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Comparison Between ELISA and Gel-filtration Assay for the Quantitation of Airway Mucins

Chan Young Shin, Suk-Jo Kang[1], Kwang Chul Kim[2] and Kwang Ho Ko*

Lab. of Pharmacology, College of Pharmacy, Seoul National University, Seoul 151-742 and [1]Center for Biofunctional Molecules, Postech, Pohang, Korea and [2]Department of Pharmaceutical Sciences, University of Maryland, School of Pharmacy, 20 North Pine Street, Baltimore, MD 21201, U.S.A.

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In this study, we developed immunoassay methods for the more convenient and effective detection of rat tracheal mucin and the results were compared with those of [^{3}H]glucosamine based gel-filtration method. A monoclonal anti-rat tracheal mucin antibody, mAbRT03, which specifically recognizes rat tracheal mucins, was used throughout in this study. To induce mucin secretion, varying concentrations of ATP (0~2 mM) were applied to the primary rat tracheal surface epithelial (RTSE) cell culture which had been metabolically radiolabeled with [^{3}H]glucosamine and the secretion of mucin was analyzed both by the immunoassay and the gel-filtration chromatography methods. For the immunoassay, the following two procedures were employed. 1) Simple ELISA; the culture spent media were directly coated onto the assay plate and the immunoreactivity with mAbRT03 was assessed from the standard curve generated with the purified rat mucin. 2) Inhibition ELISA; A known amount of the purified rat mucin was coated onto the assay plate and then ATP-stimulated culture spent media were added to inhibit the immunoreactivity with mAbRT03. The contents of mucin in the sample were calculated from the standard inhibition curve which was generated with the purified rat mucin. The assay results obtained from the immunoassays were identical with those from the gel-filtration methods. The present result indicates that ELISA can be substituted for the laborious, time-consuming gel-filtration assay in studying the regulation of airway mucin release from cultured airway epithelial cells.

Key words : Airway mucin, mAbRT03, Immunoassay

INTRODUCTION

In the airway, mucus plays an important role in the host's defense, and its proper function is believed to be attributed mainly to the quality and quantity of mucus glycoproteins or mucins which are present in the mucus. Mucins are high molecular weight (>10^6 Da) glycoproteins composed of approximately 80~90% carbohydrate and 10~20% peptides (Boat *et al.*, 1993). These airway mucins are secreted by two types of cells; goblet cells of the surface epithelium and mucous cells of the submucosal gland. Abnormal or aberrant airway mucus secretion is associated with several diseases, such as chronic bronchitis and cystic fibrosis (Boat *et al.*, 1993). However, airway mucin and its components have proven to be very difficult to study, as carbohydrates found in these molecules are heterogeneous in chain length, structure, and acidity (Samet and Cheng, 1994; Slayter *et al.*, 1984; Strous *et al.*, 1992). There is little question that a better understanding of the mechanism of regulation of mucin secretion, and physicochemical properties and functions of mucin could be invaluable in devising means for prevention and treatment of many airway diseases. Most *in vitro* studies of the regulation of airway mucin secretion have been carried out by using tracheal organ or explant cultures that contain both surface epithelial cells and submucosal glands (Adler, 1986; Reid *et al.*, 1983; Spicer and Martinez, 1984). Recently, primary hamster tracheal surface epithelial (HTSE) cell culture system has been utilized for the study of mucin secretion, since it becomes highly enriched with secretory cells at confluence (Wasano *et al.*, 1988; Wu *et al.*, 1991) and produces mucins (Kim *et al.*, 1985; Wasano *et al.*, 1988) resembling those of *in vivo* airway goblet cells. These *in vitro* culture models have been developed for various species, including hamster (Wasano *et al.*, 1988; Wu *et al.*, 1991), rat (Whitcutt *et al.*, 1988), guinea pig (Adler *et al.*, 1990), rabbit (Rearick *et al.*, 1987), dog (Van Scott *et al.*, 1988), and

Correspondence to: Kwang Ho Ko, Lab. of Pharmacology, College of Pharmacy, Seoul National University, San 56-1, Shillim-Dong, Kwanak-Gu, Seoul 151-742 , Korea

human (Wu *et al.*, 1990).

In those *in vitro* or *in vivo* systems, dialysis (Coles and Reid, 1981) and precipitation (Baker *et al.*, 1977) methods have been used to determine the mucin secretion. However, these methods are largely dependent on the uptake of radioactive precursors (e.g. [^{3}H] glucosamine or [^{35}S]sulfate), and do not specifically measure mucins because the radioactive precursors are incorporated not only into mucins but also into other macromolecules (Wu *et al.*, 1985). To separate metabolically labeled mucin from any other glycoconjugates more accurately, gel-filtration methods were employed (Cheng *et al.*, 1981; Kim *et al.*, 1987), which are most widely used at present for the study of secretion of mucin. While the gel-filtration method accurately measures the amount of high molecular mass mucins, it not only requires radiolabeling of cells but also is extremely laborious and time-consuming. On the other hand, the immunoassay might measure only a subpopulation of mucins given that mucins are extremely heterogeneous due to heavy glycosylation. Nevertheless, immunoassay methods have recently been used for measuring airway mucins (Basbaum *et al.*, 1986; Lin *et al.*, 1989; Logun *et al.*, 1991).

In this study, we developed immunoassay methods for the detection of rat tracheal mucin. Since we recently developed a mAb against high molecular mass mucins released from cultured rat tracheal epithelial cells, we intended to see whether there is any difference between the two assays. Here we report that the results from the two assays were exactly identical. The present result indicates that Enzyme Linked Immuno Sorbent Assay (ELISA) can be used for studying the regulation of mucin release from cultured rat airway epithelial cells.

MATERIALS AND METHODS

Primary rat tracheal surface epithelial (RTSE) cell culture

Tracheas were obtained from male Sprague-Dawley rats of 8 to 10 weeks of age. Preparation and plating of RTSE cells were carried out as previously described for hamster tracheal epithelial cells (Wasano *et al.*, 1988). About 0.5×10^6 dissociated cells per dish were plated on a thick collagen gel prepared in 100 mm tissue culture dishes (Falcon, Lincoln Park, NJ) as previously described (Kim *et al.*, 1996). The cells were plated in a complete medium containing 5% fetal bovine serum (Gibco, Gaithersberg, MD). The complete medium was prepared with a 1 : 1 mixture of Medium 199 and Dulbecco's modified Eagle's medium, supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), epidermal growth factor (12.5 ng/ml), 0.1 μM retinoic acid, 0.1 μM hydrocortisone, and 0.01 μM sodium selenite. The serum-depleted condition was achieved by gradually reducing the concentrations of serum in the medium: 5% on day 0; 2.5% on days 1 and 2; 1% on days 3 and 4; and 0% from day 5 to 8. Each dish was washed twice with Ca^{++}, Mg^{++}- free phosphate buffered saline (PBS) before adding new media. The serum free spent medium was collected for purification of rat tracheal mucin and stored at -70°C until required. For the stimulation of mucin secretion by ATP, cells were grown on the 24-well tissue culture plates (Falcon, Lincoln Park, NJ).

Purification of rat tracheal mucin

Rat tracheal mucin was purified from the RTSE culture spent medium by the established procedure (Kim, 1991). RTSE cell culture spent medium (600 ml) was thawed and centrifuged at 48,000×g for 20 min at 4°C to remove cell debris. The spent media were concentrated to 3 ml by ultrafiltration with Amicon stirred cell (Mw cut off; 30,000 Da, Amicon, Beverly, MA). The concentrated sample was applied to Sepharose CL-4B column (2.5×100 cm) and eluted with PBS at a flow rate of 50 ml/h by using a peristaltic pump at 4°C. Void volume fractions were pooled and concentrated to 2 ml with Centriplus (Mw cut off; 30,000 Da, Amicon, Beverly, MA). The buffer composition was changed to 0.1 M Tris acetate, pH 7.5 by desalting with PD-10 column (Pharmacia, Sweden) and the concentrate was treated with Chondroitinase ABC (0.25 U/ml) for 16 h at 37°C. The digested sample was concentrated to 1 ml with Centricon-30 (Mw cut off; 30,000 Da, Amicon, Beverly, MA) and was adjusted to 0.1% SDS and 50 mM sodium acetate, pH 7.2. The sample was heated for 5 min at 100°C and centrifuged at 12,000× g for 10 min at 4°C. The sample was applied to Sepharose CL-4B column (1.5×70 cm) and eluted with 50 mM sodium acetate /0.1% SDS, pH 7.2. Void volume fractions were pooled and stored at -70°C (purified rat tracheal mucin). The purified rat tracheal mucin was used as an immunogen for the generation of monoclonal antibody and also as a standard antigen for the immunoassay procedures.

Monoclonal antibody

Two BALB/c mice (8 weeks old) were immunized with 5 μg of the purified rat tracheal mucin and boosted for two times with equal amount of antigen at three weeks interval. The monoclonal anti-rat mucin antibody was generated by fusion of the immunized spleen cells with myeloma cells, according to the method of Kler and Milstein (Köhler and Milstein, 1975). One of the clones, mAbRT03, was characterized by Western blot, immunohistochemistry, and immunoprecipitation and used throughout in this study (Shin *et al.*, 1998). It showed strong immunoreactivity against the

high molecular weight mucus glycoproteins both *in vitro* and *in vivo*. MAbRT03 was identified as IgM type of antibody by Sigma (St. Louis, MO) immunoglobulin isotyping kit and used without further purification.

Analysis of mucin secretion by ATP

ATP was reported to stimulate mucin secretion from isolated canine tracheal epithelium (Davis *et al.*, 1992) and primary hamster tracheal epithelial cell *via* P2U receptor (Kim *et al.*, 1996). Rat tracheal epithelial cells were treated with varying concentrations of ATP and each sample was analyzed both by gel-filtration chromatography and immunoassay methods for comparison. Mucins were metabolically radiolabeled for 24 h by incubating confluent cultures (Day 5, 24-well plates, 5×10^5 cells/well) with 0.2 ml/well of a serum free complete media containing 10 μCi/ml of [^{3}H]glucosamine as previously described (Kim *et al.*, 1989). At the end of 24 h incubation, the spent media (pretreatment or PT sample) were collected and the labeled cultures were washed twice with Dulbecco's PBS without Ca^{++} and Mg^{++}, before chasing for 30 min with varying concentrations of ATP (disodium salt, Sigma, St. Louis, MO) in PBS (treatment sample or T sample). Both pretreatment and treatment samples were centrifuged to remove floating cells and stored at -70°C until assayed for their mucin content.

Gel-filtration chromatography: High molecular weight glycoconjugates excluded from Sepharose CL-4B column (Pharmacia, Sweden) and resistant to hyaluronidase were defined as mucins (Kim *et al.*, 1985), and measured as previously described (Kim *et al.*, 1987). Briefly, in order to compensate for variations in basal release rate among wells, "mucin release" during treatment was expressed as the ratio of the amount of [^{3}H]-mucin released during the treatment period to the amount of [^{3}H]-mucin released during the pretreatment period from each well (Kim *et al.*, 1987).

Enzyme linked immunosorbent assay (ELISA): Control or ATP-stimulated culture media of RTSE cells were diluted with PBS, pH 7.4 and 100 μl aliquots were coated onto 96-well assay plates for 2 h at 32°C. For the generation of the standard curve, the purified rat mucins were used as coating antigens. The plates were blocked with Blotto (5% non-fat dried milk in PBS-0.2% Tween 20) and washed with PBS-Tween. Hybridoma conditioned media were added to the wells and incubated for 1 h at 32°C. The plates were washed again and incubated with horseradish peroxidase-labeled goat anti-mouse IgM for 1 h at 32°C. Finally, the plates were washed four times and incubated with freshly prepared tetramethylbenzidine (TMB) substrate solution (Sigma, St. Louis, MO) for 10 min at room temperature. Absorbance was read at an optical density (OD) of 450 nm on a Titertrek ELISA reader (Flow Laboratory, U.K.). For inhibition assay, 50 μl aliquots of the purified rat tracheal mucin (10 ng/ml) were coated onto 96-well assay plates for 2 h at 32°C. After blocking, the plates were probed with mAbRT03 for 1 h at 32°C. At this time, varying concentrations of the purified rat tracheal mucin or RTSE culture supernatants (control and ATP-stimulated) were included in the reaction mixture for the competition of binding of mAbRT03 to coating antigens. After washing, the plates were probed with horseradish peroxidase-labeled goat anti-mouse IgM and the color was developed as above. The concentration of mucin in the sample was estimated from the standard inhibition curve obtained with the purified rat tracheal mucins.

In both cases, in order to compensate for variations in basal release rate among wells, "mucin release" during treatment was expressed as the ratio of the amount of mucin released during the treatment period to the amount of mucin released during the pretreatment period from each well (Kim *et al.*, 1987).

RESULTS

The monoclonal antibody used in this study (mAbRT-03) showed immunoreactivity against high molecular

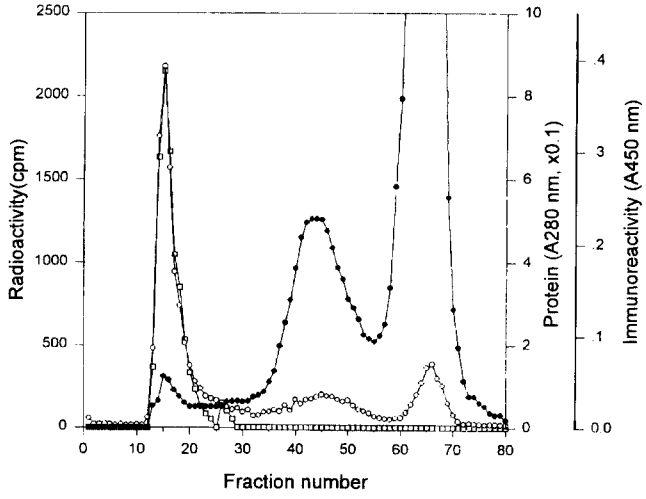

Fig. 1. Gel-filtration column profile of [^{3}H]glucosamine-labeled mucins released from rat tracheal surface epithelial (RTSE) cell cultures. [^{3}H]Glucosamine-labeled RTSE culture supernatants were loaded on Sepharose CL-4B column and eluted with 50 mM sodium acetate, pH 7.2, containing 0.1% SDS at a flow rate of 20 ml/hr. Fractions of 420 μl were collected and each fraction was analyzed for their radioactivity and immunoreactivity. The immunoreactivity was determined by ELISA as described in Materials and Methods using horseradish peroxidase-labeled goat anti-mouse IgM as a secondary antibody. The void volume peak profile which contained high molecular weight mucin showed both the radioactivity and immunoreactivity. The included volume peak which contained metabolically labeled "lower" molecular weight glycoproteins did not show any immunoreactivity. □: ELISA (Abs. 450 nm) ●: Protein (Abs. 280 nm) ○: [^{3}H]Radioactivity.

weight rat tracheal mucin (Fig. 1). When [^{3}H]glucosamine labeled rat tracheal epithelial cell culture supernatants were analyzed by Sepharose CL-4B gel-filtration chromatography, the immunoreactive peak profile was exactly superimposed with [^{3}H]glucosamine labeled high molecular weight mucin peak in the void volume fractions (Fig. 1). Also, mAbRT03 gave concentration-dependent immunoreactivity in ELISA with the RTSE culture spent media (Fig. 2).

For the immunoassay of mucin, two types of assay protocols were employed in this study. First, the spent media were adsorbed onto 96-well assay plates and

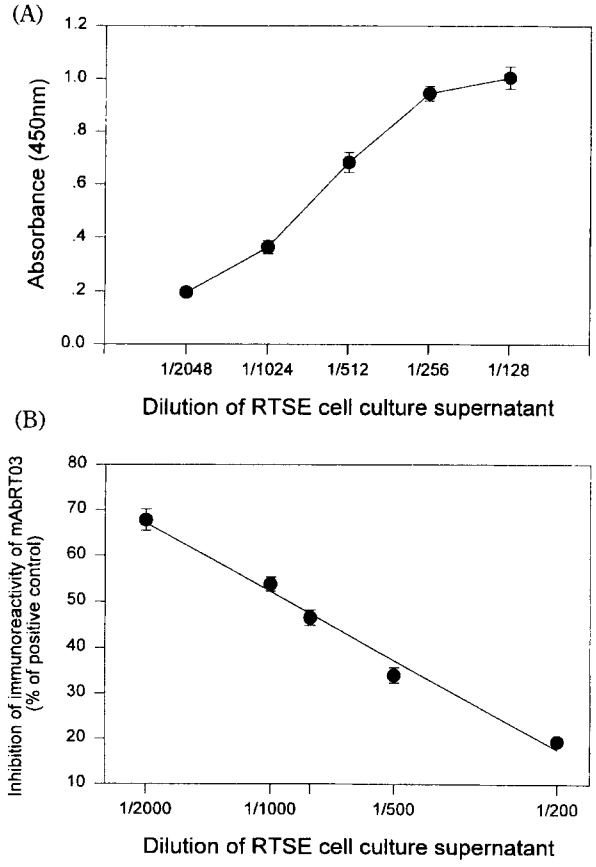

Fig. 2. A) Titration curve of RTSE cell culture supernatant constructed with the anti-rat mucin antibody mAbRT03 (1:5 dilution in Blotto) by simple ELISA. RTSE cell culture supernatants were serially diluted with PBS and coated onto 96-well ELISA plate for 2 h at 32°C. After blocking, 50 μl of hybridoma culture supernatant of monoclonal anti-rat mucin antibody was added into each well and incubated for 1 h at 32°C. Each well was probed with horseradish peroxidase-labeled goat anti-mouse immunoglobulin which was diluted 1:3,000 in Blotto. After addition of tetramethylbenzidine developing solution, the absorbance was read at 450 nm. Each data point represents mean±S.E.M. of the triplicate determinations. B) Inhibition of the immunoreactivity of mAbRT03 to precoated purified rat mucin by mucin-like antigen in RTSE cell culture supernatant. An aliquot of the purified rat mucin (10 ng/ml) was coated onto 96-well ELISA plate for 2 h at 32°C. After blocking, 50 μl of monoclonal anti-rat mucin antibody was added into each well with or without the serially diluted RTSE culture supernatant and incubated for 1 h at 32°C. The data were expressed as % inhibition of positive control (without RTSE cell culture supernatant). The extent of the inhibition of immunoreactivity was well correlated with the amount of RTSE cell culture supernatants. Each data point represents mean±S.E.M. of the triplicate determinations.

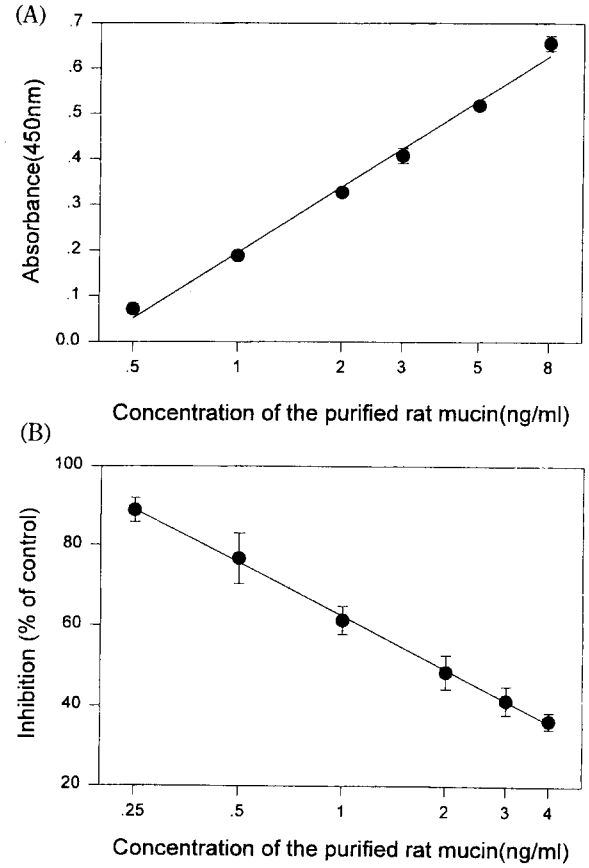

Fig. 3. A) The simple ELISA standard curve of the anti-rat mucin antibody mAbRT03 constructed with the purified rat mucin. The purified rat mucin was adsorbed onto onto 96-well ELISA plate for 2 h at 32oC. After blocking, 50 μl aliquots of the monoclonal anti-rat mucin antibodies were added into each well and incubated for 1 h at 32°C. Horseradish peroxidase-labeled goat anti-mouse IgM was used as a secondary antibody and the absorbance was read at 450 nm. Each data point represents mean±S.D. of the triplicate determinations. Linear regression of each data point generated a standard curve (r=0.9920). B) The inhibition ELISA standard curve of the anti-rat mucin antibody mAbRT03 constructed with the purified rat mucin. An aliquot of the purified rat mucin (10 ng/ml) was coated onto 96-well ELISA plate for 2 h at 32°C. After blocking, 50 μl of monoclonal anti-rat mucin antibody was added into each well with or without the varying concentrations of the purified rat mucins and incubated for 1 h at 32°C. The data were expressed as % inhibition of positive control (without purified rat mucin). The extent of the inhibition of immunoreactivity was well correlated with the amount of the purified mucin and the standard curve was generated by linear regression (r=0.9987). Each data point represents mean±S.D. of the triplicate determinations.

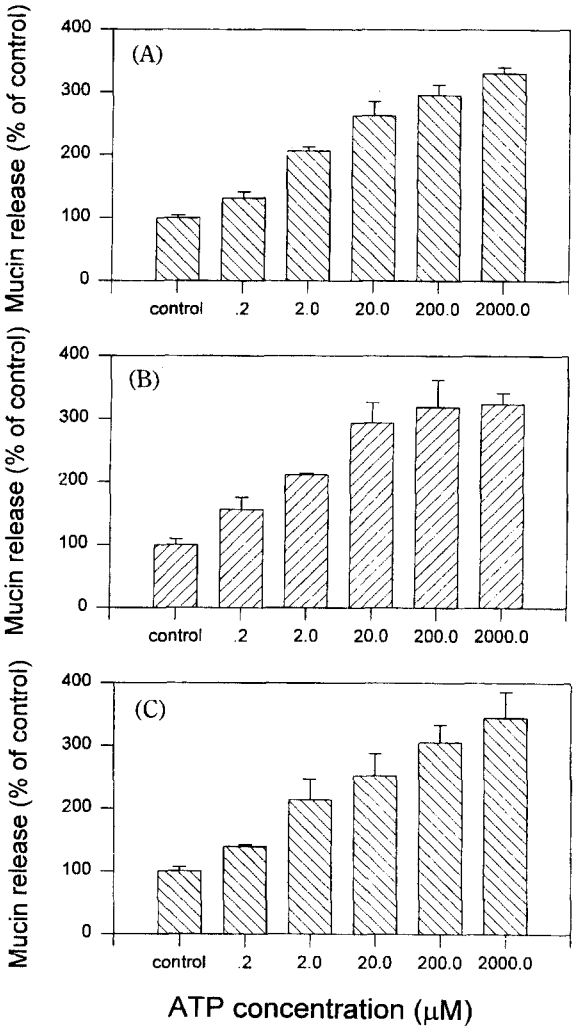

Fig. 4. Measurements of the mucin release from RTSE culture by (A) [3H]glucosamine gel-filtration column chromatography, (B) simple ELISA, and (C) inhibition ELISA methods. Effect of ATP on mucin release in rat tracheal epithelial cell culture was analyzed for radioactivity by metabolic labeling followed by Sepharose CL-4B gel-filtration chromatography. The same samples were analyzed for their immunoreactivity by simple and inhibition ELISA methods as described under Materials and Methods. The results from the above three mucin assay methods were virtually identical with one another, showing mutually superimposable graphs. ANOVA showed no statistical differences among the three assay methods. Each data point represents mean±S.E.M. of the triplicate determinations.

the immunoreactivity was measured by an indirect procedure using horseradish peroxidase-labeled goat anti-mouse IgM as a second antibody. The contents of mucin in the adsorbed sample were determined from a standard curve which was generated with the purified rat tracheal mucins as coating antigens (Fig. 3A). Second, the purified rat tracheal mucins were adsorbed onto 96-well plates and mAbRT03 was reacted with these antigens. At this time, control or ATP-stimulated media were added in the reaction mixture and the mucin contents in the spent media were determined from the extent of inhibition of immunoreactivity. The standard curve was generated by using known amounts of the purified rat tracheal mucins as competitors (Fig. 3B).

Consistent with the result obtained from HTSE cells (Kim *et al.*, 1996), ATP increased mucin secretion from RTSE cells in a dose-dependent manner.

As shown in Fig. 4, the assay results obtained from both the simple and inhibition immunoassays were virtually indistinguishable with those from the metabolic labeling followed by gel-filtration chromatography.

DISCUSSION

In the present study, immunological methods for the determination of mucin secretion from rat tracheal surface epithelial cell culture were developed. The specificity of the monoclonal antibody used in this study was well demonstrated by the comparison of the gel-filtration column profiles detected by immunoreactivity and radioactivity (Fig. 1). Since mucins are very large molecules, [^{3}H]glucosamine labeled high molecular weight mucins were eluted in the void volume fractions of Sepharose CL-4B gel-filtration column chromatography. In case of mAbRT03, the immunoreactivity was detected only in the void volume fractions. Also, the immunoreactive and the radioactive peaks were perfectly matched, demonstrating that mAbRT03 recognizes only high molecular weight mucins.

In this study, the assay results obtained from both the simple and inhibition immunoassay were identical with those from gel-filtration chromatography. When the data were analyzed by Analysis of Variance (ANOVA), each data point was not significantly different from one another. The immunoassay procedure gave several advantages over the other quantitative methods for the quantitation of mucin including gel-filtration chromatography. First, the immunoassay procedures do not require much time or equipments for the experiments. Second, with the immunoassay procedures, one can handle hundreds of samples of mucin with ease in a day, while with the gel-filtration chromatography method, dozens of samples are a practical limit. Third, the immunoassay procedures don't require the isotope, [^{3}H]glucosamine, which may cause potential health problems. Fourth, signal strength obtained with the immunoassays is much stronger than that with the gel-filtration chromatography method. For example, we could assay the culture spent medium samples which were diluted several hundred folds, while with the gel-filtration method, such diluted samples gave no reliable signal (data not shown). This means that less numbers of cells are required for the study of mucin secretion. With the im-

munoassay procedures, it should be possible to scale down the primary culture of rat tracheal surface epithelial cells, for example, from 24-well plates to 96-well plates. Fifth, with the immunoassay procedures, one can measure the absolute amount of mucin secretion by using a standard curve obtained from the purified mucin.

In this experiment, both the simple and the inhibition immunoassays gave the identical results (Fig. 4). However, there may be situations in which a test agent may cause release of other macromolecules which may interfere with the immunoassay for mucins. Therefore, the inhibition immunoassay should be more accurate and reproducible than the simple immunoassay and thus should be employed in quantifying mucins in biological samples.

In summary, we developed convenient and effective immunoassay procedures for the study of mucin secretion from the primary rat tracheal surface epithelial cell culture and demonstrated that the assay results were identical with those from the commonly used metabolic labeling followed by gel-filtration chromatography method.

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