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BIOCHEMICAL STUDY ON MUCINOUS PLEURAL EFFUSION IN A CASE OF ALVEOLAR CELL CARCINOMA OF THE LUNG

Part II. Trials for purification of glycoprotein

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Abstract——Attempts were made to separate and purify mucinous principle from the mucinous pleural effusion obtained in a case of alveolar cell carcinoma of the lung. Three different chemical methods were applied for this purpose. Chemical analysis carried out from the viewpoint of glycoprotein disclosed that the content of hexosamine was the highest of carbohydrate constituents of the preparations, and that sialic acid or fucose could not be estimated at high ratio. The preparations proved to be impure and contaminated by serum protein components. In conclusion, it seems least probable that epithelial mucin is a mucinous principle in the effusion.

INTRODUCTION

Mucinous pleural effusion has been very rarely experienced by clinicians. Only a few cases appeared in the literature. The authors have encountered this unusual manifestation in a case of alveolar cell carcinoma of the lung. Studies were conducted to elucidate the chemical characteristics of the mucin obtained in this case.

In the previous paper[1], the authors presented analytical data on the raw material. The results were characterized by increased content of protein-bound hexose. But the high viscosity and mucin clot formation of the pleural fluid could not be explained solely on the basis of elevated protein-bound hexose. Cells of alveolar cell carcinoma produce mucin, and therefore, the authors assumed that the mucin here investigated might belong to epithelial mucin which offered the closest analogy with the glycoprotein found in bronchial secreta from patients with common respiratory diseases. In our endeavors to separate and purify the substance causing high viscosity and forming mucin clot, as a first step of investigation an attempt was made to separate glycoprotein fraction possessing the chemical properties of epithelial mucin from the punctate. The present paper is concerned with the results of a few series of experiments from

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this viewpoint.

MATERIALS AND METHODS

Materials: The pleural fluid stored in a frozen state was used as a material after cellular elements having been removed by centrifugation at 4,000 rpm for 30 min at 4 C.

Analytical procedures have been previously described. In addition, protein-bound hexose[2] was determined by reaction phenol sulfuric acid[3], and uronic acid by carbazol reaction according to Dische[4] and by orcinol reaction according to Brown[5].

Methods: Three different methods were applied to the separation of glycoprotein.

1) Mucin clot formation in dilute acetic acid: A few milliliters of glacial acetic acid were added to several liters of water and the two were mixed thoroughly. The punctate was dropped into the acetic acid solution under continuous stirring. Mucin clot thus formed was picked up with a glass rod and kept in chilled water. Acetic acid solution was renewed every time mucin clot was formed. The mucin clot was washed in chilled distilled water and dissolved in slightly alkaline solution and then

Table 1. Purification procedure for mucinous principle using the method of mucin clot formation in dilute acetic acid solution

Effusion (234g)
↓
Drop into a large amount of dilute acetic acid solution under mechanical stirring
↓
Mucin clot
↓
Wash in chilled distilled water
↓
Dissolve in a minimum amount of 5 % Na_2CO_3 solution
↓
Drop into a large amount of dilute acetic acid solution
↓
Mucin clot
↓
Wash in chilled distilled water
↓
Dissolve in 5 % Na_2CO_3 solution
↓
Drop into a large amount of chilled 70 % ethanol
↓
Mucin clot
↓
Dissolve in 5 % Na_2CO_3 solution
↓
Drop into a large amount of chilled 70 % ethanol
↓
Mucin clot
↓
Dehydrate in ether-ethanol mixture (1:1, v/v)
↓
Dry in vacuo
↓
White powder (3.2g)

precipitated by adding ethanol in excess. The final step of the procedure was repeated twice. The precipitate was dried with ether-ethanol mixture (1 : 1). Three lots were obtained by this method with minor modification of the procedure (Table 1).

2) Ammonium sulfate fractionation: Saturated solution or powdered crystals of ammonium sulfate were added slowly with mechanical stirring to the diluted effusion. The final concentrations of ammonium sulfate were 40% and 100% of saturation, respectively. The respective precipitate was collected by centrifugation and dissolved in a minimal amount of distilled water. The solution of mucinous substance was dialyzed against running water for 48 hours, and then to this was added ethanol in excess. One lot was obtained for each of these concentrations.

3) Precipitation with cetyltrimethylammonium bromide (CTAB): Diluted punctate was deproteinized twice with chloroform-amylalcohol mixture according to Sevag. The deproteinized fluid was stirred and to this was added dropwise one twentieth volume of 10% CTAB solution. Developed mucin clot was washed well in distilled water and then transferred into a minimal amount of 4 N NaCl solution. The solution was stirred at room temperature for 48 hours to get a homogenous solution. The solution was centrifuged and the supernatant was dialyzed overnight against repeated changes of distilled water at 2-4 C. To the dialyzed solution an excess amount of ethanol was added and allowed to stand overnight in the refrigerator. The precipitate was collected by centrifugation and lyophilized. Five preparations were made by this method, two of which were sufficient in quantity for systemic analysis of carbohydrate. An aliquot of one preparation (No. 5G) was subjected to further fractionation with ethanol in concentrations of 50 to 70% of saturation. Each precipitate was dissolved in slightly alkaline solution and dialyzed against distilled water. The dialyzed solution was lyophilized.

RESULTS

Preparations obtained by the method 1. The preparations were white powder, and almost insoluble in distilled water, but soluble in slightly alkaline solution. The total carbohydrate content was lower than expected, but the protein was higher. The Ouchterlony's immunodiffusion revealed contamination by serum protein components in every preparation. Of individual carbohydrate constituents the content of hexosamine was the highest, and that of hexose and uronic acid followed this. DPA reacting substance was found as much as hexose but resorcinol reaction gave remarkably lower values. Fucose was detected in minute amounts only. Fishman reaction was negative but unnegligible amount of uronic acid was detected by Dische's carbazol reaction (Table 2).

Preparations made by the method 2. They were reddish brown fine flake, and contained more carbohydrate than those prepared by the method 1. Hexosamine was contained in the highest value, and hexose in the second. Sialic acid and methylpentose were detected in higher proportion than in the method 1. The preparations gave posi-

Table 2. Chemical data on preparations made by the method of mucin clot formation

Constituent	Lot number		
	No. 1	No. 2	No. 3
Nitrogen(%)	3.9	2.4	10.2
Protein(%)	41.6	42.3	85.5
Hexose(%), (Winzler)	1.2	1.2	3.1
Methylpentose(%)	0.07	0.03	0.07
Hexosamine(%)	3.3	2.3	7.2
Sialic acid(%), (DPA)	2.6	1.1	3.2
Sialic acid(%), (Svennerholm)	0.13	0.09	0.33
Hexuronic acid (Fishman reaction)	(−)	(−)	(−)
Uronic acid(%), (Carbazol)	1.6	0.9	4.5
Uronic acid(%), (Orcin)	0.6	0.4	2.1
Serum protein components	(+)	(+)	(+)

tive Fishman reaction. Contamination by serum protein components was confirmed by immunodiffusion (Table 3).

Preparations made by the method 3. Carbohydrates were contained in higher ratio than in above two methods. Neither fucose nor sialic acid was found in high ratio. Fishman reaction was positive. Contamination by serum protein components was noticed by immunodiffusion but more detailed study could not be performed because of

Table 3. Chemical data on preparations made by ammonium sulfate fractionation

Constituent	Lot	
	100% saturation	40% saturation
Protein(%)	87.0	65.5
Hexose(%)	3.5	4.0
Hexosamine(%)	8.7	8.7
Methlpentose(%)	0.62	0.5
Sialic acid(%), (DPA)	1.9	2.7
Sialic acid(%), (Svennerholm)	1.7	2.0
Hexuronic acid (Fishman reaction)	(+)	(+)
Uronic acid(%), (Carbazol)	4.1	3.4
Uronic acid(%), (Orcin)	4.5	2.3
Serum protein components	(+)	(+)

Table 4. Chemical data on preparations made by CTAB precipitation

Constituent	Lot number		
	No. 3G	No. 5G	No. 5G2
Protein(%)	14.2	53.1	68.0
Hexose(%)	11	5.6	2.5
Hexosamine(%)	27.1	19.7	2.3
Methylpentose(%)	0.08	0.15	0.18
Sialic acid(%), (DPA)	1.0	1.1	0.8
Sialic acid(%), (Svennerholm)	<1.0	1.4	
Hexuronic acid (Fishman reaction)		(+)	(−)
Uronic acid(%), (Carbazol)		4.1	
Serum albumin		(+)	

paucity of the lot. Alcohol fractioned material showed lower carbohydrate content than the original lot (Table 4).

DISCUSSION

The pleural fluid in the case described was viscous and stringy, and formed mucin clot in dilute acetic acid. Substance possessing these characteristics have been generally accepted as mucin, and are high molecular carbohydrate compounds, such as glycoprotein with high content of sialic acid or fucose (epithelial or glandular mucin)[6-9] or hyaluronate protein complex (synovial mucin)[10]. Because the authors supposed that cells of alveolar cell carcinoma might have biological properties secreting epithelial mucin just as the epithelial cells of the bronchi and bronchioles do, an attempt as a first step of investigation was made to separate glycoprotein fraction in which sialic acid or fucose was contained in high ratio. But the viscosity of the material was so high that it was impossible to separate and purify glycoprotein fraction by using electrophoretic or chromatographic methods. Therefore, several chemical methods were used in this report.

Since the study of Virchow, the separation of epithelial mucin by means of precipitation from aqueous solution with dilute acetic acid has been reported by several authors[10-14]. As a method of separation, this classical one was adopted in the present study.

As shown in the results, experiments revealed that much protein was involved into the mucinous principle when mucin clot was formed. Contamination by serum protein was remarkable and carbohydrate content was lower than expected. The analytical data were similar to those of the mucin clot made from bovine submaxillary gland[18] except for low sialic acid content. With respect to the content of carbohydrate constituents, it was high in the order, hexosamine, hexose and uronic acid. But Fishman's reaction was negative.

Ammonium sulfate fractionation has been used for the separation of glycoprotein or mucopolysaccharides by several authors[15,16]. In the present investigation, this method was used to confirm roughly whether the glycoprotein rich in sialic acid or fucose was present or not. The preparations prepared by this method gave higher content of carbohydrate than those made by the method 1. High proportion of hexosamine to protein-bound hexose as well as low values in sialic acid and methylpentose in these lots were found as in the first method. It is noteworthy that the presence of uronic acid was disclosed by both Fishman's reaction and carbazol method.

CTAB is a reagent to precipitate specifically inorganic polyanion[17,18]. Tsuiki and his associates[7,12] separated bovine salivary mucin by using this reagent. The authors also utilized this reagent for the purification of glycoprotein in the pleural fluid. Carbohydrate in the preparations obtained by this method showed the highest value of the three methods. But the preparations were apparently impure and immunodiffusion disclosed the presence of serum protein components. Of individual carbohydrate components content of hexosamine was the highest and that of hexose the second. Small amount of sialic acid and methylpentose was detected. Hexuronic acid was present but removed by further purification by ethanol fractionation.

All the preparations prepared by the three methods above described proved to be rich in hexosamine and scanty in sialic acid or fucose and to be contaminated by serum protein components. For though in other experiments which will be presented elsewhere removal of ^{125}I-labeled human serum albumin added to the raw effusion was tried according to the method of Sevag, it resulted in a failure. The affinity of protein for mucinous principle was considered too strong to remove the former from the latter by using common deproteinization procedures. Nishizawa and Pigman[13] reported that bovine submaxillary mucin is supposedly a coacervate of protein cations and glycoprotein anions, and Meyer and his associates[10] found hyaluronate protein complex in synovial mucin. In the present stage of our experiments, the significance of serum protein involved in mucin clot was not explained, and on this problem further studies will be needed.

In conclusion, it seems least probable that epithelial mucin is a mucinous principle in this effusion, because neither sialic acid nor fucose could not be estimated in high ratio. Several authors[15,19-21] found glycoprotein specific to neoplasm in ascites or pleural fluid in patients with tumors. Apart from a role played in producing high viscosity, presence of absence of such a glycoprotein will be studied in following experiments.

SUMMARY

Separation of mucinous principle from mucinous pleural effusion was carried out from the viewpoint of glycoprotein. Three different chemical methods, that is, mucin clot formation in dilute acetic acid, ammonium sulfate fractionation, and precipitation with CTAB, were used for this purpose. Obtained preparations proved to be impure

and contaminated by serum protein components. Of carbohydrate constituents of the preparations the content of hexosamine was the highest and that of hexose or uronic acid the second. Neither sialic acid nor fucose was found in high ratio. It is least probable that epithelial mucin is the principle responsible for the extremely high viscosity and mucin clot formation of the pleural effusion.

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