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Characterization of Cell-Surface Material Released from AH-66 Hepatoma Ascites Cells into an Isotonic Solution

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When AH-66 hepatoma ascites cells were incubated in an isotonic saline solution (PBS) at 0 °C for 1 h, material composed of approximately 90—95% protein and 5% carbohydrate was released without significantly affecting the viability of the cells. The material released into PBS contained no Na^+, K^+-adenosine triphosphatase and very low levels of phospholipid, cholesterol, and sialic acid, indicating the absence of contamination by fragments of the plasma membranes. The protein in the released material amounted to as much as approximately 2% of the total cell proteins and most of the protein components of the released material were identical with those of the plasma membranes as analyzed by sodium dodecyl sulfate-gel electrophoresis. The amino acid compositions of proteins of the released material were also very similar to those of the plasma membrane proteins. In contrast to the plasma membranes, however, the released material lacked periodate–Schiff-stainable glycoproteins and proteins with molecular weights larger than about 120000. The absence of sialic acid indicates that the carbohydrate chains, if any, of the proteins in the released material are incomplete. The surface structure of AH-66 cells implied by these results contrasts with that of erythrocyte membranes.

Keywords——cell-surface structure; tumor cell; cell-surface protein; AH-66 cell; released material; cell coat

Introduction

The plasma membranes of most mammalian cells are said to be covered with cell-surface material called the cell coat or glycocalyx.[1] Since the cell coat is the place where cells first come into contact with each other, it is accepted that the cell coat plays an important role in cell adhesion, cell recognition, contact inhibition, and the establishment or modulation of the antigenic properties of the cells. Furthermore, the release of the cell coat is thought to be implicated in the ability of tumor cells to escape the immune response of the host.[2] However, detailed biochemical knowledge of the structure and function of the cell coat remains incomplete, because of the lack of a procedure for the isolation of the cell coat. Histochemical studies using light and electron microscopy demonstrated that the cell coat is rich in carbohydrate and probably sialic acid.[3] According to Chiarugi and Urbano,[4] the cell coat fraction isolated from $BHK_{21/c13}$ cells by ethylenediaminetetraacetic acid treatment is composed of 50 to 60% carbohydrate; the major components of which are hexosamine, glucuronic acid, mannose, fucose and sialic acid. Graham *et al.*[5] fractionated the cell coat from the plasma membranes of NIL 8 hamster fibroblasts by both sedimentation-rate and isopycnic-zonal centrifugation. They found that the cell coat fraction was composed mainly of protein and carbohydrate and that the phospholipid content was only 10% of that in the plasma membranes. An interesting observation made by them was that fibronectin is distributed mainly in the cell coat fraction. Yamamoto and Terayama[6] prepared acid mucopolysaccharides of cell coat from various ascites cells by papain treatment and found that the pattern of acid mucopolysaccharide components of the cell coat is specific to each

hepatoma cell strain. Rittenhouse *et al.*[7] examined the optimal conditions for the selective removal of the cell coat from Ehrlich ascites tumor cells. They found that the cell coat material can be prepared from the cells by mild elution with cold isotonic buffer and that it is composed mostly of glycoproteins and glycosaminoglycans.

In order to elucidate the cell surface structure of tumor cells, we have examined cell surface proteins of the plasma membranes of AH-66 hepatoma ascites cells both by chemical modification[8] and by treatment with urea of the intact cells.[9] We have also isolated and characterized the major glycoprotein of the plasma membranes of AH-66 cells[10] and analyzed the structure of cell-surface carbohydrate moieties of that glycoprotein.[11] In the course of these studies, we found that AH-66 cells shed a part of their cell surface components into isotonic buffer. In order to elucidate further the surface structure of AH-66 cells, we examined some biochemical and enzymatic properties of the material released from AH-66 cells by treatment with phosphate-buffered saline (PBS).

Materials and Methods

Materials——Leucine-β-naphthylamide was purchased form Tokyo Kasei Kogyo Co. Carrier-free ^{125}I was from New England Nuclear.

Preparation of Material Released with PBS——AH-66 hepatoma ascites cells were grown in the peritoneal cavities of Donryu rats as previously described.[8] The cells were suspended in Dulbecco's phosphate-buffered saline (pH 7.4) and washed several times by centrifugation at $50 \times g$ for 5 min until the cells were free of red blood cells. The washed cells were incubated in PBS containing 8.1 mM sodium phosphate (pH 7.4), 1 mM $MgCl_2$ and 137 mM NaCl at 0 °C for 60 min. The solubilized material was separated from the cells by centrifugation at $1000 \times g$ for 10 min at 0 °C and the resultant supernatant was further centrifuged at $100000 \times g$ for 40 min. The supernatant was then dialyzed exhaustively against deionized water at 4 °C over a period of 24 to 36 h, followed by lyophilization. Proteins were determined by the method of Lowry *et al.*[12] with bovine serum albumin as a standard.

Plasma Membrane Isolation——Plasma membranes were prepared as described previously[8] by the zinc procedure originally introduced by Warren *et al.*[13]

Polyacrylamide Gel Electrophoresis——Electrophoresis was performed as described by Laemmli[14] using 10% polyacrylamide and 0.1% sodium dodecyl sulfate. Coomassie blue staining for protein and periodate–Schiff staining for carbohydrate were carried out as described previously.[8,10] The stained gels were scanned with a Shimadzu MPS-50L spectrophotometer equipped with a gel-scanning attachment. Apparent molecular weights were determined by comparing the electrophoretic mobility with a standard calibration curve constructed by running in parallel molecular weight markers such as trypsin inhibitor, bovine serum albumin and ribonucleic acid polymerase from *E. coli*.

Lactoperoxidase-Catalyzed Iodination of AH-66 Cells——AH-66 cells were iodinated by the procedure of Shin and Carraway[15] as described previously.[8] I^{125}-labeled plasma membranes were prepared as described above, dissolved in 2% sodium dodecyl sulfate, and run on a slab gel of 10% acrylamide at 6 mA for 20 h. After electrophoresis, the slab gel was stained with Coomassie blue, and then destained. For autoradiography the destained gel was dried, placed on an X-ray film (Kodak X-Omat R), and exposed for 7 d. The autoradiogram was scanned with a Shimadzu MPS-50L spectrophotometer equipped with a scanning attachment.

Enzyme Assays——Na^+, K^+-ATPase and Mg^{2+}-ATPase activities were determined as described by Komatsu and Fujii.[16] Glucose-6-phosphatase activity was measured according to Mörre.[17] The amount of inorganic phosphorus liberated from ATP and glucose-6-phosphate was determined according to the method of Fiske and SubbaRow.[18] Leucyl-β-naphthylamidase was assayed by incubating samples with L-leucyl-β-naphthylamine in Dulbecco's buffer (pH 7.4) at 37 °C and measuring the resulting β-naphthylamine according to the method of Goldbarg and Rutenburg.[19]

Chemical Determinations——Neutral carbohydrate was determined by means of the phenol-sulfuric acid reaction.[20] Total sialic acid was measured by using the thiobarbituric acid assay after hydrolysis of samples in 0.1 N H_2SO_4 for 1 h at 80 °C, with *N*-acetylneuramic acid as a standard.[21] Uronic acid was assayed by the carbazole method of Dische and Borenfreund[22] as modified by Bitter and Muir,[23] with glucuronolactone as a standard. Amino acids and hexosamines were determined on a Hitachi KLA-3B amino acid analyzer after hydrolysis in 6 N HCl at 110 °C for 20 h, and in 4 N HCl at 100 °C for 6 h, respectively. Total lipids were extracted with chloroform–methanol (2 : 1, v/v) at 22 °C for 24 h according to the procedure of Folch *et al.*[24] Cholesterol was determined by the method of Zlaktis and Zak[25] and the amounts of phospholipids were calculated by multiplying by 25 the values of phosphorus measured by the method of Chen *et al.*[26] Total sulfate was determined according to the procedure of Kawai *et al.*[27]

Results

Proteins Released with PBS from AH-66

When AH-66 cells were incubated in an isotonic saline solution of PBS containing 8.1 mM sodium phosphate (pH 7.4), 1 mM $MgCl_2$ and 137 mM NaCl at 0 °C, significant amounts of proteins were released from the cells. The amount of proteins released was approximately 2% of the total cell proteins, and reached a plateau after 120 min of incubation (Fig. 1). The temperature of incubation did not have a significant effect on the amount of proteins released. After 60 min of incubation, about 4% of the cells were stained with Trypan blue, as compared to the presence of 3% stained cells in untreated controls. This indicates that cell viability was not significantly affected by the incubation for 60 min. The material released by the incubation for 60 min was further characterized.

Enzyme Analysis

As demonstrated previously,[8] the specific activity of Na^+, K^+-ATPase was enriched approximately 13-fold in the purified plasma membrane fraction relative to the original cell homogenate, while no specific activity could be detected in the material released with PBS (data not shown). Neither the material released with PBS nor the plasma membrane fraction contained any detectable glucose-6-phosphatase, indicating negligible contamination by microsomal fraction. In agreement with the work of Rittenhouse *et al.*,[7] the specific activity of leucyl-β-naphthylamidase in the released material was 0.57 μmol of substrate utilized per milligram protein per hour, which is almost the same as that in the plasma membrane fraction.

Chemical Composition

The material released with PBS was composed mostly of protein (90 to 95% by weight). Table I shows the amino acid compositions of the released material and the plasma membrane fraction of AH-66 cells, as compared to that of the cell coat reported for Ehrlich cells.[7] The amino acid composition of the material released with PBS from AH-66 cells of AH-66 cells is remarkably similar to that of the plasma membranes of AH-66 cells as well as to that of the cell coat of Ehrlich ascites cells.

The carbohydrate composition of the material released with PBS was clearly distinct from that of the plasma membranes (Table II). The total carbohydrate content as well as hexosamines and uronic acid were decreased markedly in the released material compared to those of the plasma membranes. A more striking difference in carbohydrate composition was that the sialic acid content of the released material was less than one-tenth that of the plasma

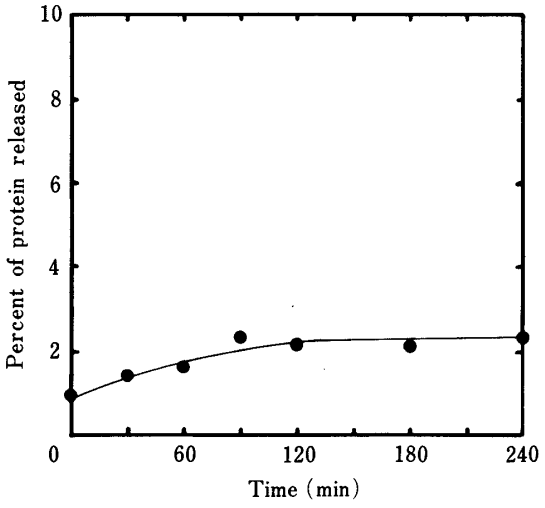

Fig. 1. Release of Proteins from AH-66 Hepatoma Ascites Cells

The cells were incubated at 0 °C in PBS containing 8.1 mM sodium phosphate (pH 7.4), 1 mM $MgCl_2$ and 137 mM NaCl. Aliquots (1 ml) were removed at the indicated times of incubation and proteins released from the cells were measured after repeated centrifugation at $1000 \times g$ for 10 min and at $100000 \times g$ for 40 min. The plots represent the average of two determinations.

TABLE I. Amino Acid Composition of the Material Released with PBS and the Plasma Membrane Fraction of AH-66 Cells

Amino acid	Material released with PBS	Plasma membrane[9]	Cell coat of Ehrlich cells[7]
		mol%	
Lys	8.2	7.9	8.3
His	2.3	2.3	2.2
Arg	5.2	5.3	5.3
Asp	10.2	12.0	10.9
Thr	5.2	5.0	5.4
Ser	5.9	5.8	5.5
Glu	12.5	12.7	12.9
Pro	5.7	5.7	5.9
Gly	8.1	7.6	7.8
Ala	7.5	8.0	7.5
Cys/2	ND[a]	ND[a]	—
Val	6.7	6.2	6.7
Met	2.3	0.4	1.2
Ile	4.9	4.4	5.1
Leu	9.0	8.3	9.2
Tyr	2.6	2.7	2.6
Phe	3.6	5.8	3.7

a) Not detected.

TABLE II. Carbohydrate Composition of the Material Released with PBS[a] and the Plasma Membrane Fraction of AH-66 Cells

Component	Material released with PBS	Plasma membrane[9]	Cell coat of Ehrlich cells[7]
		μg/mg of protein	
Neutral carbohydrate	35.5	46.7	53.4
Hexosamines	5.9	24.4	6.2
Galactosamine[b]	27%	22%	42%
Glucosamine[b]	73%	78%	58%
Sialic acid	0.6	14.9	0.8
Uronic acid	5.7	14.5	8.0
Total carbohydrate[c]	47.7	100.4	68.4
Total sulfate	26.4	47.7	9.1

a) Average of three different preparations. *b*) Calculated as percent of total amino sugar. *c*) The sum of neutral carbohydrate, hexosamines, sialic acid and uronic acid.

membranes. The presence of appreciable quantities of hexosamines, uronic acid, and organic sulfate suggests that the released material contains significant amounts of cell-surface glycosaminoglycans.

There are marked differences in the lipid contents of the material released with PBS and plasma membrane fraction from AH-66 cells (Table III). The released material contained very low levels of phospholipid and cholesterol. The weight ratio of phospholipid to protein of the released material from AH-66 cells was 0.012, which is only 5.3% of the ratio calculated for the plasma membranes. It should be noted that the phospholipid content of the cell coat reported for Ehrlich cells is much higher than that found for the material released with PBS

TABLE III. Lipid Composition of the Material Released with PBS and the Plasma Membrane Fraction of AH-66 Cells[a]

Component	Material released with PBS	Plasma membrane	Cell coat of Ehrlich cells[7]
Phospholipid[b]	0.012	0.226	0.27
Cholesterol[b]	0.006	0.120	0.005
Lipid/protein[c]	0.031	0.601	0.36
Phospholipid/protein[d]	0.015	0.291	0.35

a) Average of three different preparations. *b*) mg/mg of protein. *c*) The sum of phospholipid and cholesterol; μmol/mg of protein. *d*) μmol/mg of protein.

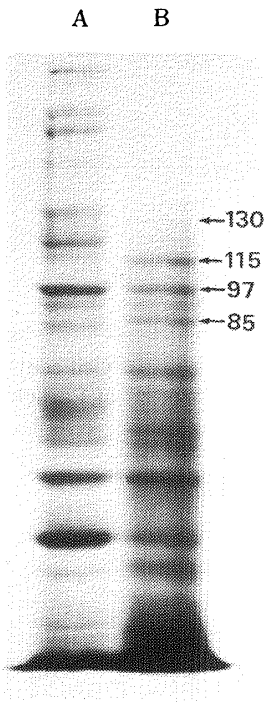

Fig. 2. Slab Gel Electrophoresis of the Material Released with PBS(B) and the Plasma Membrane Fraction (A) from AH-66 Cells

Gel electrophoresis was performed by the method of Laemmli[14] using 10% acrylamide gel containing 0.1% sodium dodecyl sulfate. Samples were dissolved in 2% sodium dodecyl sulfate containing 5% 2-mercaptoethanol, 10% glycerol and 0.06 M Tris–HCl (pH 6.8) and incubated at 100 °C for 3 min before electrophoresis. Amounts of proteins applied were 110 μg. Numerical values represent molecular weight $\times 10^{-3}$.

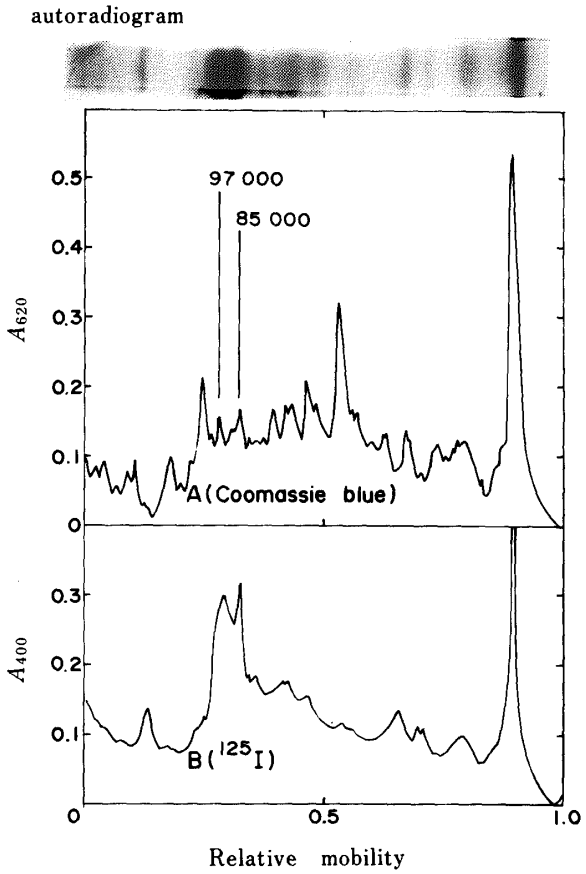

Fig. 3. Lactoperoxidase-Catalyzed Iodination of the Material Released with PBS from AH-66 Cells

Cells were radiolabeled with ^{125}I by lactoperoxidase-catalyzed iodination and the cells were subsequently treated with PBS as described in the text. The material released with PBS (150 μg of protein) was subjected to slab gel electrophoresis as in Fig. 2, stained with Coomassie blue, and then scanned at 620 nm (curve A). The gel was subsequently dried, and an autoradiogram was made and scanned at 400 nm (curve B). Numerical values represent molecular weight.

from AH-66 cells in the present study.[7] The released material also contained a very low level of cholesterol. The weight ratio of cholesterol to protein of the released material was 0.006, which is about 5% of that obtained for the plasma membranes. The very low levels of both

phospholipid and cholesterol in the released material indicate that this material is a component distinct from the plasma membranes and that contamination by fragments of the plasma membranes is negligible.

Proteins of the Material Released with PBS

The components of the material released with PBS were separated by gel electrophoresis using Laemmli's buffer system (lane B in Fig. 2). Approximately 30 distinct bands were regularly counted on the gel. A comparison of the sodium dodecyl sulfate-gel profiles of the released material and the plasma membrane fraction of AH-66 cells indicates that almost all bands of the released material had corresponding bands in the gel pattern obtained for components of the plasma membranes (lane A in Fig. 2). However, a protein with an apparent molecular weight of 115000 was detected only in the released material but not in the plasma membranes. Moreover, proteins with molecular weights larger than 120000 are scarcely present in the released material. It is also noticeable that the cell coat contained no components stainable with periodate–Schiff reagent (result not shown). Curve B in Fig. 3 shows the distribution of radioactivity over a gel after labeling of intact cells by lactoperoxidase-catalyzed iodination followed by fractionation of the material released with PBS by sodium dodecyl sulfate-gel electrophoresis. Most of the proteins in the released material were labeled, suggesting that they are located at the outer surface of the AH-66 cells. The most radioactive bands had apparent molecular weights of 97000 and 85000 on the gel.

Discussion

The chemical composition and distribution of enzyme activities of the material released with PBS from AH-66 cells were substantially similar to those of the cell coat from Ehrlich ascites cells.[7] The only remarkable difference in chemical composition is that phospholipid was almost completely absent in the material released with PBS from AH-66 cells, whereas the cell coat of Ehrlich cells was reported to contain significant amounts of phospholipid. The very low level of phospholipid in the released material of AH-66 cells indicates the absence of significant contamination of the released material by fragments of the plasma membranes.

In addition to the low phospholipid content in the released material, there are the following main differences between the released material and the plasma membrane fraction of AH-66 cells. a) Na^+, K^+-ATPase is absent in the released material. b) Cholesterol content is only 5% of the value found for the plasma membranes. c) Major glycoprotein (with a molecular weight of 130000 as determined by gel electrophoresis using Laemmli's system) stainable with periodate–Schiff reagent is absent in the released material. d) Sialic acid content is only 4% of the value of the plasma membranes. These distinguishing biochemical and enzymatic properties of the material released with PBS strongly indicate that this material is not fragments of plasma membranes but suggest that the material released with PBS is a part of the cell coat.

Most of the protein components of the material released with PBS are identical with those of the plasma membranes. To exclude the possibility that the protein in the released material is bound to the plasma membrane preparation isolated by the zinc ion procedure used in the present study, we also prepared the plasma membranes by the method of Emmelot and Bos[28] using hypotonic buffer, but the protein components isolated by the two different methods were identical as analyzed by sodium dodecyl sulfate-gel electrophoresis (result not shown).

Langley and Ambrose[29] reported that most of the sialic acid in Ehrlich cells was associated with protein rather than with lipid. In the case of liver plasma membranes,[30] sialic acid is mainly associated with the protein of the plasma membranes, forming part of a

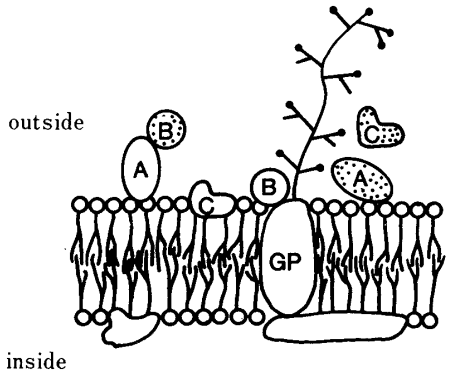

Fig. 4. Model of the Surface Structure of AH-66 Cells

The major glycoprotein (GP) with sialic acid is buried deeply in the lipid bilayer. The closed circles of carbohydrate chains indicate sialic acid residues. Cell-surface proteins (shaded) are only weakly interacting with plasma membranes and are released with PBS. Most of the cell-surface proteins released with PBS are also components of the plasma membrane proteins, but proteins with molecular weights larger than 120000 are scarcely present in the fraction released with PBS. The cell-surface proteins released with PBS have few (or short) carbohydrate chains and lack sialic acid. Proteins released with urea from AH-66 cells may be those depicted by A, B, and C, including proteins released with PBS.

glycoprotein. Previously we proved that the major glycoprotein of the plasma membranes of AH-66 cells is rich in sialic acid.[10] We demonstrated in the present study that glycoproteins stainable with periodate–Schiff reagent are not released by mild treatment with isotonic buffer and that the material released with PBS contains very low levels of sialic acid. Considering these data, it is suggested that the proteins in the material released with PBS have few carbohydrate chains and that even if they have, most of their carbohydrate chains are incomplete.

Vannier *et al.*[31] found that aminopeptidase activity is associated with integral plasma membranes in normal tissues. Takesue and Nishi[32] proved that this enzyme is buried at one end in the lipid bilayer, and catalytic subunits are localized on the outer surface of the cells. In the present study, aminopeptidase activity was found in the material released by isotonic buffer treatment. This is interpreted to mean that an enzymatically active part of an aminopeptidase may be easily released from the plasma membranes by endogenous protease during incubation of cells in isotonic buffer. In fact, Vannier *et al.*[31] demonstrated that enzymatically active aminopeptidase is released from intestinal brush border membranes by papain treatment. In order to prevent digestion by protease, 0.5 mM phenylmethylsulfonyl fluoride was added to the incubation medium, but the release of aminopeptidase activity could not be restrained. It should also be noted that the addition of phenylmethylsulfonyl fluoride has no effect on the protein species of the material released with PBS, as analyzed by sodium dodecyl sulfate-gel electrophoresis (result not shown).

In disagreement with the work of Hubbard and Cohn,[33] who demonstrated that the release of lactoperoxidase-iodinated proteins from mouse L cells into culture medium is completely inhibited below 4 °C, release of the proteins at 0 °C was observed for AH-66 cells as well as Ehrlich ascites cells. The material released with PBS from AH-66 cells is probably very labile and easily removed even at low temperature.

Based on the results obtained in our experiments described in a series of papers, we propose that the cell-surface structure of AH-66 cells is as shown in Fig. 4. While there are no peripheral proteins at the outer surface of human erythrocytes, at least 2% of the cell proteins are present at the outer surface of the plasma membranes of AH-66 cells, and are easily shed by the isotonic buffer treatment. As shown in Fig. 4, these cell-surface proteins may be only weakly interacting with the plasma membranes, (probably with the proteins), or may be in equilibrium with the released state. Urea treatment of AH-66 cells may remove cell-surface proteins, including those interacting more strongly with the plasma membranes than those released with PBS. The proteins released with PBS have few (or short) carbohydrate chains and lack sialic acid. The major glycoprotein containing sialic acid is buried in the lipid bilayer with the carbohydrate chains protruding at the outer surface of the cells. The surface structure of AH-66 cells thus depicted is quite different from that of human erythrocytes. Further

characterization of the structure and function of the cell surface material of AH-66 cells would be necessary to elucidate its physiological importance.

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