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Presence of DNA extracellular traps but not MUC5AC and MUC5B mucin in mucoid plugs/casts of infectious laryngotracheitis virus (ILTV) infected tracheas of chickens

Vishwanatha R.A.P. Reddy*, Ivan Trus, Hans J. Nauwynck

Laboratory of Virology, Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium

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ABSTRACT

Although it has been speculated that the tracheal obstructions and asphyxiation during acute infectious laryngotracheitis (ILT) are due to mucoid plugs/casts formed by mucus hypersecretion, there are no reports demonstrating this. Hence, in the present study, we first examined if the main respiratory mucins, MUC5AC and MUC5B, are expressed in the mucosae of larynx, trachea and bronchi of mock-inoculated and ILTV infected chickens. Second, the tracheas with plugs/casts were stained for mucins (MUC5AC and MUC5B) and nuclear material (traps). MUC5AC and MUC5B were produced by the mucosae of larynx, trachea and bronchi of mock-inoculated chickens. Interestingly, MUC5AC and MUC5B were exclusively present in the dorsal tracheal region of the cranial and middle part of trachea of mock-inoculated chickens. In ILTV infected chickens, the tracheal lumen diameter was almost 40% reduced and was associated with a strongly increased tracheal mucosal thickness. MUC5AC and MUC5B were scarcely observed in larynx, trachea and bronchi, and in tracheal plugs/casts of ILTV infected birds. Surprisingly, DNA fibrous structures were observed in connection with nuclei of 10.0 ± 7.3% cells, present in tracheal plugs/casts. Upon inoculation of isolated blood heterophils with ILTV, DNA fibrous structures were observed in 2.0 ± 0.1% nuclei of ILTV inoculated blood heterophils at 24 hours post inoculation (hpi). In conclusion, the tracheal obstructions and suffocation of ILTV infected chickens are due to a strong thickening of the mucosa (inflammation) resulting in a reduced tracheal lumen diameter and the presence of mucoid plugs/casts containing stretched long DNA-fibrous structures (traps) but not MUC5AC and MUC5B mucins.

1. Introduction

Avian infectious laryngotracheitis virus remains a threat to the worldwide commercial poultry industry by decreased egg production, delayed growth and mortality (Fuchs et al., 2007). ILTV belongs to the order *Herpesvirales*, family *Herpesviridae*, subfamily *Alphaherpesvirinae* and genus *Iltovirus* (Davison, 2010). ILTV replicates in the epithelial cell of the laryngeal, tracheal, and conjunctival mucosae, and invades underlying layers in a restricted manner (Garcia et al., 2013; Reddy et al., 2014). ILTV is usually highly cytolytic in the laryngeal and tracheal mucosae, which may lead to severe mucosal epithelial damage and hemorrhages. The above pathological changes cause the typical ILT clinical signs: coughing, nasal discharge, and conjunctivitis during a mild form and marked dyspnea, gasping, open mouth breathing and expectoration of bloody mucoid material during a severe form (Bagust et al., 2000; Garcia et al., 2013). Mucoid casts/plugs in the trachea obstruct airways and predispose chickens to die due to asphyxiation (Bagust et al., 2000; Linares et al., 1994).

After an acute laryngotracheits infection, ILTV can establish a lifelong latency in the trigeminal ganglion of the central nervous system (Garcia et al., 2013). Stress during rehousing with unfamiliar birds and onset of egg production cause sporadic reactivation followed by active replication of ILTV and horizontal transmission of ILTV to susceptible contact animals (Bagust et al., 2000; Fuchs et al., 2007).

Mucus is a viscoelastic and biopolymeric hydrogel, which coats the moist non-keratinized surface of mucosa. Mucus serves as a major protective layer on the mucosa, by forming a semipermeable barrier that enables the exchange of nutrients, gases and water, while being impermeable to most pathogens/foreign particles (Vareille et al., 2011; Yang et al., 2012). The mucus layer

* Corresponding author.
E-mail addresses: reddy.vishwanatha@ugent.be (V.R.A.P. Reddy), ivan.trus@ugent.be (I. Trus), hans.nauwynck@ugent.be (H.J. Nauwynck).

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thickness differs among the species, location in the respiratory tract and health status. The major components of respiratory mucus are mucins. Up till now, at least 9 mucin genes have been reported in human airway mucus, with MUC5AC and MUC5B being the major gel forming mucins (Corfield, 2015). Mucin types may change during disease (Rose and Voynow, 2006) and many respiratory viruses stimulate mucus production in respiratory mucosa (Vareille et al., 2011). During an acute ILTV infection, mucoid plugs/casts are formed in the trachea and obstruction may lead to chicken mortality (Linares et al., 1994). It has been postulated that the tracheas-mucoid plugs/casts are formed due to mucus hypersecretion, however there are no hard data proving this (Garcia et al., 2013; Linares et al., 1994).

In the present study, first we examined the expression of MUC5AC and MUC5B in the mucosae of larynx, trachea and bronchi of the mock-inoculated and ILTV infected chickens by immunofluorescence staining. Second, the tracheal lumen diameter and mucosal thickness were compared between the mock-inoculated and ILTV infected chickens to understand their role in obstruction of the trachea. Third, tracheas with mucoid plugs/casts from euthanized ILTV infected chickens showing respiratory distress were stained for MUC5AC and MUC5B and for DNA fibrous structures (extracellular network). Finally, the effect of ILTV on the formation of DNA fibrous structures from nuclei of blood heterophils was analysed (Chuammitri et al., 2009; Goldmann and Medina, 2012; Zawrotniak and Rapala-Kozik, 2013).

2. Materials and methods

2.1. ILTV (U76/1035) inoculation

Six twelve-week-old specific pathogen free (SPF) White Leghorn chickens were individually tagged and housed in two experimental rooms. Drinking water and feed were provided *ad libitum*. A pathogenic Belgian isolate of ILTV (U76/1035) was used in this study (Meulemans and Halen, 1978; Reddy et al., 2014). Before the start of the experiment, an acclimatization period of one-week was respected. At the age of thirteen weeks, three chickens (group 1) were inoculated with the virulent ILTV (U76/1035) via intratracheal (300 μl), nasal (50 μl each nostril) and ocular routes (50 μl each eye) with 10^4 EID_{50}/500 μl. The second group of three animals was mock-inoculated with PBS and served as non-infected control. This study was in agreement with the guidelines of the Local Ethical and Animal Welfare Committee of the Faculty of Veterinary Medicine of Ghent University.

2.2. Clinical signs

Clinical signs were recorded daily until 5 days post inoculation (dpi), with special emphasis on bird breathing.

2.3. Euthanasia and collection of mock-inoculated and ILTV infected larynx, trachea and bronchi, and ILTV infected trachea with mucoid plugs/casts

Larynx, trachea and bronchi were collected from three mock-inoculated and three ILTV infected chickens. Equal sized tissues were prepared, embedded in Methocel® (Fluka) and frozen at −70 °C.

In order to have a good yield of mucoid plugs/casts in ILTV infected tracheal mucosa, chickens were humanely euthanized when they showed marked gasping and open mouth breathing with an extended neck (5 dpi). During necropsy, tracheas with mucoid plugs/casts were collected in a gelatin capsule (size 000, Nova, Belgica T.O.P. nv). The schematic procedure for collecting tracheas with mucoid plugs/casts from ILTV infected chickens is illustrated in Fig. 1. Briefly, a trachea containing a mucoid plug/cast was placed vertically in a gelatin capsule. The gelatin capsule with trachea containing a mucoid plug/cast was immediately embedded in Methocel® in plastic tubes (Fluka) and were snap frozen for immunofluorescence staining.

2.4. Immunofluorescence staining for MUC5AC and MUC5B in laryngeal, tracheal and bronchial mucosae of mock-inoculated chickens

Immunofluorescence staining was performed to determine MUC5AC and MUC5B secretion in the mucosae of larynx, trachea and bronchi of mock-inoculated chickens. Cryosections of 10 μm were made from larynx, trachea and bronchi, fixed in 4% paraformaldehyde for 20 min at 4 °C and permeabilized in 0.1% Triton® X-100 for 10 min at room temperature. For MUC5AC staining, the sections were incubated with mouse anti-MUC5AC monoclonal IgG_1 antibodies as primary antibody (45M1, LifeSpan Biosciences, 1:100) and FITC labeled goat anti-mouse IgG polyclonal antibodies as secondary antibody (Molecular Probes, 1:200). For MUC5B staining, the sections were incubated with rabbit anti-MUC5B polyclonal antibodies as primary antibody (H-300, Santa Cruz Biotechnology, 1:100) and FITC labeled goat anti-rabbit IgG polyclonal antibodies as secondary antibody (Molecular Probes, 1:200). All antibodies were diluted in 10 mM phosphate buffer saline (PBS) and incubated for 1 h at 37 °C. Two washings with PBS (10 min/each) were performed after each incubation step. The nuclei were counterstained with Hoechst 33342 (Molecular Probes, 1:100) for 10 min at room temperature. The sections were then washed twice and mounted with glycerin-DABCO (Sigma).

2.5. Immunofluorescence staining for MUC5AC/MUC5B and ILTV in laryngeal, tracheal and bronchial mucosae, and trachea with mucoid plugs/casts of ILTV infected chickens

Cryosections of 10 μm were made from ILTV infected larynx, trachea and bronchi, and trachea filled with mucoid plugs/casts. ILTV infected laryngeal, tracheal and bronchial mucosae and trachea with mucoid plugs/casts were visualized for MUC5AC, MUC5B and ILTV using the same technique as for the cryosections of laryngeal, tracheal and bronchial mucosae of mock-inoculated chickens. To visualize ILTV infection in laryngeal, tracheal and bronchial mucosae and trachea with mucoid plugs/casts, cryosections were incubated with mouse monoclonal anti-ILTV gC antibodies as primary antibody (1:50) (kindly provided by Walter Fuchs, Institute of Molecular Biology, Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany) and FITC labeled goat anti-mouse IgG polyclonal antibodies as secondary antibody (Molecular Probes, 1:100). To determine if mucin-producing cells were infected with ILTV, double immunofluorescence stainings were performed for MUC5B and ILTV. Hoechst was used to visualize cell nuclei. A confocal microscope (Leica TCS SPE confocal microscope) was used for the analysis of the presence of mucin and ILTV infected cells in laryngeal, tracheal and bronchial mucosae and trachea with mucoid plugs/casts.

2.5.1. Measurement of the diameter of tracheal lumen and the thickness of tracheal mucosa

The tracheal lumen diameter and mucosal thickness were evaluated in the mock-inoculated and ILTV infected chickens by using a confocal microscope. The epithelial layer and lamina propria layer were measured as mucosal thickness (Nunoya et al., 1987). Ten randomly selected regions were considered for measurement in each chicken. The measurement was performed in tracheal rings of three mock-inoculated and three ILTV infected chickens. Student's *t*-test was used to compare the diameter of the tracheal lumen and