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Behavior of Human Serum R-Type Vitamin B_{12}-Binding Proteins (Cobalophilins) on Lectin–Sepharose Affinity Resins (41023)

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Abstract. R-Type serum vitamin B_{12}-binding proteins (cobalophilins) are a heterogenous population of glycoproteins. The reason for this appears to be due to variation in the carbohydrate moieties of the macromolecules. To support this contention we have used different sugar-specific lectin–sepharose affinity columns as a means for separating cobalophilins. Concanavalin A–Sepharose can resolve serum vitamin B_{12}-binding activity into three distinct fractions. The first two are cobalophilins and the third is transcobalamin II. The major cobalophilin fraction does not interact with concanavalin A–Sepharose, but is bound completely by both wheat germ and castor bean II lectins. Elution from these columns can be accomplished by addition of the appropriate competitive sugar to the buffer. Castor Bean I Lectin–Sepharose resolves the major concanavalin A–Sepharose cobalophilin fraction into two distinct fractions — one which does not interact with this lectin and the other which is eluted when *N*-acetylgalactosamine is added to the buffer. Furthermore, the distribution of exogenous-bound (^{57}Co) cyancobalamin between these two fractions is different when comparing certain myeloproliferative diseases in which serum cobalophilins are usually elevated. It is suggested that use of lectin–Sepharose resins may be a more definitive method for defining cobalophilin variants than is presently available.

R-Type vitamin B_{12}-binding glycoproteins or cobalophilins (1), have been separated into a number of variants (2–5). Reasons for these variants have been attributed to differences in the carbohydrate moieties of the cobalophilins rather than the apoprotein (4–8). Although apparent differences in the amount of sialic acid residues have accounted for some of the variants (6, 9), carbohydrate analysis of several purified fractions has shown that other sugar residues are also involved. (4, 7).

Anion-exchange chromatography of human serum cobalophilins can resolve cobalophilins into two fractions—transcobalamin (TC) I and III (7, 10), whereas isoelectric focusing has resolved cobalophilins into as many as five or six variants (8). These results in addition to cobalophilin variants from serum and other sources being immunologically indistinguishable (4, 7, 11) have led to some confusion in defining each variant species of the cobalophilins. Thus a reliable and reproducible procedure for resolving cobalophilins would aid in evaluating the significance of variations in serum levels of these binders that frequently occur in disease states which affect leukocyte production, such as myeloproliferative disorders (12–15). The possibility of using different sugar-specific lectin agarose resins as a suitable separation media for cobalophilins was investigated, since this approach would focus primarily on differences in the carbohydrate moieties among these glycoproteins.

Lectins and their sugar specificities used during this investigation were: concanavalin A; α-D-mannose (28), wheat germ agglutinin; *N*-acetylglucosamine (29), castor bean lectin I; *N*-acetylgalactosamine (30), and castor bean lectin II; D-galactose (31).

Materials and Methods. *Preparation of lectin–Sepharose resins.* Concanavalin A–Sepharose, cyanogen bromide Sepharose 4B, castor bean lectins I and II, and wheat germ lectin were purchased from Sigma Chemical Company (St. Louis, Mo.).

Fifty milliliters of $10^3 M$ HCl was added to 1.5 g of CNBr–Sepharose 4B and the resin was allowed to swell at room temperature for 30 min. The resin was then washed on a sintered glass funnel under vacuum with approximatley 500 ml of 0.1 *M* $NaHCO_3$ buffer containing 0.5 *M* NaCl (pH

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8.6). The resin (approximately 5 ml in packed volume) was removed from the funnel and 3–5 ml of bicarbonate buffer was allowed to remain in the suspension, making a final volume between 8 and 10 ml. Twenty-five milligrams of the desired lectin was then dissolved in 2.5 ml of bicarbonate buffer and added to the washed CNBr–Sepharose 4B suspension. The reaction was allowed to proceed for 2 hr at room temperature with intermittent stirring with a glass rod. After this time the Sepharose resin coupled with lectin was washed on a sintered glass funnel with 200 ml of 1 *M* Tris–HCl (pH 8.3) and resuspended in 50 ml of the Tris buffer for 30 min at room temperature in order to block unreacted CNBr sites. Finally the resin was washed with 500 ml of 1 *M* NaCl and kept in a 50% slurry of this solution at 4° until its use.

Chromatography of (^{57}Co) cyancobalamin-saturated serum binders on lectin–Sepharose columns. After obtaining informed consent from donors, approximately 7–10 ml of blood was obtained by venipuncture from patients and normal individuals. Serum samples from patients with polycythemia vera were obtained from the Polycythemia Vera study group at Mount Sinai Medical School in New York. After standing at room temperature for 1 hr the clot was removed by centrifugation at 3000 *g* for 10 min and the serum collected with a Pasteur pipet. To a 0.5-m aliquot of serum was added 5000 pg of high specific activity (^{57}Co) cyanocobalamin (Amersham: Arlington Heights, Ill.—sp act 220 mCi/mg) and the solution was incubated for 20 min at 37°. The labeled serum was then dialyzed overnight at 4° against 4 liters of 0.02 *M* sodium phosphate buffer (pH 6.3) containing 1 m*M* $CaCl_2$ and 1 m*M* $MnSO_4$. The radioactivity recovered from the dialysate and that amount remaining in the dialysis bag were determined using an automatic gamma counter (Searle, Model 1185) and the amount of serum-bound exogenous cyanocobalamin was calculated from the specific activity.

The labeled serum was then applied to a concanavalin A–Sepharose column (1 × 10 cm) previously equilibrated with 500 ml of the phosphate buffer. After the sample penetrated the resin, the column was eluted with phosphate buffer at a flow rate of 0.3 ml/min and 2-ml fractions were collected. Following return of the first radioactive peak to baseline levels (fraction A), phosphate buffer containing 0.5 *M* α-methyl-D-mannoside was washed through the column to remove a second radioactive peak (fraction B). After complete elution of fraction B, the column was eluted with 1 *M* NaCl which removed a final peak of radioactivity fraction C. Recoveries of greater then 95% of the applied radioactivity were routinely obtained.

Fraction A from concanavalin A–Sepharose column was then pooled and applied to other lectin–Sepharose columns (1 × 10 cm). The same procedure as described for elution of the concanavalin A–Sepharose column was followed except that the competitive sugars differed as follows: (a) wheat germ lectin, 0.5 *M* *N*-acetylglucosamine; (b) castor bean I lectin, 0.5 *M* *N*-acetylgalactosamine; and (c) castor bean II lectin, 0.5 *M* D-galactose.

Chromatography of Concanavalin A–Sepharose fractions on a sephacryl-S-200 column. A Pharmacia column 2.6 × 70 cm (Piscataway, N.J.) with flow adaptors was packed with Sephacryl-S-200 (Pharmacia) and connected to a peristalic pump (Virtis: Gardiner, N.Y.) in an upward flow direction. Approximately 2 liters of 0.5 *M* Tris–HCl buffer (pH 7.4) containing 0.5 *M* NaCl was pumped through the column at 1.5 ml/min at 4°. Four milliliters of the following calibration standards dissolved in the Tris buffer was individually analyzed on the column connected to a recording LKB 8300 Uvicord II scanner (Sweden): blue dextran (0.2%), aldolase (10 mg/ml), hemoglobin (10 mg/ml), ovalbumin (10 mg/ml), chymotrypsinogen A (10 mg/ml), and cytochrome *c* (10 mg/ml). Finally 4 ml of (^{57}Co) cyanocobalamin-labeled serum and each of the three concanavalin A–Sepharose fractions were analyzed on the column.

Results. Three distinct fractions containing vitamin B_{12}-binding activity can readily be resolved from human serum using concanavalin A–Sepharose (Fig.

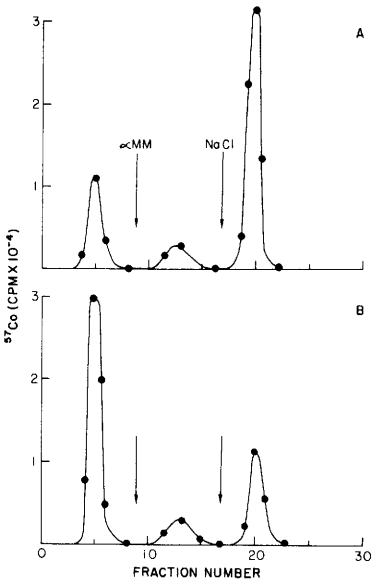

FIG. 1. Resolution of serum vitamin B_{12}-binding activity on a concanavalin A–Sepharose column. (A) Normal serum. (B) Chronic myelogenous leukemia serum. αMM, α-methyl-D-mannoside.

1A). The first fraction (A) is not retained by the resin, a second fraction (B) interacts with concanavalin A and is eluted by adding α-methyl-D-mannoside to the buffer, and a third fraction (C) is obtained by elution with 1 M NaCl. Fractions A and B behave as cobalophilins when chromatographed on a Sephacryl-S-200 column (Figs. 2B and C) while fraction C corresponds to the low-molecular-weight binder transcobalamin II (Fig. 2D). Approximately 75% of the bound exogenous vitamin B_{12} is associated with fraction C which is consistent with its identity as transcobalamin II, since it is present in normal serum primarily in an unsaturated state (4, 16, 17).

When a condition exists which results in an increase in serum cobalophilins, such as chronic myelogenous leukemia (15) or polycythemia vera (13, 18), fraction A reflects this situation by being significantly elevated (Fig 1B — Table I). Since fraction A is a vitamin B_{12}-binding glycoprotein with no apparent interaction with concanavalin A, it was of interest to examine the behavior of this fraction on other lectin–Sepharose resins. Both wheat germ and castor bean II lectins completely bound fraction A (Figs. 3A and B), with subsequent removal from the resins by addition of the appropriate competing sugar to the buffer.

On the other hand Castor Bean I–Seph-

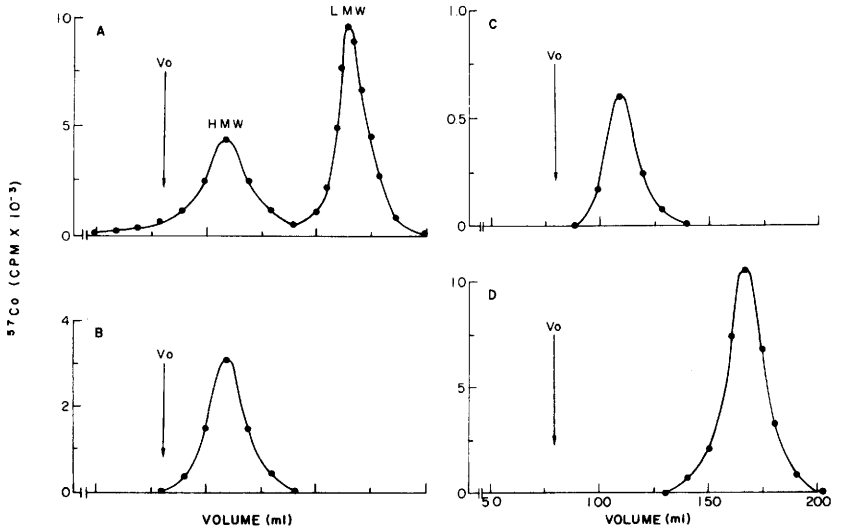

FIG. 2. Chromatography of concanavalin A–Sepharose vitamin B_{12}-binding fractions on a Sephacryl-S-200 column. (A) Unfractionated serum. (B) Con A–Sepharose fraction A. (C) Con A–Sepharose fraction B. (D) Con A–Sepharose fraction C. V_0 void volume, HMW, high molecular weight, LMW, low molecular weight.

TABLE I. DISTRIBUTION OF SERUM-BOUND ^{57}Co VITAMIN B_{12} RADIOACTIVITY ON CON A-SEPHAROSE

Normal value	Unbound B_{12}-binding capacity (pg/ml)[a]	Distribution of radioactivity on Con A–Sepharose		
		A(%)	B(%)	C(%)
1	1,920	13	8	79
2	2,080	16	8	76
3	1,680	19	11	70
4	1,460	15	11	74
5	1,610	18	11	71
6	1,450	17	9	74
	1,000–2,000	16.3 ± 1.4	9.7 ± 1.4	74.0 ± 3.0
P. Vera				
1 P	4,510	50	17	33
2 P	4,030	47	29	24
3 P	5,970	49	13	38
4 P	13,580	56	13	31
5 P	2,270	64	11	25
6 P	3,020	69	11	20
		55.8 ± 10.2	15.7 ± 4.6	28.5 ± 6.8
Chronic myelocytic leukemia				
1 C	4,540	56	24	20
2 C	10,860	75	13	12
3 C	4,500	64	14	22
4 C	4,530	47	16	37
5 C	8,415	56	18	26
		60.5 ± 10.2	16.8 ± 4.3	22.7 ± 9.0

[a] Calculated after dialysis of serum and (^{57}Co) vitamin B_{12}. Represents the amount of exogenous vitamin B_{12} which can be bound by serum.

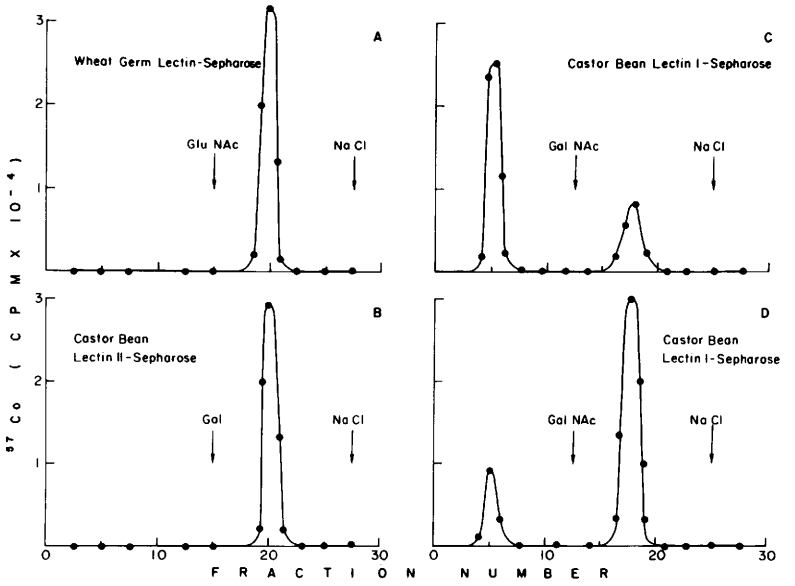

FIG. 3. Behavior of concanavalin A–Sepharose fraction A on other lectin–Sepharose resins. (A) Wheat germ lectin, (B) castor bean lectin II, (C) castor bean lectin I, chronic myelogenous leukemia serum. (D) Castor bean lectin I, polycythemia vera serum.

arose resolved concanavalin A–Sepharose fraction A into two additional fractions—one of which was not bound by this lectin and the other which was eluted when *N*-acetylagalactosamine was added to the buffer (Figs. 3C and D). Furthermore the distribution in the amount of radioactivity was different between the sera from patients with chronic myelogenous leukemia (Fig. 3C) and those with polycythemia vera (Fig. 3D). This result was consistent in five chronic myelogenous leukemia patients and six polycythemia vera patients studied (Table II).

Discussion. Heterogeneity of cobalophilins has frequently been attributed to variations in their carbohydrate moieties rather than the apoprotein (4–8). In determining their behavior on four different lectin–Sepharose affinity columns, it was possible to resolve these vitamin B_{12}-binding glycoproteins into three distinct fractions, thereby supporting the contention that variation in the carbohydrate structure could account for cobalophilin heterogeneity which has been observed by other methods (7, 8, 10). In addition, concanavalin A–Sepharose can easily separate the nonglycoprotein transcobalamin II from the cobalophilins. In fact transcobalamin II can readily be separated from the majority of serum proteins in one step, including albumin which does not bind to this resin (19). The nonspecific interaction of trancobalamin II with concanavalin A–Sepharose is apparently ionic in nature, since it can be removed with sodium chloride solution.

Chromatography of cobalophilins on various sugar-specific lectin–Sepharose resins may be used to identify different variants based on their interaction with a particular lectin. This would be an advantage over using DEAE-cellulose with stepwise elution techniques, since this method can result in artifacts (7, 20). Eventually the use of lectin–Sepharose resins for defining cobalophilin variants may prove desirable in resolving a somewhat confusing classification of these vitamin B_{12}-binding proteins (1, 4, 5, 8).

The major cobalophilin fraction which was not retained by concanavalin A–Sepharose was resolved into two fractions on Castor Bean I lectin–Sepharose. This suggests that the availability of *N*-acetylgalactosamine residues for binding to this lectin is different between these two fractions. Furthermore, a substantial difference in the distribution of the bound radioactivity between these two fractions was observed for chronic myelogenous leukemia (Fig. 3C) and polycythemia vera samples (Fig. 3D). This result is analogous to other findings in which chronic myelogenous leukemia pa-

TABLE II. COMPARISON OF CON A-SEPHAROSE FRACTION A FROM CHRONIC MYELOGENOUS LEUKEMIA AND POLYCYTHEMIA VERA PATIENTS ON CASTOR BEAN LECTIN I–SEPHAROSE

Polycythemia vera	Percentage radioactivity (^{57}Co) in unbound fraction	Percentage radioactivity (^{57}Co) in *N*-acetylgalactosamine eluted fraction
1 P	50	50
2 P	40	60
3 P	41	59
4 P	24	76
5 P	33	67
6 P	46	54
Total	39 ± 8.5	61 ± 8.5
Chronic myelogenous leukemia		
1 C	70	30
2 C	65	35
3 C	80	20
4 C	52	48
5 C	58	42
	65 ± 9.7	35 ± 9.7

tients have an elevated serum TC I cobalophilin while polycythemia vera patients exhibit an increase in TC III cobalophilin (13, 15, 21–24). It appears that increased serum levels of a cobalophilin variant (TCI) accounts for the elevation of serum vitamin B_{12} levels which is characteristic for chronic myelogenous leukemia patients (15, 25). Leukocytes from a chronic myelogenous leukemia patient also produce a cobalophilin variant that has a *N*-acetylgalactosamine content which is eight times higher than that observed for normal individuals (4, 26). This observation along with results using Castor Bean I lectin–Sepharose (Figs. 3C and D) may indicate that the processing of *N*-acetylgalactosamine residues is altered as a consequence of the disease mechanism resulting in an increase of this cobalophilin variant. This alteration may manifest itself in some glycosyltransferase reactions which have been shown to be affected by certain malignant processes (27).

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