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Vitamin B_{12}-mediated transport of nanoparticles across Caco-2 cells

G.J. Russell-Jones [a,*], L. Arthur [b], H. Walker [a]

[a] *Biotech Australia, Roseville 2069, Australia*
[b] *Westmead Hospital, Sydney, Australia*

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Abstract

Several studies have shown that Caco-2 cells have the capability to transport peptides and proteins from their apical to basal surfaces when these molecules are linked to vitamin B_{12} (VB_{12}). In this study we have extended these studies and have shown that Caco-2 cells are also able to internalize and transport VB_{12}-modifed nanoparticles from their apical to basal surfaces. Uptake and transport of nanoparticles was found to occur in both a VB_{12}-dependent intrinsic factor (IF)-independent manner as well as in a VB_{12}-dependent IF-dependent manner. Both IF-independent and IF-dependent VB_{12}-mediated uptake and transport were dependent upon the surface density of VB_{12} as a reduction in surface modification of the nanoparticles with VB_{12} resulted in a reduced level of both VB_{12}-mediated and IF-mediated uptake. At lower levels of VB_{12} modification there was no apparent non-IF-mediated uptake; however, VB_{12}–IF-mediated uptake was still measurable. These studies show that Caco-2 cell cultures are a suitable model for the study of VB_{12}-mediated uptake and transport of nanoparticles, and suggest that for effective oral uptake of VB_{12}-coated nanoparticles high surface densities of VB_{12} are required.

Keywords: Caco-2 cells; Drug delivery; Nanoparticles; Vitamin B_{12}

1. Introduction

The oral uptake of vitamin B_{12} (VB_{12}) occurs via receptor-mediated endocytosis. During this process VB_{12} is first bound to intrinsic factor (IF), a VB_{12}-binding protein that is present in the small intestine. The VB_{12}–IF complex is in turn bound to an IF receptor located on the surface of intestinal epithelial cells, and the VB_{12} is subsequently transported across the cell and enters the circula-

* Corresponding author.

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tion bound to another VB_{12}-binding protein, transcobalamin II (TcII). Recently, it has been shown that it is possible to utilize the uptake mechanism for VB_{12} to enhance the oral uptake of various peptide and protein pharmaceuticals (Russell-Jones and de Aizpurua, 1988; Habberfield et al., 1996; Russell-Jones et al., 1995a). Russell-Jones and co-workers (Russell-Jones and de Aizpurua, 1988; Habberfield et al., 1996 Russell-Jones et al., 1995b) have shown that this uptake system can potentially increase the oral uptake of molecules such as luteinizing hormone releasing hormone (LHRH) analogues, α-interferon, erythropoietin (EPO) and granulocyte colony-stimulating factor (G-CSF) which have been covalently linked to the VB_{12} molecule. The potential utility of the VB_{12} uptake system for the oral delivery of peptides and proteins is limited by three factors: (1) the capacity of the uptake system (1 nmol/dose in humans); (2) the need to covalently link the pharmaceutical to VB_{12}; and (3) exposure of the pharmaceutical to intestinal proteolytic enzymes. In an effort to overcome these problems and to maximize the potential of the delivery system, we have initiated work on the amplification of the uptake system by incorporating the pharmaceutical within biodegradable nanoparticles. This has the advantages of protecting the pharmaceutical from proteolysis within the intestine, of amplifying the uptake capacity of the oral delivery system, and of eliminating the need for conjugation of the pharmaceutical to VB_{12}.

In order to define more clearly the parameters involved in nanoparticle uptake, preliminary experiments have been conducted with an in vitro cell model of VB_{12} transcytosis. Several cell lines exist that have been shown to bind, internalize and transcytose VB_{12} in an IF-dependent fashion. There are two intestinally derived human cell lines—the CaCo-2 cell line (Dix et al., 1990; Wilson et al., 1990; Ramanujam et al., 1991a; Hassan and Mackay, 1992; Dan and Cutler, 1994) and the HT29 cell line (Schohn et al., 1991; Guéant et al., 1992)—and two kidney carcinomas—the OK cell line (from opossum kidney) and the LLCPK1 line (from porcine kidney) (Ramanujam et al., 1991b). Each of these cell lines has been shown to form a confluent monolayer in culture with the formation of tight junctions between cells. Transport has been shown to be dependent upon polarization of the cells, which occurs when the cells are grown in two-chambered tissue culture wells in which each chamber is separated by a permeable filter membrane. These cell cultures exhibit unidirectional transport of VB_{12} from the apical to basolateral chamber. Caco-2 cells have been found to be highly variable in VB_{12} transport with levels of transport ranging from 10–40 fmol per 24 h (Dix et al., 1990; Wilson et al., 1990) to 250 fmol per 4 h (Ramanujam et al., 1991a). Caco-2 cells have also been found to transport VB_{12} in an IF-independent fashion, possibly due to the expression of TcII receptors on their apical surface (Bose et al., 1997).

OK and LLCPK1 cell lines exhibit IF-dependent VB_{12} transport which is much more stable and uniform than in the Caco-2 cell line. These cell lines have a vastly different cell surface to both the CaCo-2 and HT29 cell lines as they do not possess microvilli (Ramanujam et al., 1991a).

Habberfield et al. (1996) have utilized Caco-2 cell cultures as an in vitro model for VB_{12} transport of pharmaceuticals and have shown that Caco-2 cells grown in transwells are capable of transporting VB_{12} linked to G-CSF in an IF-dependent fashion from the apical to basolateral chamber.

In the studies described below we have used Caco-2 cell cultures as an in vitro model to study the potential of using the VB_{12} transport system for oral delivery of VB_{12}-linked nanoparticles. These studies were undertaken to define the characteristics of nanoparticles that would be required for uptake via the VB_{12} uptake system. Various parameters such as the effect of nanoparticle size, VB_{12} density and nanoparticle concentration on transport have been examined.

2. Methods

The Caco-2 cell line was obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose and 10% foetal bovine serum in an atmosphere of 5% CO_2. For transport experiments, cells were grown in T75 flasks until 80–100% confluent. Following

washing with phosphate-buffered saline (PBS), adherent cells were removed by treatment with 10 μg/ml trypsin in 10 mM EDTA. Cells were immediately diluted 15-fold into fresh culture medium and counted. Cultures were seeded at $0.5–1 \times 10^5$ cells/ml in 24 wells/plate transwells (Becton Dickenson or Costar) and grown for 12–24 days. Becton Dickenson transwells had a membrane insert size of 6 mm and a pore size of 0.4 μM, an apical chamber volume of 0.5 ml and a basal chamber volume of 1.5 ml. Costar transwells had a membrane insert size of 6.5 mm and a pore size of 0.4 μM, with an apical chamber volume of 0.25 ml and a basal chamber volume of 1 ml. Cells generally reached confluence within 10 days. Transepithelial resistance (TEER) measurements were made of monolayers and only cultures with resistance greater than 250 Ω/cm^2 were used for experimentation. Prior to nanoparticle experiments all cell cultures were checked for VB_{12} transport.

$^{57}CoVB_{12}$ uptake experiments were conducted on 18–19-day-old cultures. Briefly, medium was removed from both the apical and basal chamber and then 1 ml of medium was added to the basolateral chambers. Cells in the apical chamber were washed once with PBS and a solution containing 2 μl of $^{57}CoVB_{12}$, 1 ng of unlabelled VB_{12} and 2 IU of IF in medium was added per apical well (250 μl). Cells were incubated overnight at 37°C and then apical and basal medium was removed and counted. Cells were washed once with PBS and fresh medium was added to the apical and basolateral wells. Cells were incubated overnight and the medium again removed and counted.

2.1. Preparation of VB_{12}-modified Polysciences nanoparticles

Fluorescent YG nanoparticles were obtained from Polysciences. These nanoparticles contain a fluorescein-like fluorophore and have surface carboxylic acid groups suitable for chemical modification and VB_{12} derivatization. Unwashed Polysciences nanoparticles were modified with VB_{12} by activation with EDAC (1-ethyl-3-[3-Dimethylaminopropyl]carbodiimide) in the presence of an adipyl-hydrazide derivative of eVB_{12} (e monocarboxylic acid isomer of VB_{12}) (Russell-Jones et al., 1995a). Following derivatization, unreacted, EDAC-modified carboxyl groups were blocked by incubation with 50 mg/ml glycine in 0.1 M carbonate buffer, pH 9.5. Nanoparticles were washed five times by precipitation with 0.1 M $CaCl_2$, centrifugation (5 min, $3000 \times g$, 5000 rpm), and resuspension in 50 mM EDTA. After the fifth wash, the particles were dialysed extensively against distilled water.

2.2. Binding/uptake and transport of nanoparticles by Caco-2 cells

Apical and basal chambers of Caco-2 cell cultures (22–24 days old) that were shown to be positive for $^{57}CoVB_{12}$ transport were washed once with buffer (PBS) and the basolateral wells were re-fed with 1 ml of complete medium. Dilutions of fluorescent Polysciences nanoparticles (0.25 ml) were added to the apical chamber of triplicate wells and the cultures were incubated at 37°C overnight. VB_{12}-coated particles, prepared as described previously, were added to wells with and without human IF.

Following overnight incubation, apical and basal medium was removed from the wells and the cells washed three times with PBS. The apical and basal chambers were assayed for bound, internalized, membrane-associated and transported fluorescence. Bound fluorescence was assessed by measuring the fluorescence release following incubation of the cultures with 5 mM EDTA, pH 5.0 for 15 min to release receptor-bound IF–Cbl or IF–Cbl nanoparticle complexes. Internalized fluorescence was determined following EDTA treatment (surface-bound) and lysis of the cells by incubation with a solution of 2% sodium dodecyl sulphate (SDS), 50 mM EDTA pH 8.0 for ≥ 30 min at room temperature. Finally, the membrane inserts were excised from the transwell and the fluorescence associated with the filter was assessed by lysis of the nanoparticles with 50% acetone to release the entrapped fluorochrome. All samples were adjusted to pH 8.0 with 1 M Tris–HCl pH 9.0 prior to fluorescence measurement. Fluorescence was measured on a Bio-Tek Instruments

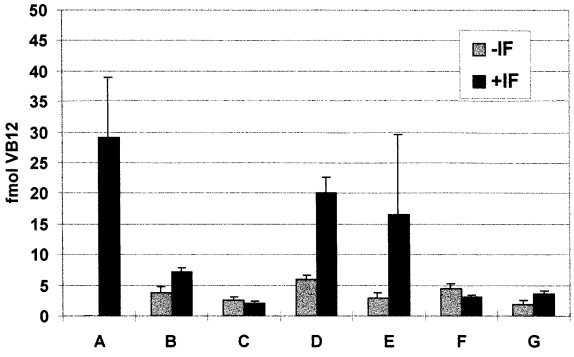

Fig. 1. Effect of cell passage on transport of $^{57}CoVB_{12}$ in the presence and absence of intrinsic factor (IF). Caco-2 cell cultures from various passages were grown for 18–19 days in culture in transwell cultures. Immediately prior to transport studies the apical and basal medium was removed and fresh medium added to the basal chamber. The apical layer of the cells was washed once with PBS, and medium containing $^{57}CoVB_{12} \pm IF$ was added to the apical chamber. Cells were cultured for a further 20 h, after which the basal medium was removed and the level of transport assessed by counting in a gamma counter.

Microplate Fluorescence Reader (FL500) at excitation 485 nm and emission 530 nm for YG nanoparticles. Data are expressed as the percentage of the total fluorescence added to the wells.

3. Results and discussion

3.1. Uptake and transport of vitamin B_{12} by Caco-2 cells

Uptake and transport of VB_{12} by Caco-2 cells has been found to be highly variable both between experimenters and between cell passages (Dix et al., 1990; Wilson et al., 1990; Ramanujam et al., 1991a). A comparison in the level of VB_{12} uptake and transport in the presence and absence of IF revealed that while the level of VB_{12} transport observed in the absence of IF was reasonably constant (2–6 fmol) (Fig. 1) a high degree of variability in IF-mediated transport (2–28 fmol) was observed between passages. In many cases the level of IF-mediated transport was no higher than the level of VB_{12} transport in the absence of IF. In contrast, transport by OK cells was much less variable (Table 1). Transport of IF in the absence of IF has also been reported by several other workers (Bose et al., 1997) and is presumably due the binding of the VB_{12} to epically located TcII. In this regard OK cells have been shown to synthesize two VB_{12}-binding proteins, haptocorrin (Hc; 66 kDa) and TcII (43 kDa). Ramanujam et al. (1991a) Ramanujam et al. (1991b) have shown that these cells secrete both proteins from the apical surface of the cells, while only TcII is

Table 1
Comparison of variability of uptake of $^{57}CoVB_{12}$ between Caco-2 and OK cell cultures

Cell line	Passage ID	Total uptake (−IF)	Total uptake (+IF)
Caco-2	4	69.8 ± 6.9	40.2 ± 0.35
	10	186.9 ± 32.5	110.7 ± 20.5
	28	60.5 ± 5.0	200 ± 28.5
OK	5	382.4 ± 29.1	458 ± 56.8
	8	413.6 ± 10.1	757.1 ± 15.8

Table 2
Uptake and transport of $^{57}CoVB_{12}$ by OK cells

Preparation	Bound	Lysed	Filter	Transport	Total uptake
VB_{12}	2.4 ± 0.6	68.3 ± 8.3	7.3 ± 2.2	338.1 ± 21.8	413.6 ± 10.1
VB_{12}+IF	8.5 ± 1.2	230.0 ± 27.8	13.2 ± 4.8	513.9 ± 34.9	757.1 ± 15.8

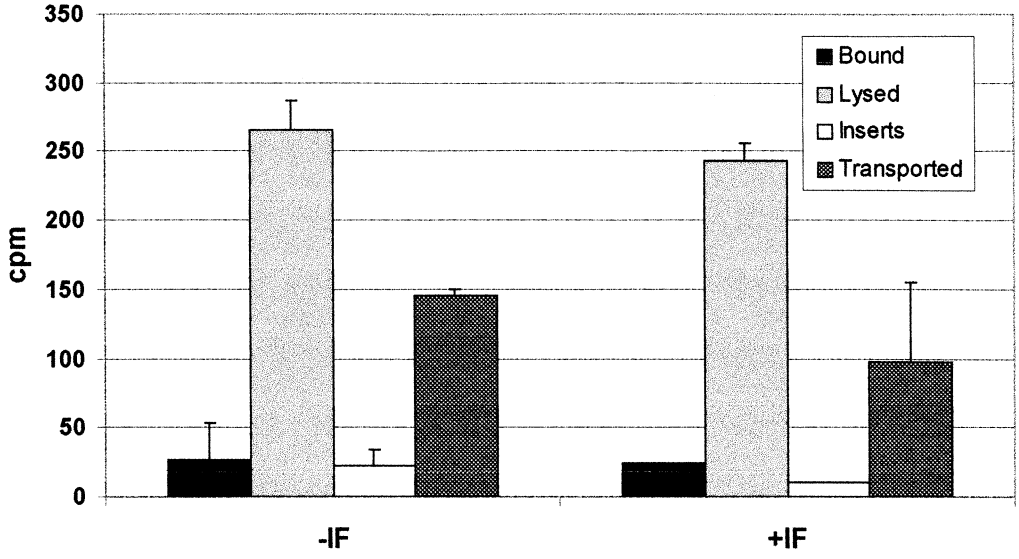

Fig. 2. Transport of $^{57}CoVB_{12}$ through Caco-2 cell cultures in the presence and absence of intrinsic factor (IF). Caco-2 cell cultures were grown for 18–19 days in culture in transwell cultures. Immediately prior to transport studies the apical and basal medium was removed and fresh medium added to the basal chamber. The apical layer of the cells was washed once with PBS and medium containing $^{57}CoVB_{12} \pm IF$ was added to the apical chamber. Cells were cultured for a further 20 h, after which the basal medium was removed and the cells washed three times with PBS. The amount of surface-bound VB_{12} was assessed by washing with 10 mM EDTA pH 5.0 for 20 min ('bound'). The cells were then lysed by addition of SDS–EDTA for 30 min ('lysed'). The filter was removed and counted ('inserts') and the amount of 'transported' VB_{12} assessed by removal of the basal medium.

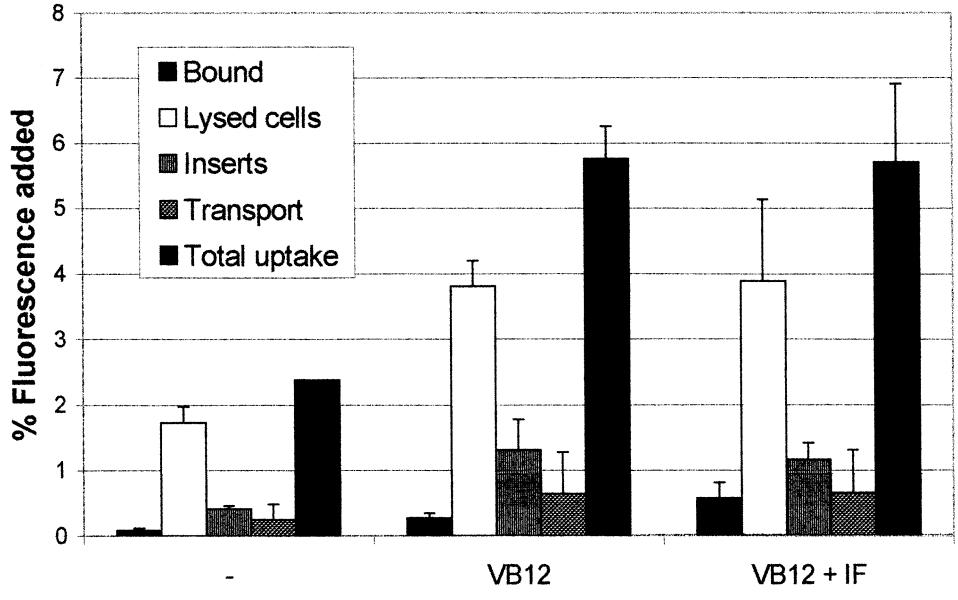

Fig. 3. Uptake of 50-nm Polysciences nanoparticles by Caco-2 cells. The extent of binding ('bound'), uptake ('lysed cells') and transport of unmodified (−), VB_{12}-coated (VB_{12}) and VB_{12}-coated particles plus IF ($VB_{12} + IF$) 50-nm Polysciences nanoparticles was assessed as described in Section 2. Using this procedure, microscopic examination of the filters revealed that nanoparticles were trapped in the filter. The fluorescence associated with the filter was assessed by lysis of the nanoparticles with 50% acetone to release the entrapped fluorochrome. All samples were adjusted to pH 8.0 with 1 M Tris–HCl pH 9.0 prior to fluorescence measurement and where necessary adjustment was made for dilution.

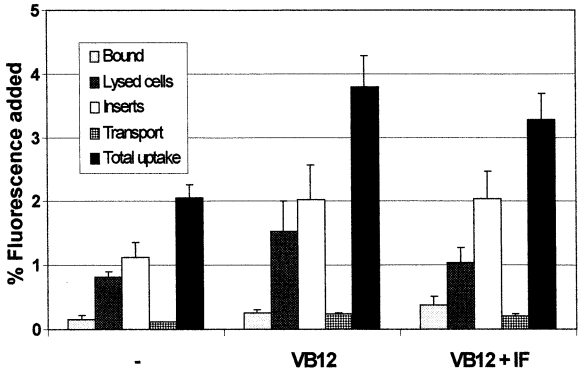

Fig. 4. Uptake of 100-nm Polysciences nanoparticles by Caco-2 cells. The extent of binding ('bound'), uptake ('lysed cells') and transport of unmodified (−), VB_{12}-coated (VB_{12}) and VB_{12}-coated particles plus IF (VB_{12} + IF) 100-nm Polysciences nanoparticles was assessed as described in Section 2. Using this procedure, microscopic examination of the filters revealed that nanoparticles were trapped in the filter. The fluorescence associated with the filter was assessed by lysis of the nanoparticles with 50% acetone to release the entrapped fluorochrome. All samples were adjusted to pH 8.0 with 1 M Tris–HCl pH 9.0 prior to fluorescence measurement and where necessary adjustment was made for dilution.

secreted from the basal surface (Ramanujam et al., 1991b). Roughly equal levels of VB_{12} binders were secreted into the apical and basal medium, with a two-fold higher level of TcII than Hc. In contrast, Ramanujam et al. (1991a) observed that Caco-2 cells only secreted TcII into the basal medium of transwell cultures with no apparent secretion of VB_{12} binders into the apical medium. Hc was found to be secreted into the cell medium of HT29 cell cultures (Schohn et al., 1991).

Table 3
Uptake of VB_{12}-coated nanoparticles (NP) by OK cells

Particle	50 nm	100 nm	200 nm	500 nm
Plain	7.8 ± 1.3	9.9 ± 1.9	8.1 ± 0.3	4.7 ± 0.1
VB_{12}NP	35.8 ± 2.4	14.1 ± 0.41	16.8 ± 1.2	8.3 ± 0.3
VB_{12}NP + IF	44.8 ± 4.0	ND	27.3 ± 2.4	ND

3.2. Localization of internalized VB_{12} within Caco-2 cells

In preliminary experiments on nanoparticle transport by Caco-2 cells, considerable fluorescence was found to be associated with the filter

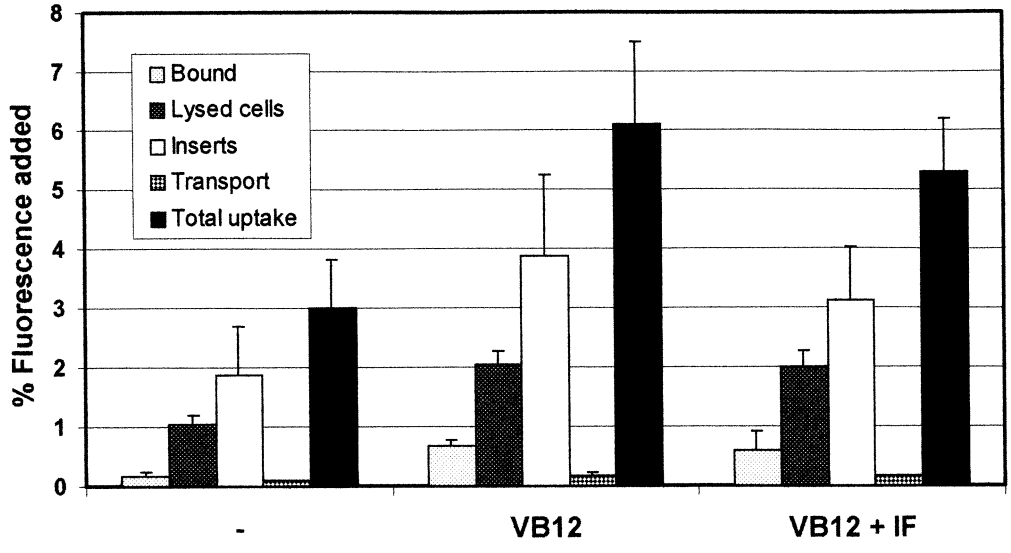

Fig. 5. Uptake of 200-nm Polysciences nanoparticles by Caco-2 cells. The extent of binding ('bound'), uptake ('lysed cells') and transport of unmodified (−), VB_{12}-coated (VB_{12}) and VB_{12}-coated particles plus IF (VB_{12} + IF) 200-nm Polysciences nanoparticles was assessed according to the methods described in Figs. 3 and 4.

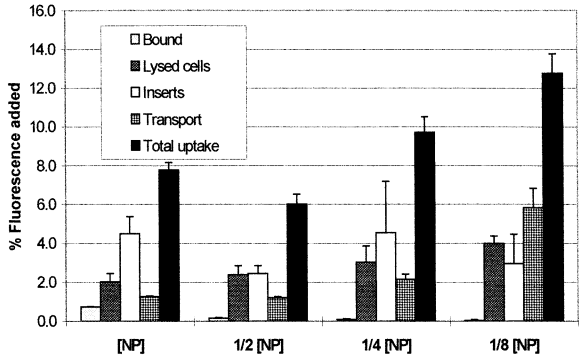

Fig. 6. Effect of dose on uptake and transport of Polysciences nanoparticles. The extent of binding ('bound'), uptake ('lysed cells') and transport of different dilutions (neat, 1:2, 1:4 and 1:8) 50-nm unmodified Polysciences nanoparticles was assessed according to the methods described in Figs. 3 and 4.

following lysis of the cells with SDS and EDTA. The possibility existed that this fluorescence was due to particles trapped in cell debris, or incompletely lysed cells, or alternatively represented particles which were trapped in the filter and could not move into the basal chamber of the filter apparatus. In order to distinguish between these possibilities, Caco-2 cells were cultured with $^{57}CoVB_{12}$–IF complexes and the cells washed with PBS, lysed with SDS–EDTA and the $^{57}CoVB_{12}$ associated with the filter counted. As can be seen in Fig. 2, the vast majority of $^{57}CoVB_{12}$ was found either within the cells (SDS–EDTA extract) or to have travelled to the basal chamber, with very little material associated with the filter (Table 2). This suggests that the fluorescence found associated with the filters after SDS–EDTA lysis did not represent cell debris, but rather was due to the presence of transported nanoparticles which had been trapped in the filter and were unable to cross to the other side of the filter.

3.3. Uptake and transport of VB_{12}-coated nanoparticles by Caco-2 cells

Preliminary experiments by Delie and Rubas (1996) on binding of Polysciences nanoparticles to

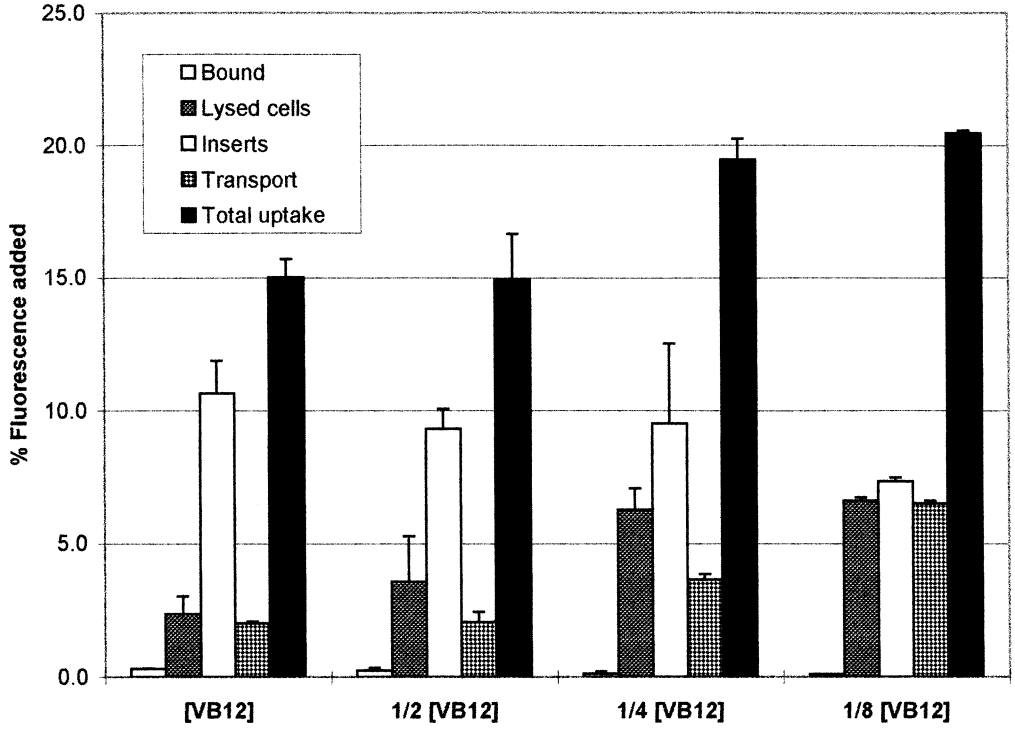

Fig. 7. Effect of dose on uptake and transport of VB_{12}-modified Polysciences nanoparticles. The extent of binding ('bound'), uptake ('lysed cells') and transport of different dilutions (neat, 1:2, 1:4 and 1:8) 50-nm VB_{12}-modified Polysciences nanoparticles was assessed according to the methods described in Figs. 3 and 4.

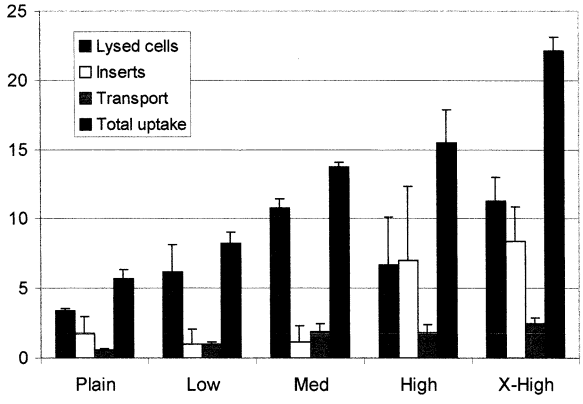

Fig. 8. Effect of VB_{12} substitution on uptake and transport of VB_{12}-coated Polysciences nanoparticles. The level of uptake ('lysed cells') and transport of nanoparticles coated with increasing concentrations of VB_{12}-modified 50-nm Polysciences nanoparticles was assessed according to the methods described in Figs. 3 and 4. For preparation of particles with different densities of VB_{12}, particles were prepared by activating with a constant amount of EDAC and increasing levels of adipyl-hydrazidyl–eVB_{12} (Low, 2.5 mg/ml; Med, 5 mg/ml; High, 10 mg/ml; X-High, 20 mg/ml). The percentages of particles within the cells ('lysed cells'), of those that had been transported to the filter ('inserts'), those transported to the basal chamber ('transport') and the sum of the above ('total uptake') are presented.

Caco-2 cells have shown low levels of non-specific binding of 100-nm particles to Caco-2 cells (1.5–3%). This level of non-specific binding was reduced with 50- and 1000-nm particles. In this study, we observed a small degree of binding, uptake and transport of 50-nm uncoated nanoparticles by Caco-2 cells (Fig. 3). When VB_{12}-modified nanoparticles were added to Caco-2 cell cultures, a higher level of uptake was observed than with uncoated particles. Uptake was further enhanced to a small degree by the addition of IF to the VB_{12}-coated nanoparticles. Addition of IF to uncoated particles did not increase uptake above background (results not shown). This shows that Caco-2 cells are able to take up and transport nanoparticles in both a VB_{12}-dependent IF-independent manner and a VB_{12}-dependent IF-dependent manner.

3.4. Transport of 50-, 100- and 200-nm Polysciences nanoparticles

Following the observation by Delie and Rubas (1996) that the level of non-specific uptake of nanoparticles varies with size, VB-mediated uptake of 50-, 100- and 200-nm particles was examined in Caco-2 cells. As the size of particles increased from 50 to 100 to 200 nm, a decrease in transport and uptake of particles by Caco-2 cells was seen (Figs. 3 and 4). Thus 4.5, 2.1 and 1.9% of 50-, 100- and 200-nm particles, respectively, was internalized and transported. The decrease in transport may be attributed to a lower ratio of particle numbers to cell numbers as the same volume of particles was added to each well. Modification of the particles with VB_{12} resulted in increases of 5.5, 1.5 and 3% in internalization and transport of 50-, 100- and 200-nm particles, respectively (Figs. 4–6). Uptake of 100- and 200-nm particles was slightly decreased by the addition of IF to 3.3 and 5.2%, respectively (Figs. 5 and 6). Similar levels of uptake of vitamin B_{12}-modified nanoparticles of varying sizes were observed in OK cells (Table 3).

3.5. Effect of dose on the uptake and transport of VB_{12}-modified nanoparticles

Previous workers who have examined lectin-mediated uptake of nanoparticles by Caco-2 cells (Lehr, 1994, personal communication) have shown that at low concentrations of nanoparticles the level of specific lectin-mediated binding was similar to non-specific binding of particles to cells. Increases in the dose of nanoparticles led to a greater increase in specific binding when compared to non-specific binding. Similar experiments comparing the effect of increasing doses of nanoparticles with and without VB_{12} are reported here. In these studies, it was found that, as the concentration of particles increased, the percentage of non-specific transport remained fairly constant in the range 2.4–4.5% (Fig. 6). In contrast, the level of specific VB_{12}-mediated uptake and transport increased from 7.3% to 10.3% (Fig. 7). It should be noted that the ratio of specific to non-specific transport, measured as 'total uptake',

also increased with an increase in the concentration of particles.

3.6. Effect of density of VB_{12} modification of particles on uptake and transport in Caco-2 cells

In the experiments described above it was found that the small level of non-specific uptake of nanoparticles by Caco-2 cells could be markedly increased by the addition of surface VB_{12}. It was therefore decided to examine the effect of ligand density on nanoparticle uptake. It can be seen from Fig. 8 that reduction in surface substitution with VB_{12} resulted in a reduced level of both VB_{12}-mediated and IF-mediated uptake. At lower levels of VB_{12} modification there was no apparent non-IF-mediated uptake; however, VB_{12}–IF-mediated uptake was still measurable.

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