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EVIDENCE THAT PANCREATIC PROTEASES ENHANCE VITAMIN B_{12} ABSORPTION BY ACTING ON CRUDE PREPARATIONS OF HOG GASTRIC INTRINSIC FACTOR AND HUMAN GASTRIC JUICE

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Crude preparations of hog gastric intrinsic factor or their own previously collected gastric juices administered with labeled vitamin B_{12} did not enhance vitamin B_{12} absorption in patients with vitamin B_{12} malabsorption secondary to pancreatic insufficiency. However, when these sources of gastric intrinsic factor were incubated with three times crystallized preparations of insolubilized bovine trypsin or chymotrypsin, the proteolytic enzymes were removed by centrifugation, and the preparations of gastric intrinsic factor were readministered to these patients, the absorption of vitamin B_{12} was markedly enhanced. Studies of hog gastric intrinsic factor before and after exposure to proteolytic enzymes failed to show any difference on Sephadex chromatography or polyacrylamide gel electrophoresis or on its affinity for vitamin B_{12} or the ileal receptor in guinea pigs. These investigations demonstrate that: (1) gastric intrinsic factor as secreted by subjects with pancreatic insufficiency or obtained from hog pyloric mucosal extracts is ineffective in promoting vitamin B_{12} absorption in patients with pancreatic insufficiency, (2) incubation of crude preparations of gastric intrinsic factor with insolubilized pancreatic proteases modified these preparations of gastric intrinsic factor in an as yet undefined manner, allowing them to enhance vitamin B_{12} absorption, and (3) in vitro studies using gut sacs or brush border preparations do not reflect the abnormality in vitamin B_{12} absorption associated with pancreatic dysfunction.

Vitamin B_{12} malabsorption occurring in patients with pancreatic exocrine insufficiency and in rats with partial pancreatic extirpation can be corrected by the administration of exogenous pancreatic extract or trypsin.[1] However, the mechanism whereby these pancreatic proteases improve vitamin B_{12} absorption has not been defined. Investigations in our laboratory have shown that gastric juice obtained from patients with pancreatic exocrine insufficiency contains immunoreactive gastric intrinsic factor (GIF) and that the administration of exogenous hog GIF does not correct the vitamin B_{12} malabsorption in these patients.[2] Studies using partially pancreatectomized rats have demonstrated that gastric homogenates obtained from these animals maintain the capacity to stimulate vitamin B_{12} uptake in intestinal sacs isolated from control rats and that the small intestine isolated from these partially pancreatectomized rats can still respond to GIF-mediated vitamin B_{12} uptake.[3] These previous studies seem to exclude at least a qualitative defect in the binding of GIF to vitamin B_{12} or in the adsorption of the GIF-vitamin B_{12} (GIF-B_{12}) complex to its small intestinal receptor as the mechanism for the vitamin B_{12} malabsorption in patients and experimental animals with pancreatic insufficiency. The present study demonstrates that pancreatic proteases act directly on crude GIF preparations or gastric juices from patients with vitamin B_{12} malabsorption and pancreatic insuiciency in an as yet undefined manner to restore absorption of this vitamin to normal.

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Methods

Vitamin B_{12} absorption was measured by the urinary excretion test using 1. 0 μg of ^{57}Co-labeled vitamin B_{12} (0.5 μc) followed ½ hr later by a parenteral flushing dose of 1000 μg of vitamin B_{12}.[4] Urine was collected for 24 hr and counted in a gamma spectrometer. The results were expressed as percentage of the dose excreted, the normal in our laboratory being greater

than 8% of the orally administered labeled vitamin B_{12}. Hog GIF (1 National Formulary XI U per capsule, E. R. Squibb & Sons, New York, N. Y.) was assayed for its vitamin B_{12}-binding capacity[5] and its ability to stimulate vitamin B_{12} uptake in guinea pig ileal tissue[6] before being administered to patients. In some tests, pancreatic extract in the form of Viokase (VioBin Corporation, Monticello, Ill.) dissolved in 0.9% sodium chloride (1 g per 10 ml), 10 mg of trypsin (Worthington Biochemical Corporation, Freehold, N. J.), or 30 ml of human gastric juice was administered concomitantly with labeled vitamin B_{12}. In some experiments hog GIF (50 mg) or human gastric juice (30 ml) was incubated for 30 min at 37°C with 100 mg of three times crystallized bovine trypsin or chymotrypsin covalently linked to carboxymethylcellulose (Miles Laboratories, Inc., Elkhart, Ind.), which had been washed 10 times to remove buffer salts and any free protease, and the insoluble protease was separated from the intrinsic factor source by centrifugation (3000 rpm for 30 min). After exposure to the insoluble protease, the GIF solutions were assayed for trypsin and chymotrypsin content by the methods of Hummel[7] and Schwert and Takenaka,[8] respectively. TAME (*p*-toluenesulfonyl-L-arginine methyl ester, Calbiochem, Los Angeles, Calif.) served as the substrate for trypsin and ATEE (*n*-acetyl-L-tyrosine ethyl ester, Sigma Chemical Company, St. Louis, Mo.) as the substrate for chymotrypsin. In vitro studies of hog GIF-B_{12} complex were performed by incubating intrinsic factor at 37°C with excess [^{57}Co]cyanocobalamin (100 μc per μg) (Amersham/Searle Corporation, Arlington Heights, Ill.) for a period of 15 min in 0.01 M K_2HPO_4, 0.75 M NaCl, *p*H 7.50. This was followed by dialysis against 1000 volumes of the equilibrating buffer, with several buffer changes to remove uncomplexed vitamin B_{12}. In all studies where GIF-B_{12} complex was compared to protease-treated GIF-B_{12} complex, GIF was first exposed to insolubilized protease and then the GIF was saturated with labeled vitamin B_{12}. Uncomplexed vitamin B_{12} was then removed by dialysis as described. The amount of intrinsic factor and nonintrinsic factor B_{12}-binding protein was determined for all studies by using antibodies to intrinsic factor obtained from the serum of a patient with pernicious anemia. The preparations of hog GIF contained 80% nonintrinsic factor (NIF) and 20% intrinsic factor (IF). The human gastric juices contained approximately 75% IF. A solution of hog IF was prepared and assayed for total B_{12} binding and the amounts of IF and NIF present. The preparation of hog IF was then divided into two equal aliquots, one of which was treated with the insoluble protease preparation. Both aliquots were then reassayed for total B_{12} binding and the amounts of IF and NIF B_{12}-binding present—again by using antibodies to IF. After exposure to the proteases, the percentages of NIF and IF did not change from the 80 to 20% ratio. Recovery of IF and NIF after treatment with the insolubilized protease preparation was virtually 100%. The hog IF preparations thus served as the proper control for the hog IF preparation that had been exposed to the insolubilized protease preparation. Secretin stimulation (1.5 gastrointestinal hormone U per kg) was performed, with 90 mM as the lower limit of normal for bicarbonate concentration in the duodenal aspirate in our laboratory.[9] Augmented betazole hydrochloride (Histalog) stimulation tests were performed (1.5 mg per kg)[10] to obtain gastric juice, which was titrated to *p*H 10 for 20 min to destroy peptic activity and retitrated to *p*H 7. The equilibrium constant for the binding of vitamin B_{12} to hog GIF was determined by the method of Hummel and Dreyer.[11] A column of G-25 Sephadex (0.9 by 60 cm) was equilibrated at 25°C with 0.01 M K_2HPO_4, 0.15 M NaCl, *p*H 7.40, containing 100 ng per ml of [^{57}Co] cyanocobalamin (100 μc per μg). A known quantity of GIF (determined by the Lowry protein assay)[12] was mixed with exactly 1.0 ml of the equilibrating buffer in an albumin-coated vessel and applied to the column. Fractions (0.50 ml) were collected and the radioactivity was counted in a gamma counter. The equilibrium constants were calculated assuming a 1:1 stoichiometry for the reactions and a molecular weight of 45,200 daltons for GIF. Care was taken to use albumin-coated glassware so that the GIF preparations were quantitatively transferred to the column. When this was not done, the equilibrium constant was falsely depressed. Gel filtration studies were performed at 4°C on hog GIF-B_{12} complex using reverse flow chromatography on Sephadex G-200 in 0.01 M K_2HPO_4, 0.75 M NaCl, *p*H 7.50. The column was 2.6 by 90 cm and the flow rate was 15 ml per hr. Fractions were collected, and then radioactivity was counted to determine the elution profile. Polyacrylamide disc gel electrophoresis was performed in the absence of sodium dodecyl sulfate in 7.5% polyacrylamide gel system at *p*H 8.2 at 2 ma per gel tube. Each gel was sliced into 1-mm discs and the radioactivity in each disc was counted in a gamma counter.

Guinea pig intestinal homogenates were prepared according to the method of Sullivan et al.,[6] and the method used for the ileal binding experiments was that of Hooper et al.[13] The distal one-half of the intestine was flushed with 0.9% NaCl (50 ml) at 4°C. The mucosa was expressed with glass slides and suspended in 10 volumes of 0.14 M NaCl, 0.0005 M KCl, 0.0025 M $CaCl_2$, 0.00125 M $MgSO_4$, 0.005 M K_2HPO_4 per wet weight at *p*H 7.40 (Krebs-Ringer phosphate buffer, $KRPO_4$) at 4°C. This mixture was homogenized for 30 sec at full speed in a Waring Blendor (Waring Products Division, Dynamics Corporation of America, New Hartford, Conn.), divided into 10-ml aliquots, and stored at −70°C until used. Immediately before use, each aliquot was thawed at 4°C and suspended by approximately 10 strokes of a motor-driven Teflon pestle. The sample was centrifuged at 20,000 × *g* for 30 min and the supernatant solution was decanted and its volume was measured. The washed pellet was suspended in a volume of $KRPO_4$-Ca^{++}/Mg^{++} equal to that of the initial supernatant solution followed by two more washings. Hog GIF and protease-treated hog GIF were mixed with a 10-fold excess of [^{57}Co]cyanocobalamin (Amersham Searle, 100 μc per μg) at 4°C, followed by dialysis for 72 hr against 1000 volumes of glass-distilled water to remove unbound vitamin B_{12}. The amount of complex in each sample was determined by measurement of [^{57}Co]cyanocobalamin. Each sample was diluted with $KRPO_4$ buffer lacking calcium and magnesium. The final concentration of the vitamin B_{12} complex in the incubation mixture was then varied from 1.0 pg (as B_{12}) to 450 pg (as B_{12}). Each reaction mixture was incubated for 180 min with a standard amount (0.20 ml) of receptor homogenate. Incubation took place at 25°C in 10- by 75-mm glass tubes that had been soaked in bovine serum albumin for 2 hr and aspirated to dryness. The reactions were quenched by filtration on 1.2 μ Millipore (Millipore Corporation, Bedford, Mass.) filters that had been soaked in bovine serum albumin for 6 hr before use. The amount of bound complex was determined by counting the radioactivity on the Millipore filters for 10,000 counts in a gamma counter. Assays were also performed in which $KRPO_4$ was replaced with $KRPO_4$ minus calcium and magnesium but with 0.001 M Na_2 ethylenediaminetetraacetate (EDTA) added. The difference between vitamin B_{12} bound to the homogenates in $KRPO_4$ and $KRPO_4$ without calcium and magnesium but with EDTA was called the EDTA-inhibitable fraction. The association constant (K_a) for the binding of hog GIF-B_{12} complex to the

ileal homogenates was determined according to the method of Steck and Wallach.[14] Double reciprocal plots were constructed by plotting $1/(IF\text{-}B_{12})_{free}$ against $1/(IF\text{-}B_{12})_{bound}$.

Studies performed in patients were carried out under carefully controlled conditions in a clinical research center ward. Informed consent was granted by all subjects and all investigations in human beings were approved by the University of Flordida Health Center Committee for the Protection of Human Subjects.

Results

The effect of trypsin on hog GIF-mediated vitamin B_{12} absorption in a patient with both pernicious anemia and pancreatic insufficiency. Figure 1 demonstrates the results of several vitamin B_{12} urinary excretion tests in this patient. This patient has well documented pernicious anemia and an impaired secretin stimulation test. She has been previously reported in detail.[15] The patient manifested vitamin B_{12} malabsorption that did not respond to the exogenous administration of 50 mg of crude hog GIF. However, normal absorption was achieved when she was given both the hog GIF and 10 mg of bovine crystalline trypsin concomitantly with the labeled vitamin B_{12}. Hog GIF, after being exposed to insoluble trypsin, effected a marked improvement in absorption. Control vitamin B_{12} absorption tests with hog GIF that had not been treated with protease preparations have continued to be abnormal. This enhancement of vitamin B_{12} absorption by these protease preparations was seen when the proteolytic enzymes were reacted with hog GIF before or after the hog GIF was complexed to vitamin B_{12}.

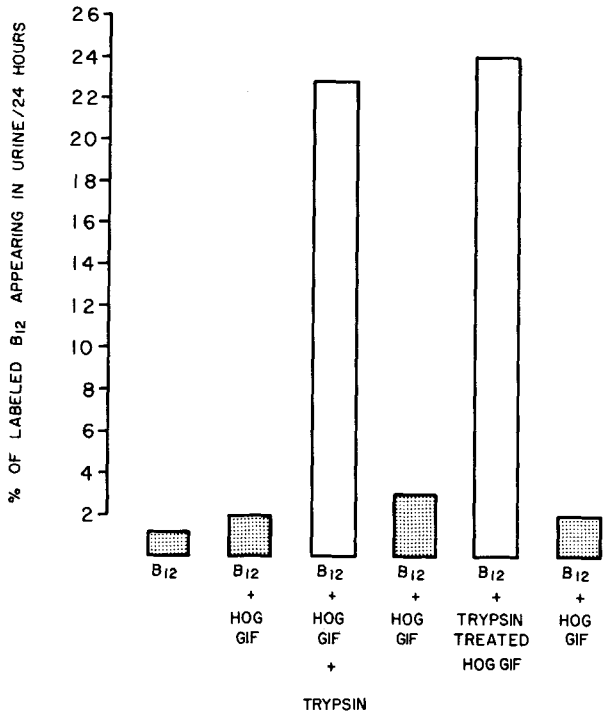

FIG. 1. Sequential vitamin B_{12} urinary excretion tests in a patient with both pernicious anemia and pancreatic insufficiency. In the first test in which trypsin was administered, the trypsin was in a soluble form and was given concomitantly with the labeled vitamin B_{12} and the hog gastric intrinsic factor (GIF). In the second trypsin test, the hog GIF was exposed to insolubilized trypsin and then given to the patient.

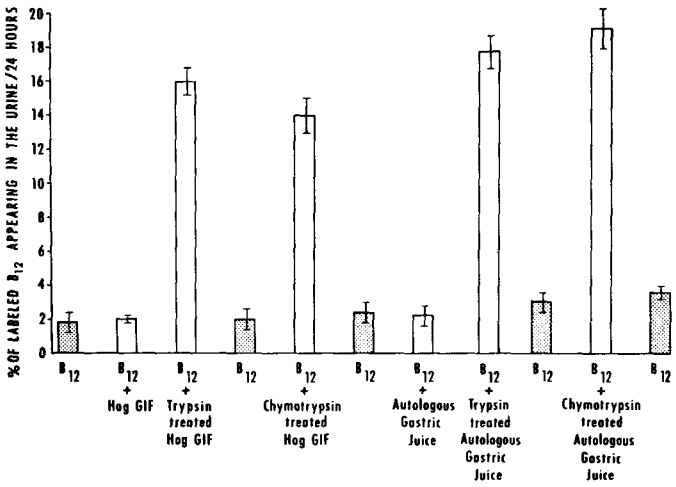

FIG. 2. Sequential vitamin B_{12} urinary excretion tests in 3 patients with pancreatic insufficiency. In those experiments in which the preparations of hog gastric intrinsic factor (GIF) or autologous gastric juice were treated with trypsin or chymotrypsin, preparations of insolubilized proteases were used.

The effect of trypsin and chymotrypsin on hog GIF- and autologous gastric juice-mediated vitamin B_{12} absorption in 3 patients with pancreatic exocrine insufficiency. All 3 patients demonstrated impairment of the secretin stimulation test and vitamin B_{12} malabsorption responsive to pancreatic extract administration. Figure 2 shows that these patients had vitamin B_{12} malabsorption unresponsive to the administration of hog GIF, but incubation of the hog GIF with insoluble trypsin or with insoluble chymotrypsin modified the crude hog GIF source in some way, so as to allow it to promote a dramatic improvement in vitamin B_{12} absorption. The gastric juices of these 3 patients with pancreatic insufficiency, when complexed to labeled vitamin B_{12}, corrected vitamin B_{12} malabsorption in patients with pernicious anemia, but did not restore absorption of this vitamin to normal levels when administered back to these same patients with pancreatic disease. However, when these same autologous gastric juices were incubated with insoluble trypsin or chymotrypsin, complexed to labeled vitamin B_{12}, and then administered back to these patients, absorption was markedly improved. The solution of hog GIF contained no detectable trypsin or chymotrypsin after the insoluble protease preparations had been centrifuged away. The assays we used can detect as little as 10 μg of enzyme per ml of sample. Because the incubations were performed in volumes of 30 to 50 ml, as much as 0.3 to 0.5 mg of trypsin or chymotrypsin could have been solubilized and not have been detected. In order to state that the insoluble protease preparations improved vitamin B_{12} absorption by acting directly on a substance in crude preparations of hog GIF and gastric juices or by acting directly on

GIF, various amount of trypsin and chymotrypsin (0.2 to 8.0 mg) were administered orally to 2 patients with pancreatic insufficiency whose vitamin B_{12} malabsorption had been corrected by incubating hog GIF or their own gastric juices with insolubilized preparations of trypsin and chymotrypsin. No improvement was noted until 4.0 mg of either trypsin or chymotrypsin were administered, thus indicating that the improvement in vitamin B_{12} absorption was not due to solubilization of small amounts of the insolubilized enzyme preparations.

Equilibrium constants for vitamin B_{12} binding to hog GIF before and after exposure to protease preparations. Figure 3 shows the elution profile on G-25 Sephadex of the equilibrium constants for vitamin B_{12} binding to hog GIF. The equilibrium constant for hog GIF was 0.30×10^{10} M^{-1}, very similar to that obtained by equilibrium dialysis.[16] Treatment with trypsin and chymotrypsin did not appreciably alter the equilibrium constant; values of 0.27 and 0.31×10^{10} M^{-1}, respectively, were obtained.

Chromatographic studies of hog GIF before and after protease exposure. Figure 4 shows that hog GIF-B_{12} complex before and after trypsin exposure displayed the same elution patterns on Sephadex G-200. Although not shown, chymotrypsin did not alter the elution pattern either.

Discontinuous disc electrophoresis of hog GIF-B_{12} complex before and after protease exposure. No differences could be detected in the electrophoretic mobility of the GIF-B_{12} complex before or after exposure to trypsin or chymotrypsin.

Double reciprocal plots of EDTA-inhibitable hog GIF-B_{12} complex binding to guinea pig ileal mucosal homogenates before and after treatment with proteases. Figure 5 demonstrates that the affinity constant (K_a) for hog GIF-B_{12} complex was 3.3×10^9 M^{-1} and for trypsin-treated complex, 7.7×10^9 M^{-1}; essentially no difference was demonstrable between the two preparations. Similarly, exposure to insoluble chymotrypsin preparations afforded an affinity constant for the hog GIF-B_{12} complex of 4.0×10^9 M^{-1}.

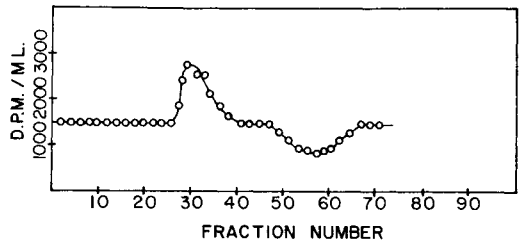

FIG. 3. Determination of the equilibrium constant for the binding of vitamin B_{12} to hog gastric intrinsic factor (GIF) by filtration over G-25 Sephadex.

Discussion

The vitamin B_{12} malabsorption associated with pancreatic dysfunction responds consistently to the administration of hog pancreatic extract or bovine trypsin.[1-3, 15] However, the exact mechanism whereby these proteases improve vitamin B_{12} absorption and where in the absorption pathway of the vitamin they act have not been previously defined.

There are many ways in which trypsin, chymotrypsin, or a contaminant in these protease preparations could improve the absorption of vitamin B_{12}: (1) the protease preparations could act directly on gastric intrinsic factor to modify the glycoprotein that would allow it to facilitate the absorption of vitamin B_{12} (2) these protease preparations could inactivate an inhibitor to vitamin B_{12} absorption that is present in gastric juice and preparations of hog GIF, (3) the proteases could inactivate an inhibitor to vitamin B_{12} absorption present in the secretions of the small intestine, or (4) these protease preparations could modify the receptor for GIF-B_{12} complex to allow attachment of the complex onto the intestinal receptor.

The present investigations in patients with vitamin B_{12} malabsorption associated with pancreatic exocrine insufficiency clearly demonstrate that preparations of three times crystallized bovine trypsin or chymotrypsin

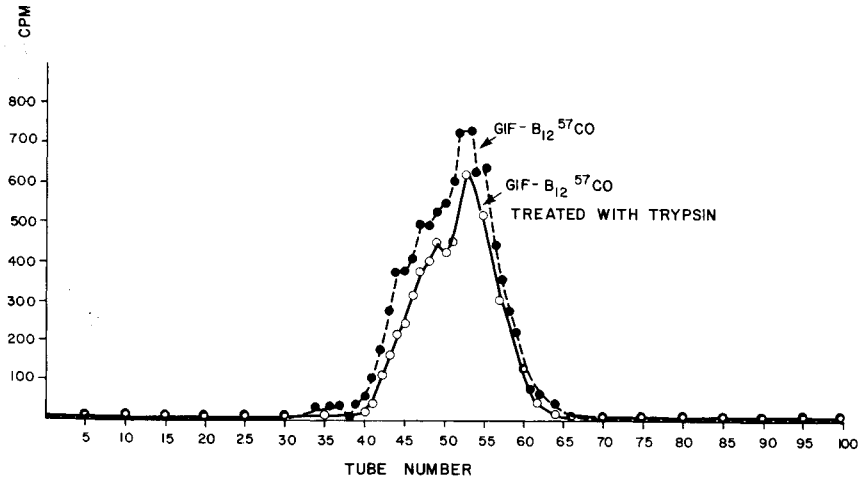

FIG. 4. Chromatography of hog gastric intrinsic factor (GIF) (before and after exposure to trypsin) complexed to labeled vitamin B_{12} on Sephadex G-200.

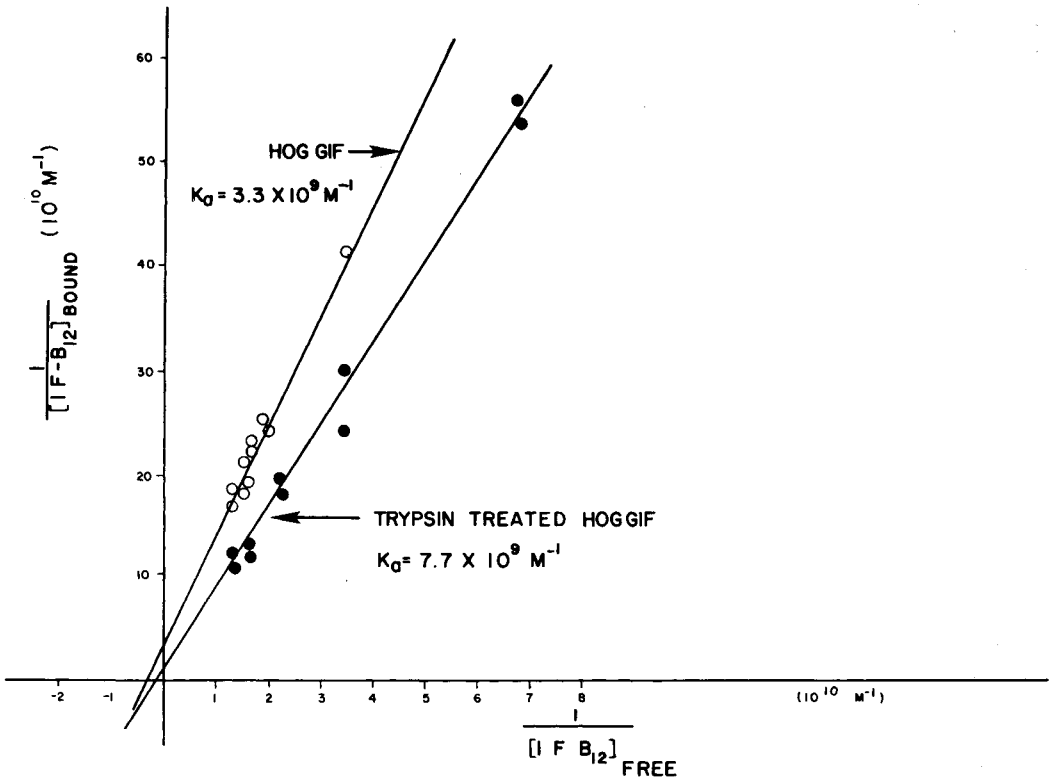

FIG. 5. Double reciprocal plots of ethylenediamenetetraacetic acid (EDTA)-inhibitable hog gastric intrinsic factor (GIF)-B_{12} complex binding to guinea pig ileal mucosal homogenates before and after exposure to trypsin.

modify crude sources of hog GIF or human gastric juice to allow these sources of GIF to promote normal vitamin B_{12} absorption.

It was fortuitous that we had the opportunity to study a patient with vitamin B_{12} malabsorption associated with both pernicious anemia and pancreatic exocrine insufficiency. This patient required the simultaneous exogenous administration of both GIF and pancreatic proteases before she was able to manifest normal vitamin B_{12} absorption. Crude sources of hog GIF incubated with insolubilized bovine trypsin were modified in some fashion so that when the GIF solution was separated from the trypsin preparation by centrifugation, the GIF preparation restored vitamin B_{12} absorption to normal levels. This same effect of both insolubilized trypsin and chymotrypsin preparations on crude sources of hog GIF was demonstrated in 3 patients with pancreatic exocrine insufficincy and vitamin B_{12} malabsorption. In addition, the gastric juices obtained from these 3 patients were complexed to vitamin B_{12} and administered back to the patients with no improvement in their capacity to absorb vitamin B_{12}. However, when aliquots of the same gastric juices were incubated with insolubilized protease preparation and the treated gastric juices were readministered to the patients, vitamin B_{12} absorption was markedly improved. Neither trypsin nor chymotrypsin could be detected after the insolubilized protease had been removed. Even when the amount of trypsin or chymotrypsin that could have theoretically been solubilized and not have been detected by our assays was administered orally to patients with vitamin B_{12} malabsorption and pancreatic insufficiency, the absorption of the vitamin was not improved. Thus these observations mitigate the possibility that retained trypsin or chymotrypsin in the GIF preparations or gastric juices was responsible for the improved absorption.

These striking observations that this pancreatic protease preparation improved vitamin B_{12} absorption by acting directly on crude GIF preparations and/or human gastric juices led us to examine where in the absorption pathway of this vitamin a need for pancreatic enzyme-treated GIF might exist.

We first compared hog GIF preparations before and after exposure to insolubilized protease preparations in respect to their equilibrium constants for vitamin B_{12} binding. No differences were detected between the preparations. These observations that the exposure to the proteases did not enhance vitamin B_{12} binding by hog GIF preparations confirmed our previous in vivo human studies, which demonstrated that complexing hog GIF preparations to vitamin B_{12} in vitro and administering this bound complex to patients with pancreatic exocrine insufficiency did not correct the vitamin B_{12} malabsorption,[2] ruling out a vitamin B_{12}-binding effect.

Our laboratory had previously shown that the intestinal receptor to GIF-B_{12} complex obtained from partially pancreatectomized rats with vitamin B_{12} malabsorption responded to GIF-mediated vitamin B_{12} uptake in a fashion comparable to the receptor isolated from control rats.[3] In addition (*unpublished observations*), we have

noted that the gastric juices from patients with vitamin B_{12} malabsorption associated with pancreatic dysfunction stimulate vitamin B_{12} uptake onto guinea pig ileal homogenates. However, the effect of pancreatic proteases on the attachment of the GIF-B_{12} complex to the ileal receptor had not been studied in a quantitative manner. In the present study we compared double reciprocal plots of EDTA-inhibitable hog GIF (before and after exposure to trypsin or chymotrypsin)-B_{12} concentrations. The affinity constant (K_a) for non-protease treated hog GIF-B_{12} complex was $3.3 \times 10^9\ M^{-1}$, for trypsin-treated complex $7.7 \times 10^9\ M^{-1}$, and for chymotrypsin-treated complex $4.0 \times 10^9\ M^{-1}$. We do not believe that the slight increase in the affinity for the ileal receptor demonstrated by the trypsin-treated GIF-B_{12} complex can account for the marked in vivo enhancement of vitamin B_{12} absorption by pancreatic protease preparations.

The present studies demonstrate that: (1) GIF obtained from hog pyloric mucosal extracts or as secreted by subjects with vitamin B_{12} malabsorption and pancreatic dysfunction is ineffective in enhancing vitamin B_{12} absorption in patients with pancreatic disease, (2) incubation of crude hog GIF preparations or gastric juices obtained from patients with pancreatic insufficiency and vitamin B_{12} malabsorption with an insolubilized protease preparation in vitro activates these preparations of GIF in an as yet undefined manner to allow them to be effective in correcting vitamin B_{12} malabsorption in subjects with pancreatic disease, and (3) in vitro studies using gut sacs or brush border preparations do not reflect the abnormality in vitamin B_{12} absorption secondary to pancreatic dysfunction.

These studies, in conjunction with previous observations from our laboratory, exonerate a defect in binding of vitamin B_{12} by GIF or an abnormality in the attachment of the GIF-B_{12} complex to the ileal receptor as the mechanism for the vitamin B_{12} malabsorption observed in patients with pancreatic disease. These studies also indicate that preparations of pancreatic proteases (trypsin, chymotrypsin, or a contaminant of these preparations) enhance vitamin B_{12} absorption by modifying crude sources of GIF in some fashion that cannot be detected by gel filtration or polyacrylamide electrophoresis. Because exposure of these sources GIF (hog pyloric mucosal extracts or human gastric juice) to the insolubilized protease preparations did not affect their capacity to bind vitamin B_{12} or their ability to attach to the ileal receptor, it is suggested that exposure to the protease preparations either prevents the inactivation of the GIF-B_{12} complex during its passage down the small intestine or facilitates the passage of the GIF-B_{12} complex through the ileal epithelial cell.

Studies using purified preparations of GIF may provide some of the answers needed to clarify fully these striking observations that treatment of crude sources of GIF with insolubilized protease preparations corrects vitamin B_{12} malabsorption in patients with pancreatic exocrine insufficiency.

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