Biochimica et Biophysica Acta, 291 (1973) 127–135

BBA 76154

ISOLATION AND CHARACTERIZATION OF AGGLUTININ RECEPTOR SITES

II. ISOLATION AND PARTIAL PURIFICATION OF A SURFACE MEMBRANE RECEPTOR FOR WHEAT GERM AGGLUTININ*

VILMA K. JANSONS AND MAX M. BURGER

Department of Biochemical Sciences, Princeton University, Princeton, N.J. 08540 (U.S.A.)

(Received July 3rd, 1972)

SUMMARY

Four different chemical extraction procedures for the isolation of wheat germ agglutinin receptor sites from L1210 cells are described. Fractionation of the biologically active material on Sephadex G-200 columns in pyridine results in two major peaks, the lower molecular weight fraction having a higher inhibitory activity. Electrophoresis in polyacrylamide sodium dodecyl sulfate gels yields four bands. The most active fraction from Sephadex G-200 has an approximate molecular weight between 40000–60000. A preliminary analysis of the active material indicates the presence of sialic acid, neutral sugars and amino sugars, including *N*-acetylglucosamine.

INTRODUCTION

Several differences in surface structure between transformed cells and their parent lines have been well documented[1]. Among the parameters studied extensively is the differential agglutinability of cells by lectins, especially concanavalin A[2–4] and wheat germ agglutinin[5–9]. Solubilization of the wheat germ agglutinin receptor site by sonification has been reported earlier[10]. We now investigate four different chemical procedures for further purification of the wheat germ agglutinin receptor site from a mouse leukemia line L1210. Description of these methods forms the first section of a two-part communication. In the second section, we characterize the isolated material by studying its specificity for the transformed state and interaction with other lectins. We als report on some experiments done with antiserum against the isolated receptor. While this work was in progress, Wray and Walborg[11] have described an entirely different procedure for the isolation of wheat germ agglutinin receptor from Novikoff ascites cells.

MATERIALS AND METHODS

Cells

L1210 cells from Dr D. Hutchison, Sloan Kettering, were maintained in

* The first report in this series was on the isolation of a receptor complex for a tumor-specific agglutinin from the neoplastic cell surface, by Max M. Burger, *Nature*, 219 (1968) 499.

DBF_1 mice by weekly intraperitoneal injections. L1210 cells in tissue culture[12] were propagated in "Falcon" flasks containing minimal Eagle medium supplemented with 10% calf serum, 10% fetal calf serum, 20% NCTC (135P) and 1% penicillin (10000 units/ml) streptomycin (10000 μg/ml). Polyoma transformed 3T3 cells (Py 3T3) and polyoma transformed BHK cells (Py HK) were grown as described[13,14]. All tissue culture media were purchased from Grand Island Biologicals.

Preparations of surface material for chemical extractions

L1210 cells were harvested, washed three times in 0.15 M NaCl and extracted with hypotonic NaCl as previously described[10]. After such a hypotonic treatment cells are not agglutinable anymore while the receptor site accumulates in the medium[10]. After centrifugation of the hypotonic incubation medium for 1 h at 105000 $\times g$, sedimenting surface material was stored at -20 °C until use. Isotope-labelled surface material from L1210 cells grown in tissue culture was obtained as follows. $2 \cdot 10^6$ cells were seeded in 75-cm^2 Falcon flasks containing 20 ml of medium described above. After incubation for 24 h at 37 °C, 200 μCi of [^{3}H]glucosamine, (D-[6-^{3}H]-glucosamine, New England Nuclear, 6 mCi/mg) were added into each flask. After additional incubation for 72 h, cells were harvested, washed and extracted with NaCl exactly as the unlabelled material. For further chemical extractions, the radioactive surface material was combined with the surface material from unlabelled mouse cells in the ratio of 1:20 on the basis of packed cell volume.

Chemical extraction procedures

Phenol

The phenol extraction procedure[15] used to isolate M and N receptors from red blood cells was adapted as follows. Surface material, 5 mg protein/ml, was suspended in 0.15 NaCl and homogenized with 5–6 strokes in a loose fitting glass homogenizer. Fresh phenol (Fisher Scientific) was added to a final concentration of 50% (v/v) and the mixture was stirred for 2 h at room temperature. After centrifugation for 1 h in a swinging bucket at 4000 $\times$ g, water phase was collected, dialyzed exhaustively against cold water and lyophilized. The lyophilized material (1 mg/ml) was dissolved in 0.15 M NaCl and extracted by adding chloroform–methanol (2:1, v/v) in equal volume and stirring for 10 min at 4 °C. Again, the water phase was collected by centrifugation, dialyzed at 4 °C and lyophilized.

Guanidine·HCl

For extraction with guanidine·HCl, surface material (5 mg/ml) was homogenized in 0.1 M phosphate buffer, pH 8.5. Guanidine·HCl (Eastman Organic Chemicals) was added to a final concentration of 6 M in 0.01 M β-mercaptoethanol (Sigma). The mixture was incubated for 1 h at 37 °C, then centrifuged for 1 h at 105000 $\times$ g[16]. Water phase was dialyzed overnight in the cold against 0.15 M NaCl, then against distilled water, and lyophilized. The lyophilized material was also extracted with chloroform–methanol as described under phenol procedure. This method was adapted from a procedure described by Gwyne and Tanford[16].

Pyridine

The solubilization method for red blood cell membranes reported by Blumenfeld and co-workers[17] was generally followed. Surface material was homogenized as described; to 5 mg protein per 1 ml, 0.5 vol. of ice-cold freshly distilled pyridine (Fisher

Scientific) was added, contents rapidly mixed by swirling and immediately dialyzed *vs* 75 ml ice-cold distilled water for 15 h. The insoluble fraction was removed by centrifugation for 1 h at 105000 × *g* while the supernate was further dialyzed against cold distilled water and then lyophilized. The lyophilized material was again extracted with chloroform–methanol.

Lithium diiodosalicylate

The method described by Marchesi and Andrews[18] for the isolation of a major glycoprotein from red blood cells, was adapted with slight modifications. Surface material (2 mg protein) was suspended in 1 ml 0.05 M Tris buffer (pH 7.5) containing 120 mg lithium diiodosalicylate/ml (Eastman Organic Chemicals). The suspension was first stirred for 15 min at room temperature then 2 vol. of cold distilled water were added and stirring was continued at 4 °C for an additional 20 min. The soluble fraction was collected after centrifugation for 90 min at 45000 × *g*, cold 50 % phenol added (v/v) and the mixture was stirred for 20 min at 4 °C. After centrifugation for 1 h at 4000 × *g*, the water phase was collected, dialyzed exhaustively against distilled water and lyophilized. The lyophilized material was extracted by suspending (1 mg/ml) in ice-cold absolute alcolhol and stirring for 1 h at 2 °C. This step was repeated three times, whereupon the precipitate was dissolved in water, dialyzed overnight in cold water and lyophilized.

Agglutination and inhibition of agglutination

Wheat germ agglutinin was prepared as previously described[6]. Removal of tissue culture cells and agglutination assays were carried out as reported in detail by Benjamin and Burger[8]. Inhibition of agglutination was set up at room temperature as follows: Varying concentrations (4–40 μg) of whet germ agglutinin in 0.05 ml physiological buffered saline were pipetted into wells of a hemagglutination tray, a constant quantity, usually 10 μg, in 0.05 ml, of wheat germ agglutinin receptor fraction was added, contents mixed with a Pasteur pipette, allowed to react for 5 min, then 0.05 ml of $2 \cdot 10^5$ cells/ml was added and mixed by gently rocking the tray. After 10 min, a drop was transferred onto a concavity slide which was inverted to form a hanging drop suspension. Agglutination was scored from 0 to +4 as previously described[8]. The minimum concentration of inhibitor necessary to fully inhibit +3 agglutination was determined. This ratio, μg agglutinin/μg inhibitor was defined as inhibitor index (*I*) and calculated on the basis of protein.

Polyacrylamide–sodium dodecyl sulfate gel electrophoresis and scintillation measurements

Wheat germ agglutinin receptor site fraction was treated with sodium dodecyl sulfate and electrophoresed as described by Inouye and Guthrie[19]. Duplicate gels, 10 cm × 0.5 cm were loaded with 100 μg of the solubilized receptor containing ^3H label and run at 8 V/cm for 6 h. After completion of electrophoresis, one of the gels was stained with 0.25 % Coomassie brilliant blue in 50 % methanol containing 12 % trichloroacetic acid. The other gel was assayed for radioactivity. Sections, about 2 mm thick, were placed in vials and 0.5 ml of 1 M ammonia added. After a few hours, 15 ml of Bray's[20] solution containing 4 % Cab-O-Sil (Cabot Corporation, Boston) were added. Counting was done in a Packard Tri-carb scintillation spectrophotometer.

Chemical analyses

Protein was determined by the method of Lowry *et al.*[21] using bovine serum albumin as standard. Amino acid and amino sugar analyses were done on a Beckman automatic amino acid analyzer. For amino acid analysis, the sample was hydrolyzed for 16 h in 6 M HCl at 110 °C while amino sugars were analyzed after 6 h of hydrolysis in 2 M HCl at 105 °C. Both samples were hydrolyzed under nitrogen. Sialic acid was determined by the method of Warren[22] after hydrolysis for 1 h at 80 °C in 0.025 M H_2SO_4. Neutral sugar was assayed by the anthrone[23] method using glucose as standard.

Affinity chromatography

Sepharose 4B was activated with cyanogen bromide following the method of Cuatrecasas *et al.*[24]. 3 ml of the activated Sepharose beads were mixed with 4 ml of 0.1 M $NaHCO_3$ containing 40 mg of wheat germ agglutinin purified by DEAE chromatography[25]. The mixture was stirred very gently for 20 h at 5 °C then packed into a column, 1 cm in diameter. The affinity column was first washed with 0.1 M sodium borate buffer (pH 8.5) in 1.0 M NaCl, then in sodium acetate buffer, (pH 4.1) in 1.0 M NaCl and equilibrated with 0.05 M Tris buffer (pH 7.4). The column was charged with 5 mg of wheat germ agglutinin receptor site fraction. Elution was started with Tris buffer, then continued with 0.1 M concentration of the wheat germ agglutinin specific hapten, *N*-acetylglucosamine. The effluent was assayed for absorption at 280 nm. Appropriate fractions were pooled, dialyzed, lyophilized and assayed for inhibition of agglutination.

RESULTS

The four extraction procedures yielded comparable results. Table I shows that both recovery of protein as well as inhibitory indices were similar. It was also found that agglutination of the virally transformed cell lines could be inhibited by

TABLE I

COMPARISON OF THE RECOVERY, INHIBITORY INDEX AND CROSS REACTIVITY OF WHEAT GERM AGGLUTININ RECEPTOR SITE FRACTION ISOLATED FROM L1210 CELLS BY THREE DIFFERENT PROCEDURES

Percent recovery was based on protein present in surface material sedimenting at 105000 × *g*. Inhibitory index (*I*) calculations are described under Materials and Methods.

Extraction procedure	*Recovery** (% *protein*)	*Bands on sodium dodecyl sulfate disc gel electrophoresis*	*Inhibitory index***	*Cross reactivity with virally transformed lines*
Phenol (50%)	5–8	4	1.1–1.5	Py 3T3, Py BHK
Guanidine·HCl	6–8	4	0.8–1.0	Py 3T3, Py BHK
Pyridine (33%)	6–10	4	0.7–1.2	Py 3T3, Py BHK
Lithium diiodosalicylate	6–10	4	1.2–1.7	Py 3T3, Py BHK

* Based on extractions of at least three separate pooled batches.

** Range of variations using different preparations of wheat germ agglutinin and receptor pools.

wheat germ agglutinin receptor fraction from L1210 cells, *i.e.* minimum concentrations of wheat germ agglutinin receptor site, fully inhibiting agglutination of L1210 cells by wheat germ agglutinin, would also affect the agglutinability of Py 3T3 and Py BHK cells to the same extent. This confirms earlier results obtained with surface material solubilized by sonification.

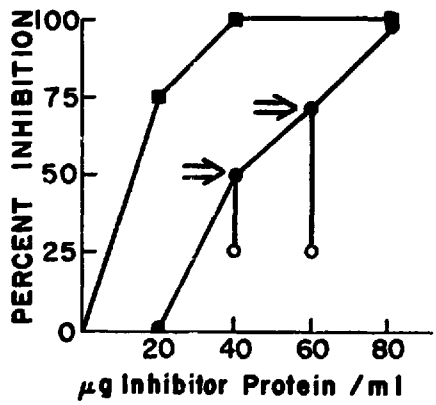

Fig. 1. Inhibition of agglutination by receptor material and reversal of inhibition. Conditions for agglutination and inhibition of agglutination are described in Materials and Methods. At arrow, additional wheat germ agglutinin was added to overcome inhibition. ■—■, 20 μg/ml wheat germ agglutinin; ●—●, 40 μg/ml wheat germ agglutinin; ○—○, additional 40 μg/ml wheat germ agglutinin at arrows.

The inhibition of agglutination by wheat germ agglutinin was reversible. The same aliquot of cells which was first inhibited to agglutinate by the addition of the necessary concentration of wheat germ agglutinin receptor site would agglutinate again when wheat germ agglutinin concentration was increased (Fig. 1). This experiment demonstrates that inhibition of agglutination depends upon agglutinin–inhibitor ratio and minimizes the effect of possible cell damage or other nonspecific inhibition of agglutination.

A comparison of the distribution of both neutral sugar and sialic acid contents during the extraction of the wheat germ agglutinin receptor shows the progressive increase in carbohydrate content when expressed on the basis of protein (Table III). Amino acid analysis revealed a distribution pattern characteristic of glycoproteins[15,17]. Aspartic acid, glutamic acid, serine and threonine and in addition also glycine accounted for about 85% of the total residues while tyrosine and phenylanine

TABLE II

CARBOHYDRATE CONTENT OF INTACT L1210 CELLS, OF SURFACE MATERIAL AND OF THE WHEAT GERM AGGLUTININ RECEPTOR SITE FRACTION

Carbohydrates were expressed as μg per mg protein measured by the method of Lowry *et al.*[21]. Neutral sugar was determined by the anthrone method, using glucose as standard[23]. Sialic acid was determined by the method of Warren[22].

Carbohydrate	*Intact cells*	*Surface material*	*Wheat germ agglutinin receptor fraction*
Neutral sugar	—	19.0	93.6
Sialic acid	2.1	8.0	21.6

concentration were about 1% each. Amino sugar analysis was done with a [^{3}H]-glucosamine-labeled wheat germ agglutinin receptor site fraction. Glucosamine, galactosamine and mannosamine were added as internal standards. Only the glucosamine concentration was found to be increased as well as exhibiting radioactivity in fractions collected in parallel.

The ^{3}H-labeled 50% phenol-extracted wheat germ agglutinin receptor site was further fractionated on Sephadex G-200, equilibrated in 33% pyridine and 0.0014 M mercaptoethanol according to Blumenfeld *et al.*[17] (Fig. 2). Two major areas were resolved: one with a sharp elution profile starting right after the void volume and another one with a broader area recorded between the elution volume of bovine serum albumin and ribonuclease used as markers. Both areas were collected, dialyzed exhaustively, concentrated by negative pressure and tested for inhibition. The lower molecular weight material (mol. wt between 40000 and 60000) had a three times higher inhibitory activity than the higher molecular weight material.

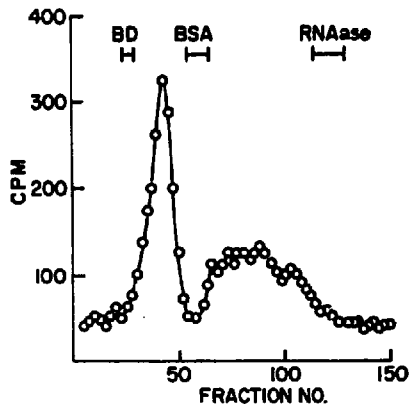

Fig. 2. Chromatography of ^{3}H-labeled wheat germ agglutinin receptor fraction on Sephadex G-200 in 33% aqueous pyridine–0.014 M mercaptoethanol. ———, denotes the elution positions of blue dextran (BD), bovine serum albumin (BSA) and ribonuclease, respectively.

Electrophoresis in sodium dodecyl sulfate–acrylamide gels yielded four bands. The mobilities of the four bands seemed identical in fractions obtained by any of the four extraction procedures as far can be judged from inspection of parallel gels stained with Coomassie brilliant blue. Wheat germ agglutinin receptor site fraction obtained after [^{3}H]glucosamine-labelling and extraction by the phenol procedure

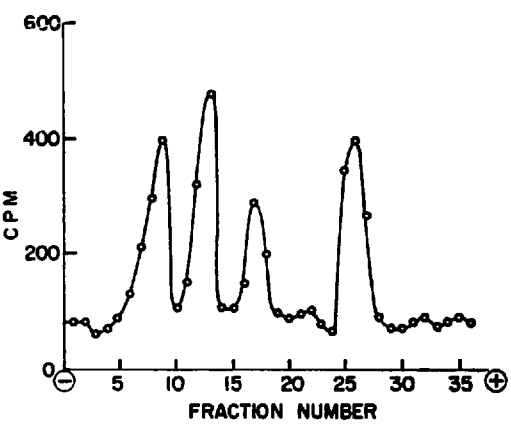

Fig. 3. Electrophoretic pattern in sodium dodecyl sulfate–polyacrylamide gel of [^{3}H]glucosamine-labeled wheat germ agglutinin receptor site fraction extracted using the phenol procedure.

also showed a similar four band pattern upon slicing a parallel gel and assaying for radioactivity (Fig. 3). This indicates that the bands revealed after staining by Coomassie brilliant blue also contain amino sugars.

The specific interaction between wheat germ agglutinin and the isolated receptor site was demonstrated also by affinity chromatography. Under the given conditions about 45 % of the lectin was found bound to the activated Sepharose beads. While a major fraction of the wheat germ agglutinin receptor site was eluted in the buffer, presumably due to overloading the column, receptor material was also retained and could be eluted as a broad peak with the specific hapten, *N*-acetylglucosamine (Fig. 4). After dialysis and lyophilization, this fraction was found to inhibit agglutination. The buffer eluted peak also had inhibitory capacity.

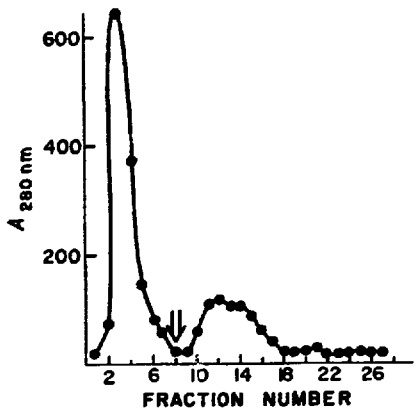

Fig. 4. Affinity chromatography of wheat germ agglutinin receptor site fraction on Sepharose B–wheat germ agglutinin column. At arrow elution started with 0.1 M *N*-acetylglucosamine.

DISCUSSION

In the characterization of membrane glycoproteins, two main types of procedures are most commonly used: solubilization of membranes by detergents[26,27] and controlled surface digestion by proteolytic enzymes[28–33]. Since irreversible binding of detergents to proteins can in many cases not be rigorously excluded and since we found all the detergents so far investigated to interfere with the agglutinability of cells at low doses, the detergent solubilization method could not be used in our case. Neither did we want to work initially with small glucoprotein fragments resulting from the digestion of cell surface by papain or trypsin. It has been shown that the biological activity both in the case of M and N receptors[34] as well as erythrocyte phytohemagglutinin receptor[35] decreased many fold in the isolated small molecular weight glycopeptide when compared to the original high molecular weight fraction. Furthermore we wanted to establish whether the wheat germ agglutinin receptor molecule was a molecular species that differed from some other agglutinin receptors which would not be possible after a proteolytic digestion of the cell surface. Other extraction methods had to be investigated therefore.

The work presented demonstrates that four different chemical extraction procedures described for red blood cell membranes can be applied to extract wheat germ agglutinin receptor sites from L1210 cells Although denaturation of protein is observed during extraction, the isolated receptor site retained the necessary structure for interaction with wheat germ agglutinin in the inhibition assay. A more

direct reaction between the isolated receptor and wheat germ agglutinin was shown by affinity chromatography where the receptor could be replaced on the wheat germ agglutinin column by the specific hapten, *N*-acetylglucosamine. Allan *et al.*[36] have recently reported the isolation of concanavalin A receptors from pig lymphocytes using a concanavalin A affinity column. These authors, however, worked with membranes solubilized by detergents.

It was also possible to obtain antiserum against the isolated wheat germ agglutinin receptor The antiserum reacted specifically with the L1210 cell surface but not the normal parent lymphocyte surface, indicating that also the antigenic *in situ* structure was maintained during isolation. A detailed description of these experiments is given in a subsequent report.

As a first approximation, the sodium dodecyl sulfate gel electrophoresis patterns seem comparable for all four methods but it is not implied that they have to be identical in the number, composition and sequence of the carbohydrate chains. Even the similarities of the inhibitory indices have to be evaluated critically. All through this work, the isolated receptor was observed to have a continuous tendency to reaggregate in the presence of aqueous buffer solutions, salt solutions and after lyophilization as already described by Blumenfeld *et al.*[17] for red blood cell membrane proteins. Since it is not known to what extent the different extraction procedures affect aggregation and since aggregation does affect inhibition of agglutination, a precise quantification is rather difficult at the present time.

From chromatography on Sephadex G-200 in 33% pyridine, a molecular weight range of 40000–60000 was calculated for the most active fraction. The less active large molecular peak eluting right after blue dextran may well be an aggregation product of the low molecular receptor together with impurities, a phenomenon known to occur in the presence of pyridine. Approximations of the molecular weight on sodium dodecyl sulfate polyacrylamide gel electrophoresis where the receptor fraction resolved into four distinct bands concur with the 40000–60000 mol. wt If the four bands do not represent distinct receptor molecules but were due to an artifact, it would be more likely that they derive from common precursors which were partially degraded by enzymes during isolation than from aggregation phenomena.

The chemical analyses demonstrate the glycoprotein nature of the receptor which contains neutral sugar, sialic acid and glucosamine. At the present state, the isolated material seems to consist of four components as revealed by disc gel electrophoresis, all containing amino sugars, including *N*-acetylglucosamine. *N*-Acetylglucosamine, the haptenic inhibitor of wheat germ agglutinin is a ubiquitous component of glycoproteins Therefore it is very possible that there are many such structures which act as receptor sites in the cell membrane. They may belong to different oligosaccharide chains attached to one or several protein backbones. Experiments are in progress, using cell surface specific label to identify which of the several glycoproteins are exposed on the cell surface and available to the agglutinin molecule

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants 10151 and 1-K4-CA-16,765, Grant P-450 from the American Cancer Society, and National Cancer

Institute Contract 71-2372 and the Anita Mestres Fund and the E. R. Squibb and Sons Fund.

The technical assistance of Mrs Kathy Jacobus and Miss Marianne Grob is gratefully acknowledged. We also wish to thank Mr L. R. Hyde and Miss B. C. Bamman, Whitehall Foundation, for amino acid analyses.

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