord with the previous finding that chickens were protected in the absence of VN and HI antibodies, and conversely chickens with circulating VN and HI antibodies were not necessarily protected (Ignjatovic & Galli, 1994). Thus, we conclude that the analysis of protection in IBV infection should be extended to include the contribution of cell-mediated immunity.

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