*IDENTIFICATION OF A TUMOR-SPECIFIC DETERMINANT ON NEOPLASTIC CELL SURFACES**

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The phenomena of invasion and metastasis may well depend largely on the chemical structure of malignant cell membranes. The work of Abercrombie, Ambrose, Weiss,[1] and others seems to leave little doubt that a change of the properties of the cell surface is responsible for loss of contact inhibition, as seen in the viral or chemical transformation from normal to neoplastic cells. Pardee has suggested that such a difference in the surface structure may also be instrumental in deranging control mechanisms, in particular the control of cell division, by modifying the permeation of regulatory substances.[2]

A lipase preparation from wheat germ was found by Aub[3] to agglutinate malignant cells exclusively. A few exceptions were later found by Aub and co-workers,[4] but this agglutination still seems to be a quite specific process for neoplastic cells. Heat resistance and periodate sensitivity of the wheat germ preparation led these authors to think that agglutination was not due to an enzymatic action of the lipase but rather to a mucopolysaccharide existing as an impurity in the lipase preparation.

The isolation and characterization of this agglutinin as a pure glycoprotein will be described in this paper. Furthermore, it will be demonstrated that the tumor-specific surface site that interacts with the agglutinin contains N-acetyl-glucosamine (GlcNAc).

Materials and Methods.—(1) *Cells for study and their preparation:* L1210 cells from Dr. D. Hutchison were maintained in ascitic form by serial transfers in 6- to 10-week-old BDF_1 mice weighing 20–25 gm (Jackson Memorial Laboratory). Baby hamster kidney cells (BHK) and their polyoma-virus-transformed variants (Py) were kindly supplied by Dr. Brian McAuslan of this department and came originally from Dr. R. Dulbecco.

(2) *Chemical and biological reagents:* Wheat germ agglutinin was obtained as an impurity in a preparation of wheat germ lipase manufactured by Pentex Inc., Kankakee, Illinois. This preparation is distributed by Calbiochem and is the same as that used by Aub. Phytohemagglutinin (kidney beans) was obtained from General Biochemicals.

The following enzymes were used in these experiments: β-glucosaminidase was purified from Jack Bean Meal purchased from Sigma. Neuraminidase (*Vibrio cholerae*) came from General Biochemicals and Behringwerke.

Chitobiose and chitodextrin were generous gifts of Dr. L. Glaser.

(3) *Assay of agglutination:* Either L1210, BHK, or Py cells (0.2 ml) at a concentration of about 5×10^6 cells/ml were mixed with agglutinin (0.02 ml), and a hanging drop of this preparation was examined with the microscope.[3] Agglutination of cells was usually determined within 5 min after adding agglutinin to the cell suspension and a serological scale of 0 to ++++ was used to estimate the degree of clumping.

(4) *Purification of wheat germ agglutinin:* Purification of the agglutinin was monitored at various stages by running samples on acrylamide gels in a Canalco electrophoresis apparatus.[5]

(I) *Extraction:* Ten grams of wheat germ lipase suspended in 500 ml of distilled water were first ground with a mortar and pestle and then homogenized in a glass homogenizer. These operations were performed at room temperature.

(II) *Heat precipitation:* One hundred-ml batches of fraction I were immersed in a 63°C water

bath for 15 min and were then centrifuged to remove the resultant precipitate. The supernatant fluid was clarified completely by passage through a glass wool filter.

(III) *Ammonium sulfate fractionation:* To 425 ml of fraction II at 0°C, 113 gm of ammonium sulfate were added and the suspension was centrifuged after 20 min of stirring. The precipitate was collected, redissolved in 14 ml H_2O, and dialyzed in the cold. A small precipitate usually formed during dialysis and was removed by centrifugation.

(IV) *Sephadex fractionation:* The dialyzed fraction III was condensed at 4°C, and 3 ml were applied to a Sephadex G-75 column (82 × 3 cm) and eluted with H_2O. The agglutinin appeared between 300 and 375 ml of effluent volume (Fig. 1).

Results.—Tests of purity of wheat germ agglutinin: After purification, the agglutinin gave a single peak with a sedimentation coefficient at 20° of 2.74*S* in the Spinco model E ultracentrifuge. The squared half-widths at various heights of the peaks obtained in sedimentation velocity runs were plotted versus time in order to obtain a diffusion coefficient for the agglutinin.[6] These gave fairly good agreement at various heights (D_{20} = 8.85, 9.00, 9.70, 8.75 × 10^{-7} cm^2/sec, bottom to top), indicating homogeneity of this preparation.

Agglutinating activity, protein concentration, and optical density at 280 mμ coincided in the elution pattern obtained from a Sephadex G-75 column (Fig. 1). Trypsin with a molecular weight of 23,800[7] came off the same Sephadex G-75 column used in Figure 1 with nearly the same elution volume, using identical conditions.

For an independent check on purity, pooled peak fractions from Sephadex G-75 chromatography were subjected to electrophoresis on a foam-rubber supporting strip.[8] Protein and agglutinating activity migrated at identical rates (Fig. 2).

Samples from the agglutinin-containing peak yielded two bands of about uniform

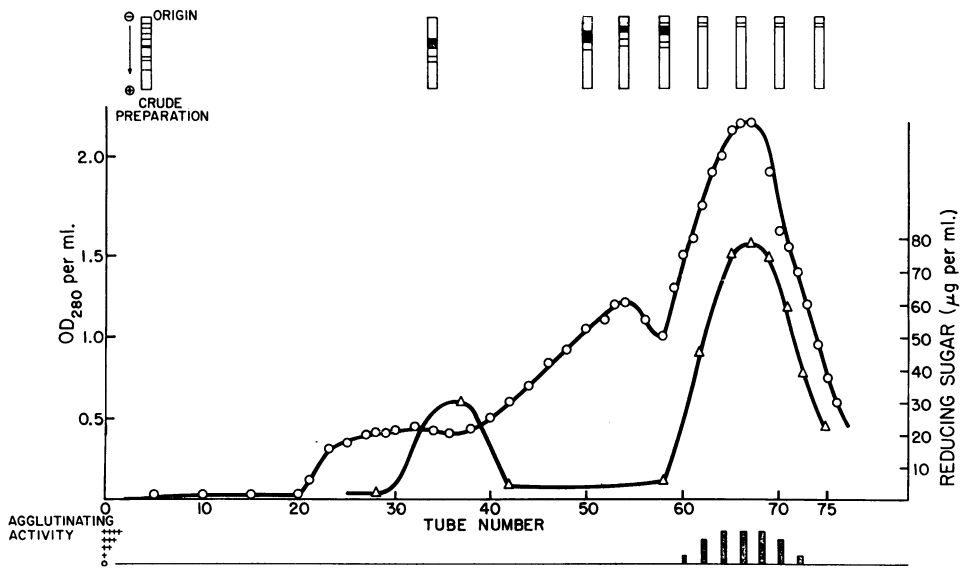

FIG. 1.—Pattern of elution from Sephadex G-75. Agglutinin from dialyzed fraction III (330 mg) was applied in 3 ml to a Sephadex G-75 column (82 × 3 cm) and was eluted with H_2O. Five-ml fractions were collected and assayed for optical density at 280 mμ (○), reducing sugar (△), and agglutinating activity (bar graph at bottom of figure). Reducing sugar values were corrected for loss during hydrolysis. Several fractions were also subjected to disk gel electrophoresis (top of figure).

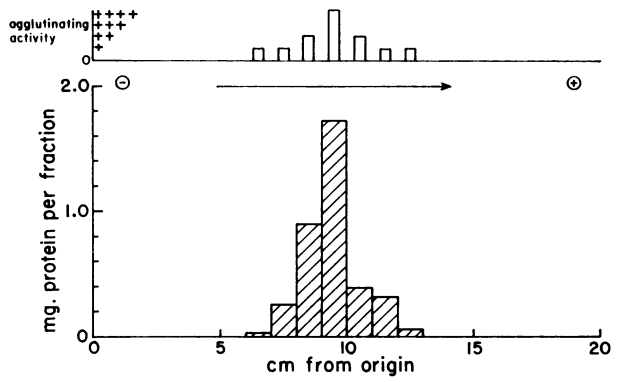

FIG. 2.—Foam-rubber electrophoresis.[8] Pure agglutinin from the peak fraction in Fig. 1 (4.1 mg) was subjected to electrophoresis on a 20-cm foam-rubber strip for 3 hr at 4°C in 0.05 *M* triethylammonium acetate, pH 6.9. Protein and agglutinating activity were determined on the fractions from 1-cm foam-rubber pieces after dialysis. Recovery of protein was 90%.

intensity on polyacrylamide gels (Fig. 1). These bands were always present during purification. They were always of the same relative intensity, regardless of the treatment given the agglutinin. The gel was sliced into 12 equal portions at the termination of the run and eluted. Agglutinating activity was found only in the slices containing the two bands. It is not yet possible to compare the biological activity of the two bands quantitatively.

We attempted several possible approaches to quantitative agglutination, but up to now, this search has been unsuccessful. It is for this reason that we cannot correlate purification steps and specific agglutination activities.

Physical properties: The absorption spectrum of the agglutinin in H_2O was typical of a protein.[9] It had a maximum optical density at 278 mμ and a minimum at 252 mμ; no absorption was observed above 300 mμ. The extinction coefficient at 280 mμ was $E^{1\%}_{1\ cm} = 15.0$. The ratio of optical densities at 280/260 mμ was 1.34. A typical fluorescence excitation maximum was observed at 280 mμ, and maximum emission was at approximately 340 mμ.[10]

Some of the physical properties are listed in Table 1. The S_{20} value is an aver-

TABLE 1

PHYSICAL PROPERTIES OF WHEAT GERM AGGLUTININ

$S_{20,w}$	2.74
$D_{20,w}$	9.2×10^{-7} cm²/sec
$\bar{v}$ (from pycnometry)	0.71 ml/gm
$\bar{v}$ (from amino acid composition)	0.72 ml/gm
$[\eta]$	8–10 ml cm^{-1} sec^{-1}

age of three runs. A molecular weight of 26,000 was calculated using the parameters in Table 1. From the same data the frictional ratio was computed to be 1.2 and the axial ratio 4 to 5.[11]

The intrinsic viscosity of approximately 9 would similarly indicate that the agglutinin has an axial ratio of 4 to 5. These numbers are compatible with an elongated protein which could be thought of as carrying two recognizing sites on each end, allowing it to serve as a bridge between cells during the agglutination process.

Chemical properties: Earlier work by Aub and his collaborators[3] showed that an agglutinin present in preparations of wheat germ lipase is distinct from lipase activity and is a large, nondialyzable molecule which can be inactivated by periodate. Aub *et al.* concluded that it might be a mucopolysaccharide. Our prelimi-

nary characterization suggested that the agglutinin is a glycoprotein. Papain[12] and pronase[13] destroyed agglutinating activity, as did treatment with phenol, cold 10 per cent trichloracetic acid, or cold 1.5 per cent perchloric acid. Agglutinin was not affected by RNase, DNase, or neuraminidase.

The amino acid composition[14] of two different preparations of agglutinin gave qualitatively and quantitatively similar results. All common amino acids were detected. The only unusual aspect of the amino acid composition found was the large amounts of glutamic acid and aspartic acid (17 and 14 residues/molecule, respectively).

Estimation of protein by the Folin method,[15] using bovine serum albumin as standard, gave 0.98 mg total protein versus 1.0 mg dry weight. The Nessler Kjeldahl N determination gave 0.75 mg, assuming a ratio of protein to N of 6.25. The microbiuret method[16] gave 1.08 mg per mg dry weight and was also calibrated with an albumin standard. The discrepancy observed with the Nessler Kjeldahl method could not be explained in full, although this may be in part due to the carbohydrate moiety. In view of the fact that the agglutinin was purified from a lipase preparation, we tested for lipids with rhodamine 6 G and Sudan Black B but were unable to detect any traces.[17]

Destruction of agglutinating activity by 0.002 M IO_4^- (ref. 18) implied that the agglutinin might contain carbohydrates. Recently another plant agglutinin, soybean hemagglutinin, was also found to contain carbohydrates.[19] Reducing sugar tests[20] indicated that about 4.5 per cent of the agglutinin was carbohydrate. This percentage was constant over the entire peak eluted from Sephadex G-75 (Fig. 1). Assays for hexosamine[21] on several preparations allowed the conclusion that if any GlcNAc were present, it had to be less than 0.3 molecules per molecule of agglutinin. From acid hydrolysates a single spot with an $R_{Glucose}$ of 1.29, reacting with a benzidine-TCA spray specific for reducing sugars, was found on paper chromatography (butanol:pyridine:water=6:4:3). This compound was destroyed by periodate. The sugar has not yet been identified, but at a concentration of 10^{-2} M it does not inhibit agglutination.

Identification of N-acetyl-glucosamine as a determinant group on the tumor cell: Under the assumption that agglutination is similar to antibody-antigen interaction, a series of possible hapten inhibitors was tested. Since sugars of the cellular membrane are the determinants in the blood group agglutination system, they were examined first. Among all known monosaccharides occurring in glycolipids and glycoproteins of membranes only one—N-acetyl-glucosamine—was found to inhibit agglutination. Inhibition was complete at 4×10^{-4} M (Table 2). Chitobiose, the disaccharide of GlcNAc, was found to be a very potent inhibitor. It was in fact more powerful per GlcNAc residue than GlcNAc itself. No other saccharide had any inhibitory action at concentrations as high as 2×10^{-2} M (e.g., mannose, glucose, galactose, mannosamine, galactosamine, N-acetyl-mannosamine, glucosaminic acid, sialic acid, L-fucose, D-xylose, and D-arabinose). The high specificity of agglutinin for GlcNAc is emphasized by the failure of glucosamine and N-acetyl-galactosamine, two compounds structurally very similar to GlcNAc, to inhibit agglutination. The lack of inhibition of UDP-GlcNAc is probably not due to substitution on C-1, since chitobiose is a strong inhibitor. It might, however, be explained as the result of steric hindrance due to the bulky nucleotide

TABLE 2

LOW-MOLECULAR-WEIGHT INHIBITORS OF AGGLUTINATION

	Concentration (mM)	Agglutinating activity
N-acetyl-glucosamine	0.4	0
	0.2	+
	0.1	+++
Chitobiose	0.06	0
UDP-N-acetyl-glucosamine	20	++++
N-acetyl-galactosamine	20	++++
N-acetyl-neuraminic acid	20	++++
Glucosamine	20	++++

The assay mixture contained 0.02 ml of purified agglutinin (fraction II) in a total volume of 0.25 ml containing 1–2 × 10^6 cells.

TABLE 3

HIGH-MOLECULAR-WEIGHT INHIBITORS OF AGGLUTINATION

	Concentration (mg/ml)	Agglutinating activity
Ovomucoid	0.010	0
	0.001	+
	0.0001	++++
Ovalbumin	1	++++
Fibrinogen	1	++++
Bovine serum alb.	1.75	++++
Hyaluronate	0.5	++++
Chitin	*Ca.* 0.3	++++

The assay mixture contained 0.02 ml of purified agglutinin (fraction II) in a total volume of 0.25 ml containing 1–2 × 10^6 cells.

group. Such an explanation is supported by the finding that higher homologues of GlcNAc, like chitodextrin, were weak inhibitors. High-molecular-weight hetero- and homopolymers containing GlcNAc, like hyaluronic acid and chitin, had no effect on the agglutination reaction (Table 3).

The assumption that GlcNAc is a part of the surface site with which the agglutinin interacts is supported also by inhibition studies with glycoproteins. Ovomucoid, a glycoprotein with a very high GlcNAc content in its oligosaccharide chain (75% of the total carbohydrate content), was the only macromolecular inhibitor found (Table 3). Its inhibitory action could be destroyed by exposure to 0.01 *M* periodate for two hours at room temperature, indicating that probably the carbohydrate moiety is responsible for the inhibitory effect.

The following finding offers indirect evidence for a sugar-containing receptor site. After a short treatment of L1210 cells with periodate (0.06 *M* periodate, 0.05 *M* sodium acetate buffer—pH 5.1, 5 min, room temperature; control without periodate) and destruction of the periodate with glycerol, agglutinability was abolished. This treatment was sufficiently gentle so that the cells were still intact and viable in terms of an eosin exclusion test. Based on these results, the site might be GlcNAc or still any sugar or surface component linked to or configurationally dependent on a periodate-sensitive compound.

Reversibility of agglutination: Agglutination was rapidly reversible. Ten to twenty seconds after addition of GlcNAc or ovomucoid, the agglutinated cell clumps began to disperse, and after approximately one minute, previously heavy aggregates appeared as homogeneous cell suspensions. The concentration of GlcNAc necessary to disperse agglutinated cells varied between 4×10^{-4} to 8 ×

10^{-4} M, while that for ovomucoid was between 3 to 10 μg/ml. No other sugar known to occur in mammalian cells was capable of reversing the agglutination.

The reversibility by GlcNAc lends support to the notion that this agglutination belongs to the class of antigen-antibody interactions and is not an enzymatic process in which the cell surface would be altered, e.g., by removing a portion of the cell membrane, rendering the cell surface sticky. Such a cell-cell adhesion which would be only indirectly dependent on agglutinin was discussed earlier by Aub as a possible mechanism.[3] However, an agglutination mechanism which depends upon an alteration of the cell surface seems to be excluded by the following experiment. Cells which were agglutinated, then dispersed with GlcNAc, and then washed clean of GlcNAc could again be agglutinated as effectively as fresh cells. This cycle of agglutination, dispersion with GlcNAc, and reagglutination could be repeated several times.

Agglutination of a cell-free system: Microsomal fractions of L1210 and Py cells (containing plasma membranes) were found to agglutinate, indicating that agglutination is not a vital process requiring the whole cell. GlcNAc was again the only inhibitor, thus pointing to a receptor similar to the one in the intact cells.

Involvement of membrane sialic acid in agglutination: The presence of sialic acid on the tumor cell membrane also seems to be required for the agglutination process. Incubation of about 6×10^6 L1210 cells with 0.2 ml neuraminidase (GBI or Behringwerke) at pH 7.3 in a total volume of 2.0 ml for 30 minutes at 37° abolished the agglutinability of the cells. No β-glucosaminidase, other glycosidase, or protease activity could be detected in the two neuraminidase preparations used. Addition of sialic acid to the enzymatically treated cells did not restore agglutinability. However, the cells recovered spontaneously from this state after about eight minutes, presumably due to the synthesis of the necessary sialic acid complement.

Discussion.—Inhibition as well as reversibility of agglutination by GlcNAc is indicative of a competitive interaction between GlcNAc and a site responsible for attachment of the agglutinin to the cell surface. Since the agglutinin itself does not contain any GlcNAc, this amino sugar should be found on the surface of the tumor cell.

The narrow specificity range among monosaccharide inhibitors found for this agglutination reaction strengthens the concept that GlcNAc is part of the agglutinin receptor site on the cell surface. The question then arises whether this is the sole sugar constituent determining the receptor site. No definite answer can be given yet to this question. Inhibitor activities of chitobiose as well as of ovomucoid as calculated per GlcNAc residue were found to be more potent than the activity of GlcNAc itself. Such a finding implies that the determining group on the cell surface contains more than only GlcNAc.

The GlcNAc carrier on the cell surface, which is involved in this agglutination, could be a glycoprotein, glycolipid, or even a mucopolysaccharide. It may be noted, however, that Hakamori and Jeanloz[22] isolated a glycolipid from a gastric adenocarcinoma which contained fucose and GlcNAc, two sugars not found or found to a smaller extent only in glycolipids from normal gastric mucosa. Although the main difference between glycolipid from normal and cancer tissue was found to be the missing fucose in the normal glycolipid, the quantitative difference in Glc-

NAc content may be a basis for the different reactivities of cancer cells with the agglutinin, if such a glycolipid is the cell surface material involved in the agglutination.[23]

Hakamori and Bloch[24] made antiserum against this glycolipid. The antibodies appeared to be directed against N-acetyl-lactosamine and to some extent against lactose, but not GlcNAc. Similarly, lactose was found to be the reactive site in cytolipin H.[25] This glycolipid occurs in cancer tissue and is thought to be the receptor for an agglutinin found in the serum of pregnant women and tumor patients.

GlcNAc inhibits the agglutination of polyoma virus-transformed BHK cells as well as chemically induced L1210 leukemia cells, indicating that the agglutinin recognizes similar groups on different neoplastic cells. GlcNAc, on the other hand, seems not to be an ubiquitous reaction site for agglutinins since the activity of another plant agglutinin—phytohemagglutinin—which reacts with the noncancerogenic BHK cells as well as L1210, Py, and erythrocytes, is not inhibited by GlcNAc.

The function of membrane sialic acid in the agglutination mechanism cannot be defined yet. Our present data seem to indicate that it is not involved in a type of antibody-antigen interaction as is GlcNAc, since sialic acid does not inhibit agglutination, even at very high concentrations.

Sialic acid on the cell surface may act by ionic interaction with agglutinin. However, such a mechanism becomes less probable in view of the fact that the presence of high salt concentrations (2.0 *M*) does not prevent the agglutination of microsomes. It is possible that the removal of sialic acid from the cell surface may loosen up the membrane structure and either initiate the loss of substances[26] containing the agglutinating site or cover up the agglutinating site by rearrangement of the membrane layers.[27] Sialic acid may not play the same role in all agglutination reactions since in the only other case examined (phytohemagglutinin-erythrocytes), it is known that after removal of sialic acid, agglutination is more effective.[28]

Normal fibroblasts (BHK) did not agglutinate in our assay system, while the same cells transformed with polyoma virus did. Among the agglutination systems described in the literature, the BHK line and its polyoma-virus-transformed counterpart should be an especially critical one for testing differences between neoplastic and normal cells. Here the mother cell is only separated by the virus transformation from the daughter cell, i.e. the formation of the GlcNAc-containing agglutination site or the rearrangement of GlcNAc-containing membrane units has to be a direct consequence of the neoplastic transformation.

Further work on the kinetics of the formation of this GlcNAc-containing site during transformation to malignancy will have to be done on a similar mother-daughter cell system. This study would be aided both by isolation of the complete site and by the development of a quantitative assay for agglutination; both projects are currently under investigation.

Summary.—An agglutinin specific for neoplastic cells was purified and was found to be a glycoprotein. The hydrodynamic properties suggest it to be an asymmetric molecule of about 26,000 mol wt.

Agglutination is rapidly reversible, indicating a haptenlike action rather than an enzymatic one. The tumor-specific surface site includes N-acetyl-glucosamine.

This conclusion is based on a unique hapten inhibition by N-acetyl-glucosamine, or chitobiose, or ovomucoid, compounds which contain N-acetyl-glucosamine. Sialic acid, believed to occur in higher amounts on surfaces of tumor cells than on normal cells, seems also to be involved in the agglutination process although it is probably not part of the specific site.

Note added in proof: H. Borberg *et al.* (in *Science*, **154**, 1019 (1966)) have just reported that phytohemagglutinin, which does not have a preference for neoplastic cells, can be inhibited with 2.3×10^{-2} *M* N-acetyl galactosamine specifically. The fact that GlcNAc is not inhibitory in this system was confirmed by these authors.

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