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Congenital Deficiency of Human R-Type Binding Proteins of Cobalamin

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INTRODUCTION

Carmel and Herbert in 1969 reported the near absence of a class of binding proteins of cobalamins (Cbl), R-type binders or cobalophilin, in two brothers [1]. R-type binders [2–6] are found in human serum, granulocytes, bile, amniotic fluid, gastric juice, saliva, tears, milk, and possibly other fluids or tissues. They are all glycoproteins but their carbohydrate composition varies, giving them different electrophoretic mobilities. Antibodies against an R binder from one source react with the R binder from any other source. Every R binder studied to date has expressed isoelectric heterogeneity. Conflicting reports (see review articles [2–6]) about the origins, significance, function, classification, and nomenclature of the R binders have been published. For the present study, we follow Grasbeck's classification system [3]. The plasma transport protein transcobalamin II, which is quite distinct from the R binders, is referred to as TC II. All other Cbl binding proteins belonging to the R class are identified by source (e.g., salivary R binder).

The original observations by Carmel and Herbert [1] contributed to the formulation of concepts of cobalamin transport [7]. We have restudied this defect to characterize it more completely by new techniques and if possible, to determine the mode of genetic transmission. The family is still the only one known of its kind, and only one affected person is living.

MATERIALS AND METHODS

Sampling

Serum was collected by the "quick-spin" method which removes the cells before clotting and avoids release of R binders from cells to serum [8]. The flow of saliva was stimulated by chewing paraffin for 10 min, and the saliva was collected on ice, filtered through gauze, and centrifuged immediately. Gastric juice was collected after stimulation with Histalog (betazola hydrochloride, Lilly, Indianapolis, Ind.). The pH was immediately raised to 11 with KOH and after 20 min, brought to 7 with H_2SO_4. Samples were filtered through gauze and divided into aliquots. Skin fibroblasts were obtained by culture of a punch biopsy for studies of Cbl uptake. Granulocytes were isolated from peripheral blood as described [9]. All samples, including tissues, were stored at −20°C until assay.

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Assay of Cobalamin

Total serum Cbl [10] and tissue Cbl [11] were measured by bioassay with *E. gracilis*. Serum was separated into fractions containing TC II-Cbl or R-Cbl by ammonium sulfate, $(NH_4)_2SO_4$ [12], and the bound Cbl was measured by bioassay [10, 13, 14].

Radioimmunoassay for TC II and R

The total amounts of TC II and R in serum and of R in saliva were measured by two previously described radioimmunoassays [15, 16], which used antibodies against pure TC II and R binder, respectively. By this method, concentrations of R binder as low as 22 ng/l could be detected.

Unsaturated B-12 (Cbl) Binding Capacities (UBBC), Total and Components

The UBBC of serum, saliva, lysed granulocytes, and the output of cultured granulocytes were measured by variants of a basic method [12]. ^{57}Co CN Cbl was added in slight excess of the amount needed to saturate all binding sites for Cbl, and free Cbl was removed by charcoal coated with protein. The radioactivity remaining represented the UBBC. In a few instances, the UBBC of individual binders were determined by labeling the binding proteins which were then separated by gel filtration with Sephadex G-200 [12]. All measures of gastric UBBC and the amount of binding to gastric R and intrinsic factor were first by saturation of binders with ^{57}Co CN Cbl and then fractionation on Sephadex G-100. The purity of the fractions was checked by reacting the contents of the two components with anti-R [17].

To measure the UBBC of serum components, Cbl binding sites were labeled, and the components containing TC II-Cbl and R-Cbl were separated by precipitation of the TC II-Cbl with 1.96 M $(NH_4)_2SO_4$ [12]. The R-Cbl was then separated into α_1-R and α_2-R by a batch anion exchange system [12]. A modification of the scheme was used to isolate the endogenous Cbl carried by TC II or R [13].

Studies of Cells in Culture

Uptake of TC II-bound ^{57}Co CN Cbl and the subsequent binding of the accumulated labeled cobalamin to intracellular proteins was studied in confluent monolayers from the proband's cultured skin fibroblasts according to techniques described previously [18]. Cells were exposed to labeled cobalamin for 72 hr. Cell free extracts were then chromatographed on Sephadex G-150 to search for the presence of intracellular binders [18].

Previously described techniques were applied for the studies of the R binders within and released by granulocytes [9].

RESULTS

Clinical Information

The propositus is the brother of the patient described by Carmel and Herbert [1] (fig. 1). A neurologic disease, classified as multiple sclerosis by two evaluating institutions, had been slowly evolving for 29 of his 52 years. Designation simply as a spastic paraparesis and dementia of unknown cause is adequate for present purposes. Since potential tissue deficiency of Cbl is an issue here, certain laboratory measurements are pertinent. The cerebrospinal fluid, urinary methylmalonic acid, CBC, and Schilling Test were all normal. The serum Cbl was below 100 ng/l in three samples tested, mean 53 ng/l. A biopsy of the sural nerve showed a demyelinating process without nerve fiber loss or fibrosis. The other members of the family studied here were healthy.

Characterization of the Defect

There was no R-type binding in the serum, saliva, cerebrospinal fluid (CSF), gastric juice, granulocytes, and the 24 or 48 hr outputs of cultured granulocytes of the

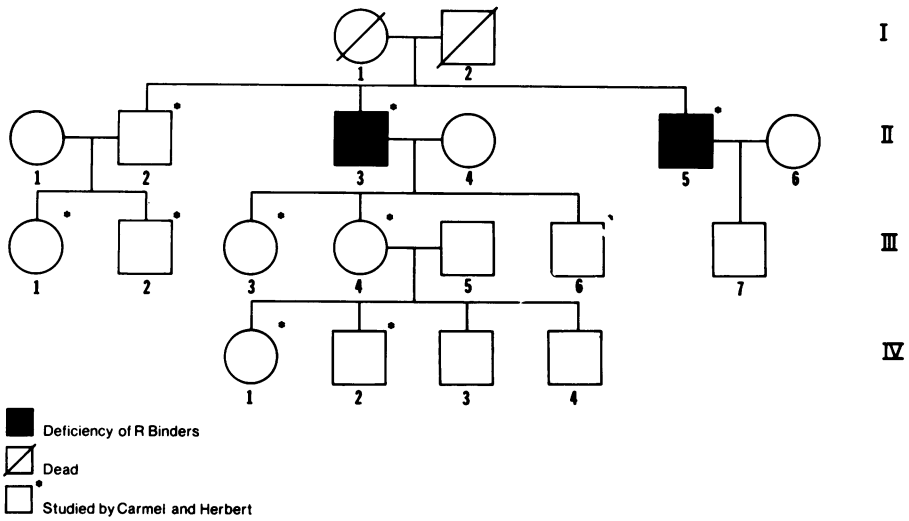

FIG. 1.—The family with congenital deficiency of R binders. Subjects II-5 (the propositus), III-3, -4, -7, IV-1, and -4 were included in the present study. Those marked with asterisk were included in the study of Carmel and Herbert [1].

propositus (table 1). The UBBC for serum given in table 1 was measured by the $(NH_4)_2SO_4$ method [12], but there was no binding for R as measured by gel filtration. The trace of apparent R binding in CSF was presumed to represent contamination since 98% of the Cbl was bound to TC II in CSF from both the propositus and control. The apparent R binder in gastric juice at the 15′ sampling and the 1%–2% binding at three other times, which are not shown, failed to react with anti-R. The low serum Cbl was due to the absence of native Cbl normally carried by R (table 1). The Cbl present was carried by TC II.

No R binder could be detected in saliva by radioimmunoassay at varied concentrations. However, a substance reacting immunologically like an R binder was found in the serum at one-fourth the expected concentration (table 1). Total R by radioimmunoassay was analyzed in four sera taken at different times after 1.0 mg of CN Cbl im. The level of cross reacting material was twice baseline in all. The possibility of an inhibitor of the assay was considered, but neither the serum nor saliva of the propositus inhibited the assay of R in normal serum or saliva. Total serum TC II was normal as were the UBBC for serum TC II and the amount of endogenous Cbl carried by TC II (table 1).

To summarize (table 1), there was no unsaturated binding capacity by R binders for several fluids or cells normally having that capacity. No native Cbl could be detected on the serum R binder, which is known to carry Cbl during natural metabolism. However, there was a molecule responding like an R binder to radioimmunoassay in serum but not in saliva.

Tissue Studies

The Cbl content of a biopsy of skeletal muscle was 15 pg/mg wet weight (normal mean 26.9, range 17–51 [11]). The biopsy was taken from a muscle group affected by

TABLE 1

CHARACTERIZATION OF THE DEFECT IN THE PROPOSITUS

	SERUM Clb (ng/l)*			LEVELS BY RADIOIMMUNOASSAY†			MISC. UBBC FOR R ONLY‡				SERUM UBBC (ng/l)§			
	Total	On R	On TC II	TC II (ng Cbl/l)	Serum R (ng Cbl/l)	Saliva R (μg Cbl/l)	Saliva (μg/l)	CSF (ng/l)	Gastric (μg/l)	WBC (ng/10^6)	Total	TC II	α_2-R	α_1-R
Propositus	90	0	72	840	155	0	0	11	3.8	0	1,310	1,240	0	0
Normal mean ± 1 SD	374 ± 111	212 ± 79	95 ± 50	917 ± 91	581 ± 61	23.4 ± 5.3	24.4 ± 7.4	10	28.7	0.57	1,250 ± 244	1,160 ± 217	45 ± 19	29 ± 15
Normal range .	227–527	91–368	40–183	586–1,410	520–642	14.8–31.2	15.8–35.4	...	...	0.15–1.05	908–1,740	940–1,370	26–64	14–44

NOTE. — Values for Cbl are expressed as weight of the vitamin per unit volume. UBBCs and the amount of a binding protein are expressed in the same way, as is conventional, but here the amount of the protein is defined by the amount of the vitamin it will carry.

* Serum Cbl by bioassay [10] and Cbl bound to proteins by removal of TC II-Cbl with 1.96 M $(NH_4)_2SO_4$[12] then bioassay of fractions [10, 13, 14].

† Total binder levels by radioimmunoassay of TC II [5] and of R [16].

‡ UBBCs of saliva and WBC which contain single Cbl binding proteins measured by adding ^{57}Co CN Cbl to excess and removing free Cbl with coated charcoal [12]. The UBBC for CSF and GJ were by gel filtration. There was only a single control value for the UBBC of CSF and gastric juice and five for lysed WBC. The apparent R detected in CSF and gastric juice appeared to be a contaminant (see text).

§ Fractional serum UBBC measured after labeling by separation of TC II-^{57}Co Cbl by 1.96 M $(NH_4)_2SO_4$ and division of remaining R-^{57} Co Cbl by anion exchange [12].

the neurologic disorder. A biopsy of the sural nerve contained 18 pg/mg. We have not established normal values for the Cbl content of peripheral nerves, but cortical fibers contain 16–50 pg/mg, mean 29.6 [11].

Cobalamin Uptake by Cultured Fibroblasts

After 72 hr of exposure to TC II-bound ^{57}Co CN Cbl, more than 90% of the ^{57}Co accumulated by the proband's intact fibroblasts was bound to intracellular proteins. Total accumulation and intracellular protein binding were identical to that observed in several control lines.

Studies of Family Members

Assuming that the propositus and one brother, II-3 and II-5, were homozygous for an abnormality which was transmitted as an autosomal recessive, family members III-3, -4, -7 (as well as III-6, not studied) would be obligate heterozygotes. Total saliva R and the UBBC of saliva, when expressed as ng/mg protein, were 1–2 SD below the normal mean in all three persons (table 2 and fig. 2). When expressed as μg/l of fluid, the UBBC and total saliva R of two-thirds of the "obligate heterozygotes" were more than 2 SD below the normal mean and the third 1–2 SD below (table 2). The values of IV-2, who could be a carrier of an abnormal gene, were low normal. The total serum R of two presumed heterozygotes were more than 2 SD below the normal mean but that of a third was normal. The UBBC for serum R, which is a measure of R only, and total serum Cbl and UBBC, which measures both TC II and R were normal in all (table 2).

Isolated granulocytes from III-7 were cultured for 24 and 48 hr, and the media was examined for content of Cbl binding proteins. There were no binding substances in the media initially. The UBBC for the lysed cells prior to culture was 0.14 ng/10^6 cells (normal mean 0.57 ng/10^6 cells). The output of binder by cultured cells was 0.02 ng/10^6 at 24 hr with no increase over the next 24 hr. Cells from five normal controls released a mean of 0.14 ng/10^6 (0.02–0.32) in 24 hr.

The 24 hr output of cultured granulocytes of subject III-7 was labeled and subjected to isoelectric focusing [8]. The components present were the same as in the output of normal granulocytes. An abnormal molecule might have been missed since it would have been detected by this technique only if it took the label.

DISCUSSION

The most obvious defect of the propositus was the absence of any capacity on the part of several body fluids and tissues to bind Cbl added in vitro to binders of the R-type. Since no *native* Cbl was found on serum R, there was also a failure to couple Cbl with an R binder of serum, TC I, in the course of in vivo Cbl metabolism. There was evidence of a substance present in subnormal amounts in serum but not in saliva that reacted either partially with antibody against R binder or reacted completely. TC II and intrinsic factor were present in normal amounts in serum and gastric juice, and both transport proteins functioned normally. The disorder seems to represent a defect in a *class* of proteins not in a single enzyme or other protein even though the proteins of the class differ in location, structure, and probably function. There was no general deficiency of plasma glycoproteins, since the amounts of thyroid binding globulin, thyroxine, ceruloplasmin, and transferrin were all normal.

TABLE 2

LEVEL OF Cbl AND BINDING PROTEINS IN ONE FAMILY

SUBJECT	SEX	AGE	TOTAL SERUM Cbl* (ng/l)	LEVELS BY RADIOIMMUNOASSAY: TC II (ng Cbl/l)	Serum R (ng Cbl/l)	Saliva R (μg Cbl/l)	Saliva R (ng/mg†)	UBBC OF SALIVA: μg/l	ng/mg†	SERUM UBBC-ng/l: TOTAL	TC II	α_2-R	α_1-R
Mean of 10 normals (±1 SD)	...	...	467 ± 157	917 ± 91	581 ± 61	23.4 ± 5.3	20.2 ± 6.2	24.4 ± 7.4	19.6 ± 8.5	1,250 ± 244	1,160 ± 217	45 ± 19	29 ± 15
II-5	M	52	90	840	155	0	0	0	0	1,310	1,240	0	0
III-3	F	16	520	1,200	310	14.0	9.3	12.7	8.4	1,150	942	59	9
III-4	F	30	468	930	325	10.4	10.1	9.5	9.2	1,010	855	36	12
III-7	M	27	315	780	550	7.6	11.2	6.2	9.1	914	712	49	16
IV-1	F	11	810	1,190	750	20.8	19.8	26.6	25.3	1,380	1,080	58	31
IV-2	M	9	858	920	760	14.8	14.4	15.8	15.4	1,170	913	42	16
IV-3	M	7	670	1,480	675	24.8	18.5	29.4	22.0	1,230	986	56	18
IV-4	M	5	770	1,400	745	21.6	19.6	24.1	22.0	1,070	830	44	16

NOTE. — Methods and normal ranges were as given in table 1 except for total serum Cbl.

* The 10 control sera of table 1 were assayed simultaneously with the corresponding protein fractions. For Table 2, 10 more were assayed in another batch to give a total of 20.

† mg of salivary protein.

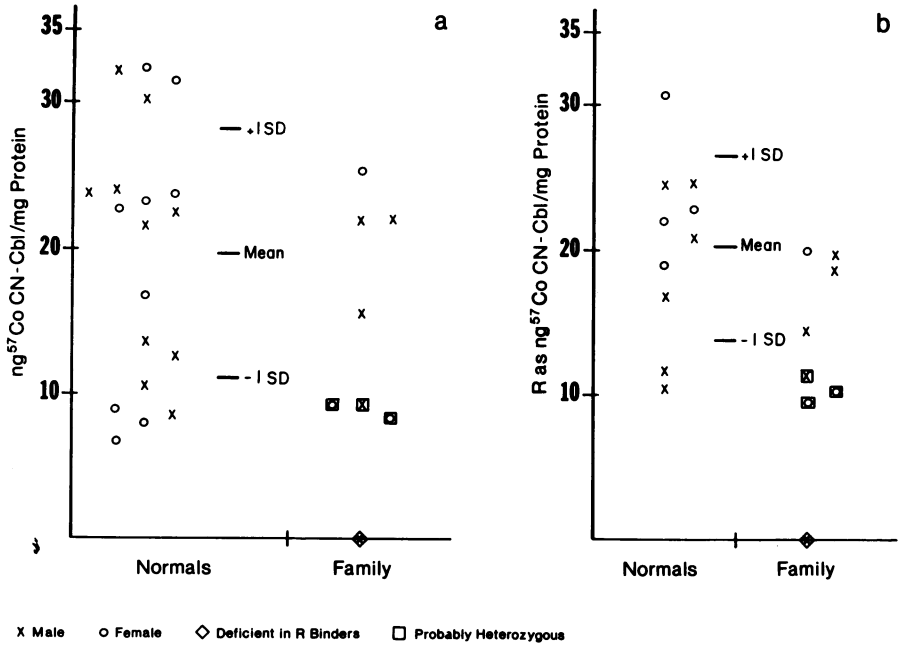

FIG. 2.—*a*, The UBBC for saliva; *b*, the total R of saliva for the members of the family. The 19 normal salivas in *a* were measured for UBBC simultaneously with those from the family. Ten of the same normal salivas which had been stored at −20°C and selected at random from the group of 19 were assayed for R content at a later date along with the R assays of family members. There was a close correlation between the UBBC and total R for any one sample of saliva. (Values are expressed as per mg protein not per liter as in table 1.)

The defect appears to be clinically benign. Although the propositus did have a poorly defined disease of the nervous system, and this system is affected by human Cbl deficiency, it is probably coincidental. There was much evidence of adequate Cbl content of several tissues, and a brother with the same defect of R binders had no neurologic disease [1]. This is in contrast to the lethal effects of the absence of transcobalamin II (TC II) unless the absence is recognized and treated [19, 20].

The present observations in serum differ slightly from those of Carmel and Herbert [1], but these differences can readily be accounted for by new and improved techniques since 1969. Thus, the earlier impression of some remaining R binding capacity for Cbl could not be supported here, and TC II is not decreased as first thought.

Some abnormality of R protein was observed in all three children of the affected persons, and in all instances, the abnormalities were multiple but in different patterns. Difficulty in measuring partial defects and variability of values among normals, however, made it impossible to state with certainty that any one person was heterozygous or that this represents a heterozygous defect. The data are consistent with autosomal inheritance, each gene being responsible for some R-protein synthesis.

SUMMARY

A family expressing the congenital absence of the R-type binders of cobalamin (Cbl), vitamin B-12, was restudied. The capacity to bind Cbl to R type binders was

absent from serum, saliva, cerebrospinal fluid, gastric juice granulocytes, and granulocyte output of the propositus. Serum R did not carry Cbl in vivo. There was no immunological R binder in his saliva, but cross reacting material was detected in his serum. Evidence of a partial expression of the defect was observed in offspring of two affected persons. There were no obvious clinical consequences of the defect.

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