Vaccine 31 (2013) 4003–4008

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Protection against avian necrotic enteritis after immunisation with NetB genetic or formaldehyde toxoids☆

Sérgio P. Fernandes da Costa[a], Dorien Mot[b], Monika Bokori-Brown[a], Christos G. Savva[c], Ajit K. Basak[c], Filip Van Immerseel[b], Richard W. Titball[a,*]

[a] College of Life and Environmental Sciences, University of Exeter, Stocker Road, Exeter EX4 4QD, United Kingdom
[b] Faculty of Veterinary Medicine, Department of Pathology, Bacteriology and Avian Diseases, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium
[c] School of Crystallography, Birkbeck College, Malet Street, London WC1E 7HX, United Kingdom

ARTICLE INFO

Article history:
Received 6 April 2013
Received in revised form 2 May 2013
Accepted 14 May 2013
Available online 29 May 2013

Keywords:
Clostridium perfringens
NetB
Pore-forming toxin
Necrotic enteritis
Chicken
Vaccine

ABSTRACT

NetB (necrotic enteritis toxin B) is a recently identified β-pore-forming toxin produced by *Clostridium perfringens*. This toxin has been shown to play a major role in avian necrotic enteritis. In recent years, a dramatic increase in necrotic enteritis has been observed, especially in countries where the use of antimicrobial growth promoters in animal feedstuffs has been banned. The aim of this work was to determine whether immunisation with a NetB toxoid would provide protection against necrotic enteritis. The immunisation of poultry with a formaldehyde NetB toxoid or with a NetB genetic toxoid (W262A) resulted in the induction of antibody responses against NetB and provided partial protection against disease.

1. Introduction

Clostridium perfringens (*C. perfringens*) is a ubiquitous bacterium that is able to colonise a variety of different biotopes and it is not unusual to find *C. perfringens* as a commensal in the normal gut microbiota in domesticated animals. However, under particular circumstances the bacterium is responsible for severe diseases. These diseases are largely a consequence of the actions of toxins on the host [1]. In addition to the four toxins used for typing *C. perfringens* strains (alpha-, beta-, epsilon-, and iota-toxin), the bacterium is able to produce a number of other toxins, including enterotoxin and perfringolysin O [2,3].

C. perfringens has been shown to cause avian necrotic enteritis (NE), a severe gastro-intestinal disease of farmed poultry [4–6]. Until recently, NE has been controlled by the addition of antimicrobial growth promoters to feedstuffs. However, in many countries national and supranational regulations now limit the addition of antimicrobials to animal feeds. Consequently, in these countries, NE is emerging as a disease which is of significant economic consequence to the poultry industry [7,8]. The disease can occur in at least two forms. The acute form of NE typically results in mortality during the last weeks of rear of broilers (week 5–6). However, many cases of NE are associated with relatively mild clinical signs [9–11]. This subclinical form of NE results in decreased digestion and absorption of feedstuffs and consequently reduced weight gain [12,13]. At least in Europe it is now believed that the subclinical NE is the most frequent form of the disease and causes the greatest economic losses to the poultry production industry [14].

Although it is clear that *C. perfringens* is the etiologic agent of NE, a wide range of host and pathogen factors can influence the severity of the disease. These factors include the nature of the feedstuff, co-infection with various *Eimeria* species and the molecular makeup of *C. perfringens* in the gut [15]. Often these factors interact with each other, and this has made the development of reliable infection models difficult [15]. The molecular basis of virulence of *C. perfringens* associated with NE is still being investigated. However, almost all *C. perfringens* isolates from cases of NE possess the *netB* gene [4,16,17] which encodes necrotic enteritis toxin B (NetB), a β-pore-forming toxin [6,18]. Pore formation by NetB can lead to cell lysis by disruption of membrane integrity and a *netB* mutant of *C. perfringens* is reported to be incapable of causing NE [6]. There

* Corresponding author at: College of Life and Environmental Sciences, Geoffrey Pope Building, University of Exeter, Stocker Road, Exeter EX4 4QD, United Kingdom. Tel.: +44 1392 725157; fax: +44 1392 723434.
E-mail address: R.W.Titball@exeter.ac.uk (R.W. Titball).

0264-410X/$ – see front matter © 2013 The Authors. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.vaccine.2013.05.063

is also accumulating evidence that other virulence factors, such as the TpeL toxin, play a role in disease [19]. The production of these virulence factors might explain the reported ability of some *netB*-negative strains of *C. perfringens* to cause NE [4,6].

Immunisation with either crude toxoids [20] or culture supernatants [21] can provide significant but incomplete protection against experimental NE. Although these vaccines are simple to prepare they suffer from the limitation that it is difficult to configure them for non-invasive dosing, for example by oral delivery. Other workers have explored the possibility of a sub-unit which is able to protect against NE, with a view towards both improving vaccine efficacy and opening the possibility of oral delivery. To date, a range of proteins derived from *C. perfringens* have been evaluated as sub-unit vaccines including alpha-toxin, glyceraldehyde-3-phosphate dehydrogenase, pyruvate-ferredoxin oxidoreductase, fructose 1,6-biphosphate-aldolase, or a hypothetical protein [22]. Immunisation with any of these sub-units provided partial protection against experimental NE. Partial protection against NE has also been reported after immunisation with *C. perfringens* large cytotoxin TpeL, endo-beta-*N*-acetylglucosaminidase or phosphoglyceromutase [23]. A more recent study in which alpha-toxin, NetB, pyruvate-ferredoxin oxidoreductase and elongation factor-Tu were compared as protective antigens concluded that NetB and pyruvate-ferredoxin oxidoreductase given with ISA71 adjuvant provided enhanced protective immunity [24]. However, it is unlikely that a licensed vaccine for widespread use could contain active toxins. Therefore, there is a need to identify non-toxic variants of these toxins.

In a previous study, mutational analysis of NetB led to the identification of several amino acids important for toxicity [18]. The mutation of tryptophan to alanine at position 262 (W262A) resulted in a significant reduction in cytotoxicity towards LMH cells, binding to LMH cells and haemolytic activity on red blood cells [18]. The W262A mutation is located within the rim domain of NetB, a region mediating binding of the toxin to the cell membrane.

In this study, we investigated whether a formaldehyde NetB toxoid or a NetB mutant (W262A) were able to induce protection against experimental NE in poultry.

2. Materials and methods

2.1. Bacterial strains and plasmids

Plasmid pBAD (Invitrogen, Paisley, UK) was used as expression vector and *E. coli* TOP10 (Invitrogen, Paisley, UK) as expression host. *E. coli* strains were grown either in Terrific Broth (TB) or Luria–Bertani (LB) agar supplemented with ampicillin (100 μg/ml) at 37 °C and shaken at 300 rpm, where appropriate.

2.2. Animals and housing conditions

Ross 308 broiler chickens were obtained as one-day-old chicks from Vervaeke-Belavi Hatchery (Tielt, Belgium, BE3031) and the parent flock had not been vaccinated with the commercial Netvax or any other *C. perfringens* vaccine. All animals were housed in the same room. The birds were reared in pens at a density of 27 animals per 1.5 m^2 on wood shavings. All pens were separated by solid walls to prevent contact between birds from different treatment groups. Before the trial, the rooms were decontaminated with Metatectyl HQ (Clim'oMedic®, Metatecta, Belgium) and a commercial anticoccidial disinfectant (OOCIDE, DuPont Animal Health Solutions, Wilmington, USA). The chickens received *ad libitum* drinking water and feed. A 23 h/1 h light/darkness programme was applied. The animal experiments were carried out according to the recommendations and following approval of the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium.

2.3. Expression and purification of NetB and NetB W262A

The expression of wild type NetB or NetB W262A, without their native signal peptides and with N-terminal His-tags for purification, was carried out in *E. coli* TOP10 cells and purification was carried out as described previously [18]. In summary, the recombinant *E. coli* carrying the pBAD-NetB expression vector was grown in TB to an optical density (OD_{595nm}) of 0.5 and expression of the toxin induced for 6 h by adding arabinose at a final concentration of 0.02% (w/v). Bacterial cells were harvested by centrifugation, lysed enzymatically using BugBuster (Invitrogen, Paisley, UK), and NetB purified by Ni-NTA chromatography columns (GE Healthcare Life Sciences, Little Chalfont, UK) according to the manufacturer's instructions. The protein was transferred into Tris-buffered saline (TBS; 20 mM Tris pH 7.5, 150 mM NaCl) using PD-10 desalting columns (GE Healthcare Life Sciences, Little Chalfont, UK) and protein concentrations measured with a UV–vis spectrophotometer (Thermo Scientific, Cramlington, UK).

2.4. Preparation of formaldehyde NetB toxoid

Wild type NetB was suspended at 400 μg/ml in TBS and formaldehyde added to a final concentration of 130 mM. After incubation for 5 days at 37 °C, the reaction was stopped by the addition of L-lysine (30 mM final concentration) and residual formaldehyde was removed by dialysis overnight against TBS by using 10 kDa MWCO Spectra/Por 6 dialysis tubing (Spectrumlabs, Rancho Dominguez, USA).

2.5. SDS-PAGE analysis

Protein purity was analysed by SDS-PAGE on precast 4–12% acrylamide-bisacrylamide gels (Invitrogen, Paisley, UK). Therefore, gels were run in MES running buffer (Invitrogen, Paisley, UK) for 45 min at 200 V and stained with SimplyBlue (Invitrogen, Paisley, UK). The Perfect Protein Marker (Merck, Darmstadt, Germany) was used as a protein standard.

2.6. Cytotoxic activity of NetB and NetB toxoids

Wild type NetB or NetB toxoids were evaluated for cytotoxicity towards a chicken hepatocellular carcinoma epithelial cell line (LMH; ATCC: CRL-2117; ATCC-LGC Standards, Teddington, UK) as described previously [18]. Briefly, LMH cells were grown on 96-well plates to approximately 70% confluency in Waymouth's MB 752/1 medium (Invitrogen, Paisley, UK) supplemented with 10% foetal calf serum in a 5% CO_2 incubator at 37 °C. Subsequently, cells were incubated with 100 μl (0.4 mg/ml) of either wild type NetB, formaldehyde NetB toxoid, or NetB W262A for 1 h at 37 °C. Effects on cell morphology were observed with the Inverso-TC optical microscope (Medline Scientific, Chalgrove, UK) and images were taken with the PowerShot S5 IS digital camera (Canon, Reigate, UK).

2.7. Measurement of antibody to NetB using ELISA

Antibody responses to NetB toxoids were determined using an enzyme-linked immunosorbent assay (ELISA). Three groups 10 of one-day-old Ross 308 broiler chickens were fed a wheat/rye-based (43%/7.5%) diet, with soybean meal (24.6% and 25.3% soybean meal in the starter and grower diet respectively) as a protein source [25]. On days 3, 9, and 15, animals were each immunised with 30 μg of either formaldehyde NetB toxoid or NetB W262A. Quil-A (50 μg;