Isolation and Characterization of an Abnormal Human Intrinsic Factor

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ABSTRACT A patient has been described previously who presented at age 13 with vitamin B_{12} (B_{12}) deficiency secondary to a functionally abnormal intrinsic factor (IF). IF has now been isolated from the gastric juice of the patient, his sister, and both parents, who are first cousins, by using affinity chromatography on B_{12}-Sepharose. Patient IF appeared normal in terms of (*a*) B_{12} binding, (*b*) mol wt, (*c*) total amino acid and carbohydrate composition, and (*d*) immunodiffusion with rabbit anti-patient and anti-normal IF sera. After adsorption with normal IF, however, anti-patient IF serum precipitated the various IFs as follows: patient IF (> 95%); mother, father, and sister IF (50%); and normal IF (< 10%). Additional adsorption with mother, father, or sister IF completely inhibited the precipitation of patient IF. The association constant determined for patient IF-B_{12} and human ileal mucosal homogenates (0.1×10^9 M^{-1}) was 60-fold lower than that determined with normal IF-B_{12} (6.0×10^9 M^{-1}). Intermediate amounts of ileal IF-B_{12} binding were observed with mother, father, and sister IF-B_{12}. These in vitro studies were supported by multiple Schilling tests, performed with a totally gastrectomized volunteer, that gave the following mean urinary excretions of [^{57}Co]B_{12}: free B_{12} (0.5%); + patient gastric juice (2.6%); + mother or father gastric juice (17%); and + normal gastric juice (26%). These studies demonstrate that the patient is homozygous and that the mother, father, and sister are heterozygous for a structurally abnormal IF that has a markedly decreased, but not absent, affinity for ileal IF-B_{12} receptors. These studies also indicate that the B_{12} and ileal binding sites are located on different portions of the IF molecule.

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INTRODUCTION

A patient has been described previously (1) who presented at age 13 with glossitis and megaloblastic anemia due to an unusual type of vitamin B_{12} (B_{12})[1] malabsorption. Classic pernicious anemia was ruled out on the basis of a histologically normal gastric mucosa, normal gastric acidity, and the absence of serum antibodies against intrinsic factor B_{12}-binding protein (IF). Generalized malabsorption was ruled out by a normal gastrointestinal radiographic examination, normal D-xylose and fat absorption studies, and negative stool examinations for ova and parasites. Isolated congenital absence of IF (2, 3), a congenital selective ileal absorptive defect associated with proteinuria (4, 5), and isolated congenital absence of plasma transcobalamin II (6) were also ruled out since (*a*) the patient's gastric juice contained normal amounts of a B_{12}-binding protein that was indistinguishable from IF in terms of its immunologic properties, its apparent affinity for B_{12}, and its behavior during standard chromatographic procedures, (*b*) normal human gastric juice corrected the patient's B_{12} malabsorption and proteinuria was absent, and (*c*) the patient's plasma levels of transcobalamin I and II were normal. Additional studies revealed that the patient's gastric juice had a decreased ability to facilitate in vivo B_{12} absorption by a subject with a total gastrectomy and a decreased ability to facilitate B_{12} binding to guinea pig ileal homogenates. Gastric juice from both parents, who

[1] *Abbreviations used in this paper:* B_{12}, vitamin B_{12}; IF, intrinsic factor vitamin B_{12}-binding protein; pseudo-B_{12}, (α-adenyl)-cobamide cyanide.

are first cousins, had an intermediate ability to facilitate in vivo B_{12} absorption.

On the basis of the studies listed above it was postulated that the patient was homozygous for a functionally abnormal IF (1). In order to test this hypothesis, IF has now been isolated from the patient and his mother, father, and sister, and the properties of these preparations have been compared with those of IF isolated from normal individuals.

METHODS

Assay of B_{12}. [^{57}Co]B_{12} (150–200 μCi/μg) and [^{58}Co]B_{12} (3 μCi/μg) were obtained from Amersham/Searle Corp. (Arlington Heights, Ill.) and diluted with nonradioactive crystalline B_{12} (Sigma Chemical Co., St. Louis, Mo.). Items containing [^{57}Co]B_{12} and [^{58}Co]B_{12} were assayed by measuring radioactivity in a Packard gamma scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Solutions of crystalline B_{12} and (α-adenyl)-cobamide cyanide (pseudo-B_{12}) (obtained from Dr. Joseph Pfiffner of Wayne State University) dissolved in water were assayed spectrophotometrically as described previously (7, 8).

B_{12} binding studies. B_{12} binding ability was assayed in 0.1 M potassium phosphate, pH 7.5, by a modification (8) of the charcoal adsorption technique of Gottlieb, Lau, Wasserman, and Herbert (9). Anti-IF blocking antibody was obtained from the serum of a pernicious anemia patient as described previously (8). Equilibrium dialysis experiments and experiments designed to measure the ability of [^{57}Co]-B_{12} to displace nonradioactive B_{12} and pseudo-B_{12} from IF were also performed as described previously (8).

Purification of IF from gastric juice. Histalog-stimulated gastric juice was collected on ice by nasogastric suction and stored at −20°C after depepsinization (8, 10). IF was isolated by using affinity chromatography on B_{12}-Sepharose as the sole purification step. This step was performed as described previously (8) except that IF was eluted from B_{12}-Sepharose with 5.0 M guanidine-HCl instead of 7.5 M guanidine-HCl. This modification (11) is advantageous since it separates IF from the R-type B_{12}-binding protein that is found in gastric juice in variable amounts. The R-type protein is not eluted with 5.0 M guanidine-HCl but can be eluated subsequently after 16 h of incubation with 7.5 M guanidine-HCl.

Saturation of B_{12}-binding proteins with [^{57}Co]B_{12}. A threefold excess (based on B_{12} binding activity) of [^{57}Co]B_{12} was added to individual B_{12}-binding proteins (1–3 μg protein/ml) in 5.0 M guanidine-HCl containing 0.1 M potassium phosphate, pH 7.5. Proteins were dialyzed subsequently for 72 h at 4°C against 2,000 volumes of 0.05 M potassium phosphate, pH 7.5, contining 0.75 M NaCl with dialysate changes at 24 and 48 h. More than 99% of unbound B_{12} is removed under these conditions. Protein preparations devoid of B_{12} were prepared in the same manner except that B_{12} was not added before dialysis.

Immunoprecipitation of IF-B_{12} in 30% $(NH_4)_2SO_4$. Test tubes containing 0.2 ml of serum, consisting of varying amounts of control and anti-IF sera, and 0.1 ml of 0.05 M potassium phosphate, pH 7.5, 0.75 M NaCl containing 500 pg of [^{57}Co]B_{12} bound to IF were incubated at 22°C for 30 min. The tubes were then placed in an ice bath and 0.25 ml of cold, saturated $(NH_4)_2SO_4$ was added. After standing for an additional 30 min, the tubes were centrifuged at 10,000 *g* for 15 min and 0.2 ml of the supernatant solution was removed and assayed for [^{57}Co]B_{12}. Less than 10% of IF-B_{12} is precipitated under these conditions in the presence of 0.2 ml of human or rabbit control sera.

Adsorption of rabbit anti-patient IF serum with normal IF. Rabbit anti-patient IF serum (10 ml) was applied to a column (0.9 cm diameter and 5.0 cm tall) of B_{12}-Sepharose containing 1.66 mg (i.e., 50 μg of B_{12} binding ability) of bound normal IF. The column was equilibrated and eluted at 20°C with 0.01 M potassium phosphate, pH 7.5, 0.14 M NaCl. The flow rate was 10 ml/h and 2-ml fractions were collected. Fractions 3 and 4 contained significantly greater antibody reactivity against patient IF-B_{12} than against normal IF-B_{12}, as judged by immunoprecipitation assays (see above), and these two fractions were pooled. Fractions 1 and 2 consisted essentially of buffer while fractions 5–7 contained significant amounts of antibody with specificity for both patient and normal IF. Pooled fractions 3 and 4 were adsorbed further by the addition of nonradioactive normal IF-B_{12} (300 ng B_{12}/ml of serum) 30 min before being used for detailed immunoprecipitation studies.

Assay of IF-B_{12} binding to intestinal mucosal homogenates. The binding of IF-B_{12} to intestinal mucosal homogenates (12) was assayed by using a Millipore filter technique (13) as modified and described previously (14). Assays were performed in Krebs-Ringer phosphate, pH 7.5, and in a modified medium in which calcium and magnesium were replaced with 0.001 M Na_2-EDTA. The difference between IF-B_{12} bound to intestinal mucosal homogenates in these two media was termed the "EDTA inhibitable" fraction. The validity of using EDTA-inhibitable B_{12} binding as a measure of specific IF-B_{12} binding to intestinal mucosal IF-B_{12} binding sites has been been demonstrated previously (14).

Schilling tests. Schilling tests were performed as described previously (1). Informed consent was obtained from the subject who had previously undergone total gastrectomy for carcinoma of the stomach. The amount of B_{12} ingested contained 0.5 μCi of [^{57}Co]B_{12}/test.

Other methods. Polyacrylamide disk gel electrophoresis (8), sodium dodecyl sulfate polyacrylamide gel electrophoresis (8), absorption spectra (8), immunization of rabbits (15), immunodiffusion (15), amino acid and carbohydrate analyses (8), and molecular weight estimation by gel filtration (14) were all performed as described previously. Human plasma transcobalamin II (16), human milk B_{12}-binding protein (15), human saliva B_{12}-binding protein (15), and human granulocyte B_{12}-binding protein (17) were isolated as described previously.

RESULTS

Purification of IF. The purification of IF from gastric juice obtained from normal subjects, the patient, and the patient's father, mother, and sister are summarized in Table I. The data presented demonstrate that all of the gastric juice preparations were normal in terms of total B_{12} binding ability and in terms of the amount and percent of B_{12} binding ability attributable to IF (18). The B_{12}-binding protein present in the various gastric juice preparations behaved normally during adsorption to B_{12}-Sepharose and during the subsequent washing and elution steps. The yield of IF obtained ranged from 59 to 87%. All of the B_{12} binding ability present in the final

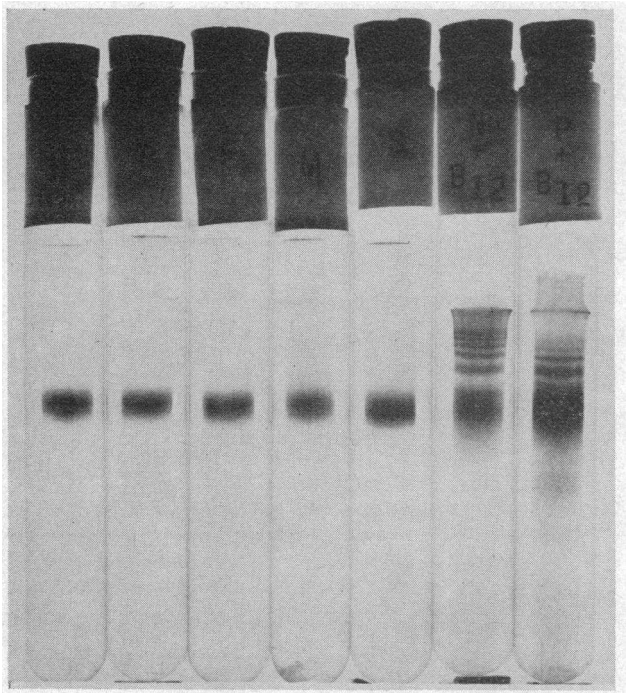

FIGURE 1 Polyacrylamide disk gel electrophoresis of the various IF preparations. Each sample contained 25 μg of protein. Protein samples devoid of B_{12} were renatured from guanidine by dialysis against H_2O for 24 h at 4°C. Protein samples saturated with B_{12} were renatured as described above except that excess B_{12} was added before dialysis. *N*, normal IF; *P*, patient IF; *F*, father IF; *M*, mother IF; *S*, sister IF; $N + B_{12}$, normal IF-B_{12}; $P + B_{12}$, patient IF-B_{12}.

preparations could be inhibited with anti-IF-blocking antibody obtained from the serum of a patient with pernicious anemia. All of the final preparations were homogeneous based on disk gel electrophoresis (see below).

Disk gel electrophoresis. When 25 μg of patient IF, normal IF, mother IF, father IF, and sister IF were subjected to polyacrylamide disk gel electrophoresis in the absence of B_{12}, single protein bands with the same mobility were observed in each case, as shown in Fig. 1. Multiple protein bands were observed, however, when patient IF and normal IF were studied as their B_{12} complexes. This phenomenon has been observed previously with normal IF and results from the fact that human IF aggregates in the presence of B_{12} under certain conditions and exists as a mixture of monomers, dimers, and higher molecular weight oligomers (8). Consistent and reproducible differences in oligomer formation between patient and normal IF-B_{12} were not observed.

Immunologic studies. Single precipitation lines with a pattern of identity were observed with purified patient IF-B_{12}, normal IF-B_{12}, father IF-B_{12}, mother IF-B_{12}, sister IF-B_{12}, and an equal mixture of patient and normal IF-B_{12} when these preparations were subjected to immunodiffusion against both rabbit anti-patient IF (Fig. 2A) and rabbit anti-normal IF (Fig. 2B) sera. Rabbit anti-patient IF serum did not give precipitation lines with human transcobalamin II nor with B_{12}-binding proteins isolated from human milk, saliva, and granulocytes. Patient IF-B_{12} was not precipitated on immunodiffusion by rabbit anti-human transcobalamin II, anti-human milk B_{12}-binding protein, anti-human saliva B_{12}-binding protein, nor control sera (data not shown).

Patient IF-B_{12} and normal IF-B_{12} were precipitated in an equivalent manner (data not presented) when quantitative immunoprecipitation studies were performed with varying amounts of serum, obtained from a pernicious anemia patient, that contained anti-IF binding antibodies. Evidence of extensive, but not necessarily complete, cross-reactivity between patient IF-B_{12} and normal IF-B_{12} was obtained when similar studies were

TABLE I

Summary of Purification of Intrinsic Factor by Affinity Chromatography

Subject	Gastric juice			5.0 M guanidine-HCl eluate from B_{12}-Sepharose			Yield
	Volume	B_{12} binding ability		Volume	B_{12} binding ability		
	ml	*μg*	*% IF**	*ml*	*μg*	*% IF*	*%*
Normals‡	6,580	491	92	64.5	358	100	79
Patient§	1,600	125	88	40.0	96.0	100	87
Father	257	24.8	96	3.7	19.6	100	82
Mother	122	6.2	75	4.1	3.2	100	69
Sister	156	9.0	88	4.0	4.7	100	59

* Based on % inhibition of B_{12} binding ability observed with pernicious anemia blocking antibody.

‡ Pooled gastric juice obtained from 23 different individuals.

§ Pooled gastric juice from 10 different collections.

TABLE II

Adsorption of Rabbit Anti-Patient IF Serum with Various IF-B_{12} Preparations

Nonradioactive item present in 80-fold excess*	Precipitation of IF-B_{12} (0.5 ng [^{57}Co] B_{12}) by 3 μl of rabbit anti-patient IF serum previously adsorbed with normal IF-B_{12}				
	Normal IF-B_{12}	Patient IF-B_{12}	Father IF-B_{12}	Mother IF-B_{12}	Sister IF-B_{12}
	%	%	%	%	%
None	3	72	43	44	44
Normal IF-B_{12}	0	66	36	38	38
Patient IF-B_{12}	0	0	0	1	0
Father IF-B_{12}	0	1	0	2	0
Mother IF-B_{12}	0	1	0	6	7
Sister IF-B_{12}	0	9	2	8	1

* Nonradioactive items were added to standard immunoprecipitation assays 30 min before the addition of IF-[^{57}Co]B_{12}.

performed with rabbit anti-patient IF and anti-normal IF sera. The results of the studies employing rabbit anti-patient IF serum are presented in Fig. 3A and suggest that this serum is slightly more reactive against patient IF-B_{12} than against normal IF-B_{12}. A similar, but opposite, difference was observed with rabbit anti-normal IF serum (data not presented). Mother IF-B_{12}, father IF-B_{12}, and sister IF-B_{12} were also completely precipitated by the two rabbit antisera although they could not be distinguished with confidence from patient IF-B_{12} nor from normal IF-B_{12}.

Immunologic differences among the various IF-B_{12} preparations were demonstrated clearly, however, when immunoprecipitation experiments were performed with anti-patient IF serum that had been adsorbed with normal IF-B_{12}. The results are presented in Fig. 3B and

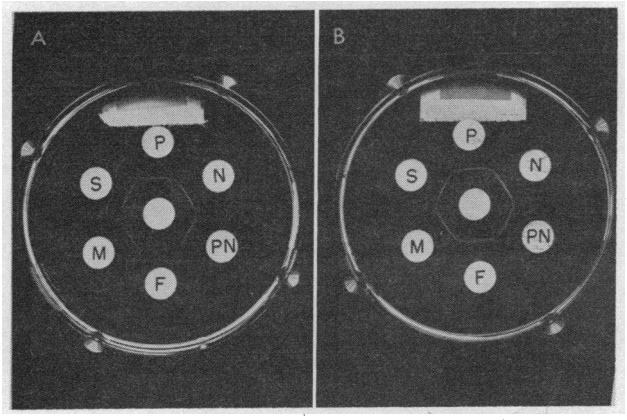

FIGURE 2 Immunodiffusion with rabbit anti-patient IF and anti-normal IF sera. The center wells contained 25 μl of (A) anti-patient IF serum and (B) anti-normal IF serum. The outer wells contained 20 μl of the various IF-B_{12} preparations (5 μg B_{12}/ml). *P*, patient IF-B_{12}; *N*, normal IF-B_{12}; *PN*, 1/2 patient IF-B_{12} + 1/2 normal IF-B_{12}; *F*, father IF-B_{12}, *M*, mother IF-B_{12}; *S*, sister IF-B_{12}.

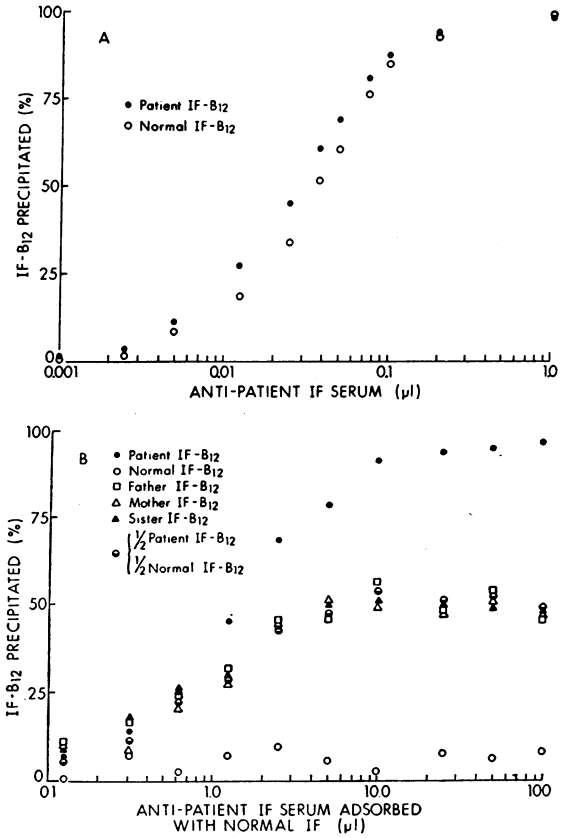

FIGURE 3 Immunoprecipitation assays with rabbit anti-patient IF sera and the various IF-B_{12} preparations. (A) Whole antipatient IF serum; (B) anti-patient IF serum after adsorption with normal IF-B_{12}.

reveal that the adsorbed anti-patient IF serum was still capable of precipitating patient IF-B_{12} completely, although its ability to precipitate normal IF-B_{12} was now negligible. Mother IF-B_{12}, father IF-B_{12}, sister IF-B_{12}, and an equal mixture of patient and normal IF-B_{12} were precipitated approximately 50% by the adsorbed anti-patient IF serum.

The data presented in Fig. 3B demonstrate that mother IF-B_{12}, father IF-B_{12}, and sister IF-B_{12} are composed of equal mixtures of normal IF-B_{12} and an immunologically distinct IF-B_{12} but the data do not enable one to determine whether these preparations contain the same immunologically distinct IF-B_{12}. In order to answer this question additional immunoprecipitation experiments were performed in which the anti-patient IF serum that had been adsorbed with normal IF-B_{12} was adsorbed further with the various IF-B_{12} preparations. The results are presented in Table II and reveal that patient IF-B_{12} and the immunologically distinct portions of mother, father, and sister IF-B_{12} all compete for the same antibodies. This observation indicates that patient IF-B_{12}

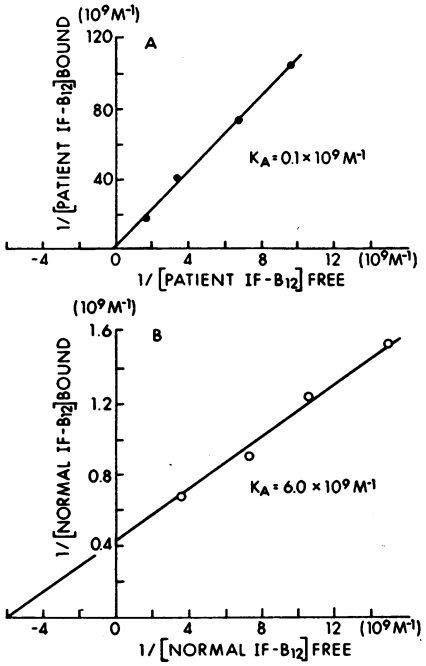

FIGURE 4 Double reciprocal plots of EDTA-inhibitable IF-B_{12}-binding to human ileal mucosal homogenate vs. IF-B_{12} concentration. (A) patient IF-B_{12}; (B) normal IF-B_{12}.

consists of a single immunologically distinct species and that mother, father, and sister IF-B_{12} consist of equal mixtures of the same immunologically distinct IF-B_{12} and normal IF-B_{12}.

A comparison of Figs. 3A and 3B reveals that the potency of the adsorbed anti-patient IF-B_{12} serum is approximately 2 orders of magnitude less than that of the unadsorbed anti-patient IF-B_{12} serum in terms of its ability to precipitate patient IF-B_{12}. This observation, together with the fact that the protein concentrations of the two sera were approximately the same, suggests that only several percent of the anti-patient IF-B_{12} antibodies present in the whole anti-serum are specific for patient IF-B_{12} as opposed to normal IF-B_{12}.

TABLE III
EDTA-Inhibitable Binding of IF-B_{12} to Human Ileal Mucosal Homogenates

Item	Amount present during incubation	B_{12} bound to ileal homogenate*	
		Range	Mean
	pg B_{12}/ml	*pg*	
Normal IF-B_{12}	200	8.80–9.33	8.99
Patient IF-B_{12}	200	0.05–0.37	0.20‡
Father IF-B_{12}	200	5.39–5.77	5.52
Mother IF-B_{12}	200	5.57–6.65	5.92
Sister IF-B_{12}	200	5.58–6.23	5.89
½ Normal IF-B_{12}, ½ patient IF-B_{12}	200	5.17–5.99	5.44
Normal IF-B_{12}	100	5.60–6.37	5.99

* Assays were performed in triplicate except for assays with patient IF-B_{12} which were performed in quadruplicate.
‡ Significantly different from zero with $P < 0.05$.

TABLE IV
Schilling Tests Performed with a Single Subject with a Total Gastrectomy

Date	[Co^{57}]B_{12} administered	Gastric juice administered*	48 h urinary excretion of [Co^{57}]B_{12}
	μg		%
10/14/70	2.0	normal	24.1
10/21/70	2.0	none	0.8
11/9/70	2.0	patient	2.8
3/25/71	1.0	normal	26.3
11/18/70	1.0	½ normal, ½ patient	17.7
3/15/71	1.0	father	17.3
3/18/71	1.0	mother	17.9
10/5/70	0.5	normal	26.6
6/7/73	0.5	normal	26.1
10/21/70	0.5	none	0.3
6/14/73	0.5	none	0.8
6/21/73	0.5	none	0.5
7/4/73	0.5	none	0.5
10/19/70	0.5	patient	3.4
6/11/73	0.5	patient	1.5
6/18/73	0.5	patient	3.4
6/30/73	0.5	patient	2.1
Means	0.5 μg [^{57}Co]B_{12} without gastric juice 0.5%.‡		
	0.5 μg [^{57}Co]B_{12} with patient gastric juice 2.6%.‡		

* The amount of gastric juice administered was such that all of the B_{12} administered could be bound to IF. Gastric juice and [^{57}Co]B_{12} were incubated together for 15 min before administration.
‡ These two mean values differ significantly with $P < 0.01$.

Ileal binding studies. The amounts of EDTA-inhibitable IF-B_{12} binding to human ileal mucosal homogenates observed with the various IF-B_{12} preparations are presented in Table III. A statistically significant amount

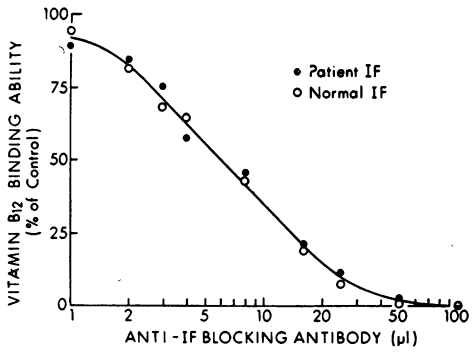

FIGURE 5 Inhibition of the B_{12} binding abilities (500 pg B_{12}) of purified patient IF and normal IF by use of anti-IF blocking antibodies obtained from the serum of a patient with pernicious anemia.

of such binding was observed with patient IF-B_{12} but the amount observed at a B_{12} concentration of 200 pg/ml was less than 3% of that observed with normal IF-B_{12}. Intermediate values (61–66% of normal) were observed with mother IF-B_{12}, father IF-B_{12}, sister IF-B_{12}, and an equal mixture of patient and normal IF-B_{12}. Similar results were observed with monkey, hog, dog, and guinea pig ileal mucosal homogenates.

The amounts of EDTA-inhibitable ileal IF-B_{12} binding observed at varying concentrations of IF-B_{12} were used to calculate values for the association constants for patient and normal IF-B_{12} and human ileal homogenates as shown in Fig. 4. Values of 0.1×10^9 M^{-1} and 6.0×10^9 M^{-1} were obtained with patient IF-B_{12} and normal IF-B_{12}, respectively. Additional human ileal binding studies have been performed and reveal that the markedly low values for B_{12} binding observed with patient IF-B_{12} are not increased when the calcium and/or magnesium concentrations in the incubation medium are increased between 10 and 100-fold or when the pH is varied between 6.5 and 9.5. Patient IF does not appear to be subjected to extensive proteolysis during incubation with human ileal homogenates since after a 3-h incubation more than 90% of unbound patient IF-B_{12} is precipitated with anti-IF serum and also elutes from Sephadex G-150 with an apparent molecular weight of approximately 60,000, i.e., the apparent molecular weight of native patient IF-B_{12}. Similar results have been reported previously with normal IF-B_{12} (14).

Schilling tests. The results of multiple Schilling tests performed with a single individual with a total gastrectomy are presented in Table IV. These studies demonstrate that the patient's gastric juice is able to facilitate B_{12} absorption in vivo although the amount of facilitation is only approximately 10% of that observed with normal human gastric juice. Intermediate levels of facilitation, i.e. 60–70% of that observed with normal human gastric juice, were observed with gastric juice from the patient's father and mother. A similar intermediate value was observed with an equal mixture of patient and normal gastric juice.

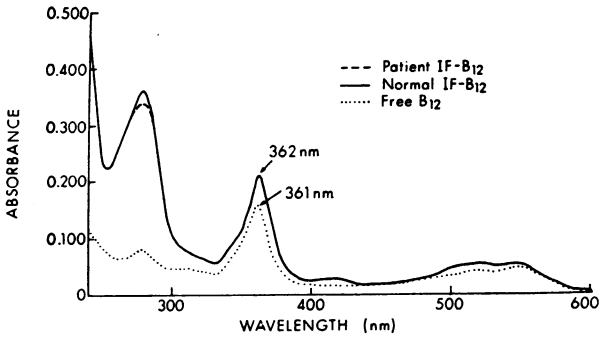

FIGURE 6 Absorption spectra of equal concentrations (7.25 μg B_{12}/ml) of patient IF-B_{12}, normal IF-B_{12}, and free B_{12}. Spectra were obtained at 22°C in 0.05 M potassium phosphate, pH 7.5, 0.75 M NaCl.

B_{12} binding studies. The ability of patient IF to bind B_{12} appeared normal when measured by a variety of techniques. The data presented in Fig. 5 reveal that the B_{12} binding abilities of patient IF and normal IF are inhibited in an equivalent manner by anti-IF blocking antibody obtained from the serum of a patient with pernicious anemia. The data presented in Table V indicate that purified patient IF and normal IF bind B_{12} at the same rate at 4°C. The rate observed was indistinguishable from the rate observed when crude patient and normal gastric juice were used as the source of IF (data not presented). Pseudo-B_{12} is displaced from both proteins at a faster rate than is native B_{12}. Similar displacement rates were observed with both patient IF and normal IF. Studies of this kind were also performed at 37°C (data not presented). The rates of B_{12} binding and displacement were significantly faster at 37°C than at 4°C but no differences between patient and normal IF were observed. The association constants for the two proteins and B_{12} were determined by equilibrium dialysis in

TABLE V

Displacement of B_{12} and Pseudo-B_{12} with [^{57}Co]B_{12} at 4°C

Item	Nonradioactive item present during 30-min preincubation	[^{57}Co]B_{12} bound at different time periods after the addition of 1,000 pg							
		0.5 min	1.0 min	2.0 min	5.0 min	10 min	30 min	120 min	
		pg		pg		pg		pg	%
Normal IF	None	126	194	310	421	463	499	489	100.0
Normal IF	1,500 pg pseudo-B_{12}	82	135	200	329	427	482	493	100.8
Normal IF	1,500 pg B_{12}	0	0	0	2	1	3	8	1.6
Patient IF	None	146	228	320	448	487	508	517	100.0
Patient IF	1,500 pg pseudo-B_{12}	86	129	210	370	422	500	501	96.9
Patient IF	1,500 pg B_{12}	0	0	1	0	2	4	9	1.7

TABLE VI
Amino Acid and Carbohydrate Composition

Item	Normal IF		Patient IF
	1st preparation	Present preparation	
	*mol/mol B_{12}**	*mol/mol B_{12}*‡	*mol/mol B_{12}*‡
Amino acid			
Lysine	20	19	19
Histidine	5	4	5
Arginine	6	6	6
Aspartic	38	38	38
Threonine	24	24	26
Serine	30	30	31
Glutamic	35	36	36
Proline	22	21	24
Glycine	20	20	22
Alanine	23	23	22
Valine	22	20	23
Isoleucine	22	20	21
Leucine	34	33	34
Tyrosine	9	9	9
Phenylalanine	10	10	10
Methionine	10	10	10
Half-cystine	6	6	6
Tryptophan	6	5	5
Total	342	334	347
(Molecular weight)	(37,500)	(36,600)	(37,900)
Carbohydrate			
Fucose	7	6	8
Galactose	6	3	3
Mannose	12	13	12
Galactosamine	3	5	5
Glucosamine	6	5	5
Sialic Acid	3	2	2
Total	37	34	35
(Molecular weight)	(6,600)	(6,100)	(6,200)
Total number of residues	379	368	382
(Total molecular weight)	(44,100)	(42,700)	(44,100)
(% carbohydrate)	(15.0%)	(14.3%)	(14.1%)

* Average of duplicate analyses. These values have been published previously (8).
‡ Single determinations.

0.1 M potassium phosphate, pH 7.5, at 4°C. Values of $1.3–1.6 \times 10^{10}$ M^{-1} were obtained for both patient and normal IF.

The spectra of equal concentrations of patient IF-B_{12}, normal IF-B_{12}, and unbound B_{12} are presented in Fig. 6. When B_{12} is bound to either patient or normal IF the spectral maximum for B_{12} shifts from 361 nm to 362 nm and the absolute absorbance at 361 nm increases by 30%. Previous studies (8) employing normal IF have demonstrated that the increase in absolute absorbance at 361 nm is due to the interaction of IF and B_{12} rather than to a possible isotope effect that could result in erroneously high values being determined for the concentration of IF-B_{12}. The absorbance of the patient IF-B_{12} complex at 280 nm was slightly lower than that of normal IF-B_{12} and resulted in an A_{280}/A_{361} value of 1.62 for patient IF-B_{12} which is lower than the value of 1.72 that was observed with normal IF-B_{12}. A value of 1.68 was obtained with a different preparation of normal IF-B_{12} (8). The significance, if any, of these differences is unknown.

Molecular weight studies. The molecular weight of patient IF appeared normal when estimated by a variety of techniques. The results of amino acid and carbohydrate analyses are presented in Table VI. By using the molecular weights of the individual amino acids and carbohydrates, it was determined that patient IF contains 44,100 g of amino acid and carbohydrate/mol of bound B_{12}. This value is not significantly different from the values of 44,100 and 42,700 g obtained with two separate preparations of normal IF. All three of these values are in good agreement with the molecular weight values of 44,000–48,000 that we have obtained previously (8) for normal IF using sedimentation equilibrium ultracentrifugation. The data presented in Table VI also fail to reveal any significant differences in the amino acid and carbohydrate composition of patient and normal IF. Minor differences can not be relied on or necessarily detected, on the basis of a small number of analyses.

When patient IF and normal IF were studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis in the presence of 1% 2-mercaptoethanol single protein bands with apparent molecular weights of 55,000 were observed in both cases.[2]

In studies employing gastric juice and gel filtration other investigators (23–25) have observed that human IF-B_{12} has a smaller apparent molecular weight than human IF devoid of B_{12}. These studies have suggested that the conformation of human IF changes to a more compact form when B_{12} is bound to it. This observation and interpretation are supported by the gel filtration experiments performed with homogeneous human IF that are presented in Fig. 7. These experiments demonstrate that both patient and normal IF devoid of B_{12} elute from Sephadex G-150 with apparent molecular weights of 70,000 while both patient IF-B_{12} and normal IF-B_{12} elute with apparent molecular weights of 61,000.[2]

When patient IF and normal IF were saturated with [^{57}Co]B_{12} for 72 h before being subjected to gel filtration multiple peaks of radioactivity were observed (data not presented) indicating that both proteins had aggregated as has been observed previously with normal IF-B_{12} (8). No differences between patient IF-B_{12} and normal IF-B_{12} were observed in terms of the amount or nature of this kind of aggregation.

[2] The molecular weight estimates obtained for human IF with sodium dodecyl sulfate gel electrophoresis and gel filtration appear falsely elevated when compared with the values obtained by amino acid and carbohydrate analyses and sedimentation equilibrium ultracentrifugation. Discrepancies of this type have been noted previously with other glycoproteins (15, 17, 19–22).

DISCUSSION

From the existing knowledge of the process of IF-mediated B_{12} absorption, it appears likely that mutations in the structural gene for IF could cause B_{12} malabsorption by a variety of mechanisms. Such mutations might, for example, result in a decrease in or absence of synthesis of IF by gastric parietal cells or in a decrease in secretion of IF into the gastric juice. Other mutations might result in IF molecules that have a decrease in or absence of affinity for B_{12} or an increase in susceptibility to proteolytic enzymes such as pepsin, trypsin, chymotrypsin, etc. Still other mutations might result in IF molecules that have as their IF-B_{12} complexes a decrease in or absence of affinity for ileal IF-B_{12} receptors. A structurally abnormal IF could also interefere with the subsequent ileal phase of B_{12} absorption. This phase is poorly understood although at some point B_{12} must be released from IF since IF does not appear to enter the portal blood (*26, 27*).

The patient described in this report represents the first documented case of B_{12} malabsorption secondary to a structurally abnormal IF. The immunologic studies performed with rabbit anti-patient IF serum demonstrate that patient IF contains at least one, though not many, antigenic determinant that is not present on normal IF. The studies performed with rabbit anti-normal IF serum suggest that the reverse is also true. These observations, together with the fact that patient IF and normal IF have very similar molecular weights and amino acid and carbohydrate compositions, are compatible with a single amino acid substitution, although this has not been proved.

Schilling tests, ileal homogenate binding studies, and immunologic studies all indicate that the patient's mother, father, and sister have gastric juices that contain equal mixtures of normal IF and a structurally abnormal IF. These studies thus demonstrate that mutations in the structural gene for IF are inherited as autosomal recessive traits. The antibody adsorption studies also indicate that the mother, father, and sister all possess the same structurally abnormal IF, an observation that is not unexpected since the parents are first cousins. These studies also indicate that the patient is homozygous for a single structurally abnormal IF.

The ileal homogenate binding studies reported here demonstrate that patient IF-B_{12} has a decreased affinity for ileal IF-B_{12} receptors and indicate that this functional abnormality is responsible for the patient's B_{12} malabsorption. The finding that this affinity is decreased rather than absent is supported by the results of multiple Schilling tests that demonstrate that the patient's gastric juice has a decreased but not absent ability to facilitate B_{12} adsorption in vivo. This small amount of activity may account for the fact that the patient did not develop clinical B_{12} deficiency until age 13, an age that is considerably greater than that (< 5 years of age) observed with patients with a complete lack of IF-facilitated B_{12} absorption (*2–5*).

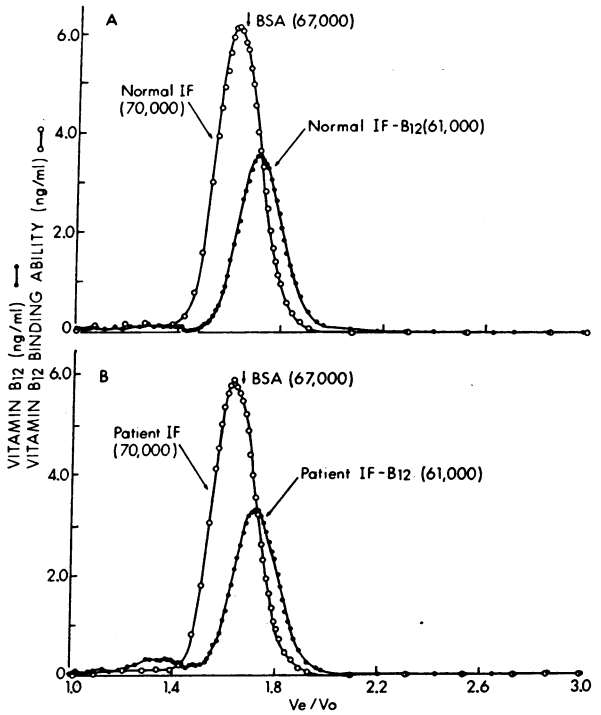

FIGURE 7 Gel filtration experiments. Protein samples were prepared in 1 ml of 0.05 M potassium phosphate, pH 7.5, 0.75 M NaCl and contained, in order of addition, IF (240 ng of B_{12} binding ability), bovine serum albumin (20 mg), blue dextran (2.5 mg), and [^{58}Co]B_{12} (83 ng). After standing for 1 h at 4°C, samples were applied to a 2.0 × 90 cm column of Sephadex G-150, equilibrated with the same buffer, and 1-ml fractions were collected. Fractions were assayed for blue dextran (A_{620}), bovine serum albumin (A_{280}), IF-B_{12} ([^{58}Co]B_{12}), and free IF (B_{12} binding ability). B_{12} binding assays were performed with [^{57}Co]B_{12} at 4°C; negligible exchange of B_{12} occurs under these conditions (see Table V). (A) Normal IF; (B) patient IF.

A number of children have been reported in the literature (*2, 3, 28–43*) as cases of B_{12} malabsorption secondary to congenital absence of IF. The diagnostic criteria have consisted in most cases of presentation with B_{12} deficiency before age 5, Schilling test results that indicate a lack of IF activity, and the demonstration of a histologically normal gastric mucosa and normal gastric acidity. From the discussion above it is clear that some, if not all, of these patients may actually represent examples of B_{12} malabsorption due to structurally abnormal IFs. In some of the cases just mentioned (*3, 38–42*) samples of gastric juice were actually shown to lack IF by studies based on assays employing either the ability of anti-IF blocking antibodies to block

[^{57}Co]B_{12} binding in the patient's gastric juice or the ability of anti-IF binding antibodies to bind to protein-B_{12} complexes formed after the addition of [^{57}Co]B_{12} to the patient's gastric juice. The fact that no IF was observed with these assays rules out the presence of a structurally abnormal IF of the kind described in this report but does not rule out the presence of a structurally abnormal IF that has a decrease in or absence of affinity for B_{12}.

In previous studies (14) it was observed that 100-fold excesses of free B_{12} or purified human IF devoid of B_{12} did not cause detectable inhibition of human IF-B_{12} binding to human ileal mucosal homogenates. These observations indicate that free B_{12} and human IF devoid of B_{12} have little if any affinity for human ileal IF-B_{12} receptors and suggest that B_{12} binding to IF results in important conformational changes in the portion of the B_{12} and/or IF molecule that interacts with the ileal IF-B_{12} receptor. The studies performed with the mutant IF reported here make it extremely unlikely, however, that the ileal IF-B_{12} receptor interacts only with the conformationally altered B_{12} molecule since the mutant IF-B_{12} complex binds poorly to the ileal IF-B_{12} receptor despite the fact that mutant IF binds B_{12} normally as judged by studies employing equilibrium dialysis, adsorption to B_{12}-Sepharose, interaction with anti-IF blocking antibodies, absorption spectra, and measurements of B_{12} binding rates and the rates of displacement of B_{12} and pseudo-B_{12}. It thus appears that the ileal IF-B_{12} receptor interacts with a portion of the IF molecule that is distinct from the B_{12} binding site although additional interaction with the B_{12} molecule is not ruled out.

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