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Protection against infectious bronchitis virus by spike ectodomain subunit vaccine

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ABSTRACT

The avian coronavirus infectious bronchitis virus (IBV) S1 subunit of the spike (S) glycoprotein mediates viral attachment to host cells and the S2 subunit is responsible for membrane fusion. Using IBV Arkansas-type (Ark) S protein histochemistry, we show that extension of S1 with the S2 ectodomain improves binding to chicken tissues. Although the S1 subunit is the major inducer of neutralizing antibodies, vaccination with S1 protein has been shown to confer inadequate protection against challenge. The demonstrated contribution of S2 ectodomain to binding to chicken tissues suggests that vaccination with the ectodomain might improve protection compared to vaccination with S1 alone. Therefore, we immunized chickens with recombinant trimeric soluble IBV Ark-type S1 or S-ectodomain protein produced from codon-optimized constructs in mammalian cells. Chickens were primed at 12 days of age with water-in-oil emulsified S1 or S-ectodomain proteins, and then boosted 21 days later. Challenge was performed with virulent Ark IBV 21 days after boost. Chickens immunized with recombinant S-ectodomain protein showed statistically significantly ($P < 0.05$) reduced viral loads 5 days post-challenge in both tears and tracheas compared to chickens immunized with recombinant S1 protein. Consistent with viral loads, significantly reduced ($P < 0.05$) tracheal mucosal thickness and tracheal lesion scores revealed that recombinant S-ectodomain protein provided improved protection of tracheal integrity compared to S1 protein. These results indicate that the S2 domain has an important role in inducing protective immunity. Thus, including the S2 domain with S1 might be promising for better viral vectored and/or subunit vaccine strategies.

1. Introduction

Infectious bronchitis virus (IBV) is a highly prevalent coronavirus of chickens that causes economic losses worldwide despite extensive vaccination. Continuous emergence of new virus serotypes results from mutation and recombination followed by selection [1]. Routinely used live-attenuated IBV vaccines, which are affected by the same evolutionary processes, not only result in vaccine-like viruses with increased virulence and persistence [2,3], but may also contribute genetic material for recombination with other vaccine or wild virus populations. We previously identified five minor vaccine virus subpopulations selected in chickens from Arkansas-Delmarva Poultry Industry (ArkDPI)-derived IBV vaccines, designated components (C) 1–5 [3,4]. The selection of these viral subpopulations within 3 days post-vaccination suggests they replicate better in chickens than the predominant virus population in the vaccine prior to inoculation [3,4].

The spike (S) protein of IBV mediates viral entry into host cells [5,6]. Its S1 subunit mediates viral attachment to host cells and induces virus-neutralizing antibodies that are important for host protective immune responses [7–9]. However, the S1 subunit shows extensive amino acid sequence variability among IBV strains, which leads to the virus's immunological escape [1,10,11]. The S2 subunit of S, responsible for membrane fusion, is more conserved among IBV strains [12]. The N-terminal portion of S2 contains immunodominant regions and a neutralizing

Abbreviations: AA, amino acids; ANOVA, analysis of variance; Ark, Arkansas; ArkDPI, Arkansas-Delmarva Poultry Industry; DOA, day of age; DPC, days post-challenge; EID_{50}, 50% embryo infectious doses; ELISA, enzyme-linked immunosorbent assay; HEK293T, human embryonic kidney 293T cells; HRP, horseradish peroxidase; IBV, infectious bronchitis virus; PBS, phosphate buffered saline; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; S, spike protein; S1, spike S1 subunit; SPF, specific-pathogen-free.

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epitope and therefore the S2 protein has been suggested for vaccine development [12,13].

Previous studies indicated that the S1 protein alone does not induce effective protection against IBV challenge. For instance, at least four immunizations with purified S1 glycoprotein were required to induce protection against nephropathogenic N1/62 strain challenge [14]. Similarly, three immunizations with KM91 S1 protein expressed by a recombinant baculovirus produced only 50% protection against virulent nephropathogenic KM91 strain challenge [15].

The S1 subunit of IBV is sufficient for attachment [5,16–19] and the S2 portion of coronavirus spike proteins has traditionally been considered to play a role only in subsequent entry [20,21]. However, a role for the S2 ectodomain in binding to cells has been demonstrated for spike proteins of Massachusetts serotype IBVs, i.e. the highly-attenuated Beaudette strain and the virulent M41 strain [22,23]. In the current study, we evaluated binding of trimeric Ark S-ectodomain compared to trimeric S1 subunit alone to multiple relevant chicken tissues. After confirming improved binding of Ark S-ectodomain, which might be explained by the presence of the S2 ectodomain altering the conformation of S1 and thus increasing its affinity for receptors, or by S2 directly contributing to interaction with receptors or co-receptors, we tested the hypothesis that immunization with recombinant soluble trimeric S-ectodomain provides more effective protection than immunization with trimeric S1 subunit alone.

2. Materials and methods

2.1. Genes and expression vectors

The amino acid sequence of S proteins representing an IBV ArkDPI vaccine subpopulation previously designated C2 (GenBank accession ABY66333) was chosen to produce recombinant proteins. C2 was strongly selected in chickens after vaccination with an ArkDPI-derived attenuated vaccine [3,4]. Its S1 is almost identical to that of the unattenuated parent ArkDPI isolate [24] and represents the consensus sequence of vaccine subpopulations rapidly positively selected in chickens after vaccination with ArkDPI-derived attenuated vaccines [2–4,25,26]. To generate recombinant S1 protein, a human codon-optimized sequence encoding C2 S1 [amino acids (AA) 19-538] was synthesized (GeneArt, Regensburg, Germany) and cloned into the pCD5 vector. To generate recombinant S-ectodomain, a human-codon optimized sequence encoding the C2 S2 ectodomain (S AA 544-1097) was cloned into the pCD5 vector already containing the S1 domain as described [22]. At the S1/S2 border, the furin cleavage site sequence RRSRR was replaced by GGGVP to avoid cleavage of the full length S-ectodomain [22]. These S1 and S-ectodomain-coding sequences were flanked by sequences encoding an N-terminal CD5 signal sequence and sequences encoding C-terminal artificial GCN4 trimerization motif and Strep-tag II for purification and detection of proteins, as described [16].

2.2. Recombinant S protein production and purification

Soluble trimeric recombinant S1 and S-ectodomain proteins were produced in human embryonic kidney (HEK) 293T cells as described [16,22,27]. In brief, the expression vectors encoding S1 or S-ectodomain were transfected into HEK293T cells and recombinant proteins purified from tissue culture supernatants 6 days post-transfection using Strep-Tactin® Sepharose columns according to the manufacturer's instructions (IBA GmbH, Göttingen, Germany). The concentration of purified proteins was determined by Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA). The purified proteins were confirmed and concentrations normalized by electrophoresis in Mini-PROTEAN®TGX Stain-Free™ Precast Gels (Bio-Rad, Hercules, CA).

2.3. Binding to tissues by protein histochemistry

The binding efficiency of S1 and S-ectodomain proteins to tissue sections prepared from healthy specific pathogen free (SPF) 40-day old white leghorn chickens was assessed by protein histochemistry as described [22,27] with minor modifications: antigen retrieval was conducted at 80 °C for 30 min, Tris buffers were substituted for phosphate buffers, slides were blocked with universal negative serum (Biocare, Pacheco, CA) instead of 10% goat serum, and the addition of most reagents and washing steps were performed by an intelliPATH FLX automated slide stainer (Biocare, Pacheco, CA). S proteins and 3-amino-9-ethyl-carbazole (AEC+; Dako, Carpinteria, CA) were added manually. Briefly, S proteins (100 μg/ml for S1 and 50 μg/ml for S-ectodomain) pre-complexed with Strep-Tactin-HRP (IBA GmbH, Göttingen, Germany) were incubated with deparaffinized and rehydrated tissue sections overnight at 4 °C. Bound S protein was visualized with AEC+ chromogenic substrate. The tissues were counterstained with hematoxylin and mounted with Lerner AquaMount (Covance, Princeton, NJ). Images were captured from an Olympus BX41 microscope with an Olympus DP71 12 mp camera.

2.4. Protection trial

2.4.1. Chickens

White leghorn chickens hatched from SPF eggs (Charles River, North Franklin, CT) were maintained in Horsfall-type isolators in biosafety level 2 facilities. Experimental procedures and animal care were performed in compliance with all applicable federal and institutional animal guidelines. Auburn University College of Veterinary Medicine is an Association for Assessment and Accreditation of Laboratory Animal Care-accredited institution.

2.4.2. Experimental design

Four groups of chickens (each $n = 16–17$) were used. Chickens were primed at 12 days of age (DOA) by subcutaneous injection in the neck region of 0.2 ml containing 10 μg of S1 (group A) or 20 μg of S-ectodomain protein (group B) emulsified in Montanide™ ISA 71 VG adjuvant (Seppic, Paris, France). Twice the amount of S-ectodomain protein was used because recombinant S-ectodomain is 1.96-times the molecular weight of recombinant S1. Thus, approximately equimolar amounts of protein were administered. Chickens in groups A and B were subsequently boosted with the same adjuvanted protein 21 days later. Control group C (non-vaccinated) was primed and boosted with PBS and the adjuvant, and group D was the unvaccinated/unchallenged control group. Chickens in groups A, B and C were challenged 21 days after boost by ocular and nasal instillation of 10^5 50% embryo infective doses (EID_{50}) of a virulent IBV Ark-type strain (GenBank accession JN861120) previously characterized [28]. Protection was evaluated 5 days post-challenge (DPC) by viral load in tears and tracheas, tracheal histomorphometry, and tracheal histopathology lesion scoring. In addition, antibodies in sera specific for IBV or S protein were determined by ELISA before prime (11 DOA), three weeks after prime (32 DOA), two weeks after boost (45 DOA) and 5 days post-challenge.

2.4.3. Viral load by qRT-PCR

Relative IBV RNA levels in tears and tracheas were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Viral RNA was extracted from individual tear samples using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA), and

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