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Journal of Cellular Biochemistry 115:1948–1954 (2014)

Journal of Cellular Biochemistry

# Transcobalamin Derived From Bovine Milk Stimulates Apical Uptake of Vitamin B12 Into Human Intestinal Epithelial Cells

Brad Hine,[1] Irina Boggs,[2] Ralph Green,[3] Joshua W. Miller,[3,4] Russell C. Hovey,[5] Rex Humphrey,[2] and Thomas T. Wheeler[2*]

[1]*CSIRO, Animal, Food & Health Sciences, Armidale, NSW 2350, Australia*
[2]*Dairy Foods, AgResearch, Ruakura Research Centre, Hamilton, New Zealand*
[3]*Department of Pathology and Laboratory Medicine, UC Davis Medical Center, Sacramento, California*
[4]*Department of Nutritional Sciences, Rutgers University, New Brunswick, New Jersey 08901*
[5]*Department of Animal Science, University of California, Davis, California*

## ABSTRACT

Intestinal uptake of vitamin B12 (hereafter B12) is impaired in a significant proportion of the human population. This impairment is due to inherited or acquired defects in the expression or function of proteins involved in the binding of diet-derived B12 and its uptake into intestinal cells. Bovine milk is an abundant source of bioavailable B12 wherein it is complexed with transcobalamin. In humans, transcobalamin functions primarily as a circulatory protein, which binds B12 following its absorption and delivers it to peripheral tissues via its cognate receptor, CD320. In the current study, the transcobalamin-B12 complex was purified from cows' milk and its ability to stimulate uptake of B12 into cultured bovine, mouse and human cell lines was assessed. Bovine milk-derived transcobalamin-B12 complex was absorbed by all cell types tested, suggesting that the uptake mechanism is conserved across species. Furthermore, the complex stimulated the uptake of B12 via the apical surface of differentiated Caco2 human intestinal epithelial cells. These findings suggest the presence of an alternative transcobalamin-mediated uptake pathway for B12 in the human intestine other than that mediated by the gastric glycoprotein, intrinsic factor. Our findings highlight the potential for transcobalamin-B12 complex derived from bovine milk to be used as a natural bioavailable alternative to orally administered free B12 to overcome B12 malabsorption. J. Cell. Biochem. 115: 1948–1954, 2014. 

**KEY WORDS:** MALABSORPTION; Caco2; COBALAMIN; MILK

Vitamin B12 (cobalamin, B12) is a cofactor required for two enzyme-mediated reactions in nucleic acid, amino acid and fatty acid metabolism, catalyzed by methionine synthase and methyl-malonyl-CoA mutase [Green and Miller, 2014]. Deficiency of B12 leads to a range of health issues [reviewed in Oh and Brown, 2003; Stabler and Allen, 2004]. Segments of the population, particularly the elderly, have low serum B12 levels [Allen, 1994; O'Leary et al., 2011], mostly due to malabsorption of B12, rather than as the result of a dietary deficiency.

Malabsorption of B12 is due to defects in the mechanisms by which B12 is taken up by the intestine [Kozyraki and Cases, 2013]. Current widely-available options for treating the consequences of B12 deficiencies caused by malabsorption are either regular, usually monthly intramuscular injections or very large daily oral doses of B12 (1 mg, compared with the recommended daily intake of 2.4 μg). Neither of these options is ideal. Daily oral B12 requires a high degree of compliance while intramuscular injections are often painful and inconvenient. Thus there is a need for alternative therapeutic options for effective delivery of vitamin B12 to susceptible populations.

Binding proteins play important roles in the uptake of vitamin B12 (see Kozyraki and Cases [2013], Green and Miller [2014] for recent reviews). Haptocorrin, which is present in saliva as well as a range of mucosal secretions, binds dietary B12 within the gastrointestinal tract. After B12 is released from haptocorrin as a

The authors declared that they have no conflicts of interest.
Grant sponsor: New Zealand Ministry of Business, Innovation and Employment; Grant number: C10×0707.
*Correspondence to: Thomas T. Wheeler, Dairy Foods AgResearch, Ruakura Research Centre, Hamilton, New Zealand.
E-mail: tom.wheeler@agresearch.co.nz
Manuscript Received: 24 April 2014; Manuscript Accepted: 2 June 2014
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 10 June 2014
DOI 10.1002/jcb.24866 • © 2014 Wiley Periodicals, Inc.

result of high pH and the action of pancreatic proteases, it is bound by intrinsic factor (IF), a glycoprotein produced in the stomach, and absorbed into intestinal epithelial cells through a receptor-mediated process that involves the protein, cubilin, present on the apical surface epithelial cells in the terminal ileum [Moestrup et al., 1998]. Two additional membrane proteins, amnionless and megalin, also play a role in the uptake of the IF-B12 complex by interacting with the cubilin-IF-B12 complex and facilitating its internalization [Kozyraki et al., 2001; Strope et al., 2004]. Following internalization in intestinal cells, at least some of the B12 leaves the cell via the ABC1 transporter before being bound to transcobalamin in the blood [Beedholm-Ebsen et al., 2010]. The transcobalamin-B12 complex is then transported via the bloodstream to peripheral tissues. Uptake of the B12-transcobalamin complex by peripheral tissues is facilitated by CD320, which is present on the surface of cells. While these processes reflect the accepted route for B12 absorption and transport, there is some evidence that transcobalamin can also facilitate uptake of B12 from the intestine. This non-canonical role for transcobalamin is suggested by earlier findings that transcobalamin purified from rabbit plasma relieved vitamin B12 malabsorption in a child with non-functional transcobalamin when it was introduced into the duodenum via an enteric tube [Barshop et al., 1990]. Consistent with this finding, the transcobalamin receptor, CD320, is present on the apical surface of intestinal epithelial cells [Bose et al., 1997]. However, the presence of transcobalamin in the intestinal lumen has not been reported.

Cows' milk contains approximately 3–4 μg/L of vitamin B12 [Watanabe, 2007], and as such represents an abundant source of vitamin B12 in a readily-available form [Russell et al., 2001]. Transcobalamin appears to be the major vitamin B12 binding protein in cows' milk [Fedosov et al., 1996], whereas the major B12 binding protein in human milk is haptocorrin/R binder [Burger and Allen, 1974; Trugo and Sardinha, 1994]. Based on the evidence that transcobalamin from one heterologous species (rabbit) facilitated the absorption of B12 in humans [Barshop et al., 1990], we herein investigated whether transcobalamin derived from cows' milk could stimulate B12 uptake in cultured bovine, mouse and human cells, including polarized human intestinal epithelial cells. We find that addition of B12 to mouse and human cells in culture in the form of a complex with bovine transcobalamin-B12 results in a greater uptake than B12 in an unbound form. Furthermore, this enhanced uptake occurs via the apical membrane of differentiated human intestinal epithelial cells.

## MATERIALS AND METHODS

### PURIFICATION OF TRANSCOBALAMIN

Transcobalamin was purified by passing pasteurized skim milk (obtained from Tatua Dairy Co-Operative Ltd, Morrinsville, New Zealand) over a cation exchange column (SP Sepharose Fast Flow, GE Healthcare) and eluting the basic proteins with a linear gradient of 0–1 M NaCl. Fractions enriched for transcobalamin, as determined by Western blotting, were then pooled and subjected to ultrafiltration against 50 mM phosphate buffer, pH 7 to concentrate the eluate and remove salt. This fraction was subjected to heparin sepharose chromatography with elution using a 0–1.1 M NaCl gradient. The fractions enriched for transcobalamin were then subjected to a third round of chromatography on Source 30S media. A final round of purification was performed by HPLC using a MonoS column. The identity of the major 43 kDa band as well as a 15 kDa trace contaminant were identified by excising the protein bands, digesting them with trypsin, and subjecting the extracted peptides to LC-tandem mass spectrometry as previously described [Smolenski et al., 2007]. The protein content of the final preparation was determined using a Direct Detect Infra-red spectrophotometer (Merck Millipore, Merck KGoA, Darmstadt, Germany). The B12 content of the transcobalamin preparations was determined by electrochemiluminescence immunoassay (ECLIA) as performed by New Zealand Veterinary Pathology (Hamilton, New Zealand) using a commercially supplied kit (Cobas-Roche, Burgess Hill, UK).

### PREPARATION OF ANTI-TRANSCOBALAMIN ANTIBODIES AND WESTERN BLOTTING

Rabbit polyclonal antibodies were raised against chemically-synthesized peptides representing regions of bovine IF, transcobalamin and haptocorrin by AusPep (Melbourne, Australia). The IF peptide, LSQGAGSHVVQNG, represents amino acids 411–423 of bovine IF (GenBank accession number XP_873306). The transcobalamin peptide, SQLKRFLEDEKRAIGH, represents amino acids 129–144 of bovine transcobalamin (NP_776620), a region that is identical, except for one amino acid, with its human orthologue. The haptocorrin peptide, DLTYWQFLSGKTPLIQ, represents amino acids 383–397 of bovine haptocorrin/R binder (XP_873322). Each of the peptides was coupled to keyhole limpet hemocyanin and injected into rabbits (single peptide per rabbit). Following immunization, IgG was purified from the serum as previously described [Haigh et al., 2008]. Each peptide was also coupled to ovalbumin to provide a positive control in Western blotting. Western blotting confirmed that each IgG had high specificity for its target (data not shown). The transcobalamin antibody was capable of detecting as little as 6 ng of purified transcobalamin by Western blotting. Subsequent Western blotting was performed as previously described [Broadhurst et al., 2005] using 0.43, 0.44, and 0.29 μg/ml of anti-IF, -transcobalamin, and -haptocorrin IgG, respectively. The signal was visualized using goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma) as the secondary antibody, and enhanced chemiluminescence.

### CELL CULTURE

The bovine MAC-T mammary epithelial cell line was a kind gift from Jeffrey D Turner, McGill University, Quebec. The human HeLa cervical carcinoma cell line and the mouse C127 mammary fibroblast cell line were each obtained from the American Type Culture Collection (ATCC, Manassas, VA). HeLa, MAC-T and C127 cells were cultured in DMEM supplemented with 10% fetal bovine serum (SAFC Biosciences, Lenexa, KS) and 1% penicillin/streptomycin (Gibco Life Technologies, Carlsbad, CA) (plus 1 μg/ml hydrocortisone for MAC-T) in six-well tissue-culture treated plates (Nunc, ThermoFischer Scientific, Roskilde, Denmark). Medium was changed every 2–3 days, and experiments were performed 1–2 days after the cells reached confluence.

Caco-2 cells (obtained as passage 35 from ATCC) were seeded onto transwell polycarbonate inserts (20 mm × 25 mm, pore size 0.4 μm, Nalgene Nunc, Penfield, NY) within six well culture plates. Cells were cultured in 1 ml of DMEM containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% non-essential amino acids (Gibco Life Technologies) that was added to inserts and incubated at 37°C and 5% $CO_2$. A further 1 ml of medium was added to the outside of the insert in each well. Medium was changed every 2–3 days. Experiments were performed after the cells had differentiated and polarized, as monitored and confirmed by measuring the transepithelial electrical resistance (TER) using an epithelial voltohmmeter (World Precision Instruments, Inc., Sarasota, FL). TER increased from 200 to 1,000 ohms between day 1 and days 14–21 post-confluence. Caco-2 cells were used for uptake experiments when the TER reached 1,000 ohms, at which time they were deemed to be fully differentiated. Differentiation was further confirmed by Western blotting using antibodies directed against two differentiation markers, villin (Cell Signalling Technology, Danvers, MA) and Muc2 (Pierce, Rockford, IL) (data not shown).

All cell culture experiments were performed a minimum of two times with each condition with an experiment assessed using a minimum of triplicate wells per condition. The results presented as the means ± standard error.

### VITAMIN B12 UPTAKE ASSAY

For experiments with MAC-T, HeLa, and C127 cells, free cyanocobalamin (vitamin B12) (Sigma) or B12 in the form of a transcobalamin-B12 complex was added to the culture medium at concentrations ranging from 1 to 9 ng of B12/ml. Cells were cultured in the presence of B12 for 16 h, then washed three times with phosphate buffered saline (PBS). The cells were then removed by trypsin treatment, centrifuged at 220 *g* and washed twice with PBS. The cells were resuspended in 0.3 ml PBS and chilled on ice for 30 min prior to lysis by sonication on ice using a probe (Vibra Cell, Sonics & Materials, Inc., Danbury, CT) in three 10 s bursts. Cell lysates were centrifuged at 800*g* for 20 min and the protein concentration and vitamin B12 content of the supernatant determined using a Bradford assay kit (Bio-Rad, Hercules, CA).

For differentiated Caco-2 cells, B12 was added in concentrations ranging from 0 to 40 ng/ml. The B12 was added either to the apical side of the polarized epithelial cell layer (on top of the inserts) or the basolateral side (in the bottom of the well). The vitamin B12 was added either in the form of free B12, IF-B12 complex, or transcobalmin-B12 complex. The IF-B12 complex was prepared by pre-incubating a 1:1 molar ratio of human recombinant IF and B12 (Cobento Biotech, Aarhus, Denmark) for 1 h at room temperature. The transcobalamin-B12 complex was the pre-existing complex isolated from bovine milk. The Caco-2 cells were incubated and processed as described above.

Statistical significance of differences between the conditions was determined by the Students *t*-test, using the "TTEST" function in Excel.

## RESULTS

### VITAMIN B12-BINDING PROTEINS IN BOVINE MILK

We first sought to determine which B12 binding proteins are present in cows' milk. Western blotting of whole milk and several subfractions using antibodies directed against bovine IF, transcobalamin and haptocorrin revealed that only transcobalamin could be detected (Fig. 1). This result is consistent with an earlier report suggesting that transcobalamin is the main vitamin B12 binding protein present in bovine milk [Fedosov et al., 1996]. Further, these results demonstrate that the transcobalamin is present in a fraction enriched for basic proteins by cation-exchange chromatography.

### PURIFICATION AND B12-BINDING ACTIVITY OF TRANSCOBALAMIN FROM BOVINE MILK

A total of 1.6 mg of transcobalamin was prepared from 54 L of pasteurized milk using four successive rounds of chromatography (Fig. 2). The purity of the preparations was estimated to be up to 97%

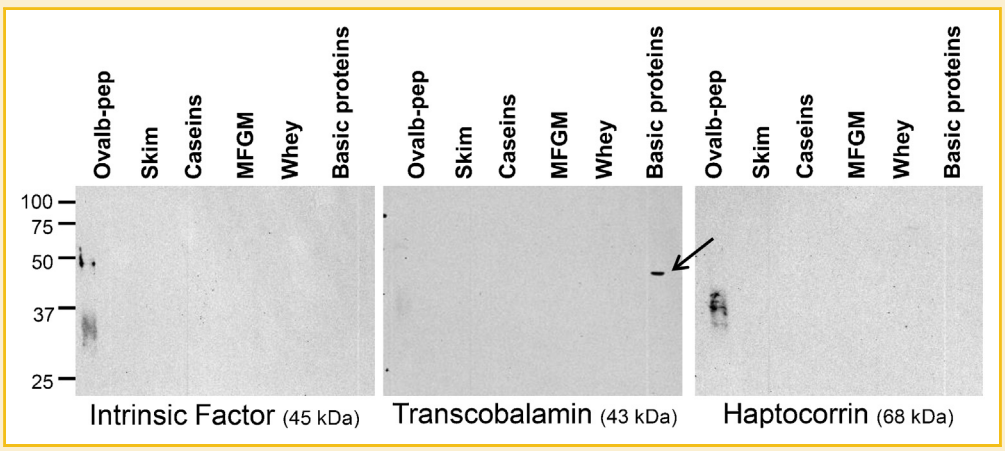


**Fig. 1.** Detection of vitamin B12-binding proteins in bovine milk and its sub-fractions. Western blotting was performed on bovine pasteurized skimmed milk and its sub-fractions, which were produced as previously described [Smolenski et al., 2007]. The left panel was probed for intrinsic factor, the middle panel for transcobalamin and the right panel for haptocorrin, using procedures and antibodies produced as described in the Materials and Methods Section. The MW markers are indicated on the left panel, and the positive signal for TC is indicated by an arrow.

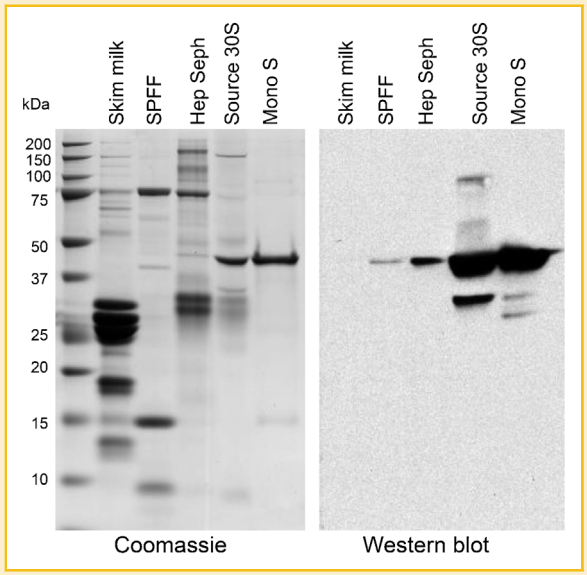


**Fig. 2.** Purification of transcobalamin from bovine milk. Milk was subjected to successive rounds of chromatography as described in the Materials and Methods section. The proteins present after each step (chromatography with SPFF, heparin sepharose, Source S and Mono S media, respectively) were analyzed by SDS–PAGE followed by staining with Coomassie blue (left panel). For Coomassie blue staining, the amount of total protein loaded in lanes 1–5 was 20, 5, 5, 2, and 2 μg, respectively. The relative amount of transcobalamin present after each of the purification steps was visualized by Western blotting using an anti TC antibody (right panel). For Western blotting, a total of 2 μg of protein was loaded in each lane.

by densitometry of Coomassie blue stained gels (data not shown). This approach does not discriminate between apo- and holo-transcobalamin. The identity of the major 43 kDa protein in the final preparation as transcobalamin was confirmed by Western blotting and mass spectrometry. The lesser intensity bands at 30 and 35 kDa are most likely proteolytic fragments of transcobalamin. The trace amount of a 15 kDa protein was (trace is singular) identified as fibroblast growth factor binding protein (GenBank accession number 27805911) by mass spectrometry of the excised gel band. Analysis of the vitamin B12 and protein concentrations of the preparation established an average molar ratio of B12:transcobalamin of approximately 1:6 ($16 \pm 3\%$ occupancy of the binding site, $n = 6$). Incubation of the purified transcobalamin preparation with excess free B12 resulted in no significant increase in its non-dialyzable B12 content.

### STIMULATION OF B12 UPTAKE INTO CULTURED CELLS BY TRANSCOBALAMIN DERIVED FROM BOVINE MILK

The ability of the transcobalamin preparations to enhance uptake of vitamin B12 was tested in cell lines from three species; bovine MAC-T mammary epithelial cells, mouse C127 mammary epithelial cells and human HeLa cervical epithelial adenocarcinoma cells. Cells were cultured for 16 h in the presence of a range of concentrations of free B12 or B12 complexed to transcobalamin, prior to measuring the amount of B12 taken up by the cells. Uptake of B12 by each cell type was substantially higher ($P < 0.02$ for all dosages) and dose-dependent when added as a complex with transcobalamin (Fig. 3). The binding of B12 to the intestinal cell wall requires calcium, which would be removed by EDTA. The levels of B12 uptake were unaffected by washing the cells with EDTA prior to harvesting, suggesting that the B12 was internalized into the cells rather than bound to the cell surface (data not shown).

### STIMULATION OF B12 UPTAKE INTO CULTURED POLARIZED HUMAN INTESTINAL EPITHELIAL CELLS BY TRANSCOBALAMIN

We next investigated the ability of the transcobalamin preparation to enhance the uptake of B12 into differentiated Caco-2 cells with distinct apical and basolateral surfaces. Vitamin B12 was added at a range of concentrations to either surface prior to measuring the amount of B12 taken up by cells after 16 h. The vitamin was added either in the form of free B12, as a complex with IF, or as a complex with transcobalamin. As shown in Figure 4a, uptake of free B12 from either the apical or basolateral surface was saturated at approximately 150 pg B12/mg of total cellular protein (Fig. 4a), in contrast to the much lower levels observed for free B12 in the other cell lines tested (Fig. 3). The presence of IF did not significantly alter the level of B12 uptake from the apical side of the cells compared with the uptake of free B12, whereas uptake from the basolateral side was significantly decreased ($P < 0.03$ at all dosages) to approximately 90 pg B12/mg protein due to the presence of IF (compare Figs. 4a and b). A dose-dependent increase in the uptake of B12 from the apical surface was determined when vitamin B12 was added to the cells as a complex with transcobalamin, which approached saturation at 1,200 pg/mg. This uptake was approximately eightfold that observed for free B12 (compare Figs. 4a and c) and is highly significant ($P < 0.005$ for all dosages). A somewhat lesser increase was observed via the basolateral side, which was approximately threefold greater than that for free B12 and was significant ($P < 0.03$) for all the dosages above 2 ng/well.

## DISCUSSION

In these studies, we have demonstrated that vitamin B12 is taken up much more effectively as a complex with bovine transcobalamin compared with its free form, in cultured cells from cattle, mice and humans. These data therefore show that bovine transcobalamin, which a multiple sequence alignment shows has 67% and 72% amino acid sequence identity with its murine and human orthologs, respectively (data not shown), is active in stimulating B12 uptake among these species. The enhanced uptake of transcobalamin-B12 complex by polarized Caco-2 human intestinal epithelial cells occurred via both the apical and basolateral cell surfaces. Notably, uptake via the apical surface was particularly high, which contrasts with an earlier study showing a sixfold higher level of uptake of B12-transcobalamin complex from the basolateral surface of Caco-2 cells compared with the apical side [Bose et al., 1997]. Uptake from the basolateral surface is compatible with the canonical cellular uptake of B12 from plasma through the CD320 receptor. However, substantial transcobalamin-facilitated uptake via the apical surface has not been previously reported. The results of both our study and that of Bose et al. [1997] suggest that a functional transcobalamin-

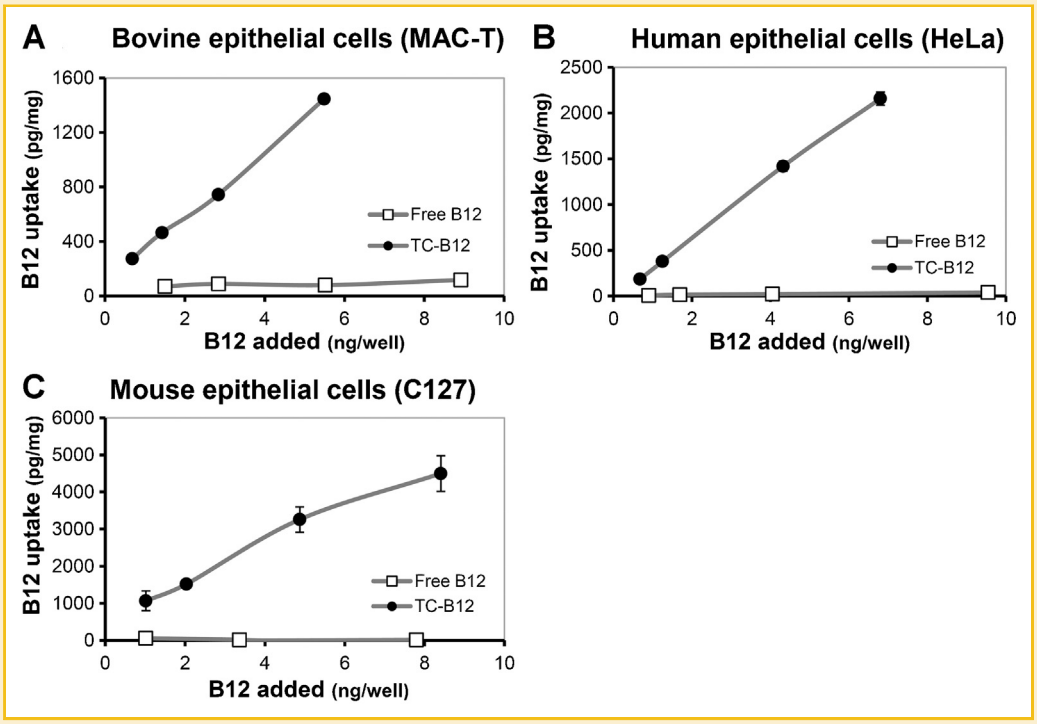


**Fig. 3.** Uptake of vitamin B12 by cultured cells. The extent of uptake of vitamin B12 was determined in bovine MAC-T (A), human HeLa (B) and mouse C127 (C) cell lines. The vitamin was added either in the form of free B12 or as transcobalamin–B12 complex at the indicated amount, and the B12 taken up by the cells measured as described in the Materials and Methods section. Each data point represents the mean value of three separate experiments ± standard error.

facilitated uptake mechanism exists on the apical as well as the basolateral surface of intestinal epithelial cells. The difference in results between our study and that of Bose et al. with respect to the relative levels of uptake from each surface of the cells may be a consequence of differences in study design, where we used much higher amounts of unlabelled B12, and obtained approximately a 600-fold greater level of uptake compared with the Bose et al. study. The relatively high level of uptake of free B12 we recorded at both the apical and basolateral surfaces of Caco-2 cells compared with the other cell lines suggests that these cells possess a distinct mechanism for uptake of B12 complexes. The high uptake in the absence of added B12-binding protein suggests that either culture media-derived or endogenously produced B12-binding proteins are involved, as has been previously suggested for glial cells [Pezacka et al., 1992]. The enhanced uptake of transcobalamin-B12 complex, but not IF-B12 complex from the apical surface suggests that the uptake mechanism from the apical surface is distinct from that on the basolateral surface, and is not mediated by cubilin.

While the cyano co-ordination group present on the free form of B12 is almost certainly different from that present on the

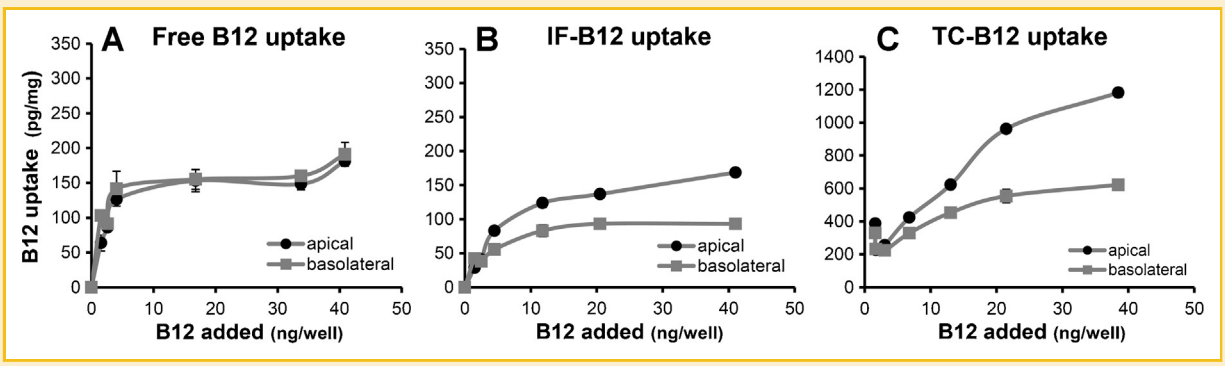


**Fig. 4.** Uptake of vitamin B12 into differentiated Caco-2 cells. Cells were differentiated on porous transwell inserts and the extent of vitamin B12 uptake was measured when it was added from either the apical (upper) or basolateral (lower) side. The B12 was added either as free B12 (A), a B12–IF complex (B), or as a B12–transcobalamin complex (C). The amount of internalized B12 was assessed as described in the Materials and Methods section. Each data point is the mean of three separate experiments ± standard error.

endogenously bound form of B12 prepared from milk, this is very unlikely to have affected uptake, since the avid binding of cyanocobalamin to its cognate transport proteins is well documented [e.g., Bose et al., 1997]. The concentrations of B12 used in our study are somewhat higher than normal levels in human blood (~0.5–1 ng/ml) but are comparable to intestinal contents (meat contains 30–80 ng/mg B12 [Watanabe, 2007]).

The stimulation of free vitamin B12 uptake by Caco-2 cells as well as the B12-IF and transcobalamin complexes was saturable, suggesting that in each case uptake occurs via a receptor-mediated mechanism. In the case of IF-B12 complex, the uptake is likely to be mediated via cubilin, its canonical receptor that is present in the intestinal mucosa [Levine et al., 1984; Moestrup and Verroust, 2001]. The lower level of uptake of IF-B12 complex compared with transcobalamin-B12 complex suggests that cubilin is present only at low abundance on the apical surface of the Caco-2 cells compared with intestinal tissue. In the case of the transcobalamin-B12 complex, the situation is likely more complex. Differentiated Caco-2 cells express the transcobalamin receptor, CD320, on their apical surface [Bose et al., 1997] although its expression by normal intestinal cells in vivo and its abundance on the apical surface has not been established. On the other hand, the intestinal mucosa is known to express megalin, a cell surface receptor that facilitates uptake of a range of proteins in association with cubilin [Moestrup and Verroust, 2001]. Both these proteins have been previously linked with uptake of transcobalamin in the kidney tubule [Yammani et al., 2003; Birn, 2006; Abuyaman et al., 2013]. Our results suggest that a similar transcobalamin-CD320/megalin mechanism may be an important adjunct pathway for vitamin B12 uptake from the intestinal lumen.

Evidence exists to support transcobalamin-mediated uptake of B12 from the intestinal lumen in vivo. Malabsorption of vitamin B12 was observed in a child carrying an inactivating mutation in the transcobalamin gene, which was subsequently overcome by oral administration of rabbit transcobalamin-B12 complex, but not by free B12 [Barshop et al., 1990]. This observation requires further investigation through clinical studies as well as the use of isolated intestinal tissue and whole animal models.

Our finding of enhanced vitamin B12 uptake via the apical surface of polarized human intestinal epithelial cells when presented as a bovine transcobalamin-B12 complex strongly suggests that bovine transcobalamin facilitates B12 uptake. This finding presents the possibility that transcobalamin in bovine milk may also facilitate enhanced uptake of the vitamin from the diet. Malabsorption of B12, particularly in the elderly, is a significant clinical issue [Green and Miller, 2014], and the mechanisms involved in this process are currently not well understood. The findings presented here offer a new perspective by which to understand the responsible mechanisms, and also offer a possible therapeutic approach for overcoming such issues. Supporting this concept, breastfed infants are known to have a lower cobalamin (B12) status compared to infants fed formula derived from bovine milk [Hay et al., 2008], and dairy products are known to be a good source of bioavailable B12 [Tucker et al., 2000].

The low maximum saturation of transcobalamin extracted from cows' milk (16%) suggests that its B12 binding activity may be compromised by current standard factory processing procedures. It is possible that the yield of transcobalamin-B12 complex can be improved through optimization of pasteurization conditions. Given the need for alternative treatments of B12 malabsorption, oral supplementation with B12 in the form of a complex with transcobalamin derived from bovine milk may provide a cost-effective practical alternative to current therapies.

## ACKNOWLEDGEMENTS

The authors acknowledge the technical assistance of Paul Harris, Marita Broadhurst, Rachel Brunt and Michael Agnew for protein purification. This study was supported by New Zealand Ministry of Business, Innovation and Employment (C10×0707) (Defence Proteins in Milk), awarded to T.T.W.

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