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Vitamin B12-mediated uptake of erythropoietin and granulocyte colony stimulating factor in vitro and in vivo

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Abstract

Proteins are normally very poorly absorbed from the intestine as the intestinal epithelial cells act as a barrier to the uptake of all but the smallest of molecules. We have used the natural uptake mechanism for Vitamin B_{12} (VB_{12}) to increase the absorption of proteins from the gut. VB_{12} was conjugated to two recombinant human therapeutic proteins, granulocyte colony stimulating factor (G-CSF) and erythropoietin (EPO). In an in vitro model of VB_{12} uptake, high levels of VB_{12}-G-CSF conjugates were shown to be transported across the epithelial cell monolayer when compared to free G-CSF. In in vivo experiments in rats, both VB_{12}-EPO and VB_{12}-G-CSF conjugates were transported into the circulation from the gut lumen. Uptake was sufficient to generate a therapeutic response in animals receiving VB_{12}-G-CSF, but not in those administered G-CSF alone. These preliminary findings have important implications in the oral delivery of peptide and protein pharmaceuticals. Copyright © 1996 Elsevier Science B.V.

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1. Introduction

Despite numerous advances in the study and treatment of many diseases of man, which have been aided by the recent developments in genetic engineering, most protein drugs must still be given to patients *by injection*. Thus, the many millions of diabetics in the world must inject themselves with their daily dose of insulin. Similarly, haemophiliacs must be injected with the protein, Factor VIII, or the new protein drug erythropoi-

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Table 1
Structure and characteristics of VB_{12}-protein conjugates

Conjugate	Chemistry	IF binding, K_a (M^{-1})	% Retention protein bioactivity	
			In vitro	In vivo
GBC-1	'e'VB_{12}-CONH$(CH_2)_{12}$NHCO$(CH_2)_2$S-S-G-CSF	2.0×10^8	11.3	63.4[a]
GBC-2	'e'VB_{12}-CONH$(CH_2)_2$S-S$(CH_2)_2$NHCO-G-CSF	3.6×10^8	31	85[c]
GBC-3	eVB_{12}-CONHNHCO-G-CSF	8.9×10^7	48	89[c]
EBC-1	eVB_{12}-CONHNHCO(CH)CONHNHCO-EPO	6.7×10^8	n/d	17[b]

n/d, not determined. The percentage of protein bioactivity was determined by comparison to unmodified EPO or G-CSF in triplicate assays.
[a]Significantly different from G-CSF ($P < 0.01$)
[b]Significantly different from EPO ($P < 0.001$)
[c]Not significantly different from G-CSF.

etin (EPO). The injection of such drugs is often associated with considerable inconvenience, trauma and patient discomfort, particularly in children. The ability to administer protein and peptide pharmaceuticals orally would have significant advantages over injection, including increased patient comfort and compliance, as well as reduced medical costs. Unfortunately the majority of proteins, including insulin, EPO and Factor VIII, cannot be administered orally as the intestinal wall forms an almost impenetrable barrier excluding all but the smallest of molecules (Levine et al., 1980; Dix et al., 1990; Smith et al., 1992; Takada et al., 1994). In this report we describe the potential use of the intestinal uptake pathway for VB_{12} to breach the intestinal barrier. Vitamin B_{12} is a large water soluble molecule (molecular weight 1356) which cannot be absorbed directly from the intestine as it is too big to diffuse across the intestinal wall. Instead, vitamin B_{12} must first complex to a carrier protein, intrinsic factor, which is recognised by a receptor located on the intestinal cells lining the gut wall and is subsequently transported across the cell into the circulation. We present preliminary data to show that this uptake pathway can be used to co-transport proteins bound to the vitamin B_{12} from the intestine to the circulation following oral administration.

Vitamin B_{12}-mediated transport of proteins was studied using two recombinant human proteins: granulocyte colony stimulating factor (G-CSF), a 18 800 Da protein that induces the proliferation and release of neutrophilic granulocytes to the bloodstream (Morstyn and Burgess, 1988); and EPO, a 29 543 Da glycoprotein which stimulates the maturation of erythroid progenitor cells into mature erythrocytes (Krantz and Goldwasser, 1984).

2. Methods

2.1. Preparation of vitamin B_{12} conjugates

Three VB_{12}-G-CSF and one VB_{12}-EPO conjugate were prepared as described previously (Russell-Jones et al., 1995). The structure of the linkage and the intrinsic factor (IF) affinity of the conjugates is described in Table 1.

2.2. Caco-2 cell cultures

The Caco-2 cell line was grown on 25 mm diameter microporous membrane inserts, with a pore size of 0.45 μm and a density of 1.6×10^6 pores/cm^2 (Falcon Labware). After trypsinization with PBS containing 0.05% trypsin and 0.53 mM EDTA, cells were seeded on the inserts at a cell density of 1.8×10^5 cells/cm^2. Cells were cultured for 21 days in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum, 1% minimum essential medium (MEM) non-essential amino acids, 1000 U/ml penicillin,

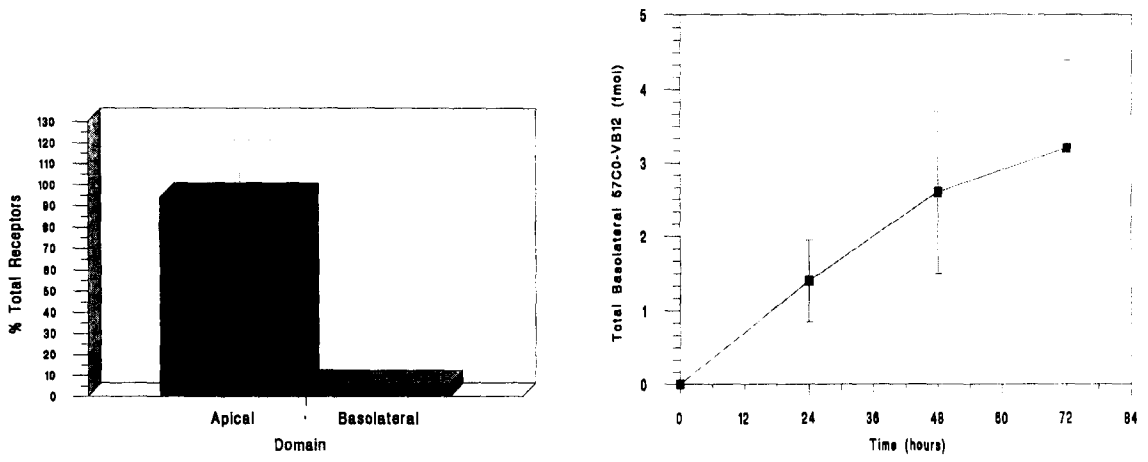

Fig. 1. (a) Distribution of the IF receptor in Caco-2 monolayers. Binding of IF was assessed after 6 h at 4°C, using IF-^{57}Co-VB_{12} complex. The percentage of total cell binding located in each membrane domain is shown; points are mean ± SEM ($n = 6$). (b) Transport of VB_{12} across fully polarized epithelial monolayers of the human enterocyte cell line, Caco-2. Specific transport of ^{57}Co-VB_{12} from the apical to the basolateral domain of the Caco-2 monolayers. Preformed IF-^{57}Co-VB_{12} complexes (100 pM or 250 fmol) were added to the apical chamber of confluent Caco-2 cell cultures. Cells were incubated for 72 h at 37°C with 250 fmol IF-^{57}Co-VB_{12}^2. Total transport of ^{57}Co-VB_{12} from the apical to basal chamber was assessed at 24, 48, and 72 h (Fig. 1b). Specific transcytosis was measured in the presence and absence of 10 nM cold IF-VB_{12}. Data presented represents specific transport (IF-^{57}CoVB_{12}) minus non-specific transport ([IF-^{57}CoVB_{12}] − [IF-VB_{12}]). Data are expressed as the mean ± SEM ($n = 6$).

100 μg/ml streptomycin and 0.3 mg/ml glutamine, at 37°C under a 5% CO_2 atmosphere. Confluent monolayers were tested for integrity and tight junction formation by measuring [^{14}C]-inulin diffusion and transepithelial electrical resistance (TEER) using an EVOM-F epithelial voltameter (World Precision Instruments, New Haven, CT). Inulin transport was monitored throughout the duration of each experiment and was compared to TEER measurements. Changes in TEER measurements corresponded with inulin transport and were used to determine the integrity of the cell monolayers and disruption of tight junctions. TEER values ranged from 400 to 500 Ω cm^2 throughout the study. All studies were conducted under sterile conditions.

2.2.1. Characterization of IF-mediated VB_{12}-binding to Caco-2 cells

The distribution of the IF-receptor was determined on fully polarized Caco-2 cell monolayers. Cells were incubated with IF-^{57}Co-VB_{12} complexes (250 fmol) as a solution in serum free DMEM with 1% BSA, and binding was assessed after 6 h incubation at 4°C. The % of total cell binding located on each membrane domain was then calculated (Fig. 1a).

2.2.2. Specific IF-mediated transport of ^{57}Co-VB_{12} across Caco-2 monolayers

Preformed IF-^{57}Co-VB_{12} complexes (100 pm or 250 fmol) were added to the apical chamber of confluent Caco-2 cell cultures. Total transport of ^{57}Co-VB_{12} from the apical to basal chamber was assessed at 24, 48, and 72 h (Fig. 1b). Data presented represents specific transport (IF-^{57}CoVB_{12}) minus non-specific transport ([IF-^{57}CoVB_{12}] − [IF-VB_{12}]).

2.2.3. Transport of VB_{12} G-CSF across the human enterocyte cell line, Caco-2

Samples of non-conjugated G-CSF, and VB_{12}-G-CSF conjugates (GBC-1, GBC-2, GBC-3), corresponding to 5.3 pmol of G-CSF, were added to the apical domain of Caco-2 cultures. The conjugates were preincubated overnight with a 12-fold molar excess of porcine intrinsic factor (Sigma, ILL) prior to addition to cultures. The apical and basal levels of G-CSF were determined after incubation for 24 h at 37°C (Fig. 2). Determination of

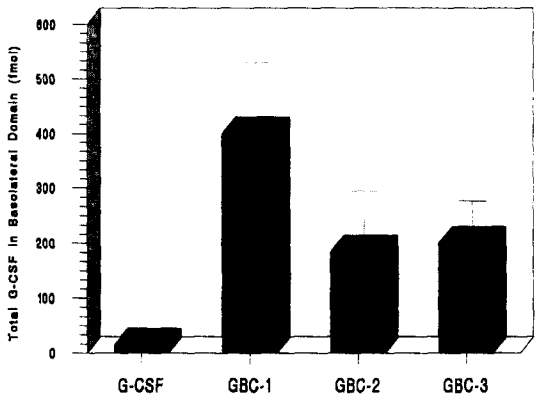

Fig. 2. Transport of VB_{12} G-CSF across the human enterocyte cell line, Caco-2. Levels of non-conjugated G-CSF, and VB_{12}-G-CSF conjugates (GBC-1, GBC-2, GBC-3) transported to the basal chamber of confluent, impermeable Caco-2 monolayers. Data are expressed as the mean ± SEM ($n = 3$).

VB_{12}-G-CSF conjugate transport was conducted with 5.3 pmol (2 nM) of the protein and between 4.4 and 6.6 pmol (1.76 and 2.64 nM) of the conjugated VB_{12}. Levels of transported G-CSF were determined in a G-CSF Elisa from R&D systems (Minneapolis, MN), deriving a standard curve for each conjugate as appropriate. Comparison of the basolateral levels of the conjugates to the non-conjugated G-CSF was carried out using unpaired Student's 2-tailed t-test, GBC-1 ($P < 0.01$), GBC-2 ($P < 0.1$), GBC-3 ($P < 0.05$).

2.3. Intraduodenal uptake of $Co^{57}VB_{12}$ or VB_{12}-G-CSF and VB_{12}-EPO complexes

Male Sprague Dawley rats (250 g) were given ^{57}Co-VB_{12} (20 ng and 6.6×10^5 DPM), in 220 μl PBS with 0.1% BSA (IF, VB_{12}, free) (Sigma, St. Louis, MO). For intraduodenal dosing, minor surgery was performed (Seetharam et al., 1985). Rats were starved for approximately 18 h prior to surgery. Under Nembutol anaesthesia (50 mg/kg), a 3–4 cm midline incision was made, and a 2 mm incision was then made in the exposed duodenum 1 cm from the pylorus. A 10 cm silastic catheter was advanced 8 cm into the duodenum which was secured in place by a purse string suture. For bolus dosing into the duodenum, a 1 cm^3 syringe was connected to the end of the catheter and sample was administered. The catheter was withdrawn and the suture closed tightly. For infusion dosing, the sample was placed in an Alzet™ mini-osmotic pump, Model 2001D (Alza, Palo Alto, CA) infusing 9 μl/h for 24 h. The pump was then attached to the free end of the catheter and placed in the peritoneal cavity. The abdominal muscle was closed with a running silk suture and the skin incision was closed with wound clips. Rats recovered from the anaesthesia on a warming pad. For all studies, the rats were housed in metabolic cages and allowed food and water ad libitum. The amount of VB_{12} absorbed was determined from counting total ^{57}Co measured in the faeces over a 48 h period (Fig. 3).

2.3.1. Plasma levels of EPO in rats after intraduodenal infusion of non-conjugated EPO (EPO) and VB_{12} conjugated EPO (EBC-1)

Alzet™ mini-osmotic pumps were surgically implanted into rats and were primed to deliver various formulations. Over a 24 h period rats received (i) vehicle (EPO formulation buffer, 200 mM sodium citrate, pH 7.0, 100 mM NaCl), (ii) non-conjugated EPO in vehicle delivered at 21 units/h (a total of 12.6 μg/kg (1500 units/kg) of EPO over 24 h), (iii) EBC-1 infused at 175 ng/h (equivalent to 21 units EPO per hour); conjugated VB_{12} was delivered at 8 ng/h. Blood samples (500 μl) were

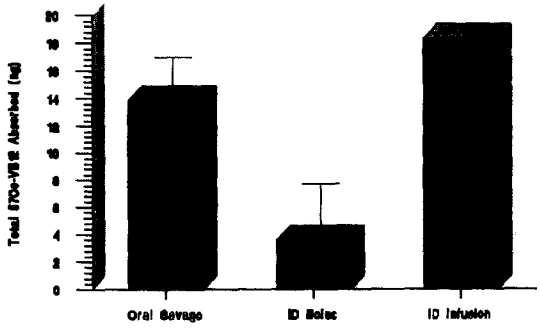

Fig. 3. Validation of the in vivo model for the intraduodenal infusion of the VB_{12}-GCSF conjugates using ^{57}Co-labelled VB_{12}. A comparison was made of $^{57}CoVB_{12}$ uptake after administration of a 20 ng dose of $^{57}CoVB_{12}$ to the gut by three different routes: oral gavage, intraduodenal bolus and intraduodenal infusion over 24 h. The data are the mean ± SEM ($n = 4$).

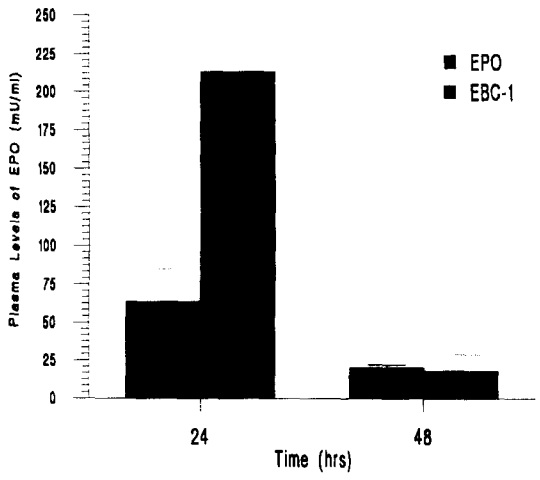

Fig. 4. Plasma levels of EPO in rats after intraduodenal infusion of non-conjugated EPO (EPO) and VB_{12} conjugated EPO (EBC-1). The data are expressed as the mean ± SEM ($n = 4$). Rats received vehicle alone, non-conjugated EPO in vehicle, or VB_{12}-EPO conjugate in vehicle. No detectable EPO could be measured in the serum of animals receiving vehicle alone (data not shown).

taken via the tail artery, the hematocrit determined and plasma samples collected by centrifugation at 12000 rpm (11750 × *g*) for 15 min. Plasma levels of EPO were determined using the EPO ELISA from R&D Systems. Standard curves were established using EPO or EBC-1 as appropriate (Fig. 4).

2.3.2. Plasma levels of G-CSF and total white blood cell levels following intraduodenal infusion of VB_{12}-G-CSF conjugate

Alzet™ mini-osmotic pumps were surgically implanted into rats in such a way as to deliver a constant infusion of non-conjugated G-CSF and GBC-1 over 24 h. Plasma levels of the protein therapeutic (assayed using the R&D Systems G-CSF ELISA) and total white blood cell levels in the rats were determined (Fig. 5).

2.4. Determination of the IF binding affinity of VB_{12} conjugates

IF binding affinity of the VB_{12} conjugates was determined by a competitive binding analysis with porcine IF and ^{57}Co-VB_{12} (Mathan et al., 1974). Non-conjugated VB_{12} had a K_a of $6.0 \pm 0.9 \times 10^9$.

2.5. Bioactivity of VB_{12}-conjugates

In vitro bioassay of the conjugated G-CSF was determined by measuring the stimulation of mitogenesis in mouse bone marrow cells using [^{3}H]-thymidine (Jensen-Pippo et al., 1995). The in vivo bioassay of VB_{12} conjugated G-CSF was determined by measuring the total white blood cell elevation in hamsters, in response to a subcutaneous administration of the conjugate (Jensen-Pippo et al., 1995). In vivo bioactivity of the VB_{12}-EPO conjugates was determined by measuring the stimulation of erythrocyte production in exhypoxic polycythemic mice using ^{59}Fe incorporation as the marker (Cotes and Bangham, 1961). The bioassay results are reported as percent retention of bioactivity compared to the non-conjugated protein (Table 1). Data is presented as the mean of three measurements performed by the Quality Assurance Analytical group at Amgen. Conjugates of VB_{12} and recombinant protein were chosen for intraduodenal administration based upon their ability to bind the VB_{12} transporting protein, intrinsic factor, and the retention of bioactivity of the conjugated protein.

3. Results and discussion

Proteins must be chemically linked to vitamin B_{12} (VB_{12}) transport system to be able to carry proteins from the intestine to the circulation. A total of three VB_{12}-G-CSF conjugates (GBC1–3) and one VB_{12}-EPO conjugate (EBC-1) were prepared (Russell-Jones et al., 1995). Prior to in vitro and in vivo testing for vitamin B_{12}-mediated transport, the VB12 conjugates were analysed to determine if they had retained both their ability to bind to intrinsic factor (IF) (an essential requirement for uptake; see above), and their functional activity (bioactivity) (see Table 1). Conjugates formed between G-CSF and VB_{12} were still able to bind to IF, although with a lower affinity than native VB_{12} (compare values for K_a of 2.0×10^8, 3.6×10^8, 8.9×10^7, and 6.7×10^8 for GBC-1, 2,

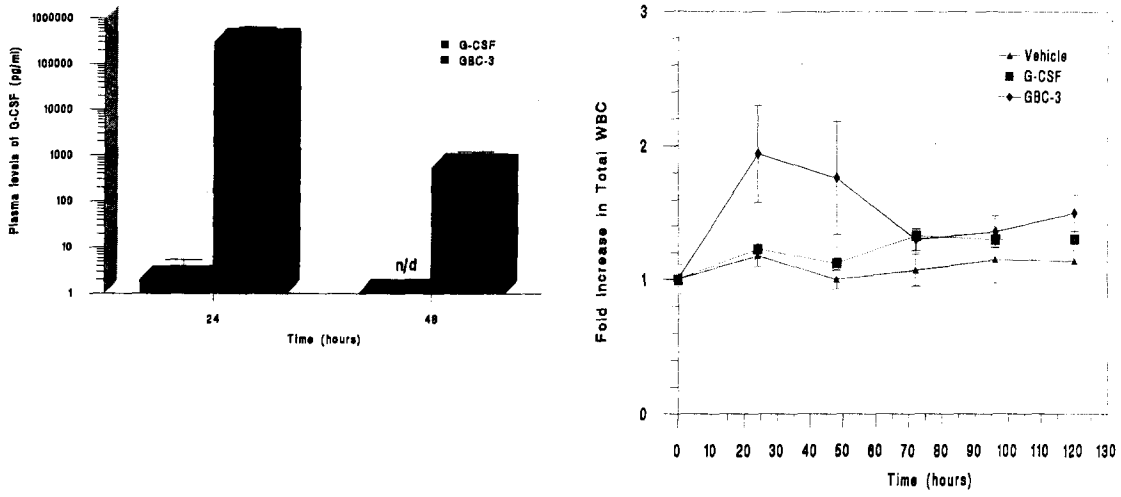

Fig. 5. Plasma levels of G-CSF and total white blood cell levels following intraduodenal infusion of VB_{12}-G-CSF conjugate. (a) Plasma levels of G-CSF were determined for rats receiving G-CSF or GBC-3 by intraduodenal pump infusion. G-CSF levels were determined by ELISA. The data are the mean ± SEM ($n = 4$). n/d non-detectable levels. (b) Increase in white blood cell numbers in rats receiving various solutions intraduodenally over 24 h. Groups were: (i) Vehicle (distilled water (DW), pH 3.25 with HCl) (▲), (ii) G-CSF control, non-conjugated G-CSF in vehicle, dosed at 360 μg/kg (■), (iii) GBC-3 in vehicle, dosed at 360 μg/kg (◆). The pump infusion into the duodenum (9 μl/h for 24 h) was as described for Fig. 3. Blood samples were taken via the tail artery. Data are normalised to show the fold increase relative to the starting WBC levels for each individual animal. The white blood cell determination was with a Sysmex F-800 microcell counter (Baxter, Irvine, CA). The data are the mean ± SEM ($n = 4$).

3, and EBC-1, respectively, with a K_a value of 6.0×10^9 for native VB_{12}). The VB_{12}-G-CSF conjugates exhibited a slightly decreased bioactivity in vitro, but were found to have good in vivo bioactivity when tested subcutaneously in hamsters (Table 1).

Before testing the VB_{12}-mediated transport of the VB_{12}-G-CSF and VB_{12}-EPO complexes in vivo, transport was assessed in an in vitro cell model. A number of human and animal cell lines exist which, when grown in vitro, show polarized (unidirectional) IF-dependent transport of VB_{12} across the cell (Hidalgo et al., 1989; Wilson et al., 1990). One of these, the Caco-2 cell line, a colon cancer cell line, shares similarities with the human small intestinal cell (enterocyte; Hidalgo et al., 1989; Le Bivic et al., 1990; Wilson et al., 1990; Rubas et al., 1993). Under the incubation conditions described above, these cells exhibited a high degree of polarity in the distribution of VB_{12}-IF receptor (IF-R) which was predominantly exposed on the apical or lumenal domain of the cells (Fig. 1a). The IF-R was almost undetectable on the basolateral or serosal side of the cells, as would be expected considering that IF-R binds IF-VB_{12}, which is only present in the lumen of the gut. The complete polarity observed in our studies differs from some other Caco-2 systems (Dan and Cutler, 1994), where mixing of the IF-R between the apical and basal domains suggested incomplete polarity of the filter-grown cells. These cells were found to transport VB_{12} complexed to IF from their apical surface (the lumenal side of the cell) to their basal surface (the serosal side of the cell). Transcytosis was specific, and occurred at a rate of 0.05 fmol/h (Fig. 1b). This system was selected as an in vitro model for studies on the VB_{12}-mediated transport of VB_{12}-G-CSF conjugates across the human enterocyte. Transcytosis was specific and occurred without loss of monolayer integrity as demonstrated by ^{14}C-inulin diffusion and TEER (data not shown).

When free G-CSF was added to the apical chamber of Caco-2 cell cultures, only very low levels of G-CSF were found to cross the monolayer. After 24 h of incubation (Fig. 2) only

14.13 ± 4.4 fmol, from a total of 5.3 pmol G-CSF (0.27%) added to the apical chamber, had crossed to the basolateral domain. In contrast, when VB_{12}-GCSF conjugates were added to the apical chamber in the presence of IF, a total of 398 ± 112 fmol, 187 ± 89 fmol and 200 ± 59 fmol of the 5.3 pmol of G-CSF added as VB_{12}-conjugates GBC-1, GBC-2 and GBC-3 respectively, were transported to the basolateral domain over the same period. This represents VB_{12}-mediated transport of 7.5%, 3.5% and 3.8% of the added G-CSF (for GBC-1, 2 and 3 respectively) over 24 h of culture.

Following the successful demonstration of VB_{12}-mediated transport of G-CSF in vitro, the potential of the VB_{12}-delivery system to increase the bioavailability of a protein therapeutic administered via the oral route was examined in vivo. In order to avoid the hostile environment of the stomach (i.e., proteolysis by pepsin, etc.), conjugates were administered by infusion into the duodenum. Comparable methods for the delivery of therapeutics beyond the stomach are currently used clinically (Marshall, 1979). Validation of the in vivo, intraduodenal (id) model with ^{57}Co-VB_{12}, showed that the id infusion method compared favourably with oral gavage feeding in terms of uptake (Fig. 3).

The therapeutic activity of EPO (Egrie et al., 1986) is normally assessed by the increase in total red blood cell numbers (hematocrit). The one-time dosing regimen of EBC-1 that was used in these studies was unlikely to increase hematocrit, so this method could not be used to demonstrate bioactivity. Serum EPO was therefore measured by an EPO ELISA. The quantity of EPO reaching the systemic circulation (following 24 h of id infusion) was four-fold greater when administered as a VB_{12} conjugate than that seen for non-conjugated EPO (compare 57 ± 22.4 mU/ml for EPO and 213 ± 33 mU/ml for EBC-1) ($P < 0.05$) (Fig. 4). 24 h after cessation of the id infusion there was no measurable difference in the plasma levels of animals receiving EPO or EBC-1. Although hematocrit levels were monitored, there was no measurable elevation, despite a four-fold increase in plasma levels of the protein after intraduodenal infusion of EBC-1 as compared to EPO ($P < 0.05$ by Student's t-test).

More pronounced effects were seen following the intraduodenal infusion of the VB_{12}-G-CSF conjugate, GBC-3 (Fig. 5a). Infusion of GBC-3 for 24 h into the duodenum, resulted in highly elevated plasma levels of the protein (299 ± 160 ng/ml) as compared to non-conjugated G-CSF (1.94 ± 1.94 pg/ml). Plasma levels of the therapeutic in the animals receiving GBC-3 were still elevated at 48 h (554 ± 308 pg/ml), compared to non-detectable levels for non-conjugated G-CSF. As expected the total white blood cell numbers were not affected in the group receiving non-conjugated G-CSF (Fig. 5b), but a two-fold increase in total white blood cell number was seen in the GBC-3 group at 24 h ($P < 0.05$ for comparison of GBC-3 with vehicle, and for the comparison of GBC-3 with rhG-CSF). The data indicate that when G-CSF is conjugated to VB_{12} it can be systemically delivered from the gut to the circulation via the VB_{12} uptake pathway. The material which reached the circulation was found to be biologically active as it stimulated an increase in the numbers of white blood cells.

Apart from enhancing uptake of G-CSF, conjugation of G-CSF to VB_{12} also appeared to reduce the susceptibility of G-CSF to cleavage by intestinal proteases such as chymotrypsin and trypsin (Jensen-Pippo et al., 1995). It is possible that conjugation of VB_{12} to G-CSF sterically prevented the protease from binding to the G-CSF. Binding of IF (45 000 Da) to the conjugated VB_{12} would further protect the protein from proteolysis.

Large molecular weight proteins and peptides normally have very low or non-detectable bioavailability from the oral route (Takada et al., 1994). This is due not only to the hostile environment in the gut but also to the barrier effect of the enterocyte lining (Smith et al., 1992). The results of our studies suggest that when therapeutic proteins (such as EPO or G-CSF) are conjugated to VB_{12} the uptake of these proteins is greatly increased. It was found that the limited uptake capacity for VB_{12} seen with single bolus oral administration of VB_{12} (1–4 nmol per feed in humans, or 30–100 pmol per feed in rats) could be overcome by chronic infusion of the VB_{12}-G-CSF and VB_{12}-EPO conjugates. These findings should have a significant impact on the potential route of

delivery of these pharmaceuticals, and may provide a means for replacing the current method of injection of patients with the much more preferable oral administration of these materials.

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References

Cotes, P.M. and Bangham, D.R., Bio-assay of erythropoeitin in mice made polycythaemic by exposure to air at a reduced pressure. *Nature*, 4793 (1961) 1065–1067.

Dan, N. and Cutler, D.F., Transcytosis and processing of intrinsic factor-cobalamin in Caco-2 cells. *J. Biol. Chem.*, 269 (1994) 18849–18855.

Dix, D.J., Hassan, I.F., Obray, H.Y., Shah, R. and Wilson, G., The transport of Vitamin B_{12} through polarized monolayers of Caco-2 cells. *Gastroenterology*, 88 (1990) 1272–1279.

Egrie, J.C., Strickland, T.W., Lane, J., Aoki, K., Cohen, A.M., Smalling, R., Trail, G., Lin, F.K., Browne, J.K. and Hines, D.K., Characterization and biological effects of recombinant human erythropoietin. *Immunobiology*, 172 (1986) 213–224.

Hidalgo, I.J., Raub, T.J and Borchardt, R.T., Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology*, 96 (1989) 736–749.

Jensen-Pippo, K.E., Whitcomb, K.L., DePrince, R.B., Ralph, L. and Habberfield, A.D., Enteral bioavailability of human granulocyte colony stimulating factor conjugated with poly(ethylene glycol). *Pharm. Res.*, 13 (1996) 102–107.

Krantz, S.B. and Goldwasser, E., Specific binding of erythropoietin to spleen cells infected with the anaemia strain of Friend virus. *Proc. Natl. Acad. Sci. USA*, 81 (1984) 7574–7578.

Le Bivic, A., Quaroni, S., Nichols, B, and Rodriguez-Boulan, E., Biogenetic pathways of plasma membrane proteins in Caco-2, a human intestinal epithelial cell line. *J. Cell Biol.*, 111 (1990) 1351–1361.

Levine, J.S., Nakane, P.K. and Allen, R.H., Immunocytochemical localisation of human intrinsic factor in: the non-stimulated stomach. *Gastroenterology*, 79 (1980) 493–502.

Marshall, K., in G.S. Banker and C.T. Rhodes (Eds.), *Modern Pharmaceutics*, 1979, p. 10, Marcel Dekker, New York.

Mathan, V.I., Babior, B.M. and Donaldson, R.M. Jr., Kinetics of the attachment of intrinsic factor-bound cobamides to ileal receptors. *J. Clinical Invest.*, 54 (1974) 598–608.

Morstyn, G. and Burgess, A.W., Haematopoietic growth factors: a review. *Cancer Res.*, 48 (1988) 5624–5637.

Rubas, W., Jezyk, N. and Grass, G.M., Comparison of the permeability characteristics of a human colonic epithelial (Caco-2) cell line to colon of rabbit, monkey, and dog intestine and human drug absorption. *Pharm. Res.*, 10 (1993) 113–118.

Russell-Jones, G.J., Westwood, S.W. and Habberfield, A.D., Vitamin B_{12} mediated oral delivery systems for Granulocyte-Colony Stimulating Factor and erythropoietin. *Bioconjugate Chemistry*, 6 (1995) 459–465.

Seetharam, B., Presti, M., Frank, B., Tiruppathi, C. and Alpers, D.H., Intestinal uptake and release of cobalamin complexed with rat intrinsic factor. *Am. J. Physiol.*, 248 (1985) G326–G331.

Smith, P.L., Wall, D.A., Gochocco, C.H. and Wilson, G., Routes of delivery: Case studies: Oral absorption of peptides and proteins. *Adv. Drug Delivery Reviews*, 8 (1992) 253–290.

Takada, K., Nakahigashi, Y.T.T., Kishimoto, S., Ushirogawa, Y. and Takaya, T., Pharmacological activity of tablets containing recombinant human granulocyte colony-stimulating factor (rhG-CSF) in rats. *Int. J. Pharm.*, 101 (1994) 89–96.

Wilson, G., Hassan, I.F., Dix, C.J., Williamson, I., Shah, R., Mackay, M. and Artusson, P., Transport and permeability of human Caco-2 cells: An in vitro model of the intestinal epithelial cell barrier. *J. Control. Rel.*, 11 (1990) 25–40.