A VERSATILE IMMUNOADSORBENT CAPABLE OF BINDING LECTINS OF VARIOUS SPECIFICITIES AND ITS USE FOR THE SEPARATION OF CELL POPULATIONS

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ABSTRACT

A procedure for cell fractionation using lectin-affinity chromatography is described. It consists of a single affinity adsorbent, hog gastric mucin blood group A+H substance covalently coupled to Sephadex or Sepharose, to which lectins of various specificities can bind. The complex formed, lectin ⇄ hog A+H substance-Sephadex, then serves as an affinity probe for isolating and fractionating cells. The lectins from *Ulex europaeus*, *Lotus tetragonolobus*, *Helix pomatia*, *Dolichos biflorus*, and *Phaseolus lunatus* were used with the same blood group substance as adsorbent. The affinity columns retained erythrocytes with blood group specificity for the adsorbed lectin and thus fractionate cells in mixtures. Cells as well as lectins are eluted by specific sugar inhibitors. Mixtures of two kinds of cells can be separated when the proportion of the adsorbed cells is not too low.

KEY WORDS affinity chromatography · cell surface · immunoadsorbents · blood group antigens · cell fractionation on lectins

Lectins interact with specific carbohydrate structures of glycoproteins or glycolipids, both in solution and on cell membranes, and have therefore been useful in the isolation, identification, and characterization of soluble glycoproteins and cell surface receptors containing sugars (25, 34). The separation of intact cells with the aid of lectins to distinguish cells bearing different surface carbohydrates, notably by affinity chromatography, is only beginning to be exploited. The first attempt (11) was made by attaching concanavalin A to nylon fibers which could then separate mouse thymocytes from mouse erythrocytes. Nylon fibers derivatized with Con A were also used (22) to isolate tumor cells which were shown to be more immunogenic than the parent tumor cell population. In other studies, cells were fractionated on lectin immobilized on agarose beads. For instance, a column of *Helix pomatia* lectin coupled to Sepharose 4B selectively retained neuraminidase-treated T cells and thus could be used in the separation of T and B cells (19); and *Lens culinaris* lectin bound to Sepharose 2B specifically adsorbed HeLa cells (23). Lectins free in solution have also been used to separate cells by differential agglutination (38).

Problems have been encountered with the use of lectin-affinity chromatography. One is that for the preparation of each distinct affinity adsorbent, a separate covalent coupling of lectin is required. In some instances, the cells with specific receptors are not retained by the affinity adsorbent, while in

others the cells are so strongly bound that they can not be eluted readily (11, 33). Purified lectins must be used for preparing the insoluble adsorbent, and some laboratories may not be equipped to purify lectins routinely; not all lectins are commercially available.

The present study describes a simple and general procedure for rapid cell fractionation which gives highly reproducible results. Human erythrocytes are used as a prototype but the method is potentially applicable to many other cell types. The procedure utilizes a single affinity adsorbent, hog gastric mucin blood group A+H substance covalently coupled to Sephadex or Sepharose, with lectins of various specificities passed through the column as crude extracts; the column is then washed free of unrelated proteins, a first step which essentially leaves purified lectin on it. The lectin $\rightleftarrows$ hog A+H substance-Sephadex is then used as an affinity probe for isolating and fractionating cells. The versatility of the approach derives from the multiplicity of sugar determinants on the hog gastric blood group A+H glycoprotein. Five lectins of different specificities were used with the same blood group substance as adsorbent. The capacity of the affinity adsorbent for retaining cells was high as was the total mean recovery, and the retained cells were readily eluted even after they were in the column for 48 h. A great advantage of the method is that even if the cells are very tightly bound to the lectin, they can nevertheless readily be eluted with sugar haptens which compete with the blood group substance $\leftrightarrows$ lectin bonds though they may be unable to dissociate the lectin-cell bonds.

MATERIALS AND METHODS

Reagents and Immunological Methods

Antisera used were anti-A Chris D_2 (31) and human anti-B 310_4 (1). The lectins were used as 10% saline extracts of *Dolichos biflorus*, *Lotus tetragonolobus*, and lima bean and of the albumin gland of *H. pomatia* (16). The extract of *Ulex europaeus* seeds was precipitated with $(NH_4)_2SO_4$ to separate lectin I from lectin II (27, 37). Crude *Ulex* lectin I, which is precipitated at 40% $(NH_4)_2SO_4$, was used in the fractionation experiments. Erythrocytes were outdated units from the blood bank of Presbyterian Hospital, N. Y., except for A_2 cells, which were from the New York Blood Center through the courtesy of Dr. Pablo Rubinstein.

Hemagglutination assays were done with the Takatsy microtitrator (Cooke Engineering Co., Alexandria, Va.) using 0.025 ml loops and suspensions containing 8×10^7 erythrocytes/ml. Equal volumes of the erythrocyte suspension and four hemagglutinating units of lectin or of antibody dilutions were mixed, placed at room temperature for 1 h, and read. The four hemagglutinating units were determined previously by titrating the lectin or antibody solution using the same number of erythrocytes.

The erythrocyte number was estimated from a calibration curve constructed as follows: the erythrocytes were washed four times with 0.15 M NaCl, 0.01 M sodium phosphate buffer (PBS)[1], pH 7.0, and resuspended in the same diluent to make suspensions ranging from 0.2 to 2%; the number of cells was determined with a microscope, using an improved Neubauer hemocytometer. The erythrocyte suspensions (0.1 ml) were lysed with 0.1% aqueous solution of sodium carbonate (0.4 ml), and the optical density of the clear lysate was read at 541 nm in a spectrophotometer (28). A calibration curve was then made by plotting erythrocyte number determined in the microscope against absorption at 541 nm. An optical density of 0.650 corresponds approximately, in terms of hemoglobin concentration, to 10×10^7 erythrocytes/ml of erythrocyte suspension, provided the cells are derived from a healthy donor (29).

For estimation of numbers of cells in the effluent and eluate fractions of the lectin-affinity columns, the cells in each fraction were washed with appropriate buffers and resuspended in 0.15 M NaCl (0.5–1.0 ml) and lysed as above. The absorption at 541 nm was determined and the number of cells was calculated from the calibration curve. In this way, an estimate of cells in a large number of fractions can be performed rapidly and reliably. However, the method is not very sensitive since 3×10^6 erythrocytes/ml gives an absorption at 541 nm of only 0.026. Thus, a concentration of $> 10^6$ erythrocytes/ml cannot be determined accurately.

Hog gastric mucin (HGM) was obtained commercially (Wilson Laboratories, Chicago, Ill.) and purified as described (20, 35). Briefly, 100 g of mucin powder was suspended in 3 l of H_2O (~30 mg/ml), and the pH was raised to 8.2 with 265 ml of 0.1 M $NaHCO_3/Na_2CO_3$ buffer. The mixture was stirred overnight under toluene, then centrifuged at 2,000 rpm for 3 h at 4°C. The supernate was extensively dialyzed against distilled water to remove low molecular weight materials, ultracentrifuged at 12,000 rpm for 90 min, and treated with 95% ethanol to a final concentration of 65% (vol/vol) in the presence of 1% sodium acetate. The precipitate was washed with 95% ethanol and dried *in vacuo* over P_2O_5 and NaOH. The yield varied from 60 to 70%.

[1] Abbreviations used in this paper: BGS, blood group substance; DB, *Dolichos biflorus* lectin; LFuc, fucose; DGal, galactose; DGalNAc, N-acetyl-D-galactosamine; DGlcNAc, N-acetyl-D-glucosamine; DMan, mannose; HGM, hog gastric mucin; HP, *Helix pomatia* lectin; LBL, lima bean lectin; LCL, *Lens culinaris* lectin; LT, *Lotus tetragonolobus* lectin; PBS, 0.01 M sodium phosphate-buffered saline, pH 7.0; UE I, *Ulex europaeus* lectin I.

Preparation of Immunoadsorbent Columns

The purified HGM blood group A+H glycoproteins, 105.8 mg in 20.0 ml of 0.15 M NaCl–0.01 M $NaHCO_3$, were coupled to 20 ml of swollen settled Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, N. J.) activated with 1 g of cyanogen bromide (2, 9). The amount of blood group substance bound to Sephadex was 15.3 mg. The resulting insoluble immunoadsorbent was stirred for several hours with excess 0.1 M glycine in 0.1 M Na_2CO_3 to destroy residual activated groups on the Sephadex. Disposable syringes (5.0 ml) were fitted with polyethylene discs (Bell-Art Products, Penawnock, N. J.) and packed with 3–4 ml of the Sephadex containing covalently coupled HGM blood group A+H substance (Sephadex-BGS). The columns were washed with 0.15 M NaCl–0.01 M $NaHCO_3$, followed by 0.01 M Na acetate buffer, pH 4.0, and equilibrated with PBS. HGM was also coupled to Sepharose 4B in the same manner but flow rates were not satisfactory

Crude extracts of *Ulex* lectin I, *L. tetragonolobus, D. biflorus, Phaseolus lunatus* (lima bean), or *H. pomatia* were then applied continuously to the HGM-Sephadex column, and 1.0-ml fractions were collected until the titer against O erythrocytes (with *Ulex* lectin I and *L. tetragonolobus*) or A_1 erythrocytes for *Dolichos*, *H. pomatia*, or lima bean of the effluent equaled that of the initial extract; ~2–3 ml of crude *Ulex* I or *H. pomatia* and 4–5 ml of *Dolichos* or lima bean extracts were required to saturate 3.0 ml of HGM-Sephadex, corresponding to 15–30 μg N, 12–24 μg N, 16–20 μg N, and 20–25 μg N of *Ulex* I, HP, *Dolichos* and lima bean lectins, respectively. The amount of *Lotus* lectin bound was not calculated. The columns were then washed with PBS until the absorbance at 280 nm was <0.010.

The affinity adsorbent thus contained blood group substance covalently coupled to Sephadex beads and bound lectin in equilibrium (reversibly) to the immobilized blood group substance. Thus, it may be represented as lectin ⇆ HGM-Sephadex.

Cell Fractionation

Human erythrocytes were washed four times in PBS, resuspended in the same buffer to a concentration of 10–20 × 10^6/ml and then applied (total 38–114 × 10^7) to each 3–4 ml column of lectin ⇄ blood group substance–Sephadex. The columns were washed with 2.0-ml aliquots of starting buffer at a flow rate of ~0.5 ml/min until the effluent was virtually cell free. Bound cells were removed by competitive inhibition, using stepwise elution with 2.0-ml aliquots of PBS containing 1.0 mg/ml of hog A+H blood group substance. Unless stated otherwise, all experiments were performed at room temperature. A complete separation and elution of the bound cells was accomplished in a few hours.

Analysis of Erythrocyte Fractions

Unfractionated or mixed, unretained and BGS-eluted erythrocyte populations were studied with respect to agglutination by lectins or antibodies of known specificity. The effluent fractions were adjusted to 8 × 10^7 cells/ml. The eluted cells were washed twice with PBS containing 1.0 mg/ml of hog A+H substance to remove the lectin which was co-eluted with the erythrocytes from the HGM-Sephadex column, then washed four times with PBS to rid them of HGM, and resuspended to 8 × 10^7 cells/ml of PBS. The eluted cells showed normal shape without evidence of agglutination when examined under the microscope, provided they were washed as described above. If the eluted cells were washed only with saline, they formed significant numbers of clumps probably because of the co-eluted lectin, since the aggregated cells could be dissociated with the sugar for which the lectin was specific. The two populations of cells after separation were examined visually and microscopically for purity by adding the lectin specific for the minor population. For instance, a mixture of 0.5 × 10^7 O erythrocytes + 32 × 10^7 A_1 erythrocytes clearly showed clumps of O erythrocytes with 4 hemagglutinating units of Ulex and Lotus lectins (25 μl) + 10^7 cells (25 μl). Thus a 1–2% contamination was readily detectable.

RESULTS

Separation of erythrocytes by lectin-affinity chromatography was first attempted with purified or crude lectin preparations attached directly to the CNBr-activated Sephadex or Sepharose beads. Although some A_1 and O erythrocytes were retained specifically in the DB and UE-I resins, respectively, the number of cells bound were insufficient for characterization, even using different concentrations of lectins (0.1–8.0 mg/ml settled beads) and volumes of adsorbents (3–20 ml). In addition, bound cells could not be eluted with specific sugar or with HGM-A+H blood group substance, unless the beads were stirred mechanically, but then hemolysis became a problem. Retention of cells was also unsuccessful on a preparative scale when *Dolichos* or *Ulex* lectin I was coupled to N-hydroxysuccinimide ester of succinylated aminopropyl Sephadex (10).

Retention of cells was accomplished by passing them on a column to which a lectin was adsorbed to blood group substance immobilized to Sephadex G-25. Human erythrocytes may be retained by a HGM-A+H blood group substance-Sephadex column, depending on the specificity of the lectin adsorbed to the conjugated blood group substance. Results on the specificity of the affinity columns using five lectins are shown in Table I. If the HGM-Sephadex column is saturated with the H specific lectin from the 40% $(NH_4)_2SO_4$ fraction of crude *U. europaeus* seed extracts, or with *L.*

TABLE I

Specific Fractionation of Cells in Mixtures on Affinity Lectin Columns

Column*	Cells applied to column		Cells in effluent		0.1 M DGal§		Elution with HGM 1.0 mg/ml		Yield‖	Total recovery
	No. × 10^{-7}	Type‡	No. × 10^{-7}	Type‡	No. × 10^{-7}	Type‡	No. × 10^{-7}	Type‡		
									%	%
Ulex I ⇄ HGM-Sephadex	19.0	A_1	18.5	A_1						97
	19.0	O	12.3	O			6.5	O		99
	38.0	50% A_1	28.5	A_1			7.0	O	37	93
		50% O		O						
	8.0	50% A_1	3.9	A_1			3.7	O	93	95
		50% O								
DB ⇄ HGM-Sephadex	19.0	A_1	11.2	A_1			7.5	A_1		98
	19.0	O	18.0	O						95
	6.4	A_1					6.2	A_1		97
	12.8	50% A_1	6.5	O			6.0	A_1	94	98
		50% O								
LBL ⇄ HGM-Sephadex	19.0	A_1	11.0	A_1			7.0	A_1		95
	19.0	O	18.3	O						96
	6.4	A_1					6.2	A_1		97
	12.8	50% A_1	6.6	O			6.1	A_1	95	99
		50% O								
HP ⇄ HGM-Sephadex	19.0	A_1	5.2	A_1			12.3	A_1		92
	19.0	O	19.0	O						100
	38.0	50% A_1	24.8	A_1			11.8	A_1	49	96
		50% O		O						
Lotus ⇄ HGM-Sephadex	19.0	O	19.3	O			4.2	O		97
	19.0	A_1	18.3	A_1						
	4.0	A_2	1.8	A_2			2.3	A_2		100

* 3.0 ml columns

‡ A_1- and O-type red cells are determined with H-specific lectins (*Ulex* and *Lotus*) and with anti-A reagents (*Helix pomatia* and *Dolichos biflorus* lectins and anti-A antibodies). A_2 cells are agglutinated by H- and A-specific lectins and anti-A antibodies but not by *Dolichos biflorus* lectin.

§ Elution with DGal was always done prior to HGM. Dash indicates that no cells were detected in the spectrophotometric assay.

‖ Adsorbed cells removed/cells of same specificity added × 100.

tetragonolobus lectin, O but not A_1 cells are adsorbed and specifically eluted from the column. If, however, the same adsorbent is equilibrated with A-specific lectins, such as those from the seeds of *D. biflorus* and *P. lunatus,* or from the snail *H. pomatia*, O erythrocytes are not retained and pass through the Sephadex; the bound cells, which are readily eluted with HGM or with 0.1 M DGalNAc, but not by 0.1 M DGal or 0.1 M L-Fuc, are agglutinated by anti-A reagents (HP, DB, and LBL lectins, and anti-A antibodies) but not by H-specific lectins (*U. europaeus* and *L. tetragonolobus*). Untreated Sephadex and HGM-Sephadex fail to bind cells.

The loading capacity of 3.0 ml of settled beads, determined by deliberately passing excesses of erythrocytes over the specific lectin-affinity Sephadex column at room temperature, is in the range of 6.5×10^7 to 7.5×10^7 cells, except for HP ⇄ HGM-Sephadex which retains 12.3×10^7 cells (Table I). At 4°C, approximately the same number of cells is bound, but it is necessary to keep the cells in contact with the beads for at least ½ h to obtain a stable cell-bead binding, otherwise most cells come through in the effluent if they are applied continuously.

Artificial mixtures of two types of erythrocytes were applied to the column to determine whether these lectin-affinity columns could distinguish erythrocytes for which the lectin is specific from those which are not. The elution pattern of a representative separation experiment using *Ulex* lectin I ⇄ HGM-Sephadex is depicted in Fig. 1 and in Table I. Table I also shows the results with DB, LBL, and HP columns. 10.1 ml of erythrocytes are placed over 3.0-ml columns and washed or eluted with 2.0-ml aliquots of PBS or PBS containing 1.0 mg/ml of HGM, respectively. With *Ulex* I, only O cells are retained, while with DB, LBL, and HP-affinity columns, only A_1 cells are

bound, as could be predicted from the experiments in which O and A_1 cells were chromatographed separately. If the number of specific cells applied is equal or below the capacity of the column, then the number of cells bound and eluted is approximately the same regardless of whether they are applied singly or as a mixture with cells not containing the specific determinant. For instance, when the *Ulex* lectin ⇄ HGM-Sephadex column is overloaded with 19×10^7 O cells, either as a single suspension or mixed with 19×10^7 A_1 cells, 6.5×10^7 and 7.0×10^7 cells are specifically retained and eluted, respectively. The remaining specific cells are recovered in the effluent. The yield of specific cells bound is 37%, but the low value is due to the deliberate excess of specific cells applied since, if the mixture contains 4.0×10^7 O and 4.0×10^7 A_1 cells, the number of O cells in the eluate is 3.7×10^7 (93% yield). High specific yields are also obtained with DB- and LBL-columns where the number of specific cells (A_1) corresponds to the capacity of the column (Table I). With HP ⇄ HGM-Sephadex, the capacity is $\sim 12 \times 10^7$ cells, and thus the column retains 11.8×10^7 A_1 cells when it is run with 38×10^7 cells containing equal parts of A_1 and O cells. However, if the mixture contains a proportionally large excess of irrelevant cells, then the yield of adsorbed cells is decreased. This is shown in Table II for the *Ulex* lectin I ⇄ HGM-Sephadex system. Thus, when the mixture contains 4.0×10^7 O (12.5%) and 32.0×10^7 A_1 cells, only 1.8×10^7 O (45% specific yield) erythrocytes are retained, as compared with 3.8×10^7 or 3.7×10^7 (93% specific yield) if the same number of O cells is applied in the absence of A_1 cells or mixed with 4.0×10^7 A_1 cells, respectively. No erythrocytes are retained by the column with a mixture containing 0.5×10^7 (1.5%) O cells and 32.0×10^7 A_1 cells; however, if the same number of O cells is run alone, then 60% of the cells are recovered in the eluate. An amount smaller than 0.5×10^7 was not tested because of the difficulty in detecting 10^6 cells/ml in the spectrophotometric assay. Nevertheless, it seems that the decreased apparent ability of the column to bind specific cells when these cells are mixed with a large excess of irrelevant ones is probably due to mechanical interference of the latter. With erythrocytes the obvious mechanical factor would be the formation of rouleaux.

This mechanical interference, however, is not a serious limitation to the technique when it is used to detect small proportions of cells in mixtures since, in the latter instance, if the total amount of cells applied is three times as much while maintaining the same relative proportion of A_1 (98.5%) and O (1.5%) cells, then O cells could be detected (Table II). Despite the large excess of irrelevant

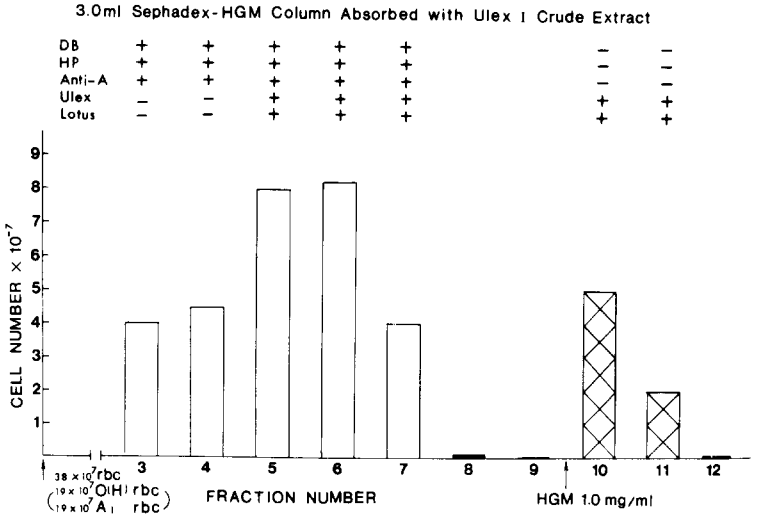

FIGURE 1 Representative stepwise elution profile obtained with a mixture of A_1 and O erythrocytes on a 3-ml *Ulex* lectin I ⇄ HGM-Sephadex column. The agglutination pattern of each fraction with anti-A reagents (DB, HP, and anti-A antibodies) and with H-specific lectins (*Ulex* and *Lotus*) is shown; (+) indicates agglutination with four hemagglutination units of each reagent and (−) for no agglutination with the same dose. Each fraction was 2 ml.

TABLE II

*Recovery of Retained and Eluted Cells from a Ulex ⇄ HGM-Sephadex Adsorbent Column**

No. of cells applied × 10^{-7}			Cells‡ in effluent			No. of cells eluted§ × 10^{-7}			
				Type					
A_1	+	O	No. × 10^{-7}	A_1	O	A_1	O	Yield¶	Total cell recovery
								%	
		16	9.5		+	−	6.5		100
16.0		16.0	25.7	+	+	−	7.0	44	102
		4.0		−	+	−	3.8		95
4.0		4.0	3.9	+	−	−	3.7	93	95
32.0		4.0	32.9	+	−	−	1.8	45	96
4.0		1.0	4.0	+	−	−	1.0	100	100
32.0		1.0	32.2	+	−	−	0.6	60	99
4.0		0.5	3.8	+	+	−	0.3	60	91
		0.5	—‖	−			0.3		60
32.0		0.5	31.0	+	−	−	—‖	0	95
96.0		1.5	93.1	+	−	−	1.0	66	94

* 3.0 ml column

‡ A_1 and O cells typed as in Table I: (+) indicates agglutination with specific reagents, and (−) for no agglutination. Agglutination scored using the unaided eye.

§ These cells were of O-type because they were agglutinated by *Ulex* and *Lotus* extracts and not by *Dolichos* lectin or by anti-A antibodies; they were eluted with hog gastric mucin (1.0 mg/ml).

‖ Cells could not be detected by the spectrophotometric assay; however, cells could be seen under the microscope after pelleting them by centrifugation, although they were not counted.

¶ Adsorbed cells eluted/cells of same specificity added × 100.

cells, they do not seem to contaminate significantly the specifically eluted ones, inasmuch as the eluted cells are agglutinated only by H-specific reagents.

Since two fractions of differing immunochemical specificity are obtained by fractionating A_2 blood group glycoproteins on *Lotus* lectin–Sepharose immunoabsorbent (35), attempts were made to fractionate A_2 erythrocytes (from two individuals) on the *Lotus* lectin ⇄ HGM-Sephadex adsorbent. Although the column retains A_2 cells (Table I), they are indistinguishable serologically from the effluent cells and from the original cell population as assayed with *Lotus*, *Ulex* I, *Dolichos*, and *H. pomatia* lectins, and with anti-A antibodies. Similarly, the *Ulex* ⇄ HGM-Sephadex column fails to fractionate A_2 erythrocytes into different cell populations. Thus, these experiments indicate that both A and H determinants are present on the same cell.

Although *Ulex* lectin I is considered to be H specific (6, 27, 37), this lectin also agglutinated B erythrocytes although to a lesser extent than O or A_2 cells. It could therefore be argued that B cells represent a heterogeneous population of cells with varying proportions of H determinants. If this were so, one might obtain a fraction of cells enriched in H determinants by passing B cells on *Ulex* I ⇄ HGM-Sephadex. However, the fraction of B cells bound and eluted from the column behave similarly to the original cells when assayed with *Lotus* and *Ulex* extracts, suggesting that the receptors for *Ulex* lectin I are present on all cells. It is of interest that purified *Ulex* lectin I also precipitates with B substances of human and of horse origin (37), although the precipitation is not inhibited by blood group B-specific oligosaccharides. It is not clear whether the interaction of *Ulex* lectin I with B cells or with B substances is due to weak binding to B determinants or due to the presence of H determinants of incompletely synthesized B chains.

The mean total cell recovery is >95% and the ranges of cell recovery vary minimally from experiment to experiment, as seen by the results of 18 separate fractionation experiments (Table I). Furthermore, no appreciable hemolysis is observed either in the effluent or in the eluted cell fractions, provided that fresh (1–7 d old) erythrocytes are used.

DISCUSSION

This paper describes a simple and general method

for the rapid fractionation of erythrocytes of different antigenic specificities by lectin-affinity chromatography. Human erythrocytes are used, but the method is potentially applicable to many other cell types. It consists in coupling hog gastric blood group A+H substance to Sephadex G-25 which then serves as an immunoadsorbent for reactive lectins (or antibodies), which, in turn, may interact with cells. A similar "sandwich"-type principle for separation of cells has also been achieved (40) using glass beads coated with antigen, followed by antibody, then passing cells containing the corresponding surface antigen.

The main advantage of the method described here is that the adsorbent, HGM-Sephadex, can be used for a variety of lectins of various specificities because of the multispecificities of HGM blood group substance; this glycoprotein has blood group A and blood group H determinants and also terminal nonreducing DGlcNAcα1 → 4DGalβ1 → 4 and DGalNAcα1 → 3DGalNAcα1 → and, in addition, it has determinants from incompletely synthesized chains (12, 24). Thus, D-Man, D-Gal, D-GalNAc-, D-GlcNac, and L-Fuc-binding lectins may interact with HGM. Examples, in addition to those in Table I, are: Con A (26), ricin and *Ricinus* agglutinin (32), soybean agglutinin (36), *Aaptos* lectins (4), and many others (cf. reference 34). In principle, anti-A serum or antibodies that cross react with HGM can also be used. The use of HGM, which is commercially available, lends considerable versatility to the method.

Crude lectin preparations rather than purified materials are suitable for adsorption since, in effect, the lectins (or antibodies) are isolated from extraneous compounds by affinity chromatography on HGM-Sephadex, provided they can interact with HGM. This may be important in many laboratories because purified lectins are expensive, and some are not commercially available.

The cells are readily eluted with specific sugars or glycoproteins, unlike the findings with affinity methods employing lectin directly attached to the beads. For instance, A_1 cells retained in the lima bean lectin ⇄ HGM-Sephadex are easily eluted with a solution of HGM; however, bound cells were not released (33) with the following solutions: 50 mM glycine-HCl-0.15 M NaCl (pH 5.0), 50 mM $NaHCO_3$–0.15 M NaCl (pH 9.0), or 0.2 M KSCN using LBL coupled directly to Sepharose. A hapten-induced replacement of cells under physiological conditions, even with mechanical aid, has been difficult, if not impossible, in the case of Con A-adsorbent (11). The inability to dislodge bound cells may be due to strong secondary interactions between cell and bead (11). In other instances, however, cells can be eluted from the immunoadsorbent without major difficulties (19, 23). A possible explanation for the ease in eluting cells using the approach described in this paper is that HGM blood group glycoprotein intercalates between the bead and the lectin-erythrocyte complex, possibly preventing the development of the postulated secondary forces. The physical characteristics of blood group substances may facilitate the retention and release of cells from the beads, since these glycoproteins appear to be molecules with a flexible configuration approaching that of a random coil (8) having molecular weights, ranging from 5×10^5 to several millions (references 8, 5, cf. 39). Furthermore, elution with HGM or with a specific ligand inhibits the binding of the lectin to the HGM-Sephadex and to the cell, thus contributing to the removal both of the lectin and of the cell from HGM-Sephadex. In addition, examination of the erythrocytes bound to *Ulex* I ⇄ HGM-Sephadex G-25 and to *Ulex* I–Sepharose 4B under the microscope (Fig. 2) shows that under the latter conditions, cells seem to be attached in the pores as well as on the surface of the beads. It is possible that this has some bearing on the inability of these cells to be eluted and might also result in damage to more delicate cell membranes such as those of lymphocytes.

An important consideration for cell sorting by affinity methods is the complete recovery of bound cells since, if some are retained even after elution, one may be losing a functionally unique population. Table I shows that the cells applied to the column were quantitatively recovered (92–100%) in good agreement, for example with that (~95%) of human lymphocytes fractionated on a Sephadex G-200 column containing rabbit anti-human Fab (7). Other reported techniques for the fractionation of erythrocytes (40, 33) or T and B cells (13, 30, 14) fail to recover cells quantitatively.

The yield of specific cells retained by the affinity columns varied from 0 to 100% according to the relative proportion between specific and irrelevant cells and with the total number of irrelevant cells in the mixture as shown in Table II for *Ulex* lectin I ⇄ HGM-Sephadex. If the proportion of specific cells is low (1.5%) and the amount of irrelevant ones (32×10^7) several times the capacity of the column, then no cells were retained. However, 66% of cells were specifically bound and eluted

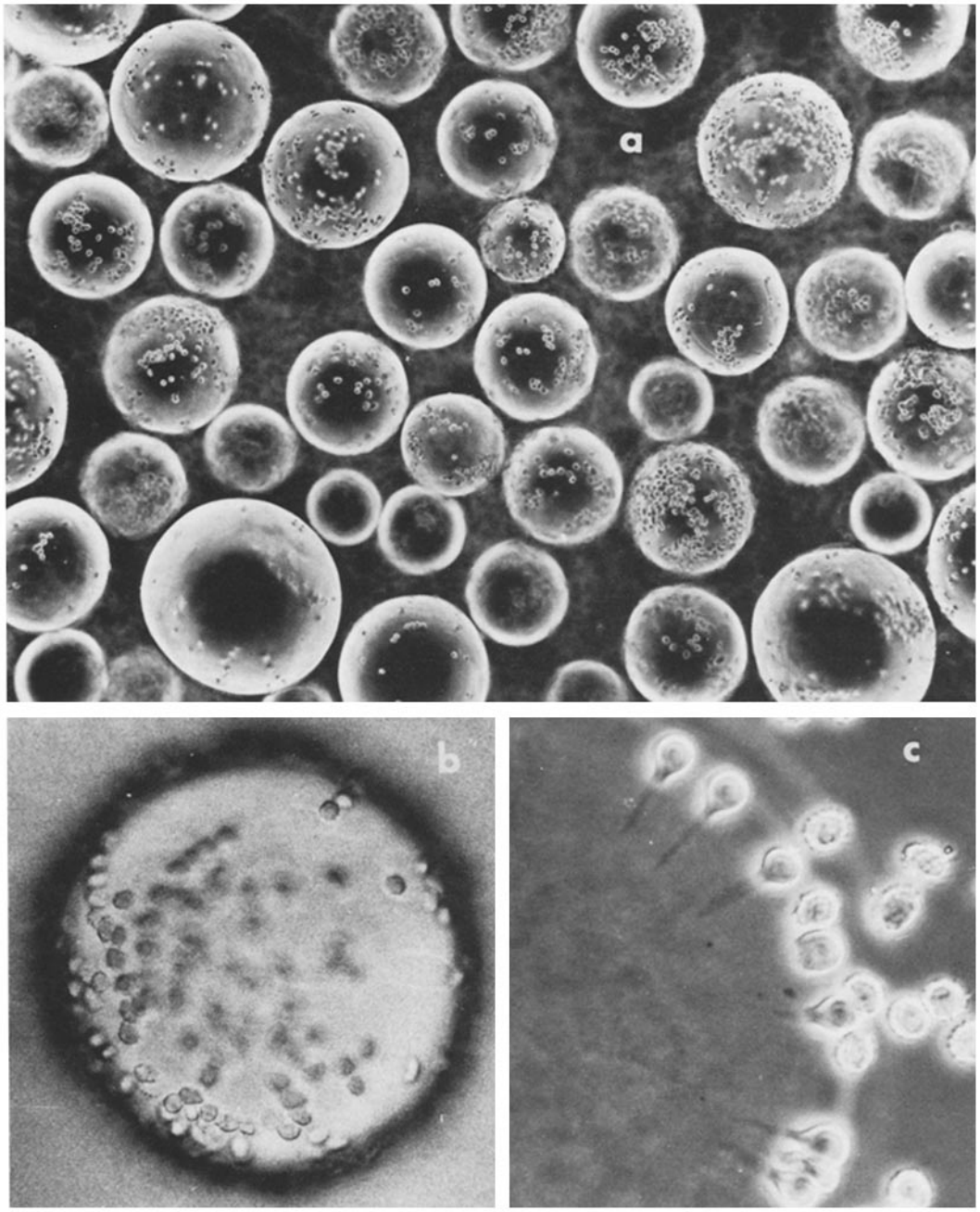

FIGURE 2 Binding of O erythrocytes by *Ulex* lectin I ⇄ HGM-Sephadex G 25 (*a* and *b*) and by *Ulex* lectin I-Sepharose 4B (*c*). *a*, × 125; *b*, × 325; *c*, × 650.

when the total number of cells in the same mixture was three times higher; this may be an important consideration when one attempts to enrich specific cells present in low concentration in a population.

The loading capacity of the affinity-adsorbent that had been recycled up to 20 times did not change. For recycling, the cell ⇄ lectin ⇄ HGM-Sephadex was eluted with a solution of HGM

until all erythrocytes were completely removed from the column, washed with PBS and a crude seed or snail extract passed over the HGM-Sephadex to readsorb lectin. The HGM-Sephadex beads are stable for many months, even years, when kept at 4°C in the presence of 0.02% Na azide without appreciable loss of their lectin-binding capacity.

The lectin-affinity columns retain approximately the same maximum number of cells/ml of beads (Table I) except for the *H. pomatia* lectin ⇄ HGM-Sephadex, which retains significantly larger numbers of cells. This is probably related to the hexavalence of the HP lectin (16, 18) since this increases the chances of interaction with the cell receptors. The lima bean and the *Lotus* lectins are either di- or tetravalent (3, 21) and the *Dolichos* agglutinin probably has four combining sites per molecule (17). The valence of *Ulex* I is not known. Since the same batch of HGM-Sephadex was used in all experiments, the amount of each lectin necessary to saturate the same volume of settled beads did not vary significantly (see Materials and Methods) and thus it is unlikely that the HP-column retains more cells because of a higher concentration of protein per Sephadex bead. Furthermore, this multivalent interaction increases the affinity of HP to erythrocytes as compared with other lectins. Thus, the K^a of HP for A_1 cells is 1×10^{10} M^{-1} (15), a much higher value than that for LBL, 7.2×10^6 M^{-1} (33), and for other lectins (cf. reference 25).

It is of interest that, if the cell separation experiments were performed at 4°C, instead of at room temperature, the loading capacity of the column did not change, provided that the cells were kept with the adsorbent for several minutes before washing. Similar dependence on temperature has been observed in the binding of HeLa cells to *L. culinaris* lectin (LC) immobilized on Sepharose (23) since, at room temperature, or at 37°C, the LCL-Sepharose beads were covered with cells within 10–30 min, while at 4°C, it took twice as long to bind the same amount of cells.

The method for erythrocytes purification and fractionation described in this paper, based on differences in cell surface glycoproteins or glycolipids, may prove very useful for fractionating other cells, such as lymphocytes, bacteria, or protozoa.

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