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Optimized polypeptide for a subunit vaccine against avian reovirus

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ABSTRACT

Avian reovirus (ARV) is a disease-causing agent. The disease is prevented by vaccination with a genotype-specific vaccine while many variants of ARV exist in the field worldwide. Production of new attenuated vaccines is a long-term process and in the case of fast-mutating viruses, an impractical one. In the era of molecular biology, vaccines may be produced by using only the relevant protein for induction of neutralizing antibodies, enabling fast adjustment to the emergence of new genetic strains. Sigma C (SC) protein of ARV is a homotrimer that facilitates host-cell attachment and induce the production and secretion of neutralizing antibodies. The aim of this study was to identify the region of SC that will elicit a protective immune response. Full-length (residues 1–326) and two partial fragments of SC (residues 122–326 and 192–326) were produced in *Escherichia coli*. The SC fragment of residues 122–326 include the globular head, shaft and hinge domains, while eliminating intra-capsular region. This fragment induces significantly higher levels of anti-ARV antibodies than the shorter fragment or full length SC, which neutralized embryos infection by the virulent strain to a higher extent compared with the antibodies produced in response to the whole virus vaccine. Residues 122–326 fragment is assumed to be folded correctly, exposing linear as well as conformational epitopes that are identical to those of the native protein, while possibly excluding suppressor sequences. The results of this study may serve for the development of a recombinant subunit vaccine for ARV.

1. Introduction

Avian reovirus (ARV) is a member of the *Orthoreovirus* genus in the family Reoviridae [1,2]. It is associated with a number of diseases [3], the most prominent being viral arthritis syndrome (tenosynovitis) which is characterized by swelling of the hock joints and lesions in the gastrocnemius tendons [4], and causes considerable economic loss to the poultry industry [5]. Susceptibility to ARV occurs mostly in young (1–2 weeks of age) chickens [6–8]. The control of viral tenosynovitis in broiler chicks is conferred by antibodies that are transferred to the progeny following vaccination of maternal flocks [9]. The available live-attenuated and inactivated vaccines for ARV are based on the s1133 strain [10], as well as isolated strains belonging to a single serotype [11]. However, those vaccines are not effective against the diverse ARVs found in the field [11–13]. Sequencing of the sigma C (SC) protein of ARV isolates for genetic characterization enabled their division into genotypes [11,13–15]. Vaccination based on a mixture of the four representatives of ARV genotypes conferred protection against all tested viruses from the four genotypes [13]. The outer capsid cell attachment protein, SC of ARV, encoded by the S1 gene, is a relatively small protein of 326 amino acids [16], a homotrimer with a tertiary structure consisting of two domains: the "head", which is located at the C-terminal end of the protein, and the "shaft", at the N terminus. The crystal structure of the C-terminal domain and of residues 117–326 has been resolved [17,18]. SC elicits reovirus-specific neutralizing antibodies [19,20], making it a suitable candidate for a recombinant subunit vaccine.

Indeed, efficient recombinant vaccines have been developed in the past for a number of viruses, including vaccines for hepatitis B [21] and for papillomavirus [22] for humans, as well as infectious bursal disease (IBD) [23] and egg drop syndrome [24] for chickens and hemorrhagic enteritis virus for turkeys [25]. SC has been expressed in various expression systems, including bacteria [17,18,26–28], baculovirus [29,30], yeast [31], plants [32] and

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Table 1
Designed oligonucleotides for amplification of sigma C (SC) fragments.

Label[a]	Direction	Primer sequences (5′ → 3′)	Expected size (bp)
SC122–326	Forward	GGGGGATCCGACGGAAACTCCACTGCC	669
SC122–326	Reverse	GGGCGGCCGTTAGGTGTCGATGCCGG	
SC192–326	Forward	GGGGGATCCTCGGCGGAGGCTCAACTAATGC	459
SC192–326	Reverse	GGGCGGCCGTTAGGTGTCGATGCCGG	

[a] Oligonucleotides were designed to amplify the gene encoding SC122–326 and SC192–326 partial proteins; the 5′ ends of the oligonucleotides were designed to create restriction enzyme sites BamHI–EagI, BamHI–EagI, and EcoRI–EcoRI (underlined), respectively, after PCR amplification.

mammalian cells [33]. Recombinant SC proteins have been used for diagnostics to distinguish between strains [26–28,30,34]. Anti-SC antibodies have been shown to neutralize the virus in cell lines [28,31]. In a previous study, SC expressed in bacteria showed only weak immunogenicity [26,27]. The objective of the current study was to determine the fragment of the SC protein that will induce a high level of neutralizing antibodies toward the production of an efficient recombinant subunit vaccine against ARV.

2. Materials and methods

2.1. Expression of the SC protein

Three cDNA fragments of SC from the vaccine strain s1133 encoding SC residues 1–326, 122–326 and 192–326 were produced by polymerase chain reaction (PCR) with specifically designed oligonucleotides (Table 1). Fragments 122–326 and 192–326 were cleaved with restriction enzymes BamHI and EagI introduced into the primers during synthesis, and were cloned into the expression vector pET28a (Novagen, Darmstadt, Germany), which included a purification tag containing six consecutive histidine residues at the N terminus. The sequence of the insert was confirmed by DNA-sequence analysis (Hy-Labs, Rehovot, Israel). For expression, *Escherichia coli* strain BL21 (DE3) was freshly transformed with the plasmid. Cultures were grown aerobically at 37 °C to an optical density (OD) at 600 nm of 0.6–0.8. The cultures were cooled to below 25 °C, and expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubating for 3 h at 25 °C. Harvested cells were resuspended in 40 ml cold resuspension buffer (4.29 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, 0.1% v/v Tween-20) and frozen at −20 °C [18]. Bacteria were lysed by sonicating three times for 10 min each time (Sonics, Taunton, MA, USA), centrifuged (10,000 × *g* for 15 min at 4 °C) and the pellet was discarded. SC residue 1–326 was expressed as described previously [27]. The expressed SC122–326 protein fragment was purified in a Ni-NTA agarose column according to the manufacturer's instructions (Qiagen, Hilden, Germany). The purified protein was dialyzed overnight against phosphate buffered saline (PBS) at 4 °C. The expressed SC proteins were detected by 12% SDS–polyacrylamide gel electrophoresis (PAGE). The amount of SC protein was estimated by comparison with a standard curve of known amounts of bovine serum albumin run on the same gel.

2.2. Birds and vaccination

Specific pathogen-free (SPF) birds (Charles River/SPAFAS) were hatched and raised in a sterile hatchery. At 1 week of age, the birds were randomly separated into six groups of 10 birds each. Each bird was vaccinated with 100 μg SC protein mixed 1:1 (v/v) with Freund's adjuvant to a final volume of 1 ml. Birds were vaccinated intramuscularly and subcutaneously at 14 and 28 days of age as described in Table 2.

Blood was withdrawn 2 weeks after each of the vaccinations and serum was separated and kept at −20 °C.

2.3. Enzyme-linked immunosorbent assay (ELISA)

To determine the anti-SC antibody titer, SC was used as the antigen in an ELISA. The antigen was diluted in coating buffer (0.397 g Na_2CO_3, 0.732 g $NaHCO_3$, 250 ml double-distilled water pH 9.6) and incubated in an ELISA plate (Nunc, Rochester, NY, USA) for 24 h at 4 °C. Each subsequent step was followed by three washes with 0.05% Tween-20 in PBS. Serum from birds vaccinated with the tested proteins or controls were serially double-diluted (1:100–1:800,000) in a blocking buffer (5% w/v skim milk, 0.05% Tween-20 in PBS) and incubated for 1 h. The secondary antibody, peroxidase-conjugated rabbit anti-chicken IgG (Sigma, Rehovot, Israel), diluted 1:7000, was added and the mixture was incubated for 1 h. The substrate o-phenylenediamine dihydrochloride (Sigma) was then added. OD was measured by ELISA reader (Thermo Scientific Multiskan RC, Vantaa, Finland) at 450 nm. The endpoint titer was determined as the last dilution for which the OD was still positive (relative to the negative control in the ELISA).

The level of anti-ARV antibodies in the sera of vaccinated birds was determined by a commercial ELISA (IDEXX® Laboratories, USA) according to the manufacturer's instructions. OD values were measured at 650 nm. Sample-to-positive (S/P) ratios greater than 0.2 were considered to be positive for ARV (S/P ratio = (sample mean OD − negative control mean OD)/(positive control mean OD − negative control mean OD)).

2.4. Cell-proliferation assay

Spleens were collected from birds 42 days post-vaccination and macerated with a syringe plunger through a screen sieve to obtain a single-cell suspension in PBS. Splenocytes were suspended in RPMI 1640 supplemented with 2% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (10 ng/ml) (Biotech Industry, Bet Haemek, Israel). Cells (1×10^6/well) were seeded in 96-well culture plates. Concanavalin A (ConA; 5 μg/ml), lipopolysaccharide (LPS; 5 μg/ml) or ARV (5 μl/well, at a titer of $10^{6.6}$) (Sigma–Aldrich) were added as stimulators in triplicate and incubated for 48 h. Cell titer blue (CTB) assay was performed by adding 20 μl CTB reagent (Promega, Madison, WI, USA) to each well. The cells were then incubated for 6 h at 37 °C under 5% CO_2. Color intensity of the CTB reagent was quantified by fluorometer at excitation/emission wavelengths of 560 and 590 nm,

Table 2
Vaccination program.

Group	Vaccine at 14 d[a]	Vaccine at 28 d[a]
1	ARV s1133[b]	ARV s1133
2	PBS + adjuvant[c]	PBS + adjuvant
3	SC1–326	SC1–326
4	SC122–326	SC122–326
5	SC192–326	SC192–326
6	SC122–326	ARV s1133

[a] Days of age.
[b] Positive control.
[c] Negative control.

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