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Parameters influencing intestinal epithelial permeability and microparticle uptake *in vitro*

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Abstract

The hypothesis that, *in vivo in situ*, villous uptake of 2 μm latex microparticles involves changes at enterocyte tight junctions (TJs) was tested using Caco-2 cells on porous membranes. Epithelial permeability was measured by transepithelial resistance (TER) and particle numbers in surface, intraepithelial and sub-epithelial compartments by microscopy. Apical particle or medium addition initially closed TJs, but this was subsequently reversed in particle-treated groups. Peristaltic onward movement of a bolus was simulated by removing apical particles after an exposure period and leaving the remaining particles to interact with the epithelium: this produced marked TJ loosening during the interaction period. For particle exposure groups, the early similarity with particle numbers *in vivo* taken up in young adult rats became less marked with time, although bolus removal counteracted this tendency. The TJ response to vasoactive intestinal polypeptide (VIP) was time-dependent. Adsorbed and intraepithelial particle numbers increased with particle exposure time; epithelial-associated microparticle aggregation varied with treatment and submembranous particles were seen in all groups. Correlation between TER changes and particle numbers suggests TJ loosening may be important in microparticle uptake. This Caco-2 model gives epithelial particle numbers that approximate well to published figures for microparticle uptake *in vivo* and allows effective microenvironmental manipulation.

Keywords: Caco-2; Latex microparticles; Time; Transepithelial resistance (TER); VIP; Rat small intestine

1. Introduction

Small intestinal uptake and translocation of particles to secondary organs is relevant to drug delivery systems and environmental release of possibly harmful substances that could affect humans or their food chain. Uptake of particles with diameters in the micrometer range may occur through Peyer's patch microfold (M) cells (Ermak et al., 1995; Thomas et al., 1996; Beier and Gebert, 1998) or through villous epithelium (Hodges et al., 1995; McClean et al., 1998; Hillyer and Albrecht, 2001). The latter could imply loosening of apical tight junctions (TJs, Bjork et al., 1995; McCullough et al., 1995; Wiesner et al., 2002), important in the formation of epithelial barriers (Aijaz et al., 2006). Testing this hypothesis allows a shift from *in vivo* to *in vitro* models, such as Caco-2 cells (Delie and Rubas, 1997), which can be examined in isolation from other parts of the intestinal wall. Caco-2 cells, although derived from a human colonic adenocarcinoma, resemble small intestinal enterocytes (Beaulieu, 1999). Transepithelial resistance (TER) has been used to study epithelial permeability and reflects changes in TJs (Madara, 1998). The current paper addresses three aspects of this model, namely its responses to microparticle administration and its use for comparison with *in vivo* uptake or changes in the microenvironment.

There are few reports on the uptake of inert microparticles, although the interaction between cultured epithelial cells and bacteria has been described (Lytton et al., 2005; Tyrer et al., 2006). A temperature-dependent transcytotic uptake mechanism was deduced for nano- and micro-particles by McClean et al. (1998). This study and that of Tyrer et al. (2002) plated Caco-2 cells on membranes with pores too small to allow passage of microparticles of the size relevant to the current work, avoiding cell growth through the pores to form a bilayer, but hampering passage of particles through the epithelium. Little information is

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available on *in vitro*/*in vivo* comparisons or the extent to which onward peristaltic movement of an administered bolus limits the time to which any area of the epithelium is exposed to the particles. This can be simulated by removing the particle-laden apical fluid from the Caco-2 surface, described below as 'bolus removal' experiments. An *in vitro* cell model can also be used to explore microenvironmental effects in isolation. These include the addition of gastrointestinal hormones such as vasoactive intestinal polypeptide (VIP) at physiological levels. The gastrointestinal neurotransmitter VIP is a 28-amino acid peptide that signals through the VPAC receptors via activation of protein kinase A and phospholipase C to influence the gene transcription of the cell, enhancing gut relaxation and promoting sphincter opening (Delgado et al., 2004).

The hypothesis addressed in the current paper is that the *in vivo* microparticle uptake which occurs at villous epithelium, and not Peyer's patches, is explained by an opening of enterocyte tight junctions. This study has three aims, namely

(a) to explore *in vitro* epithelial permeability after exposure to microparticles
(b) to compare directly *in vitro* and *in vivo* microparticle uptake for a range of exposure times and also after microparticle bolus removal, thereby simulating the peristaltic onward movement of intestinal contents
(c) to use the *in vitro* model to explore the effect of the gut microenvironment, by the administration of the gastrointestinal hormone VIP.

2. Materials and methods

2.1. Protocol summary

These experiments explored the effects on Caco-2 cells of 2 μm diameter fluorescent latex particles ('particle exposure' and 'bolus removal' experiments) and of microenvironmental change ('hormone' experiments). 'Particle exposure' experiments varied the sampling time after treatment, namely 'particle time' for exposure to latex particles or 'medium time' for exposure to medium, the latter as a control. In 'bolus removal' experiments, excess particles were removed after a range of particle times, and the cells incubated for a further period to allow those already associated with the Caco-2/membrane unit to interact for a further period of time ('interaction time').

The groups studied and numbers of experiments are given in Table 1 and insert numbers in Tables 2 and 3. Data for particle exposure time included results from all groups of Caco-2 cells without other treatment (Table 1). Data from the *in vitro* experiments were compared with the previously reported *in vivo* results of the uptake of particles of the same size and composition (Smyth et al., 2005; Doyle-McCullough et al., 2007).

2.2. Caco-2 cell culture

Caco-2 cells (ECACC, Wiltshire, UK) between passages 30–45 were cultured at 37 °C in a humidified 5% CO_2 atmosphere. Cells were seeded at a density of 1×10^6 cells per Transwell® polyester membrane insert, 6-well format, with 3 μm pores (Corning Costar). Basic culture medium (BCM) consisted of Dulbecco's Modified Eagle's Medium supplemented with 10% foetal bovine serum, 1% non-essential amino acids and 1% penicillin–streptomycin (all obtained from Gibco BRL). For most experiments, cells were cultured for 21 days during which time the medium was changed every 48–72 h. For the assessment of the time taken by the Caco-2 cells to reach confluence, the TER was noted for one experiment from day 5 to day 21 ($n = 3$ wells) and for another experiment for days 21, 32 and 47 ($n = 6$ wells): these cells were not used in further experimentation reported in this paper. Samples without an even layer of cells covering the entire insert were also excluded.

Table 1
Treatments and times of exposure (min) to particle (P) and medium (M) in the nine experiments included in this paper

Experiment no.	Experiment description	Timeline (min) after particle or medium exposure				
		5	30	60	90	120
1	Exposure	P	P	P		P
				M		M
2	Exposure		P			
3	Exposure	P	P	P		
4	Exposure	P	P	P		
5	Exposure	P	P			
6	Bolus removal	P	P	P	P	P
7	Bolus removal	P	P	P	P	P
		M	M	M	M	M
8	VIP/exposure		P			
9	VIP/exposure		P			
	n values for wells	P = 56	P = 107	P = 32	P = 14	P = 20
		M = 16	M = 16	M = 6	M = 4	M = 5

Entries for P and M for experiments 7 and 8 include TER values after particle exposure, both with and without subsequent interaction time. A further two experiments were carried out on the effect of culture period on TER over 21 days ($n = 3$) and 32 and 47 days ($n = 6$), these are not included in this table as these cell layers were not exposed to either particles or control medium.

Table 2
Transepithelial resistance (TER) as a measurement of epithelial permeability (mean ± S.E.M.) after exposure to medium (top) or particles (middle) and in bolus removal experiments (bottom)

Groups (experiment numbers)	Timeline (min) and TER values (Ω cm^2)						
Treatment/time (min)	**0**	**5**	**30**	**60**	**65**	**90**	**120**
	Day 21						
Medium exposure (mean of 1, 7)							
5 (n=16)	645 (19)	801 (24)					
30 (n=26)	622 (17)		813 (25)				
60 (n=6)	874 (49)			975 (76)			
90 (n=4)	1021 (57)					1189 (52)	
120 (n=5)	761 (43)						872 (29)
Particle exposure (mean of 1-9)							
5 (n=56)	802 (52)	955 (61)					
30 (n=107)	933 (47)		1015 (42)				
60 (n=32)	1322 (134)			1312 (122)			
90 (n=14)	1280 (115)					1363 (86)	
120 (n=20)	1116 (142)						1156 (131)
Bolus removal (mean of 6-7)							
5/60 (n=14)	1139 (175)	1379 (205)			1014 (106)		
30/60 (n=17)	1038 (148)		1046 (121)			759 (29)	
60/60 (n=13)	1637 (255)			1549 (236)			990 (97)

Column 1 outlines treatments and time of exposure: n values are given in brackets. TER data in green or orange boxes refer to values following exposure to particles or medium respectively, for the time given in row 2. Bolus removal experiments have TER values after initial particle exposure (green boxes) and also after a further time for interaction, following particle removal and medium addition (orange boxes).

Table 3
Changes in particle distribution and numbers with different treatments

Particle time/treatment (min)	A (total adsorbed particles)	E (intraepithelial particles)		Percentage aggregation of cross-wire subtotal
		Total particle numbers	Proportion of total particle count	
Caco-2 cells				
5	286	135	32	15
30	1038†	1072†	51	17
60	477†	590†	55	18
90	1847†,‡,§	2737†,‡,§	60	32†,‡,§
120	2716†,‡,§,*	5045†,‡,§,*	65	36†,‡,§
Bolus removal				
5/60	20†,§	217†,§	92	22
30/60	203‡,*	332‡,*	62	29
60/60	3550$^{\blacktriangle}$	8650$^{\S,\blacktriangle}$	71	11$^{\blacktriangle}$
VIP				
10 min VIP + 30P	460	2366	84	24
30 min VIP + 30P	514	2007	80	20
PBS + 30P	770	1477	66	17
Medium + 30P	467	1334	74	17

The following symbols denote the time from which it is significantly different ($p \leq 0.05$) or shows a trend ($p \leq 0.1$, in brackets); †from 5 min, ‡from 30 min, §from 60 min, *from 90 min and $^{\blacktriangle}$from 120 min. $n \geq 6$ for all groups.

2.3. Assays, processing and particle counting

2.3.1. TER

Transepithelial resistance (TER) was recorded using a Millicell®-ERS instrument (Millipore). Changes in TER were observed either sequentially (measurements taken before and after experimental procedures) or comparatively (comparison of measurements taken at similar times after different treatments). TER measurements are described here either in full or by including descriptors in brackets: for example, before cell seeding, the TER of the Transwell® insert membrane with BCM was measured giving a value for TER (insert membrane). An average of the values for TER (insert membrane) per experiment was subtracted from subsequent measurements of TER (insert membrane and Caco-2 layer) to determine the resistance for the cell layer alone (Wells et al., 1998): values for these two types of TER were compared to explore whether the former was influencing the outcome. On day 21 of culture prior to any experimentation, the TER for the epithelium was measured, TER (d21): only wells with values of at least 70 Ω higher than the TER (insert membrane) were considered confluent. TER measurements reported were described in $\Omega\,cm^2$ (Wells et al., 1998), to allow for the surface area of the epithelium, as follows: $4.5\,cm^2 \times$ (TER measured − TER of insert membrane).

2.3.2. Particle counting

The epithelia were stained with 3.5 μg/ml FM1-43FX membrane stain (Cambridge Bioscience) for 5 min, washed with 0.1 M phosphate-buffered saline (PBS) and fixed in 3% glutaraldehyde for a minimum of 20 min. Thereafter, they were mounted on to coded microscope slides with Vectashield mounting medium (Vector Laboratories Ltd.) and sealed around the edges.

Particle numbers were assessed by fluorescence microscopy, using an Olympus BH-2 fluorescence microscope at a total magnification of 125×, and particle distribution by confocal (CF) microscopy using a Leica TCS SP system. All assays were carried out 'blind' on coded samples. Slides were stored in minimum light conditions until counted. Where counts for different experiments were carried out by different observers, each was trained by the same experienced observer, who also spot checked a proportion of the values for the others.

Particle numbers associated with the epithelial layer were counted by a 'crosswire' method. This involved assessing the number of particles per field of view along two full diameter 'virtual' lines perpendicular to each other, superimposed on the membrane. In addition, all particles were categorised as either single (non-touching) or as aggregates (touching or within one cell width of each other). Since this cross-wire protocol consistently sampled 26% of the total particles on the entire epithelial-covered insert (data not shown), all cross-wire particle data was increased by a factor of 3.85 to estimate the value for the whole insert.

Particle distribution was assessed by confocal microscopy orthogonal views to subdivide the particle numbers for the whole insert into those adsorbed (A) on to the epithelial surface and those within the epithelium (intraepithelial, E): this avoided overestimation of E. To do this, a *z*-series was taken of three, random particle sites/sample for three coded epithelial samples/group. Particles with less than 50% of their profile below the apical cell surface were classified as A: the number of these was subtracted from the total epithelial particle count to provide an estimate of E.

The fluid in the well below the membrane was retained for counting of submembranous particles (S) i.e. those that had passed through the epithelium. Fluid from each well was individually processed and mounted on to coded microscope slides and the total number of particles counted.

2.4. Particle experiments and their control groups

Particle exposure experiments were carried out on day 21 of culture. After TER (d21) was recorded, 1 μl containing 5.68×10^6 of 2 μm diameter (±S.D. 0.089 μm) Fluoresbrite® yellow-green (YG) latex particles (Polysciences, Inc., Warrington, PA) was added to the insert fluid in the apical compartment: 1 μl of basic culture medium was added to control wells. Cells were then incubated for the allotted particle/medium time (Tables 1 and 2; 5, 30, 60, 90 and 120 min) after which time the TER (post-particles) or TER (post-medium) reading was taken.

2.5. Bolus removal experiments

These experiments were carried out to simulate peristaltic onward movement of microparticles and comprised a 'particle time' followed by an 'interaction time'. After a particle time, carried out as for exposure experiments, the excess particle-containing medium was removed from the upper well and 1.5 ml of BCM was added. The plates were returned to the incubator for an interaction time and a final value was recorded for TER (bolus removal), prior to sampling, processing and counting as above. The experimental conditions were varied in two ways: (a) particle exposure times of 5, 30 or 60 min followed by a constant interaction time of 60 min; (b) particle exposure time constant at 30 min, followed by interaction times of 30, 60 and 90 min. Results for each group could be compared with those from exposure groups in two ways; for example, results for the 30 min exposure/60 min interaction bolus removal samples were compared with those for both the 30 and 90 min exposure group.

2.6. Comparisons with in vivo situation

The number of particles/1000 cells was calculated from the values for 5, 30 and 90 min after particle administration for both *in vitro* and *in vivo*.

In vitro values: (a) the number of adsorbed particles per well diameter, i.e. 50% of cross-wire count multiplied by the proportion of adsorbed particles; (b) the number of intraepithelial particles per well diameter, i.e. 50% of cross-wire count multiplied by proportion of intraepithelial particles; (c) the number of cells per insert diameter (3000 cells), obtained by dividing the insert diameter by the mean cell diameter, the latter from confocal microscopy.

In vivo values: these were taken from previously reported data on (a) the mean number per intestinal circumference of adsorbed particles on mucosal villous enterocytes for small intestinal segment 2 (5 min: 38, 30 min: 70, 90 min: 54); (b) mean number of particles in enterocytes per circumference for small intestinal segment 2 (5 min: 5, 30 min: 17, 90 min: 12; Smyth et al., 2005; Doyle-McCullough et al., 2007); (c) mean number of enterocytes per circumference (3926 cells, Carr et al., 1997).

2.7. Comparison of 'bolus removal' data with in vivo data

Calculation of the number of particles/1000 cells was carried out as described above in 2.6, for the bolus removal group for 30 min exposure/60 min interaction.

2.8. Hormone experiments

Vasoactive intestinal peptide (VIP, Sigma) was diluted in sterile PBS to a physiological concentration (20 pM, Gozes and Furman, 2004). On day 15 of culture the TER was recorded for plates to be exposed to VIP, aliquots of hormone were defrosted just before use and added to the apical surface every 24 h for the remaining culture period. Equivalent volumes of PBS vehicle or medium were used as controls.

On day 21 of culture, a reading for TER (after 5 days VIP, PBS or medium exposure) was taken. Thereafter, VIP was again added and cells were incubated for 10 or 30 min, after which time a further TER reading was taken (hormone addition). Particle addition experiments for a 30 min time point were then carried out on all groups and processed and analysed as above.

2.9. Data handling and statistics

TER data were subjected to statistical testing in two ways: by analysis of raw data; and by analysis of changes in TER, the latter calculated by subtracting one TER from another. TER data were analysed by ANOVA and particle numbers by Mann–Whitney *U*. The text of the results deals almost entirely with significant differences within and across groups, with relevant *p* values quoted; details of statistical results are also given in the figures.

3. Results

3.1. Caco-2 cells

Confocal microscopy showed that Caco-2 cells in a confluent epithelial layer were 17 μm high, and 8 μm by 10 μm wide, with a 3 μm subsidiary layer often present below the insert membrane. The TER increased over 21 days to $816 \pm 17\ \Omega\,cm^2$ ($n = 9$), implying adequate confluence. Thereafter ($n = 6$) it increased further to $1016 \pm 37\ \Omega\,cm^2$ at 32 days, but there was no significant further increase at 47 days ($1082 \pm 34\ \Omega\,cm^2$). Over the nine experiments reported here (Table 1), the mean TER (d21) for the untreated Caco-2 cultures was $993 \pm 36\ \Omega\,cm^2$. The variation was unrelated to the TER of the insert membrane in 'blank wells' prior to cell seeding (TER insert membrane).

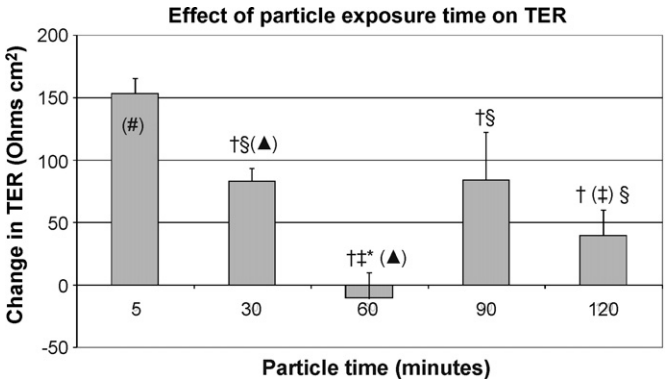

Fig. 1. Changes in TER (mean ± S.E.M.), for all particle exposure time points. Data are calculated from Table 2, columns 3–8 (TER post-particle exposure) cf. column 2 (TER pre-particle treatment). The zero line signifies no change after particle addition. Results of statistical tests are given as significant differences, $p \leq 0.05$ (or strong trends in brackets $p \leq 0.1$): #a change from TER (pre-particle exposure), †from 5 min, ‡from 30 min, §from 60 min, *from 90 min and ▲from 120 min.

3.2. Particle addition to cell layers (experiments 1–9)

While the addition of particles to blank insert membranes caused no change in TER (data not shown), their addition to Caco-2/membranes increased TER at 5 min (Table 2, columns 3–8, compared with column 2; Fig. 1), with a subsequent decrease, most obvious at 60 min ($p = 0.0001$–0.016). The addition of medium, as a particle control, produced the initial increase in TER but not the subsequent decrease.

The number of particles adhering to (A) and within (E) the epithelium was time-dependent (Table 3, $p = 0.002$–0.0002), with $5 < 30 = 60 < 90 < 120$ min and the percentage of particle aggregates on and in the Caco-2 epithelial layer increased significantly at 90 min exposure and thereafter ($p = 0.0002$–0.05). Submembranous (S) particles, which had passed through the cell layers, were identified to a similar extent in all groups, with a range of values of 5 ± 3 to 496 ± 314 particles.

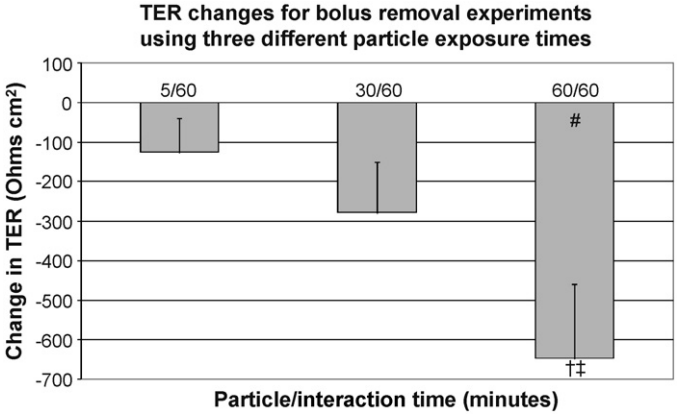

Fig. 2. Changes in TER (mean ± S.E.M.) for bolus removal experiments. Bars show total effect of particle and interaction time: changes are calculated from Table 2; columns 6–8 TER (post-both treatments), cf. column 2 TER (d21). The zero line signifies no change after treatment with both particle addition and interaction time. Significant differences ($p \leq 0.05$) are as follows: #a difference between TER (d21) and TER (post-both treatments), †from 5/60 and ‡from 30/60.

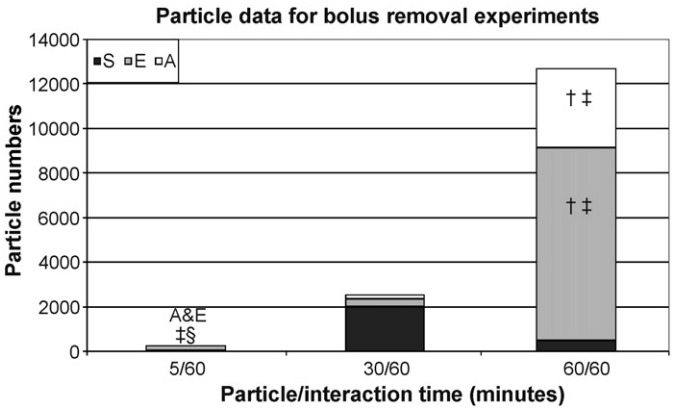

Fig. 3. Particle numbers in bolus removal experiments. Subepithelial (S), intraepithelial (E) and adsorbed (A) particle numbers. Significant differences ($p \leq 0.05$) from other time points are shown as: †a change from 5/60, ‡from 30/60 and §from 60/60. See Table 3.

3.3. Bolus removal (experiments 6–7)

In the peristalsis-simulating bolus-removal experiment groups there was a decrease in TER over the whole experiment (Table 2, columns 6–8 compared to column 2; Fig. 2) which was significantly greater than any TER change seen after standard particle addition ($p = 0.04$–0.0001). The extent of this total TER reduction increased with the time for which cultures were exposed to particles, but not with the length of the interaction time (data not shown), despite the fact that the decrease in TER occurred during the latter period (Table 2, columns 6–8 compared to columns 3–5).

The number of particles taken up in bolus-removal experiments (Table 3, Fig. 3) was dependent on particle exposure time. The 60 min exposure/60 min interaction time group was the only one to show an increase in epithelial particles compared to both particle and total time equivalent groups, in this case 60 and 120 min ($p = 0.002$ in both cases). Further to this, the percentage of aggregates was decreased at this time point when compared to that seen after 120 min particle exposure.

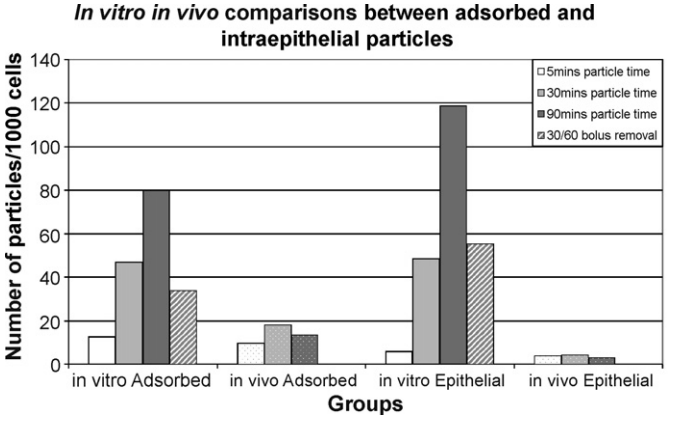

Fig. 4. Comparison of *in vitro in vivo* models for the number of particles/1000 cells adhering to or taken up into the epithelium. *n* values are as follows—*in vitro* 5 min: 11, 30 min: 23, 90 min: 9, bolus removal 30/60: 11; *in vivo* 5 and 30 min: 6 (Smyth et al., 2005; Doyle-McCullough et al., 2007), 90 min: 3 (Doyle-McCullough et al., 2007).

3.4. Comparisons of in vitro data with previous in vivo particle uptake (experiments 1–9)

Comparison of *in vitro* and *in vivo* data (Fig. 4) showed that, at 5 min, there were twice as many adsorbed as intraepithelial particles in both. By 30 min, although *in vivo* values had changed little, the corresponding *in vitro* values had both increased substantially, in particular the intraepithelial numbers. By 90 min, *in vivo* particle numbers remained virtually unchanged. However, the effect for *in vitro* groups depended on whether bolus removal was included or not. Although the standard particle exposure 90 min data showed a further substantial increase from the levels at 30 min, there was no such increase for the 30 min exposure/60 min interaction bolus removal group.

3.5. Particle uptake in VIP pre-treated cell layers (experiments 6–7)

There was no difference in TER between groups treated by daily addition of VIP from day 15 to day 20 and untreated groups (data not shown). However, after the final administration of VIP on day 21, 10 or 30 min prior to particle addition, the change in TER was time-dependent (Table 4; columns 5–6 compared with column 3; Fig. 5). The combined effect of VIP and subsequent particle addition produced no overall change in TER, however, this masked opposite changes seen following VIP and subsequent particle exposure between the two VIP exposure time points. For the 10 min exposure, there was an early strong increase in TER, produced by the final VIP administration, and

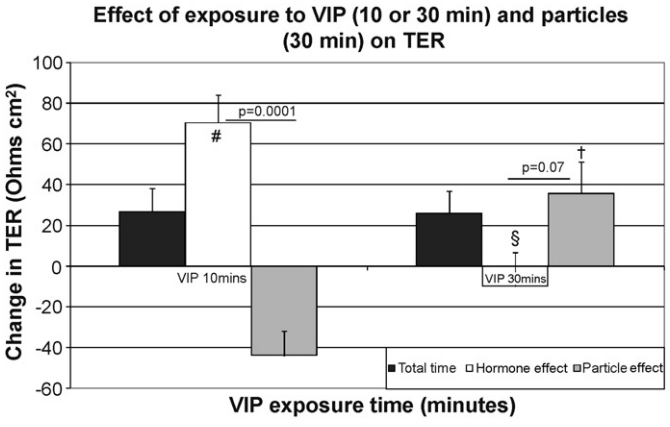

Fig. 5. Changes in TER (mean ± S.E.M.) of effect exposure time to VIP on 30 min particle exposure. Data for total time bars are calculated from Table 3, columns 7–8 TER (post-particle exposure) cf. column 3 TER (d21), VIP effect ('Hormone effect' bars) are columns 5–6 TER (post-final VIP exposure), cf. column 3 TER (d21) and subsequent particle effect ('Particle effect' bars) from columns 7–8 TER (post-particle exposure), cf. columns 5–6 TER (post-final VIP exposure). Significant differences ($p \leq 0.05$) are: #a change from TER (d21); §for changes in hormone effect with time and †for particle effect. The 20 pM 30 min is also the only group with a similar particle response to groups not treated by hormones (cf. Fig. 1, 30 min bar). Differences between VIP and particle effect are shown by '*p*' values on histogram.

Table 4
Transepithelial resistance (TER) as a measurement of epithelial permeability (mean ± S.E.M.) for Caco-2 cell layers pre-treated with VIP or control groups and subsequent particle exposure

Groups (experiment number)		Timeline (min) and TER values (Ω cm^2)				
Treatment/time (min)	**Untreated 21 day comparator**	0 Day 21	10	30	40	60
VIP (mean of 8-9)						
10min VIP+30P (n=8)	368 (11)	367 (20)	437 (18)		393 (20)	
30min VIP+30P (n=9)		390 (42)		381 (30)		416 (43)
VIP controls (mean of 8-9)						
30P (n=6)		368 (11)		422 (7)		
PBS+30P (n=12)	368 (11)	435 (20)		467 (22)		
Medium+30P (n=12)		412 (17)		435 (20)		

Column 1 outlines treatments and times: *n* values are in brackets. TER data in grey boxes refer to values following VIP exposure, the first being after short-term exposure daily for 5 days and the second after the final 5 or 30 min exposure. TER data in yellow boxes refer to values following short-term exposure daily for 5 days to PBS or medium. Green boxes refer to data gathered following particle exposure, for the time given in row 2.

the subsequent opposite effect of particle addition while, for the 30 min VIP exposure, the reverse was true (Fig. 5). The particle numbers reflected the lack of change of the final TER (Table 3).

4. Discussion

Before discussing the significance of the results, comment is needed on some aspects of the methodology. The dimensions of the Caco-2 cells conform to literature values (Delie and Rubas, 1997). The TER values of 399–1662 Ω cm^2 correspond adequately to those of Delie and Rubas (1997) and Ingels and Augustijns (2003), given accepted inter-laboratory variation. The data must be affected by the use of a 3 μm pore size, required for 2 μm particles to reach the lower well. These pores are larger than those used by McClean et al. (1998), Tyrer et al. (2002), Bravo et al. (2004) and Mukherjee et al. (2004), who thus avoided bilayer formation (Delie and Rubas, 1997). This submembrane epithelial component in the current study is only 3.3 μm, but may affect the passage of particles, perhaps even simulating the tissues found underlying the epithelium *in vivo*.

The current study focuses on the use of TER. While this, unlike mannitol passage, does not provide information on membrane integrity (Mukherjee et al., 2004), it does measure transepithelial resistance to passive ion flow (Madara, 1998) and is often used as a description of TJ permeability (Delie and Rubas, 1997). The variability of TER (d21) was addressed by referring each subsequent TER to this value, in line with the use of percentages (McClean et al., 1998) or control-referenced normalisation (Turner et al., 2000), rather than using raw data (Delie and Rubas, 1997; Bravo et al., 2004; Mukherjee et al., 2004). In the current paper, the comparison of TER values with different 'pre-treatment' or 'comparator' values proved useful by showing, for instance, that the lack of final change in TER in the VIP/particle experiments may hide two changes of equal but opposite effect.

The first aim of the current work, the study of *in vitro* epithelial permeability after microparticle exposure, was prompted by earlier reports of predominantly villous uptake *in vivo in situ* (Hodges et al., 1995; Smyth et al., 2005; Doyle-McCullough et al., 2007) rather than Peyer's involvement (Ermak et al., 1995; Thomas et al., 1996; Beier and Gebert, 1998). Several mechanisms could explain villous uptake, including uptake at villous tips (Volkheimer, 1977), or passage through or between the cells (Cartwright-Shamoon et al., 1995). The paracellular pathway could involve the opening of TJs (Bjork et al., 1995; Hodges et al., 1995; McCullough et al., 1995; Wiesner et al., 2002), which have gate and fence functions around contiguous cells, related to the presence of specific families of membrane, scaffolding and cytoskeletal proteins (Cereijido et al., 1993).

The TJ closure and subsequent loosening after latex particle addition in the current study, is at variance with the lack of change after addition of poly-DL-lactide particles (McClean et al., 1998), although the apparent different outcome could be related to their use of raw TER values; to possible changes induced by taking TER measurements every 5 min for 2 h; and to different biological responses associated with the type of particle used. TER may be altered by factors other than TJs changes, such as distension of the intercellular space, as seen during high rates of fluid passage between cells, when the epithelial resistance is lowered (Pappenheimer, 1987). These alterations could be related to the increased permeability with time in exposure experiments, amplified by the interaction period of the bolus removal experiments. However, the decrease in TER change with time, for particle exposure groups, coincides with an increase in intraepithelial particle numbers, suggesting some correlation between the two. These particle-induced changes in TER are in line with the decreases following exposure to inorganic substances (Ferruzza et al., 1999), to organic agents (Stenson et al., 1993; Wells et al., 1998; Ma et al., 1999; Araki et al., 2005), and to other

substances known to loosen TJs (Gonzalez-Mariscal et al., 2005). More information is needed on how various materials interact with TJs and their protein constituents (Smyth et al., 2005; Gonzalez-Mariscal and Nava, 2005).

The second aim addresses the gap in the literature on direct comparison of *in vitro* and *in vivo* microparticle uptake, despite a range of *in vitro* studies used to explore the *in vivo* intestinal situation (Artursson et al., 2001; Masungi et al., 2004). One study that examined interaction of nano- and micro-spheres with both Caco-2 and rat or rabbit isolated intestinal tissue did not compare the *in vitro* and *in vivo* results (McClean et al., 1998). The current experiments confirm the usefulness of the Caco-2 model by the similarity between particle numbers *in vitro* and *in vivo*, particularly at 5 min. The fact that bolus removal experiments improves this comparison at later time points implies that this more closely approximates to the *in vivo* peristaltic situation and shows that more information can be gained on the epithelial permeability by changing the parameters of the model.

The third aim addresses the use of the *in vitro* model to explore the effect on microparticle uptake of other challenges to the epithelium, by the use of the gastrointestinal hormone VIP, which is known to increased the expression of ZO-1 TJ protein (Neunlist et al., 2003). The TJ closure seen 10 min after exposure to VIP is in line with that reported 10–60 min after a higher dose (Blais et al., 1997). Factors other than dose will also be relevant, including VIP half-life (Gololobov et al., 1998), the effect on cleavage routes of the *in vitro* environment and the need to position the hormone nearer to the basolateral position of the relevant receptors (Anderson et al., 2003).

Finally, the hypothesis being addressed is the likelihood that the *in vivo* microparticle uptake occurring at villous epithelium and not Peyer's patches is explained by an opening of enterocyte tight junctions. The results demonstrate that the initial TJ response to most stimuli is immediate closure, possibly protective, followed by varying degrees of opening, the extent of which may be dependent on the microenvironment. Since microparticles form one of the stimuli that produce these changes in TJs, the hypothesis has not been disproved: more work is needed to confirm the role of villous TJs in the uptake of large particles.

In conclusion, Caco-2 cells grown to confluence on a membrane with 3 μm pores allow the study of the interaction between the cells and microparticles and gives results comparable to those obtained from *in vivo in situ* experiments. The model also shows the sensitivity of the TJs to environmental changes, such as the addition of hormone.

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