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ISOLATION AND CHARACTERIZATION OF AGGLUTININ RECEPTOR SITES

III. STUDIES ON THE INTERACTION WITH OTHER LECTINS

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SUMMARY

1. A wheat germ agglutinin receptor fraction isolated from surface membranes of leukemia cells failed to interact with concanavalin A but did interact to a lesser degree with two other agglutinins.

2. A preparation of concanavalin A that was not multivalent but presumably monovalent and which blocked the agglutination of susceptible cells by intact concavanalin A had no effect on the agglutinability of the same cells by wheat germ agglutinin.

3. Antiserum against the isolated wheat germ agglutinin receptor fraction from leukemia cells did not react with the normal lymphocytes nor did it react with red blood cells of syngeneic mice.

4. The same antiserum against the wheat germ agglutinin receptor fraction inhibited the agglutination of L1210 (leukemia), Py 3T3 and Py BHK cells by wheat germ agglutinin but had no effect on the agglutination by concanavalin A of Py 3T3 or Py BHK cells.

5. It was concluded that at least some lectin receptors are chemically and topographically on the membrane distinct from each other.

INTRODUCTION

The previous communication described the isolation of wheat germ agglutinin[1,2] receptor sites[3] from the surface of L1210 cells, *i.e.* a mouse leukemia line. Some of the questions which arise and can be answered once such a preparation becomes available are the following:

(1) Do most other agglutinins with specificities directed towards other carbohydrates than that of the wheat germ agglutinin (di-*N*-acetylchitobiose) also bind to the wheat germ agglutinin receptor or can some agglutinins be found which do not interact with the wheat germ agglutinin receptor? Concanavalin A[4–6] is of special interest since the interaction between this lectin and the cell surface has been correlated with growth regulation of transformed cells[7].

(2) What is the topographical surface relationship between receptor sites if they occur as distinct structures?

(3) Since wheat germ agglutinin was shown to agglutinate L1210 cells but not normal lymphocytes, it was interesting to investigate whether antibodies against the wheat germ agglutinin receptor site would react with the source of the receptor preparation, *i.e.* the L1210 cell but not the lymphocyte surface.

(4) Is the antigenic site of the wheat germ agglutinin receptor the same as the agglutinin receptor? If the antiserum against wheat germ agglutinin receptor from L1210 is found to inhibit agglutination of these cells, would there be any similar inhibition also of other cell lines? The following report attempts to answer these questions.

MATERIALS AND METHODS

Animals

BDF_1 male mice (18–22 g) were used as carriers of the leukemia line and as donors of red blood cells and normal lymphocytes. Young adult male rabbits were supplied by Cortelyou's Farm, Rocky Hill, N.J.

Cells

L1210 cells, a mouse leukemia line (kindly supplied by Dr Dorris Hutchison, Sloan Kettering, N.Y.) was maintained by weekly intraperitoneal injections of $1 \cdot 10^5$ cells per animal. Normal lymphocytes were obtained from lymph nodes. Polyoma virus transformed mouse fibroblasts (Py 3T3) were grown in Dulbecco–Vogt medium, supplemented with 10% calf serum. All nutrients were purchased from Grand Island Biologicals. Mouse cells were washed three times in cold phosphate-buffered saline and kept at 5 °C until use. Tissue culture cells were released as described earlier[2].

Lectins

Wheat germ agglutinin was prepared as previously described[8]. Concanavalin A was purchased from California Biochemicals. Trypsinization of concanavalin A was carried out as previously described[7]. Phytohemagglutinin M was obtained from Difco, while *Lens culinaris* lectin[9–11] was a gift from Dr M. Leon, St. Luke's Hospital, Cleveland, Ohio.

Production of antiserum

Wheat germ agglutinin receptor site fraction (4 mg/ml water) isolated by the phenol extraction procedure and emulsified with complete Freund's adjuvant (1:1, v/v) by forcing the suspension through a 18-gauge needle several times, was injected intramuscularly into rabbits. Each rabbit received 0.5 mg antigen weekly for four weeks, followed by a rest of three weeks, then by another four week series of injections.

Assays

Complement fixation tests were based on the procedure described by Schmidt[12] with details given later in the text. Complement fixation ingredients were purchased from Gibco, *N*-acetylglucosamine from Calbiochem and di-*N*-acetylchitobiose was kindly given by Dr V. Reusch. Agglutination assays were done following the procedure worked out in this laboratory[13], with a modification of the scoring system. The

degree of agglutination of cells ranging from + 1 given as + 1 in ref. 13, corresponds to what is referred to here as 25 % agglutination, *etc.* Conditions for use of crystalline chymotrypsin (Sigma), trypsin soybean inhibitor (Worthington Biochemicals) and Pronase (Sigma) are given in legends to figures. Inhibition of agglutination was described in a preceding communication[3].

RESULTS

Interaction of the isolated wheat germ agglutinin receptor site fraction with other lectins

It has been reported that wheat germ agglutinin receptor sites from L1210 inhibit agglutination also of other lines, *e.g.* Py 3T3[3,14]. Since L1210 cells agglutinate poorly with concanavalin A, the Py 3T3 line was chosen to study interactions between wheat germ agglutinin receptor and concanavalin A as well as phytohemagglutinin M[8,15] and *Lens culinaris* lectin. The isolated wheat germ agglutinin receptor inhibits agglutination by wheat germ agglutinin better than that by any other lectin tested. Fig. 1 shows that a four-fold concentration of the wheat germ agglutinin receptor fraction fully inhibiting agglutination by wheat germ agglutinin has no effect on the agglutination by phytohemagglutinin M or concanavalin A. The agglutination by *Lens culinaris* lectin is inhibited to some extent.

Spatial and chemical distinctions of wheat germ agglutinin and concanavalin A receptors

The relationship of the wheat germ agglutinin receptor site to that of concanavalin A was tested also in another system. It has been shown that chymotrypsin treated concanavalin A does not agglutinate susceptible cells, *e.g.* Py 3T3, but rather prevents them from agglutinating with intact concanavalin A to the same extent[7].

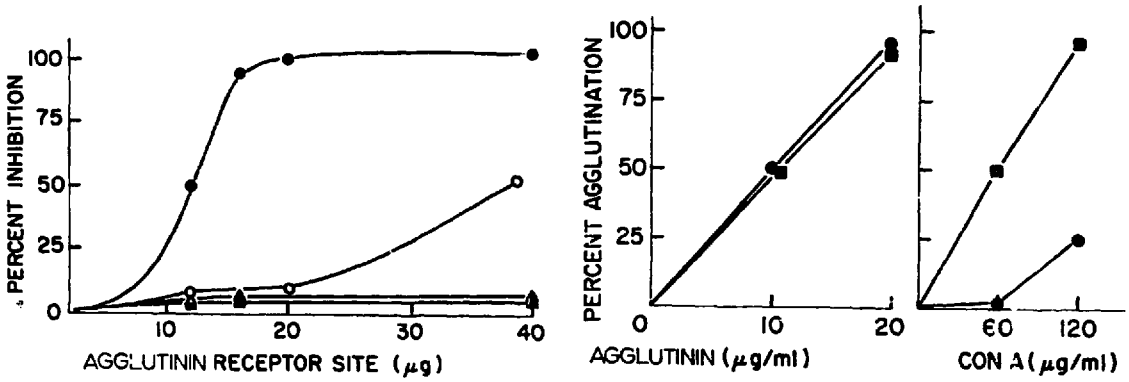

Fig. 1. Interaction of wheat germ agglutinin receptor site from L1210 cells with the agglutination of Py 3T3 cells by wheat germ agglutinin, phytohemagglutinin M, concanavalin A and *Lens culinaris* agglutinin. Concentrations of lectins required to give + 3 on agglutination scale are for wheat germ agglutinin 10 μg/ml, for phytohemagglutinin M 6 μg/ml, for concanavalin A 160 μg/ml and for *Lens culinaris* agglutinin 10 μg/ml. Agglutination with concanavalin A was scored after 15 min while that for all other lectins after 5 min. Inhibition of agglutination was carried out as described earlier[3]. ●—●, wheat germ agglutinin; ▲—▲, concanavalin A; ■—■, phytohemagglutinin M; ○—○, *Lens culinaris* agglutinin.

Fig. 2. Effect of chymotrypsinized concanavalin A (con A) on the agglutination of Py 3T3 cells by intact concanavalin A and wheat germ agglutinin. Conditions for trypsinization: 10 mg concanavalin A: 1 mg chymotrypsin, incubated for 5 h at 37 °C in phosphate buffer (pH 7.0). Cells were preincubated with chymotrypsinized concanavalin A for 10 min and controls with phosphate-buffered saline before agglutinations with wheat germ agglutinin or concanavalin A were carried out. ●—●, Preincubation with 60 μg/ml chymotrypsinized concanavalin A in phosphate-buffered saline. ■—■, Preincubation with phosphate-buffered saline.

This is precisely what is expected of a concanavalin A molecule which is operationally defined as still binding (K. D. Noonan, unpublished results) but not agglutinating anymore. In the present experiment, Py 3T3 cells were treated with such a chymotrypsinized concanavalin A preparation and then tested for agglutinability with both concanavalin A and wheat germ agglutinin. While the concanavalin A agglutination titer decreased markedly, there was no difference between the normal and treated cells when tested for agglutinability with wheat germ agglutinin (Fig. 2). This is the predicted result if a monovalent agglutinin would still bind to its receptor which is far enough apart from the receptor for wheat germ agglutinin.

Agglutinability of normal and transformed lymphocytes

The differential agglutinability of L1210 cells and normal lymphocytes has been previously reported[1]. In an attempt to quantify the difference we found that while L1210 cells agglutinate with 10 μg wheat germ agglutinin/ml; a 40-fold higher concentration is necessary to agglutinate normal lymphocytes. Since such a high concentration of wheat germ agglutinin caused cells to lyse and clump nonspecifically, it was not possible to achieve complete and specific agglutination. Trypsinization (0.08 % for 5 min) of lymphocyte surface, a treatment which in the 3T3 *versus* Py 3T3 system resulted in identical agglutination titers[2], had no effect on normal lymphocytes. Pronase treatment (0.08 % for 5 min), however, decreased the concentration of wheat germ agglutinin necessary for 50 % agglutination about four-fold.

Interaction of antiserum against wheat germ agglutinin receptor with:

Related normal cells

Upon injection of wheat germ agglutinin receptor fraction into rabbits complement fixing antisera were obtained. The immune sera showed high specificity for the L1210 line versus normal lymphocytes or red blood cells from the same strain of mice (Fig. 3). There was a very small residual complement fixing activity by normal lymphocytes which was cell number dependent and could not be absorbed out differentially. Surface area differences between L1210 and the two types of normal cells were more than compensated by assaying the normal cells in ten-fold higher concentrations.

Wheat germ agglutinin and wheat germ agglutinin haptens

Complement fixation by wheat germ agglutinin receptor and the antiserum could be inhibited by preincubating the antigen with wheat germ agglutinin. Since the agglutination by wheat germ agglutinin is inhibited by *N*-acetylglucosamine and di-*N*-acetylchitobiose[8, 16], these two compounds were also included as inhibitors of complement fixation in the same system but found to have no effect (Fig. 4).

Agglutination of L1210 and Py 3T3 cells by wheat germ agglutinin and concanavalin A

Treatment of L1210 cells with the antiserum was found to inhibit agglutination by wheat germ agglutinin while an identical treatment with preimmunization serum had no effect on the agglutinability. The same was also found when, instead of L1210, Py 3T3 cells were used. However, when Py 3T3 cells were preincubated with the same sera but tested for agglutinability by concanavalin A, there were no differences between the preimmunization and the immune sera (Fig. 5). Here in both cases, the titers were those usually obtained with nontreated cells[6].

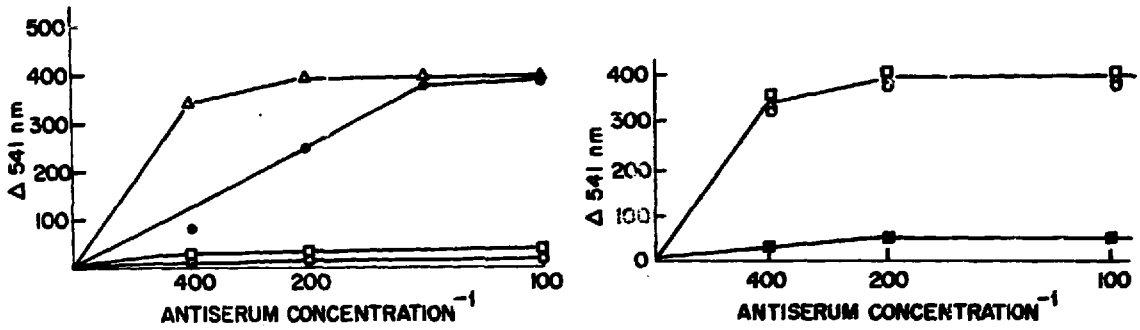

Fig. 3. Complement fixation by wheat germ agglutinin receptor (Ag), intact L1210 cells, normal mouse lymphocytes and red blood cells. Antiserum produced in rabbits against the isolated wheat germ agglutinin receptor fraction from L1210 cells. Reaction mixture contained 0.1 ml of each of the following ingredients when applicable: L1210 (10^6/ml), normal lymphocytes or red blood cells (10^7/ml), appropriate dilution of antiserum inactivated for 30 min at 60 °C and guinea pig complement (1:40). Complement fixation was allowed to proceed for 18 h at 4 °C, then indicator cells were added, allowed to react for 45 min at 37 °C, the mixture was centrifuged to sediment cells and released hemoglobin was measured at 541 nm. All components were tested for anticomplimentary activity or other interference with the complement fixation. Δ—Δ, wheat germ agglutinin receptor (4μg/reaction mixture); ○—○, Normal mouse lymphocytes; ●—●, L1210 cells, □—□, Mouse red blood cells.

Fig. 4. Effect of wheat germ agglutinin and haptenic inhibitors on complement fixation by isolated wheat germ agglutinin receptor and its antiserum. Wheat germ agglutinin receptor was preincubated with agglutinin (1:4, w/w) for 18 h at 5 °C, or antiserum (1:40, 1:100, 1:200) was preincubated with equal volumes of 0.1 M *N*-acetylglucosamine or 0.1 M di-*N*-acetylchitobiose. Complement fixation assays were done as described for Fig. 1. □—□, wheat germ agglutinin receptor; ○—○, wheat germ agglutinin receptor + di-*N*-acetylchitobiose; Δ—Δ, wheat germ agglutinin receptor + *N*-acetylglucosamine; ■—■, wheat germ agglutinin receptor + wheat germ agglutinin.

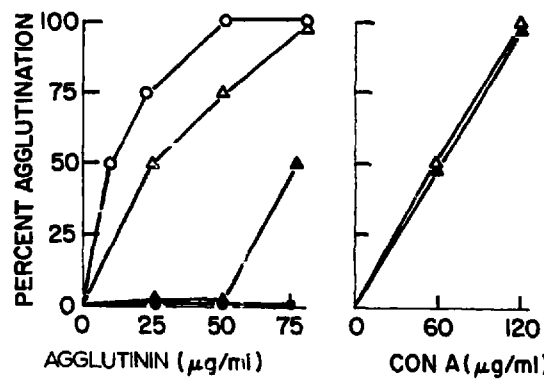

Fig. 5 Effect of antiserum against wheat germ agglutinin receptor site on agglutination of Py 3T3 and L1210 cells by wheat germ agglutinin and concanavalin A (Con A). $2 \cdot 10^6$ cells/ml were incubated in an equal volume of 1:200 dilution in phosphate-buffered saline of either control or immune sera for 20 min at 37 °C. Aliquots were taken and assayed for agglutination as previously described[15]. ○—○, L1210 cells preincubated with preimmunization serum; Δ—Δ, Py 3T3 cells preincubated with preimmunization serum; ●—●, L1210 cells preincubated with serum against wheat germ agglutinin receptor; ▲—▲, Py 3T3 cells preincubated with serum against wheat germ agglutinin receptor.

DISCUSSION

The L1210 cell has a distinct surface structure, part of which is the wheat germ agglutinin receptor site which seems to be absent on the normal mouse lymphocytes. This is a different situation from that present in other normal-transformed pairs.

While trypsinization of *e.g.* normal 3T3 fibroblast line renders it agglutinable with wheat germ agglutinin to the same extent as Py 3T3 cells, trypsinization of normal lymphocytes did not change the surface so that cells became agglutinable by wheat germ agglutinin. Pronase treatment stimulated the agglutinability of lymphocytes nevertheless to a minor degree.

The finding that antiserum against wheat germ agglutinin receptor from L1210 did not react with the normal lymphocytes enforces the differences suggested in agglutination systems with wheat germ agglutinin. While the agglutination reaction depends both on the presence of an available site and conditions that allow the cells to clump together, reaction with antiserum is measuring binding only and is therefore more restricted to availability of a surface antigen only. It is interesting that although red blood cells react with wheat germ agglutinin, the receptor sites are antigenically different from those of L1210 cells. Antiserum against wheat germ agglutinin receptor from L1210 surface did not fix complement with red blood cells from the same mice. Moreover, although wheat germ agglutinin, on the basis of haptenic inhibition has been shown to react with *N*-acetylglucosamine or di-*N*-acetylchitobiose-like sites, these two compounds were not found to act as haptenic inhibitors in the antigen-antibody system. We believe therefore that the antiserum against the isolated wheat germ agglutinin receptor is not directed against the same chemical determinants as the wheat germ agglutinin. This may explain why the antiserum seems to be more specific for tumor cells and does not interact with red blood cells.

In addition to wheat germ agglutinin receptors, the L1210 surface structure possesses receptors for a variety of lectins. The ones chosen here are representatives for different haptenic groups. Concanavalin A is inhibited by α-methyl mannosides, phytohemagglutinin M by *N*-acetylgalactosamine, *Lens culinaris* agglutinin both by *N*-acetylglucosamine and α-methylmannoside. The isolated wheat germ agglutinin receptor fraction provides a tool for the comparison of carbohydrate structure of this receptor and others of different determinants, also present on cell surfaces. One should, however, bear in mind the best haptenic inhibitor does not necessarily have to be a part of the actual receptor on the cell surface presumably due to different average conformations of the monosaccharide in solution and bound in an oligosaccharide structure. For example, *N*-acetylgalactosamine is a haptenic inhibitor for phytohemagglutinin M although it is not present in the receptor isolated from red blood cells[17].

The finding that the wheat germ agglutinin receptor fraction isolated by the phenol process did not inhibit agglutination by phytohemagglutinin M cannot be interpreted at the present time to mean that phytohemagglutinin M and wheat germ agglutinin receptors do have to be distinct structures in the L1210 cell membrane. While such a situation is conceivable, the negative results could also be due to degradation during extraction of the wheat germ agglutinin receptor using the phenol procedure. Work is in progress studying the interactions of the above mentioned lectins and wheat germ agglutinin receptor sites isolated by other procedures[3]. Preliminary results indicate that both the pyridine and lithium diiodosalicylate isolated wheat germ agglutinin receptors can indeed also inhibit agglutination by phytohemagglutinin M.

The failure to inhibit concanavalin A agglutination by isolated wheat germ

agglutinin receptor site emphasizes additionally the specificity of the inhibition reaction. Since L1210 cells do not agglutinate with concanavalin A, they may be lacking the concanavalin A receptor sites provided these are not cryptic. Wray and Walborg[18] found upon treatment of Novikoff ascites tumor cells with papain followed by pronase and fractionation on a DEAE column that concanavalin A and wheat germ agglutinin inhibitory activites were in separate fractions differing with respect to sialic acid. Although sialic acid is on one hand necessary for agglutination by wheat germ agglutinin[19] and on the other might have easily cleaved off during Wray and Walborg's fractionation (enzymatic digestion, trichloroacetic acid treatment), their results suggest that concanavalin A and wheat germ agglutinin receptors seem to be on distinct oligosaccharide residues.

The failure to inhibit agglutination of concanavalin A susceptible cells by a preincubation with antiserum against wheat germ agglutinin receptor suggests that the concanavalin A receptor is also topographically distinct from the wheat germ agglutinin site. While Py 3T3 cells exposed to wheat germ agglutinin receptor antiserum could not be agglutinated by wheat germ agglutinin, there was no effect on the agglutinability by concanavalin A. The implication from such an experiment is reinforced by yet another experiment where chymotrypsinized or presumably monovalent concanavalin A, while inhibiting the agglutination by intact concanavalin A, had no effect on agglutination by wheat germ agglutinin.

From earlier collaborative work (M. Inbar, L. Sachs and M. M. Burger, in preparation) on the susceptibility of the agglutination of 3T3 cells to trypsin, we reached tentatively the same conclusion. Ozanne and Sambrook[20] have reached similar conclusions since essentially no competition could be observed in the binding of ^{121}I-labeled concanavalin A and wheat germ agglutinin to intact cells. The multiple pieces of evidence given in this report for the chemical and topographical identity of the concanavalin A and wheat germ agglutinin receptors on Py 3T3 cells are the most comprehensive at the present time. It strongly suggests to us that a surface membrane is a mosaic consisting of at least two, perhaps a few, although not too many, glycoproteins[21].

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