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Detection of Genetic Variation with Radioactive Ligands. III. Genetic Polymorphism of Transcobalamin II in Human Plasma

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The cobalamins, including vitamin B_{12} (cyanocobalamin), are essential vitamins required by all mammals. They act as cofactors for enzymes involved in protein synthesis, carbohydrate and fat metabolism, and in conjunction with tetrahydrofolate, in nucleic acid synthesis [1]. In nature, the cobalamins are most often found in association with binding proteins and virtually never occur free or in an unbound state.

Proteins binding vitamin B_{12} are found in human gastric secretions, blood plasma, cerebral spinal fluid, tears, saliva, and amniotic fluid (reviewed in [2]). The best characterized of these, gastric intrinsic factor, is required for uptake of dietary B_{12}. In plasma, two cobalamin binding proteins are found, transcobalamin I (TC I), an α-globulin, and transcobalamin II (TC II), a β-globulin. Transcobalamin III (TC III), another β-globulin, is found in serum and some plasma preparations but appears to be an artifact of the anticoagulant used, presumably being a constituent of granulocytes released in vitro [3, 4].

TC I, TC III, the salivary binder, and B_{12}-binding proteins from other tissues—together known as the "R"-binders or cobalophilins—are antigenically and biochemically similar to each other (i.e., they are glycoproteins, approximately one-third carbohydrate) and distinct from TC II (not a glycoprotein; reviewed in [5]). Purified TC I and TC III have a molecular weight of 60–65,000 [6], while TC II is thought to have a molecular weight of 38,000 [5]. The transcobalamins bind 1 mol of vitamin B_{12} per mol of protein.

The ratio of TC I to TC II is roughly 1:1, based on binding capacity [4], although 90% of endogenous cobalamin is bound to TC I, possibly because the TC II:B_{12} complex is rapidly cleared from circulation [7]. As a measure of their concentration, the total binding capacity of the plasma transcobalamins is 2 ng cyanocobalamin/ml (1.5×10^{-9} M) [4, 8]. By comparison, the concentration of other plasma transport proteins (e.g., transferrin, transcortin, or thyroxine-binding globulin [9]) is on the order of 10^{-7} or 10^{-6} M; the transcobalamins are clearly trace constituents of plasma.

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TC II, like intrinsic factor, is essential for transport and utilization of the cobalamins. The congenital absence of TC II is associated with severe megaloblastic anemia in infancy [10–12], and TC II enhances the cellular uptake of vitamin B_{12} in vitro (e.g., see [13, 14]). In contrast, the congenital absence of the R-binders is without apparent clinical consequence [15], and no distinct physiologic properties have been demonstrated in vitro (a role in cobalamin excretion or as an antibacterial agent has been proposed [16]).

As part of a general study of transport proteins, we conducted an electrophoretic survey of vitamin B_{12}-binding proteins in human plasma. We report here the detection of genetically determined electrophoretic variants of one class of these proteins, TC II. These variants have mobility characteristic of a β-globulin, are autosomally inherited, and have been found at polymorphic levels in all populations tested. Portions of this work have been reported earlier [17]. The technique applied in this study, polyacrylamide gel electrophoresis (PAGE) autoradiography of plasma labeled in vitro with ^{57}Co-vitamin B_{12}, a procedure particularly well adapted to the study of trace binding proteins, has been described previously [18].

MATERIAL AND METHODS

Blood Samples

Blood samples were drawn in sodium citrate or Na_2-EDTA vacutainers, centrifuged, decanted within 1 hr, and stored at 3°C for testing within 48 hr or stored frozen until use. As discussed subsequently, heparin is unsuitable as an anticoagulant, and serum, though acceptable, makes interpretation of phenotypic patterns difficult.

Blood samples, from adult donors with informed consent or from children with parental consent, were from Caucasians, American Blacks, Chinese, and Japanese from the San Francisco Bay region. Additional plasma samples in acid-citrate-dextrose (ACD) were supplied by M. S. Schanfield (Irwin Memorial Blood Bank, San Francisco) and by H. M. Cann (Stanford University Medical Center) whose samples were from a large, extended Puerto Rican family and from Guatemalan Indians. Plasma samples from two sisters with TC II deficiency and their mother were contributed by C. R. Scott (University of Washington, Department of Medicine, Seattle). Sodium citrate plasma from a female rhesus monkey was obtained by venipuncture, and ACD plasma from a male chimpanzee was a gift of M.-C. King (University of California, San Francisco).

Vitamin B_{12} Labeling

Plasma or serum samples were labeled in vitro by the addition of ^{57}Co-cyanocobalamin (288 Ci/mmol, Amersham-Searle, Arlington Heights, Ill.) to a final concentration of 0.5–1.5 ng/ml (usually 0.5 ng/ml) and incubated at 37°C for ½ hour before electrophoresis or overnight at 3°C. Radiochemical purity of the stock ^{57}Co-cyanocobalamin was tested before and after a series of experiments by thin layer chromatography and autoradiography on cellulose acetate using a 0.05 M potassium phosphate (pH 6.5) acqueous solvent with unlabeled vitamin B_{12} as control. Radiochemical purity was greater than 95% in all cases.

Electrophoresis and Autoradiography

PAGE was conducted in 7.5% polyacrylamide slab gels (Bio-Rad apparatus, Richmond, Calif.) using a Tris-HCl-glycine buffer system, upper buffer pH 9.1, stacking gel pH 7.4, and separating gel pH 9.3 at 3°C [18]. Samples of 10 μl each were loaded and electrophoresed in parallel. After testing a series of modifications (see Results), it was determined that the best resolution of B_{12}-labeled electrophoretic variants was achieved by addition of 0.01% EDTA to

the upper buffer and the gel buffers. Following electrophoresis, the gel was dried on filter paper and exposed to X-ray film for 1–2 weeks. Microdensitometry of X-ray films was conducted using a Joyce Automatic Recording Microdensitometer.

Ammonium Sulfate Separation

A batch process was used to separate the various transcobalamin fractions. One-half ml of fresh serum was mixed with 0.4 M K_2HPO_4 plus ^{57}Co-cyanocobalamin (0.32 ng/ml) and incubated at ambient temperature for 30 min. Sufficient $(NH_4)_2SO_4$ was then added to make the solution 24% (w/v). The sample was shaken and incubated for an additional 30 min before centrifugation at 300 *g* for 15 min. The precipitate was resuspended in 1 ml of 0.2 M sodium phosphate buffer, pH 8.0, and dialyzed against this buffer overnight; the supernatant was concentrated to 1 ml by vacuum dialysis against this buffer. Both fractions were then subjected to PAGE autoradiography to determine which contained the polymorphic vitamin B_{12}-binder. Using this separation procedure, Carmel [19] found that nearly 100% of TC II is precipitated, while over 85% of TC I/III remains in the supernatant.

RESULTS

Preliminary Observations

PAGE autoradiography of samples labeled with ^{57}Co-cyanocobalamin consistently revealed genetically determined variation in electrophoretic patterns between individuals. Additional variation, dependent upon anticoagulant used and details of the electrophoresis system, was also noted. (1) For nearly all samples tested, distinct, reproducible bands were seen in the region midway between the origin and albumin. (2) The clarity of these bands was greatly enhanced by the addition of 0.01% EDTA to gel buffers. (3) In the case of serum samples, and occasional plasma samples, a further set of faint bands (not influenced by EDTA) were found to overlap the primary bands but had slower mobility. (4) Heparinized plasma samples produced only a dense, distorted band just below the origin (see fig. 1). The significance of these preliminary findings is considered in the Discussion; except where noted, work described subsequently is based on the use of sodium citrate or EDTA-plasma and an electrophoresis system containing EDTA.

Population and Family Studies

Plasma samples from Caucasians, American Blacks, and Orientals were screened to determine the phenotype of their B_{12}-labeled transcobalamin (referred to as TC II; see Discussion). Results are given in table 1. In all individuals tested, either two distinct, labeled bands or a set of four such bands were observed in the mid-gel region (fig. 2). (Three bands are seen when the two central bands overlap). To date, at least eight phenotypic patterns, diagrammed in figure 3, have been distinguished. Although the relative distance between any pair of bands in two-banded patterns is constant, their electrophoretic mobility can vary in stepwise fashion from "cathodal," just entering the mid-gel region, to "anodal," approaching albumin. Patterns with four bands appear to be the simple sum of two dissimilar two-banded types, and these patterns can be duplicated by mixing plasma one-to-one from individuals with appropriate two-band phenotypes. Samples retaken at intervals of up to 1 year from several individuals, representing most observed phenotypes, produced patterns identical to those originally observed.

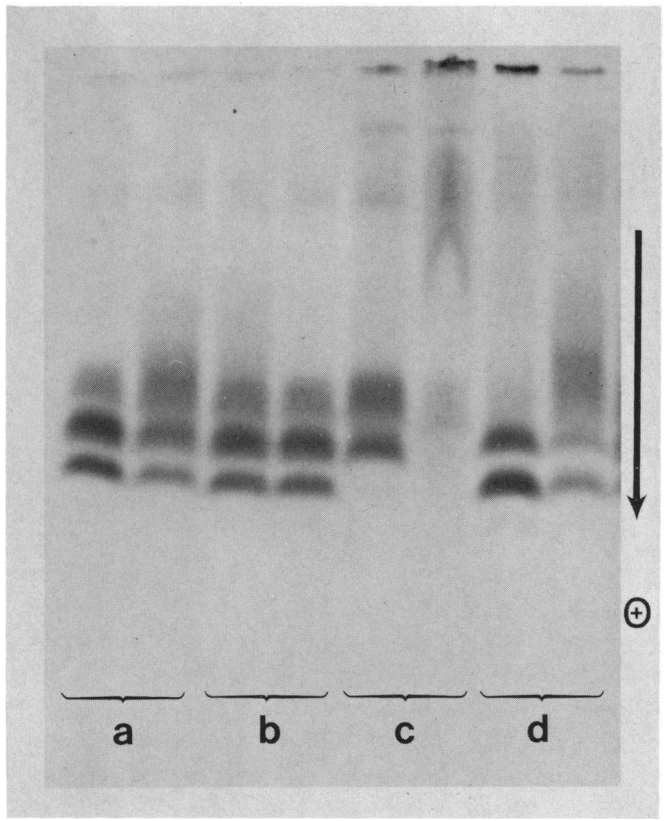

FIG 1.—Autoradiograph of human serum and plasma samples labeled with ^{57}Co-vitamin B_{12} (1 ng/ml final, 21 day exposure, 5% polyacrylamide gel). Each pair of samples are serum and plasma from the same individual drawn consecutively. Sample types and donor's TC II phenotype are (*left* to *right*) *a*, sodium citrate plasma, serum, 3-4; *b*, serum, sodium citrate plasma, 3-4; *c*, serum, heparinized plasma 4-4; and *d*, sodium citrate plasma, serum, 3-3.

The simplest model explaining these phenotypic patterns is a genetically determined, autosomally inherited polymorphism with at least four codominant alleles at one locus. Thus, individuals with a pair of binding proteins are homozygotes, and heterozygotes are those with four bands, combining two distinct homozygote types. Referring to this as the TC II locus, observed alleles are *TC II*^{1}, . . ., *TC II*^{4}, named from the most anodal pair of bands to the least.

Family data in table 2 confirm this model. Males with four-band phenotypes were common, and male-to-male transmission was not infrequent. Inheritance of the most common alleles was seen in one or more instances, and no discordant offspring phenotypes were found. Offspring ratios were consistent with simple Mendelian inheritance of an autosomal gene (table 3).

Based on this genetic model and the nomenclature noted above, observed and expected phenotype numbers and gene frequencies for the surveyed populations (unrelated adults) are given in table 1. Observed phenotype numbers do not differ

TABLE 1

TC II PHENOTYPE NUMBERS AND GENE FREQUENCIES

POPULATIONS	PHENOTYPES																GENOTYPES					
	1-4		2-2		2-3		2-4		3-3		3-4		4-4		Total							
	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	*TC II*^{1}	*TC II*^{2}	*TC II*^{3}	*TC II*^{4}	χ^2	df
American Blacks	. . .	. . .	7	5.70	33	38.7	14	10.85	71	65.72	32	36.85	6	5.16	163	163	. . .	.187	.635	.178	3.25	3
Caucasians	1	0.94	. . .	0.059	1	2.1	3	1.77	36	37.08	66	62.54	24	26.38	131	131	.004	.015	.531	.450	0.44*	2
Guatemalan Indians	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	131	129.57	77	80.06	14	12.37	222	222	. . .	. . .	.764	.236	0.36	1
Orientals	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	34	37.25	77	70.45	30	33.30	141	141	. . .	. . .	.514	.486	1.22	1

* First four classes pooled for computation of χ^2 (including all genotypes with *TC II*^{1} allele, expected sum = 1.51).

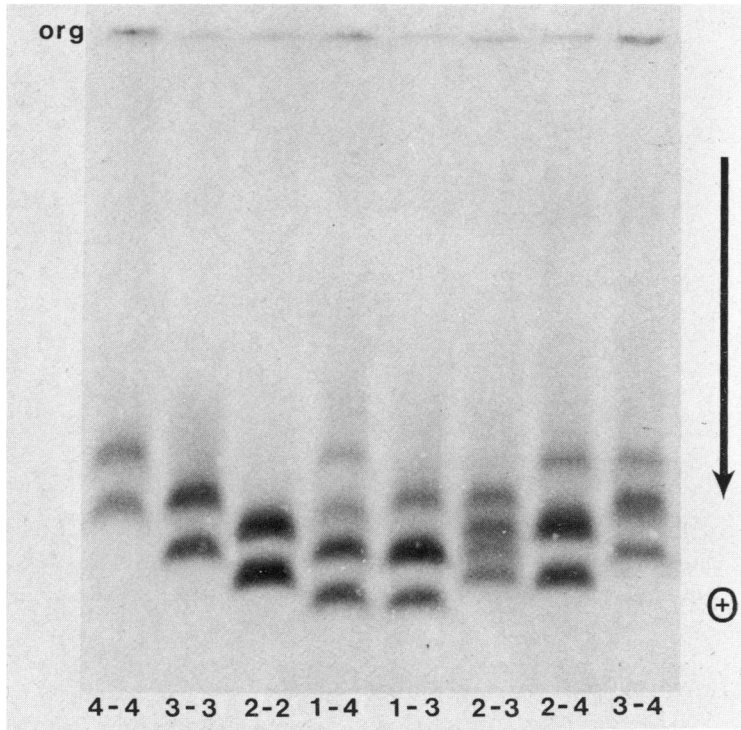

FIG. 2. — Autoradiograph of sodium citrate plasma samples from Caucasian donors of representative TC II phenotypes labeled with ^{57}Co-vitamin B_{12} (1 ng/ml final, 21 day exposure, 5% gel). Org = origin of lower gel.

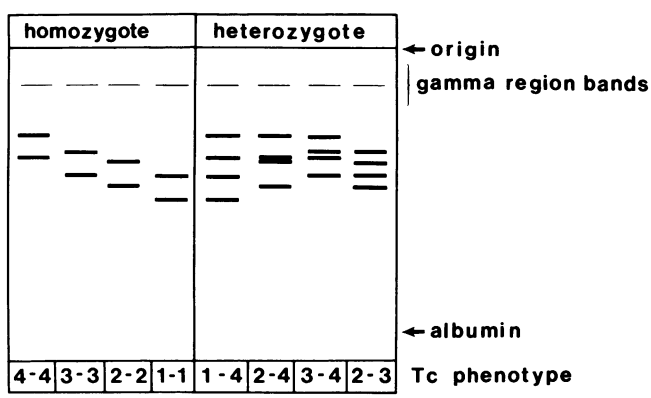

FIG. 3. — Diagram of TC II phenotypes observed to date (*TC II*^{1} seen in heterozygote state only).

TABLE 2

INHERITANCE OF TC II ALLELES. SUMMARY OF DATA FROM AMERICAN BLACKS AND CAUCASIANS, PUERTO RICANS, AND GUATEMALAN INDIANS

TC II Types			Progeny Types				
Male	Female	No. Families	4-4	3-4	3-3	2-4	Total Progeny
4-4	3-4	1	6	1	· · ·	· · ·	7
4-4	3-3	2	· · ·	5	· · ·	· · ·	5
4-4	2-3	1	· · ·	1	· · ·	3	4
3-4	4-4	2	5	3	· · ·	· · ·	8
3-4	3-4	9	5	15	7	· · ·	27
3-4	3-3	8	· · ·	16	10	· · ·	26
3-4	1-4	1	1	1	· · ·	· · ·	2
3-3	4-4	1	· · ·	8	· · ·	· · ·	8
3-3	3-4	12	· · ·	25	19	· · ·	44
3-3	3-3	16	· · ·	· · ·	68	· · ·	68
Sum		53	17	75	104		192

significantly from those expected under an assumption of Hardy-Weinberg equilibrium. Two alleles, *TC II*^{3} and *TC II*^{4}, occurred in high frequency in all populations tested, achieving frequencies of greater than 40% in Caucasians and Orientals. *TC II*^{2}, though uncommon in Caucasians and absent from these Orientals, was moderately frequent in Blacks. The rare allele, *TC II*^{1} was detected (as TC II 1-4) in only one independently ascertained individual, a Caucasian, but was also present in her sister (TC II 1-3) and has been repeatedly observed in a large pedigree from Salt Lake City, Utah (Cavalli-Sforza et al., to be published). We also have preliminary evidence for a fifth allele in American Blacks, whose product has mobility intermediate to TC II-2 and TC II-3. In all, this is a highly polymorphic, widely distributed genetic marker with considerable ethnic variation.

TABLE 3

TEST OF HETEROGENEITY OF RECIPROCAL CROSSES [30] FROM FAMILY DATA IN TABLE 2

TC II Types		Segregations		
		χ^2	df	$P(>)$
4-4	3-4	3.57	1	.05
3-4	4-4	0.5	1	.3
Sum		3.27	1	.05
3-3	3-4	0.82	1	.3
3-4	3-3	1.38	1	.2
Sum		2.06	1	.1
3-4	3-4	0.63	2	.7

NOTE. — Sum 3-4 × 4-4, $\chi^2 = 0.08$; df = 1; $P > .3$. Sum 3-4 × 3-3, $\chi^2 = 0.14$; df = 1; $P > .7$.

Relation to Other Plasma Proteins

Because the polymorphic vitamin B_{12}-binding protein is in exceptionally low concentration (less than 2×10^{-9}M [17]) and because its electrophoretic patterns are unlike those of other β-globulins, a reasonable supposition is that the polymorphism is not one previously detected by other methods. This assumption was confirmed by a series of immunologic, electrophoretic, and genetic tests comparing this protein with other known polymorphic plasma proteins, concluding that the transcobalamin variants are distinct from β_2-glycoproteins II and III, glycine-rich β-glycoprotein, haptoglobin, hemoglobin, and transferrin [17]. We also note that in this particular PAGE system, vitamin B_{12} does not bind to albumin, to prealbumin, or to lipoproteins, and that free B_{12} (in sucrose) does not enter the lower gel. Further, a mid-gel protein binding free cobalt, detected using PAGE autoradiography with $^{57}CoCl_2$, did not overlap any of the transcobalamin bands and appears to be monomorphic in these populations.

Molecular Aspects

There is indirect evidence (see Discussion) that the polymorphic vitamin B_{12}-binding protein is transcobalamin II. Two additional findings conclusively confirmed this suspicion. First, in several repeat trials, the polymorphic protein was found exclusively in the precipitate fraction following ammonium sulfate precipitation as expected of TC II. Second, and more significantly, the B_{12}-binder was completely absent from plasma from the children with hereditary absence of TC II established earlier by unrelated methods. The children's mother has a TC II-4 phenotype with reduced binding capacity and presumably is a heterozygote for a silent allele.

An explanation for the difference in electrophoretic mobility of the TC II variants should distinguish between charge and size (or conformation) differences. Electrophoretic procedures themselves can provide useful information on this question. The electrophoretic patterns of the B_{12}-labeled proteins (e.g., fig. 3) are relatively independent of total acrylamide concentration (%T), suggesting the protein bands in any particular pair and the pairs themselves differ only in charge, not size [20]. To test this hypothesis, labeled plasma samples representing most observed phenotypes were electrophoresed in slab gels of 5%, 7.5%, and 10% T, respectively, under otherwise identical conditions. The mean mobility of each band (R_f) relative to the fast TC II-3 band is shown in table 4. The table confirms that changes in acrylamide concentration do not profoundly alter TC II band patterns, therefore the proteins comprising these bands must be roughly equivalent in size. A more refined analysis of the original data [17], based on the expected linearity of the plot of log (R_f) vs. %T [20], further supports this conclusion.

An additional feature of the TC II allele products observed following PAGE autoradiography—apparent differences in binding affinity for vitamin B_{12}—may be of physiologic significance. Microdensitometry of X-ray autoradiographs revealed that the two TC II-4 bands, when seen in heterozygote combination with other TC II alleles, were consistently less dense than their companion bands if less than saturating amounts of label were applied (fig. 4; see also fig. 2). On the other hand, bands produced by *TC II*^{4} homozygotes were generally as dense as those produced by other

TABLE 4

ELECTROPHORETIC MOBILITY OF TC II BAND PAIRS RELATIVE TO TC II-3-FAST (each ± .01 SEM)

BAND	POLYACRYLAMIDE CONCENTRATION		
	5%	7.5%	10%
4:			
Slow	.83	.81	.82
Fast	.94	.92	.93
3:			
Slow	.90	.90	.90
Fast	1.00	1.00	1.00
2:			
Slow	.96	.96	.97
Fast	1.06	1.07	1.08
1:			
Slow	. . .	1.00	1.02
Fast	. . .	1.09	1.10

homozygotes. On this basis, we tentatively conclude that TC II-4 proteins bind less ligand when in competition with other TC II proteins and thus may have lower affinity for cyanocobalamin.

Animals Other than Man

PAGE autoradiography of plasma from rhesus monkey and chimpanzee revealed B_{12}-labeled band patterns, each with two bands, reminiscent of human patterns. The chimpanzee pattern, in particular, was nearly identical to the human TC II-3 pattern.

DISCUSSION

PAGE (polyacrylamide gel electrophoresis) autoradiography of samples labeled in vitro with physiologic amounts of ^{57}Co-vitamin B_{12} revealed considerable phenotypic variation in the major plasma transcobalamin with mobility intermediate to albumin and the origin. Genetically determined, polymorphic electrophoretic variants of this protein are found in Caucasians, American Blacks, and Orientals; this is a marker with considerable diversity and wide distribution.

The difficulties originally encountered in optimizing our electrophoresis system were the following. (1) Serum samples produced autoradiographic patterns with considerable "contamination" in a region overlapping the polymorphic band patterns. The contaminating binders displayed independent, individual variation in electrophoretic mobility and were absent from or greatly diminished in plasma. (2) Heparinized plasma, or EDTA plasma to which heparin was added, produced significant distortion in the patterns of the polymorphic protein due to heparin binding. (3) EDTA in the electrophoresis buffers materially enhanced the separation and clarity of the polymorphic patterns.

It is most probable that the "contaminant" observed in serum samples is TC III

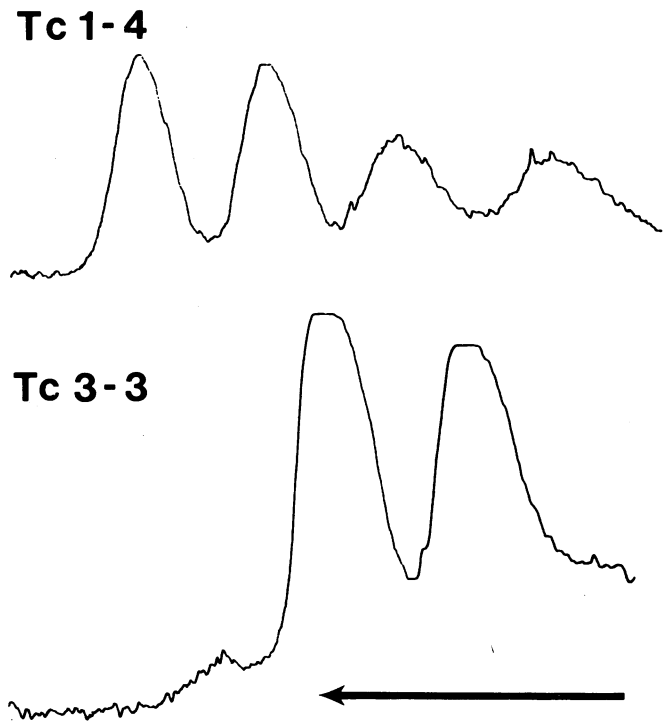

FIG. 4.—Microdensitometry tracing of an X-ray autoradiograph of two sodium citrate plasma samples labeled with ^{57}Co-vitamin B_{12} (1 ng/ml final, 14 day exposure, 5% gel).

since it has the same electrophoretic mobility, and variation in the amount present in serum compared to plasma parallels the amount of TC III reportedly released from granulocytes [4]. Further, the binding of heparin is a commonly accepted property of TC II (e.g., see [21]), but at least one report [22] suggests that TC III may bind heparin as well.

Finally, a frequently cited difficulty in dealing with the transcobalamins, primarily TC II, is their tendency to aggregate. TC II complexes at low salt concentrations [23], when partially purified [24] and possibly in vivo [25]. EDTA may inhibit complex formation as may sodium citrate [21, 25]. Thus use of EDTA-containing buffers appears to prevent aggregation prior to or during electrophoresis.

Polymorphic Transcobalamin

The preceding argues that TC II is the polymorphic protein, a conclusion corroborated by our failure to detect the protein in plasma from a child with congenital absence of TC II and by the removal of the polymorphic protein from normal serum by ammonium sulfate precipitation. Therefore, we feel justified in referring to this as the TC II locus with alleles *TC II*^{1}, . . . , *TC II*^{4}. Our failure to detect B_{12}-labeled proteins with mobility characteristic of TC I probably stems from the fact that this protein is

nearly saturated with endogenous B_{12} and in vitro exchange with unbound vitamin is extremely slow [26].

Electrophoretic Patterns

The consistent appearance of two equally dense and equally spaced B_{12}-labeled bands produced by plasma from TC II homozygotes, of all allelic types, and the simple additive nature of heterozygote patterns provides information about the molecular composition of these proteins. First, the equal density of band pairs under varying conditions of B_{12} saturation and widely varying conditions of storage, implies that any particular set of bands is composed of proteins of roughly equal concentration and B_{12} affinity. This constancy is not usually observed in proteins that differ as the result of some simple post translational event such as deamidation or a change in carbohydrate content [27, 28]. Band pairs are always equally spaced, even though varying in overall mobility from individual to individual, a feature that is invariant or "monomorphic" across TC II types. Finally, evidence obtained by comparing relative mobilities, in gels of varying acrylamide concentration (table 3), and the apparent absence of hybrid molecular forms in heterozygotes, suggests that all observed bands do not differ substantially in molecular weight or conformation.

Although post-translational events cannot be excluded as an explanation for the observed patterns, we feel a genetic explanation is more probable. One simple model which accounts for these patterns postulates that each electrophoretic band is composed of two protein subunits, one coded for by the polymorphic locus and the other determined by two distinct, monomorphic loci, each expressed in every individual. Homozygote TC II patterns, at least in the presence of EDTA, would thus be of the form $\alpha\beta$ plus $\alpha\gamma$, with α the polymorphic subunit and β, γ the monomorphic subunits. Since affinity differences exist between TC II variants (fig. 4), it is likely that the α subunit binds the ligand.

Selective Significance

Transcobalamin II is essential for transfer of cobalamin from the intestine to plasma, for plasma transport, and for uptake of the TC II:B_{12} complex into recipient cells [11, 13, 14]. Thus genetic variation may have significant physiologic consequences, as illustrated in the extreme by the morbidity associated with its congenital absence. The product of the *TC II*[4] allele, which occurs in high frequency, appears to have reduced affinity for ^{57}Co-cyanocobalamin. Though pharmacologically active, cyanocobalamin is not one of the commonly occurring forms of cobalamin. Methyl-, adenosyl-, and hydroxo-cobalamin are the endogenous forms found in human plasma [29]; therefore, conclusions about affinity differences in the transcobalamins must eventually extend to these substances. We speculate that differences in affinity or capacity of the TC II allele products, as exemplified by TC II-4, or differences in their interaction with cellular receptor sites, may have selective significance and may be involved in maintenance of the TC II polymorphism.

SUMMARY

We detected genetically determined, electrophoretic variants of vitamin B_{12}-binding

proteins, most probably transcobalamin II, in human plasma. Polymorphic variants were observed in all populations tested; the two most common alleles (of at least four detected to date) attain frequencies of greater than 40% in Caucasians and Orientals. The variants are autosomally inherited and are seen as doublets in homozygotes, and four-banded patterns, the sum of two dissimilar homozygote patterns, in heterozygotes. The technique used in this survey, polyacrylamide gel electrophoresis (PAGE) autoradiography of plasma and serum labeled in vitro with ^{57}Co-vitamin B_{12} is particularly applicable to the study of trace proteins such as the transcobalamins (10^{-9}M). Possible functional variation in the TC II allele products is described, and the selective significance of this worldwide polymorphism is considered.

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