Innate Immune Response of the Pig Laryngeal Mucosa to Endotracheal Intubation

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Abstract

Objective. The aim of this study was to measure the effects of endotracheal intubation on innate immune response within the pig laryngeal mucosa.

Study Design. Prospective controlled basic science study.

Setting. The animal experiments and analyses were conducted at the University of Bristol.

Samples and Methods. Eighteen pigs, matched at the major histocompatibility complex (MHC), were used in the study. The pigs were divided into 9 pairs. One of each pair (9 pigs in total) was intubated with an endotracheal tube under general anesthesia for 90 minutes. Two days later, pinch biopsies were taken from the supraglottis (specifically the false cords) and subglottis of both pigs. The experiment was repeated 8 more times. Based on quantitative immunohistochemistry, percentage areas of positive staining for CD172a, CD163, MHC class II, CD14, and CD16 were calculated separately for the epithelium and lamina propria of each biopsy.

Results. Total areas of laryngeal mucosa (epithelium and lamina propria) expressing CD172a and coexpressing CD163 and CD172a were significantly reduced at 2 days following endotracheal intubation (P = .039 and P = .037, respectively). MHC class II expression and MHC class II coexpression with CD172a were similarly reduced following intubation (P = .003 and P = .005, respectively). In the supraglottis, MHC class II coexpression with CD16 and CD14 was also reduced following endotracheal intubation (P = .037).

Conclusions. Our results indicate that endotracheal intubation reduces the number of innate immune cells within the upper airway mucosa. This may be an important first step in a cascade leading to chronic wound and scar formation causing airway stenosis.

Keywords

intubation, major histocompatibility complex, endotracheal
and bacterial infections.\textsuperscript{10-12} Given these observations, we predicted that endotracheal intubation presents a sufficient environmental challenge to alter the normal laryngeal immune architecture within 48 hours and that this may represent a sentinel event leading to the cascade of altered wound healing, scar formation, and stenosis within the upper airway.

To test this hypothesis, we aimed to measure the effects of endotracheal intubation on the innate immune response within the larynx of pigs matched at the major histocompatibility complex (MHC). The pig larynx is similar to that of humans in size, anatomy, and, importantly, the detailed immune architecture of its mucosal lining.\textsuperscript{13-15} For these reasons, pigs have been used extensively by our group and others to study laryngeal immunology and the effects of endotracheal intubation.\textsuperscript{13,16-20} However, to date, there have been no studies of the laryngeal mucosal immune response to endotracheal intubation.

\section*{Materials and Methods}

\subsection*{Animals and Operative Procedures}

This study was conducted with national and local research ethics board approval (UK Home Office PPL 30/1786). National Institutes of Health (NIH) minipigs specifically inbred for the MHC loci (c/c haplotype) were used (N = 18; mean ± SD: weight, 32 ± 10 kg; age, 19 ± 4 weeks). The pigs were divided into 9 pairs and housed together for at least 2 weeks prior to any interventional procedure to reduce any environmental effect. One of each pair was anesthetized for 2 weeks prior to any interventional procedure to reduce any environmental effect. One of each pair was anaesthetized for 90 minutes before being extubated and recovered.

Then 48 hours later, each pig (one previously intubated as described below and the other not intubated at all) was induced, and before the larynx was intubated with an endotracheal tube, pinch biopsies were taken from the supraglottic, and before the larynx was intubated with an endotracheal tube. Atracurium (Glaxo, Middlesex, UK) was used as muscle relaxant at an initial dose of 1 mg/kg. Anesthesia was maintained with isoflurane (Baxter Healthcare Ashworth, UK), with the cuff placed into the subglottis. Intubation and Anesthetic Procedures

Nine pigs were premedicated with intramuscular ketamine (10 mg/kg; Vetco, Dublin, Ireland) and azaperone (2 mg/kg; Janssen-Cilag, Pfärgasse, Austria). Anesthesia was induced by intravenous propofol (Abbott Laboratories, Kent, UK). Intubation was performed with a Macintosh blade placed into the vallecula and a 5.5-mm cuffed endotracheal tube (Smiths Medical, Ashworth, UK), with the cuff placed into the subglottis. Anesthesia was maintained with isoflurane (Baxter Healthcare Corporation, Cambridge, UK) and 50:50 oxygen:nitrous oxide (BOC, Manchester, UK) administered through an endotracheal tube. Atracurium (Glaxo, Middlesex, UK) was used as muscle relaxant at an initial dose of 1 mg/kg.

\subsection*{Intubation and Anesthetic Procedures}

Biopsies were prepared and stained according to our usual techniques.\textsuperscript{21} Biopsies were orientated on cork discs to allow sectioning at right angles to the laryngeal epithelium, covered in OCT media (Tissue-Tek, Sakura; Raymond A. Lamb, Eastbourne, UK) and snap frozen in liquid nitrogen. Samples were stored at –80°C until processed. Each frozen biopsy was mounted in a cryostat (Bright Instruments, Cambridge, UK) and 5-μm serial sections cut and placed onto multisport glass slides (Hendley, Loughton, UK). The slides were air-dried for 1 hour at room temperature, then fixed in acetone for 10 minutes before being stored at –20°C until processed. Each tissue sample was blocked with 5% pig and goat serum in phosphate-buffered saline (PBS) for 60 minutes at room temperature in a humidity chamber. The blocking solution was washed off with PBS before primary antibodies were applied at optimum concentration. All primary antibodies were kindly gifted by Dr K. Haverson, University of Bristol. Combinations of the following primary antibodies were used: CD16 (IgG1, G7), CD14 (IgG1, MIL2), CD163 (IgG2b, SwNL517.2), swine cluster 3 (also known as CD172a; IgG2a, 74-22-15 or IgG1, 6F3), and MHC class II (IgG2a, MSA3). Primary antibodies were incubated at 4°C in a humidity chamber overnight and then washed 3 times for 10 minutes in PBS. After the slides were dried, isotype-specific secondary goat anti-mouse conjugated to FITC, Alexa Fluor 633, and TRITC, or Texas Red (SouthernBiotech, Birmingham, Alabama) was added in combination and incubated for 1 hour at room temperature, then washed 3 times in PBS. The slides were then carefully dried and mounted with glass cover slips and Vectashield mounting fluid (Vector Laboratories, Burlingame, California). Between 6 and 10 fields (depending on the size of the biopsy) at ×20 or ×40 magnification were viewed and digitized with a Leica DMRA fluorescence microscope (Leica, Wetzlar, Germany) and an ORCA-ER monochrome camera (Hamamatsu) with the image capture application Q-Fluoro (Leica). Separate grayscale images were digitized with single-channel filters K3 (FITC), N3 (TRITC), and Y5 (Alexa Fluor 633), where appropriate, and false-colored to green, red, and blue color-channels, respectively, then recombined to create a true-color image. Positive staining was defined as being above background intensity with Image J analysis software (NIH, Bethesda, Maryland; http://rsb.info.nih.gov). Percentage areas of positive staining for red, green, and blue and all possible combinations (yellow, magenta, cyan, white) were calculated with a macro as previously described.\textsuperscript{22} The basement membrane was easily visible by eye, and for each tissue section, positive staining was calculated separately for the epithelium and lamina propria. Our group has previously demonstrated that this method produces results comparable to those obtainable by flow cytometry.\textsuperscript{23}

\subsection*{Data Analysis}

Statistical analysis was performed with SPSS (version 16.0; SPPS Inc, Chicago, Illinois). We have divided the mucosa into 2 areas consistent with standard nomenclature:\textsuperscript{24} (1) the epithelium, which sits above the basement membrane and can be considered to be analogous to the epidermis of the skin, and (2) the lamina propria, which sits below the basement membrane and is analogous to the dermis of the skin. The percentage area of positive staining in the epithelium and lamina propria was $\log_{10}(n+2)$ transformed to produce...
normally distributed data. The effect of endotracheal intubation was analyzed with an independent-sample t test containing previous intubation and laryngeal subsites as categorical variables. Levene’s test for equality of variance was performed and statistical significance set at \( P < .05 \).

Results

Using quantitative immunofluorescence, we identified that the total area of laryngeal mucosa (by which we mean the total area of epithelium and lamina propria contained in the biopsy) expressing CD163, CD172, MHC II, CD16, and CD14 was determined by quantitative immunofluorescence. MHC, major histocompatibility complex.

To investigate the effects of endotracheal intubation on the supraglottis and subglottis, expression of each fluorophore was calculated as a percentage of total area separately for each subsite within the larynx. Changes in protein expression in the supraglottic mucosa (both the epithelium and the lamina propria; Figure 2) following endotracheal intubation reflected those of the larynx as a whole: namely, MHC class II expression (\( P = .003 \)) and MHC class II coexpression with CD172a were similarly reduced following intubation (\( P = .006 \)).

Discussion

Using a panel of fluorescent antibodies against cell surface proteins, we quantified the number of cells belonging to the innate immune system within the laryngeal mucosa. Our results indicate that the number of innate immune cells within the larynx is reduced following endotracheal intubation under general anesthesia.

Our objective was to study the early (within 48 hours) immune responses to endotracheal intubation (under anesthesia), since these are likely to be most amenable to novel therapeutic interventions. Therefore, in this work, we have not examined the effects of endotracheal intubation on the innate and adaptive immune responses over the longer term. Furthermore, we designed the study to look at the most early events following endotracheal intubation rather than the response to tissue injury; our intention is to identify mechanisms that may lead tissue injury to occur. For this reason, our work differs from others\(^9,25,26\) in that we have neither reach statistical significance in the epithelium (data not shown).

In contrast, in the supraglottic lamina propria, CD172a (\( P = .039 \)) and CD172a and CD163 coexpression (\( P = .021 \)) were significantly reduced with intubation, but levels of MHC remained unchanged (\( P > .05 \)) (Figure 3). Within the supraglottis, significant reduction in protein expression was observed only for MHC class II with endotracheal intubation (\( P = .047 \); Figures 4 and 5). The representative images of the supraglottis demonstrate strong and ubiquitous MHC staining without intubation (Figure 5A) but not following intubation (Figure 5B). There was no reduction in expression when the supraglottic epithelium and lamina propria were analyzed separately (data not shown).
artificially created a wound nor waited for a macroscopic wound to appear in the mucosa. Our study also differs from others in that we have taken biopsies for immunofluorescence histochemistry rather than measured secreted cytokines. This approach has enabled us to look at the cells of the immune system in situ within the mucosa.

A possible limitation of this study is that we are unable to distinguish whether our observed results are due to the effects of anesthesia, intubation, or a combination of the two. This could have been addressed by having a third control group of pigs who are maintained under anesthesia with a device akin to the laryngeal mask. Unfortunately, a laryngeal mask has not been developed for pigs, so this additional control was not possible in this experimental model. Furthermore, since intubation for any period of time is invariably maintained under anesthesia, it may be argued that a pragmatic approach is to accept these 2 elements to be part of the same intervention.

To explain our observations of protein expression, we need to first understand which cells within the immune system express these proteins. First, we consider our observation that MHC class II expression is decreased at 48 hours following endotracheal intubation and that its coexpression with CD172a, CD14, and CD16 was also decreased (Figure 5). MHC II is expressed by mature dendritic cells (DCs), the so-called professional antigen-presenting cells, together with other costimulatory molecules on their surface. Following maturation, DCs migrate to regional lymph nodes where, via their MHC complexes, they cause T-cell activation and proliferation—the hallmark of the adaptive immune response. Our observation may therefore indicate a reduction in the activation of
DCs or recruitment of antigen-presenting cells into the laryngeal mucosa following intubation.

To explain this observation, we can look at the role played by PGE2. This cytokine is of interest for 3 reasons: (1) PGE2 secreted by stromal fibroblasts suppresses DC maturation\(^{29}\); (2) PGE2 increases at 12 to 72 hours following injury to the laryngeal mucosa\(^9\); and (3) fibroblasts recruited into the laryngeal mucosa following injury become resistant to PGE2, which would usually reduce their migration.\(^30\) The result is the accumulation of fibroblasts at the site of injury, which has been described as a key event in scar formation and, ultimately, laryngeal stenosis.\(^9\)

Another property of PGE2 is that in addition to reducing the number of DCs within the mucosa, as described by Shiraishi et al, PGE2 also reduces the amounts of proinflammatory cytokines secreted by these cells.\(^{29}\) Since proinflammatory cytokines, such as interferon \(\gamma\)\(^{31}\) and IL-12,\(^32\) provide strong chemotactic signals that cause immune cells to migrate and accumulate in the mucosa, this role of PGE2 may also explain why we observed a decrease in CD172a expression and its coexpression with CD163 in the laryngeal mucosa following intubation. CD172a is expressed on all myelocytic cells and is increasingly expressed as monocytes mature into macrophages.\(^{33-35}\) whereas CD163 is expressed by macrophages located within the lamina propria of mucosal surfaces—this has been observed in the gastrointestinal and uterine tracts, but it has not been looked for in the larynx.\(^{36,37}\)

The results that we present here indicate that endotracheal intubation under general anesthesia reduces the number of innate immune cells within the laryngeal mucosa. It is conceivable that laryngeal mucosal injury in the context of a suppressed mucosal immune cell response and increased fibroblast accumulation creates an environment ripe for chronic wound and scar formation. Nonetheless, further work is required to fully characterize the innate mucosal immune response to endotracheal intubation in the larynx. Specifically, our understanding of the role played by macrophages within the mucosa is evolving to show a much more complex picture than previously thought. We know that macrophages reside in mucosa, even in the absence of apparent inflammation or infection, where they act as the “front-line” defense against immune challenges by phagocytosing cellular debris and pathogens and presenting antigens to the adaptive immune system.

In summary, this article provides new insights into the etiology of postintubation laryngeal injury where either injury or anesthesia (or a combination of the two) creates an environment in which the concentration of immune cells within the mucosa is reduced. This work builds on previous work on laryngeal injury and stenosis.

**Author Contributions**

Owain R. Hughes, substantial contribution to data collection, data analysis and writing of the manuscript; Sarah M. Ayling, substantial contribution toward design of the study, data collection, data analysis, and reviewing and revising the manuscript; Martin A. Birchall, significant contribution to design of the study, data collection and reviewing and revising the manuscript.

**Disclosures**

**Competing interests:** Owain R. Hughes, Operavox Ltd—director; Martin A. Birchall, Videregen Ltd, ReNeuron Plc—consultant.

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**References**


