A MEMBRANE-BOUND HAEMAGGLUTININ FROM CULTURED HAMSTER FIBROBLASTS (BHK 21 CELLS)

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1. Introduction

The formation of intercellular adhesions by suspended BHK 21 cells is stimulated by treatment of the cells with neuraminidase [1]. One of a number of possible explanations for this effect is that adhesion of these cells may involve 'recognition' of desialysed complex carbohydrate (expected to have terminal galactosyl or *N*-acetyl-galactosaminyl residues) by a cell surface protein [1,2]. Such an interaction would be analogous to the recognition of serum asialo-glycoproteins by a protein from the surface membrane of liver parenchymal cells [3]. Support for this 'sialyl acceptor' model of fibroblast adhesion comes from the observation that desialysed bovine submaxillary mucin is an inhibitor of the aggregation of neuraminidase-treated 16C cells [4], and that the aggregation of BHK 21 cells is inhibited by glycopeptides sensitive to galactose oxidase, isolated from the surface of BHK cells [5]. Properties of ricin-resistant BHK 21 variants further indicate a role in adhesion for galactosyl or *N*-acetyl-galactosaminyl residues [2].

The rabbit hepatic protein that specifically binds asialoglycopeptides has been shown to have lectin-like properties [6]. Lectin-like carbohydrate binding proteins, which may be involved in intercellular adhesion [7,8] have been isolated from the cellular slime moulds *Dictyostelium discoideum* and *Polysphondylium pallidum*. It has also been proposed that lectins with β-D-galactoside specificity may mediate cell fusion in cultures of chick embryonic myoblasts [9] and of a permanent line of rat myoblasts [10].

Proteins with similar specificity have also been found in the electric organ tissue of *Electrophorus electricus* and in several mammalian tissues and tissue cultures [11].

We now report the detection of a haemagglutinin in cultures of BHK 21 cells. This agent, which is assayed by its ability to agglutinate formalinized rabbit erythrocytes, appears to be membrane bound and its agglutinating activity can be inhibited by sugars related to D-galactose.

2. Materials and methods

2.1. *Cell culture*

BHK 21 cells were grown at 37°C in roller culture bottles, in Eagle's medium, Glasgow modification, supplemented with 10% foetal calf serum and 10% tryptose phosphate broth.

2.2. *Cell fractionation*

Each roller culture bottle was washed twice with 50 ml 20 mM phosphate-buffered saline, pH 6.4 (PBS) and the cells scraped off into 20 ml of the same buffer. The cell suspension was then centrifuged at 600 × *g* for 5 min. The packed cells were resuspended in 8 vol. of PBS and homogenized in a Potter-Elvejhem Teflon grinder, sonicated briefly in a Geos soni-bath and the homogenate (H) centrifuged at 13 500 × *g* for 15 min. The pellet (P_1) was resuspended in PBS and the supernatant (S_1) was centrifuged for 90 min 80 000 × *g* to give a pellet (P_2) and a supernatant (S_2). P_2 was resuspended in 10^{-3} M $MgSO_4$, 10^{-3} M Tris (pH 8.6) and layered over 14.5% Ficoll containing 10^{-3} M $MgSO_4$, 10^{-3} M Tris (pH 8.6) [12] and centrifuged for 2 h at 125 000 × *g*. The white band of plasma

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membrane (PM) at the interface was removed with a syringe fitted with a J-shaped needle and diluted with 9 vol. of PBS and the pellet of endoplasmic reticulum (ER) was resuspended in PBS. Both were centrifuged at 300 000 X *g* for 60 min and the pellets resuspended in PBS.

2.3. *Solubilization of membranes*

Only P_2 was solubilized. This was achieved by addition of a suitable volume of a 10% w/v solution of sodium deoxycholate in water [13] to a suspension of P_2 in water to give a final concentration of 2% sodium deoxycholate. After 15 min at room temperature the mixture was centrifuged at 100 000 X *g* for one hour and the supernatant used as the soluble fraction.

2.4. *Elution from columns of Sepharose 4B*

Samples of solubilized membranes were eluted with 1% sodium deoxycholate from columns of Sepharose 4B (1.5 X 24 cm, void volume 20 ml) equilibrated with 1% sodium deoxycholate. Fractions, 3.5 ml were collected and tested for agglutinating activity.

2.5. *Red blood cells*

Rabbit red blood cells were collected in either heparin or citrate. Human cells were outdated blood bank stocks. Cells were formalinized using the method of Butler [14].

2.6. *Haemagglutination assay*

Haemagglutinating activity was assayed at room temperature. A 50 μl sample of the test material was diluted serially two-fold in PBS in V-shaped microtitre wells and then 50 μl of a 2.5% suspension of formalinized red blood cells were added to each well. After sealing with parafilm the plate was agitated briefly then left for 2 h at which time agglutination was determined. The highest dilution giving definite agglutination was regarded as containing one haemagglutination unit (HU)/50 μl (fig.1). Sugars were tested for inhibitory activity on samples containing 2–4 HUs.

3. Results

3.1. *Distribution of agglutinin in subcellular fractions*

Table 1 shows the activity of the haemagglutinin in various fractions of BHK cells. The plasma membrane fraction has the highest specific activity although the endoplasmic reticulum fraction has a higher total activity. The activity in the soluble fraction may be due to small membrane vesicles as centrifuging for longer times usually results in the disappearance of most of the activity from this fraction.

3.2. *Inhibition by sugars*

The agglutinating activity of extracts containing 2–4 HUs could be completely inhibited by addition of 1 mM D-fucose. Several other saccharides also inhibited but at higher concentrations (table 2). The saccharides which inhibited the agglutination reaction were similar to those found to inhibit haemagglutination by Discoidin 1 [15].

3.3. *Gel-filtration*

Samples solubilized in 2% sodium deoxycholate lost none of their agglutinating activity and in some cases their activity was increased.

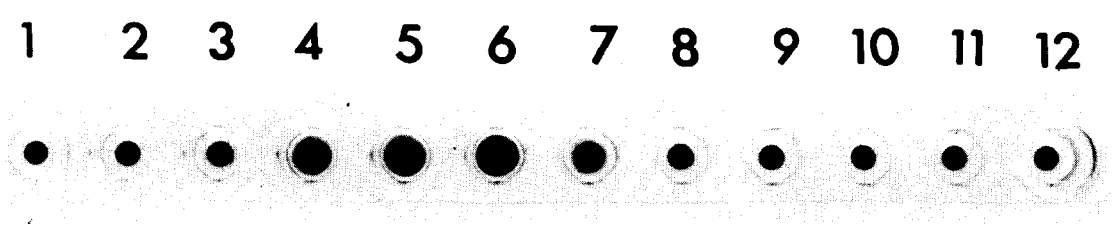

Fig.1. Haemagglutination assay in microtitre wells. Well 1. Control well without extract showing button of unagglutinated cells. Wells 2–12. Series of two-fold dilutions of extract showing mat of agglutinated cells. The agglutination patterns are similar to those of classical antibody haemagglutination, showing less agglutination at high concentrations of extract. Well 7 would be taken as the end-point and the titre of the extract would be 64 HU/50 μl.

Table 1
Agglutinating activity in various sub-cellular fractions

Fraction	Specific activity (HU/mg protein)	Activity yield (%)	Protein yield (%)	Relative specific activity % activity/% protein
H	14.8×10^2	100	100	1
P_1	4.5×10^2	15	51	0.29
P_2	44.9×10^2	50	16.5	3.03
S_2	10.0×10^2	22.5	33	0.68
ER	43.1×10^2	35	12.1	2.92
PM	51.9×10^2	15	4.2	3.75

Foetal calf serum had no detectable agglutinating activity

Table 2
Effect of saccharides on agglutination of rabbit erythrocytes by BHK 21 agglutinin

Sugar	Concentration for complete inhibition of 2–4 HUs (mM)
D-Fucose	1
N-acetyl-D-galactosamine	2
Thiodigalactoside	2.5
α-Lactose	10
L-Fucose	50
D-Melibiose	50
N-Acetyl-D-glucosamine	75
D-Galactose	100
α-Me-D-galactoside	> 100
β-Me-D-galactoside	> 100
α-Me-D-mannoside	> 100

Figure 2 shows the fractionation of solubilized membranes by elution with 1% sodium deoxycholate from a column of Sepharose 4B. All of the agglutinating activity was eluted in the void volume. Active fractions were pooled and the total recovery of activity and the purification relative to the column input were calculated (table 3). Frazier et al. [15] found that Discoidin would bind to columns of Sepharose and thus could be eluted specifically with 0.3 M D-galactose. This was not the case with the agglutinin from BHK cells. No detectable activity was eluted by 0.3 M D-galactose, also an extremely weak inhibitor of the haemagglutinin.

The agglutinin was also tested against formalinized human blood cells groups A, B, AB and O. The results were negative in every case, even with the most con-

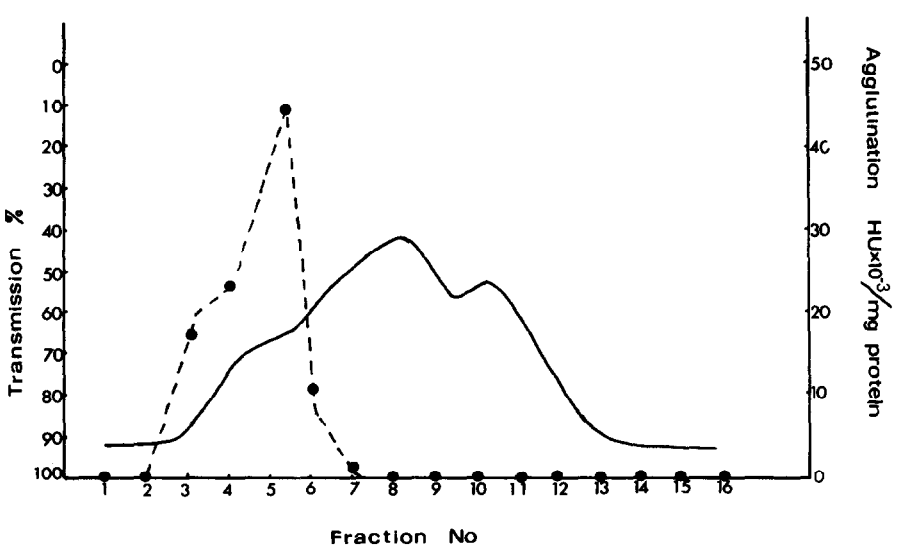

Fig.2

Table 3
Recovery of activity from Sepharose 4B column

	Total activity (HUs)	Activity yield (%)	Total protein (mg)	Protein yield (%)	% Activity/ % protein
Column input	81 920	100	15	100	1
Pooled fractions	56 000	68	2.5	16.5	4.12

centrated extracts. With non-formalinized rabbit erythrocytes agglutination occurred but lower titres were obtained

4. Discussion

The results indicate that BHK 21 cells contain an agglutinin which is inhibited by specific saccharides, although we must stress that its nature and function are presently unknown. Clearly proteins with a variety of activities (including for example biosynthesis) towards complex carbohydrate could act as agglutinins if they possessed multiple specific binding sites for oligosaccharide sequences. Although the agglutinin from BHK cells is inhibited by galactose derivatives, it remains a possible candidate for the carbohydrate binding protein implicit in the sialyl-acceptor model of fibroblast adhesion. The close similarity of its hapten inhibitors to those of Discoidin suggests it could have a similar role. Rosen et al. [7] have shown that the synthesis of Discoidin correlates with the development of cohesiveness in the slime mould and suggest that it is directly involved in intercellular adhesion. The observation, that the highest specific activity of the BHK agglutinin is in the plasma membrane fraction, is consistent with an intercellular function. Furthermore Yamada et al. [16] have recently reported that the major cell surface glycoprotein of chick embryo fibroblasts (CSP), which is also an agglutinin, can restore the adhesiveness of transformed fibroblasts. It is not yet clear whether there is any relationship between the haemagglutinin reported here and cell surface proteins such as CSP and the large, external, transformation-sensitive (LETS) glycoprotein reported by Hynes and Bye [17]. However preliminary experiments indicate that BHK cells transformed by polyoma virus, although less adhesive [18] have as much agglutinin as the non-transformed parent cell. They also indicate that mild trypsinization of intact cells does not reduce the yield of agglutinin in homogenates whereas CSP, LETS and similar proteins in other cell types [19–21] are highly trypsin sensitive.

Purification of the agglutinin may enable us to reach a clearer understanding of its function. The apparently high molecular weight obtained from Sepharose fractionation (exclusion mol. wt $5–20 \times 10^6$) may be due to aggregation of the agglutinin or its interaction with endogenous carbohydrate. These possibilities remain to be tested.

Acknowledgement

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Fig.2. Fractionation of BHK 21 agglutinin on Sepharose 4B. 15 mg of membrane protein in 5 ml of 2% sodium deoxycholate were added to a column of Sepharose 4B (24 × 1.5 cm, void volume 20 ml) and eluted with 1% sodium deoxycholate. Fractions (3.5 ml) were collected and analysed for haemagglutinating activity. Solid line is percent transmission at 280 nm as measured by an LKB Uvicord spectrophotometer. Dotted line is haemagglutinating activity on rabbit erythrocytes.

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