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Original article

An enzyme-linked immunosorbent assay (ELISA) for the determination of mucin levels in bronchoalveolar lavage fluid

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Abstract

Introduction: A method to measure the mucin concentration in bronchoalveolar lavage (BAL) fluid was developed to aid efforts to identify pharmacologically the mechanisms that modulate pathophysiological mucin secretion. Mucins are the major macromolecular components of mucus. In the airways, mucus is the first line of defense against inhaled microorganisms (infection) and particulates (irritation). **Methods**: An enzyme-linked immunosorbent assay (ELISA) was developed, comparing two monoclonal anti-mucin antibodies (A10G5 and 45M1) raised to human mucin, to quantify the mucin in BAL fluid from animal models of pulmonary inflammation. To validate the ELISA method, rats were exposed to ovalbumin (OVA, in sensitized rats), lipopolysaccharide (LPS), vanadium pentoxide (V_2O_5), or saline. One hundred microliters of BAL fluid was analyzed for mucin concentration. Pooled BAL fluid from untreated rats was used as an internal "plate standard", as a standard mucin that cross-reacts with A10G5 was unavailable. **Results**: We found both antibodies reacted with rat, human, and guinea-pig mucin; where the 45M1 antibody also reacted with the mucin in porcine BAL, while A10G5 did not. We determined the mucin concentration in each BAL fluid sample relative to the standard, defined as a mucin concentration of 100 plate units. BAL fluid from LPS (218 ± 25 plate units, $n=5$), OVA (386 ± 31, $n=3$), V_2O_5 (1208 ± 450, $n=6$) challenged rats displayed significantly elevated mucin concentration over their saline controls (126 ± 22, $n=12$). Subsequently, the 45M1 antibody displayed immunoreactivity with a commercially available crude preparation of porcine stomach mucin, allowing us to calculate the concentration of mucin directly compared to the known concentration of the porcine stomach mucin standard. Both the 45M1 and A10G5 based ELISA assays detected higher mucin content in the saline challenged rat than the saline challenged guinea pig BAL. **Discussion**: The recent availability of the 45M1 antibody and the use of the crude purification of porcine stomach mucin as a reference standard should allow for direct comparison of mucin concentration in BAL (and other fluids).

Keywords: ELISA; A10G5; Guinea pig; Lavage; Methods; Mucin; Porcine; Rat; 45M1

1. Introduction

In many human airway diseases, excessive abnormal mucus is retained in the airways. The resulting mucostasis leads to an obstruction situated within the airway lumen and a loss of ventilatory function (Wells & Richardson, 1997). Mucus glycoproteins (mucins) are the major macromolecular components of mammalian mucus. Twenty human mucin genes have been cloned and named as MUC1-4, 5B, 5AC, and 6-19 (Chen et al., 2004). Of the five gel-forming mucin genes currently identified (MUC2, MUC5AC, MUC5B, MUC6, and MUC19), MUC5AC and MUC5B make up an estimated 97% of the weight of the gel-forming mucins in airway mucus (Hovenberg, Davies, Herrmann, Linden, & Carlstedt, 1996b; Kirkham, Sheehan, Knight, Richardson, & Thornton, 2002). In the airways, MUC5AC and MUC5B appear to be respectively produced primarily by epithelial goblet cells (Hovenberg, Davies, & Carlstedt, 1996a) and submucosal glands (Wickstrom, Davies, Eriksen, Veerman, & Carlstedt, 1998). Although, the glands and goblet cells can produce both types (Roger, Gascard, Bara,

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Dulmet, & Brink, 2001; Wickstrom et al., 1998). The amounts of these two mucins can be altered significantly in diseased airways (Henke, Renner, Huber, Seeds, & Rubin, 2004; Kirkham et al., 2002; Sheehan, Howard, Richardson, Longwill, & Thornton, 1999) and are likely responsible for the abnormal secretions found in asthma, chronic obstructive pulmonary disease (COPD), chronic bronchitis, and bronchiectasis (Matsui, Randell, Peretti, Davis, & Boucher, 1998; Rogers, 2000). Estimates of mucin concentration in airway secretions range from 0.6 mg/ml in health (Fahy et al., 1993) up to 40 mg/ml in a gel plug taken from an asthmatic airway (Sheehan, Richardson, Fung, Howard, & Thornton, 1995). Accordingly, drugs that normalize mucin secretion are of interest as remedies for these diseases.

In the past, many ELISA methods have been developed to measure airway mucin secretion (Basbaum et al., 1986; Fahy et al., 1993; Lin, Carlson, St.George, Plopper, & Wu, 1989; Sosse-Alaoui et al., 1998; St.George et al., 1985; Steiger, Fahy, Boushey, Finkbeiner, & Bashaum, 1994; Tesfaigzi, Fischer, Martin, & Seagrave, 2000). However, these methods depend on anti-mucin antibodies and mucin containing standards that are incompletely characterized or not widely available. Herein, we describe a direct-binding ELISA method (Crowther, 2001), using a widely available mucin standard and compare the results of the ELISA using two different anti-mucin primary antibodies, A10G5 (a proprietary antibody) and the commercially available 45M1. The ELISA was developed to measure the relative differences in mucin content in bronchoalveolar lavage (BAL) fluid from multiple animal species. The mucin ELISA was validated with BAL fluid from lipopolysacharide (LPS), ovalbumin (OVA), and vanadium pentoxide (V_2O_5) challenged rats.

2. Methods

All studies using animals were done in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act in a program approved by the American Association for the Accreditation of Laboratory Animal Care. Protocols used in these studies were approved by the Animal Care and Use Committee of Schering-Plough Research Institute.

2.1. ELISA (A10G5 antibody)

Mucin concentration in BAL fluid was determined and expressed in relative plate units, with 100 plate units defined as the concentration of mucin found in BAL fluid pooled from naive rats. To create the rat BAL mucin plate standard, the lungs of anesthetized Sprague–Dawley rats were lavaged with two X 3-ml volumes of saline. Multiple samples from naïve Sprague–Dawley rats were pooled together to create the mucin standard. All lavage fluids were centrifuged (7 min, 4 °C, 350 *g*) and the supernatants were stored at −80 °C until assayed.

The unchallenged rat BAL fluid was used as a plate standard because no commercially available mucin was reactive with the A10G5 anti-mucin antibody. The A10G5 antibody has been shown to react with guinea pig and monkey mucins (C. Basbaum, personal communication) as well as rat (Steiger, Hotchkiss, Bajaj, Harkema, Basbaum, 1995) and dog (Chen & Yeates, 2000) mucins. Serial two-fold dilutions of the plate standard out to 1:2048 (100 μl/well) were conducted on a 96 well plate (Becton Dickinson, Microtest 96) with phosphate buffered saline (PBS, Mediatech, 21-030-CM). Similarly, samples with unknown mucin concentration were serially diluted from 1 : 1 to 1 : 2048, in duplicate (100 μl/well). The plates were incubated at room temperature for 90 min and then washed three times with Wash buffer (PBS with 0.1% surfactant Tween 20, Acros Organics, 233360010). Next, 200 μl of Blocking buffer (17% Sea block, Pierce, 37527 in PBS) was added to each well and incubated for 60 min. The plates were then washed three times with Wash buffer. Afterwards, 100 μl of mouse monoclonal antibody obtained from C. Basbaum (A10G5 an IgG_1, 1 : 300 in Blocking buffer) was added to the wells for 30 min. Washes with Wash buffer were repeated, followed by the addition of 100 μl of biotinylated, polyclonal, goat anti-mouse IgG (human, swine, rat, and guinea pig IgG-absorbed, Zymed, 65–6440, diluted 1 : 5000 in Blocking buffer). After incubating for another 30 min, the plates were washed again followed by a 30 min incubation with purified streptavidin–horseradish peroxidase (HRP) conjugate (Biosource SNN2004, diluted 1 : 5000 in Blocking buffer, 100 μl/well). Finally, 100 μl of tetramethylbenzidine (TMB) reagent with peroxide (Pierce, 34021) was added and incubated for 5 min. The reaction in the wells was then stopped by addition of 50 μl of 2M sulfuric acid. The optical density of the wells was determined at an excitation wavelength of 450 nm (giving optical density readings that corresponded to mucin concentration) and a reference wavelength of 540 nm on the SpectraMax 250 (Molecular Devices) spectrophotometer.

2.2. ELISA (45M1 antibody)

The 45M1 ELISA followed the same protocol as the A10G5 ELISA except that the 45M1 antibody (Neo-Markers, MS-145-P1) at a 1:100 dilution was substituted for the A10G5 antibody and a crude purification of porcine stomach mucin (Sigma-Aldrich, M-2378, St. Louis, MO) was used as the standard starting at a 100 μg/ml concentration with serial two-fold dilutions out to 1 : 2048.

2.3. Data analysis

For each 96 well plate, the optical density at 450 nm minus the optical density at 540 nm (OD_{450}–OD_{540}) for each well was calculated. Mean OD_{450}–OD_{540} versus the mucin concentration (or dilution ratio) for each serially diluted standard sample pair was plotted (standard curve) for