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Transepithelial Transport of Large Particles in Rat: a New Model for the Quantitative Study of Particle Uptake

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Abstract

The transport of large particles across the intestinal mucosa and the mechanisms of transfer of the particles into the body are still little understood.

Fluorescent polystyrene latex particles (2 μm diam.) were administered orally to young male Sprague–Dawley rats in doses of $2{\cdot}33 \times 10^3$, $2{\cdot}33 \times 10^6$ and $2{\cdot}33 \times 10^9$ particles. After 60 min, Peyer's patches and Peyer's patch-free tissues were collected from the small intestine and colon. A novel technique was used to exclude non-translocated particles adherent to the mucosal surface; the intestinal epithelium was stripped from the intestine by immersion in Hanks' balanced salt solution containing 1·5 mM EDTA. Particles in solubilized samples of intact and epithelium-stripped Peyer's patches and Peyer's patch-free intestinal tissue and colon were quantified by fluorescence microscopy. The location of particles within the intact and epithelium-stripped gut samples was revealed by confocal microscopy. Particles were shown to have been taken up along the entire length of the small and large intestines via both Peyer's patches and the normal intestinal epithelium. The number of particles detected in the distal region was greater than in the proximal part of the small intestine, although the difference was not statistically significant.

This study has revealed that large numbers of non-translocated particles adhered to the mucosal surface resulting in a high background count. The assay system was considerably improved by the epithelium-stripping technique. The process of transepithelial uptake is a potentially important route of uptake of toxic, immunologically active and radioactive substances. These particles are much larger than the conventionally accepted upper limit for absorbed materials.

The normal gastrointestinal tract in the adult was previously thought to be an impenetrable barrier to the uptake and transport of large particles (Jankowski et al 1994). However, over the last three decades experimental data have accumulated showing that large particles can be transported from the intestinal lumen across the gut wall into the circulation (Volkheimer & Schulz 1968; Warshaw et al 1971; Jani et al 1992; Simon et al 1995). Several investigations have shown that Peyer's patches are the major sites through which particles traverse the intestinal epithelium (LeFevre & Joel 1984; Sass et al 1990; Pappo et al 1991; Jepson et al 1993). These findings have been linked to the specialized structure of the epithelium overlying Peyer's patches, as it contains the unique M cells which have the capacity to take up a wide range of macromolecules, bacteria and viruses (Wolf & Bye 1984; Amerogen et al 1994; Clark et al 1994; Regoli et al 1995).

However, there is now evidence that the Peyer's patch-free villous absorptive region of the intestine also has the capacity to take up particles ranging from 11 nm to 2 μm (Kataoka et al 1989; Hodges et al 1995). So far, most conventional experiments have relied on oral administration of identifiable particles, which have subsequently been counted in solubilized samples of Peyer's patches, Peyer's patch-free intestinal tissue and colon. The validity of these studies is a matter of concern because many of the particles counted in samples of the intestinal wall and Peyer's patches might only have been adherent to the mucous layer. In that position they would lie on the luminal surface of the epi-

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thelium, and so would be external to the intestinal wall, but they would be attached to the tissue samples and would remain in the tissue digests, particularly after short-term exposure to particles, which is of interest in this study. After solubilization they would be counted as if they had been within the intestinal wall. Such contamination by particles adherent to the surface mucosa would make it impossible to quantify accurately particles transported across the gut epithelium into the submucosa. This might differ in tissues excised from particle-treated rats after long-term exposure.

We have adapted and applied a novel method to remove particles adherent to the surface mucosa of samples of intestine, and so have been able to quantify accurately translocated particles within the remaining tissues. The technique has been employed in the rat to determine uptake through Peyer's patches and the intervening mucosa in the small bowel, and also across the wall of the colon.

This method is proposed as an accurate quantitative means of study of the uptake of large particles across the mucosal barrier of the intestine. The method has been used to demonstrate the preferential site of uptake of particles into the gut wall.

Materials and Methods

Microspheres

Experiments were performed with non-degradable, monodispersed, fluorescent, Fluoresbrite plain yellow-green, polystyrene latex particles, 2·139 μm in diameter (Polysciences, Northampton, UK).

Animals

Male CD Sprague–Dawley rats (Charles River, Kent, UK), 150–200 g, six-weeks-old, were used. Rats were acclimatized in the laboratory under standard conditions for one week before the study and had free access to SDS (Witham, Essex) RMI (SQC) pellet diet and tap water throughout. All animal treatment was performed by a licensed investigator in accordance with regulated procedures under the Animals (Scientific Procedures) Act (1986).

Experimental procedures

Animals were fasted for approximately 16 h, with free access to water. Particle suspension in distilled water (0·5 mL containing $2{\cdot}33 \times 10^3$ (n = 5), $2{\cdot}33 \times 10^6$ (n = 5) and $2{\cdot}33 \times 10^9$ (n = 11) particles) was then administered by gavage. Control animals (n = 7) were dosed with distilled water (0·5 mL). After dosing the animals had free access to water but were denied food. To prevent contamination with particles, control and experimental animals were housed in separate rooms and different doses were given with clean needles. Animals were killed by CO_2 asphyxiation 60 min after dosing. To minimize contamination, the skin of each rat was removed before opening the abdomen and the instruments used to dissect the animals were cleaned between each autopsy. Disposable materials were used between each autopsy. To remove the gut contents, the entire intestine was excised and its lumen was flushed twice with ice-cold phosphate-buffered saline (40 mL), containing (mM) 96 NaCl, 1·5 KCl, 8 KH_2PO_4, 5·6 Na_2HPO_4, pH 7·4.

Tissue samples

After removal of the gut contents, two identical sets of tissue samples were taken from each animal. Each set of tissue samples consisted of three Peyer's patches from the most proximal and the most distal regions of the small intestine. Although Peyer's patch tissues were carefully dissected free from adjacent tissues, a small amount of villous tissue and underlying musculature was probably included in the samples excised. Samples of the small intestine between Peyer's patches were carefully excised from the proximal and distal ends of the small intestine and samples of the ascending colon were also excised. One set of tissues was prepared for quantitative study of particles transferred across the epithelium into the mucosa or Peyer's patches of the intestine and for examination by light microscopy. The other set of tissue samples was prepared for examination by confocal microscopy.

Removal of non-translocated particles (epithelium stripping)

To exclude non-translocated particles adherent to the mucosal surface of the intestinal epithelium, intestinal epithelial cells were dissociated from segments of the intestine by a modification of the method of Fox et al (1985) and Tepperman et al (1993). All tissue samples taken from the first set were weighed in separate containers immediately after excision. Each sample was then washed for 2 h in ice-cold phosphate-buffered saline, in its own container, before epithelial stripping. Each sample of intestine from each control and particle-treated rat was washed twice in each of two buffer solutions, ice-cold phosphate-buffered saline and ice-cold phosphate-buffered-saline plus 1 mM dithiotreitol, to remove intestinal mucus (Ferraris et al 1992). Each washing was performed for 30 min. Before changing to new buffer the tissue sample was agitated by vortex mixing for 30 s. After washing, each tissue sample was transferred to a separate plastic tube containing citrate buffer (mM,

96 NaCl, 1·5 KCl, 8 KH_2PO_4, 5·6 Na_2PO_4 and 27 $Na_3C_6H_5O_7.2H_2O$, pH 7·4; 10 mL) to loosen attachment of the epithelial cells (Ferraris et al 1992). The tubes were then placed in a shaking water bath (37°C, 80 rev min^{-1}) for 15 min.

For dissociation of intestinal epithelial cells the tissue samples were then removed from the citrate buffer and transferred to individual clean plastic tubes containing Hanks balanced salt solution (Gibco Laboratories) supplemented with 1·5 mM EDTA and 0·5 mM dithiotreitol, pH 7·4 (epithelial-cell-dissociating solution). The samples were then placed in a shaking water bath (37°C, 80 rev min^{-1}) for 60 min. The removal of calcium by EDTA and citrate results in loss of protein-mediated, calcium-dependent adhesion of cells to each other and to the basement membrane (Ferraris et al 1992). The tissues were removed from the epithelial-cell-dissociating solution and extensively washed twice (30 min each) with phosphate-buffered saline. They were then blotted dry. After each incubation and each washing period the tissue samples were agitated by vortex mixing for 30 s to remove adherent particles.

Particle quantification

After epithelium stripping, half of each tissue sample was prepared for histological examination and the other half was weighed, placed in 70% industrial methylated spirit for 24 h and then solubilized in potassium hydroxide (15%, 10 mL) for at least four days (Simon et al 1994). Each sample of solubilized tissue was filtered through a black-back gridded filter (Millipore, UK; pore size 0·8 μm). The intensity of fluorescence of the particles was not affected by the alcohol fixation or by the potassium hydroxide treatment. Filters were placed on glass slides and mounted in Hydromount (National Diagnostics, Atlanta, GA). Separate sets of glass slides and cover slips were used for each control and experimental sample. Particles on the filters were readily detected against the black background of the filter by fluorescence microscopy and were counted. The number of particles detected in each sample was expressed as the number of particles (g tissue)$^{-1}$ (fresh weight).

Technique control for quantification of non-translocated particles

Tissues from animals in the control group were treated in exactly the same way as described for particle-treated rats. To assess the number of particles which might have adhered to the tissue samples during the epithelial cell dissociation and washing steps, i.e. particles released by the dissociation technique which might then have adhered to the newly exposed submucosal tissues, intestinal tissues from undosed animals were excised, weighed and incubated in the epithelial-cell-dissociating solutions previously used for corresponding tissues from dosed animals. The intestinal tissues from undosed animals were referred to as technique-control tissues. After epithelial cell dissociation for 60 min the technique-control tissues were washed, fixed, solubilized and examined for the number of particles as previously described for the experimental tissues.

Quantification of particles in intact intestinal tissues

To compare the numbers of particles taken up by the epithelium-stripped tissues and non-epithelium-stripped tissues, four groups each of five animals were treated with a suspension of latex particles or distilled water in the same doses and exposure period as the study described above. After sampling of the gut tissues each sample of tissue from each animal was washed twice in ice-cold phosphate-buffered saline. The tissue sample was washed for 30 min and then agitated for 30 s before and after changing the phosphate-buffered saline. The tissue was then fixed in industrial methylated spirit and solubilized in potassium hydroxide solution (15%). After complete tissue solubilization, the sample was prepared and counted by the method described above.

Morphological analysis

The location of the fluorescent particles within the other identical tissue samples was determined by confocal microscopy. The industrial methylated spirit-fixed tissues from both intact and epithelium-stripped intestine were mounted on glass slides with Hydromount and were examined by scanning confocal microscopy (TCS4D; Leica, Heidwourd, Germany). FITC-filter was used at $\lambda = 488$ nm. Depth of tissue was scanned until the limit of detection (162 μm depth) of the microscope. The intact and epithelium-stripped gut tissues were also examined by light microscopy, using conventional paraffin sections stained with haematoxylin and eosin.

Statistical analysis

Quantitative data were compared using the non-parametric Kruskal–Wallis test.

Results

Particles were detected within the Peyer's patches and the intervening Peyer's patch-free tissues of the small intestine and in the colon of treated animals.

The numbers of particles detected in all epithelium-stripped samples of intestinal tissue of rats treated with $2{\cdot}33 \times 10^9$ particles were greater than in the tissues of those given the lower concentrations ($P < 0{\cdot}02$; Table 1). In addition, the numbers of particles in the epithelium-stripped samples of the intestinal tissues from rats treated with $2{\cdot}33 \times 10^6$ particles were generally larger than those from rats treated with $2{\cdot}33 \times 10^3$ particles, although the difference was not significant. There was no significant difference between the numbers of particles translocated across the intestinal epithelium of Peyer's patches and Peyer's patch-free tissues derived from animals given $2{\cdot}33 \times 10^9$ particles. The numbers of particles in the gut tissues obtained from animals given the same treatment varied. Significantly higher numbers of particles were detected in samples of detached epithelium; again most were present in the tissues from animals dosed with $2{\cdot}33 \times 10^9$ particles (Table 2).

No contaminating particles were found in tissues taken from control animals. The numbers of particles detected in technique-control tissues corresponding to all particle-treated tissues were always found to be low; the numbers of particles did not exceed 1% of the value in experimental tissues (Table 3) and were therefore ignored.

The number of particles in the gut tissues obtained from non-epithelium-stripped or intact gut tissues was greater than in the epithelium-stripped tissues ($P < 0{\cdot}02$), as shown in Table 4.

The histological appearance of the samples of small intestine after epithelium dissociation showed that the intestinal epithelial cells were removed from the villous tip to the lower villous-upper crypt region, leaving the villous core completely denuded of cells. Complete removal of the overlying epithelium from Peyer's patches by the epithelial-cell-dissociating solution was also demonstrated (Figure 1).

Confocal microscopy revealed translocated particles within the intestinal wall in both tissues of Peyer's patches and Peyer's patch-free samples of the small intestine and also in the colon in both intact and epithelium-stripped tissue (Figure 2).

Discussion

The findings revealed that 2-μm polystyrene particles were translocated across the intestinal epithe-

Table 1. Number of 2-μm latex particles in intestinal tissues taken from rats 60 min after oral administration of distilled water or latex particles.

Particle treatment	Proximal Peyer's patches	Distal Peyer's patches	Proximal small intestine	Distal small intestine	Colon
Distilled water (n = 7)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
$2{\cdot}33 \times 10^3$ particles (n = 5)	8 ± 8 (0·02)	18 ± 18 (0·07)	6 ± 4 (0·69)	28 ± 17 (2·92)	68 ± 65 (4·42)
$2{\cdot}33 \times 10^6$ particles (n = 5)	136 ± 96 ($3{\cdot}66 \times 10^{-4}$)	16 ± 12 ($5{\cdot}79 \times 10^{-5}$)	346 ± 344 (0·04)	162 ± 101 (0·02)	114 ± 68 ($7{\cdot}41 \times 10^{-3}$)
$2{\cdot}33 \times 10^9$ particles (n = 11)	14 740 ± 10 672 ($3{\cdot}97 \times 10^{-5}$)	17 797 ± 9607 ($6{\cdot}45 \times 10^{-5}$)	9709 ± 13 790 ($3{\cdot}42 \times 10^{-3}$)	78 522 ± 28 027 ($8{\cdot}19 \times 10^{-3}$)	58 572 ± 25 073 ($3{\cdot}81 \times 10^{-3}$)

The number of particles is shown for each region of epithelium-stripped intestinal tissues examined. Results are presented as the number of particles (g tissue)$^{-1}$ (mean ± s.e.m.). The numbers in parentheses are percent dose taken from each whole region of the intestinal tissue.

Table 2. Number of 2-μm latex particles detected in the epithelium-detached fraction of each region of intestinal tissue taken from rats 60 min after oral administration of distilled water or latex particles.

Particle treatment	Proximal Peyer's patches	Distal Peyer's patches	Proximal small intestine	Distal small intestine	Colon
Distilled water (n = 7)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
$2{\cdot}33 \times 10^3$ particles (n = 5)	0 ± 0	4 ± 4	0 ± 0	286 ± 283	5·4 ± 4
$2{\cdot}33 \times 10^6$ particles (n = 5)	48 ± 48	1230 ± 1057	88 ± 56	328 ± 308	10 ± 3
$2{\cdot}33 \times 10^9$ particles (n = 11)	1559 ± 704	12 550 ± 10 790	88 442 ± 44 517	1 438 140 ± 1 382 010	14 105 ± 5746

Results are presented as the number of particles (g tissue)$^{-1}$ (mean ± s.e.m.).

Table 3. Number of 2-μm latex particles detected in technique-control tissues of undosed rats incubated in the epithelial-cell-dissociating solution of corresponding tissues derived from rats 60 min after being dosed with latex particles.

Particle treatment	Proximal Peyer's patches	Distal Peyer's patches	Proximal small intestine	Distal small intestine	Colon
$2{\cdot}33 \times 10^3$ particles (n = 5)	0 ± 0	32 ± 32	4 ± 4	2 ± 2	0 ± 0
$2{\cdot}33 \times 10^6$ particles (n = 5)	38 ± 24	42 ± 26	16 ± 4	8 ± 8	6 ± 4
$2{\cdot}33 \times 10^9$ particles (n = 11)	61 ± 28	232 ± 115	101 ± 45	220 ± 82	41 ± 17

Results are presented as the number of particles (g tissue)$^{-1}$ (mean ± s.e.m.).

Table 4. Number of 2-μm latex particles in non-epithelium-stripped or intact intestinal tissues obtained from rats 60 min after gavage of distilled water or latex particles.

Particle treatment	Proximal Peyer's patches	Distal Peyer's patches	Proximal small intestine	Distal small intestine	Colon
Distilled water (n = 7)	18 ± 0	0 ± 0	8 ± 8	0 ± 0	7 ± 7
$2{\cdot}33 \times 10^3$ particles (n = 5)	33 ± 25	22 ± 15	34 ± 13	172 ± 125	24 ± 22
$2{\cdot}33 \times 10^6$ particles (n = 5)	19 ± 12	39 ± 21	1585 ± 1372	1262 ± 766	59 ± 28
$2{\cdot}33 \times 10^9$ particles (n = 11)	8 206 850 ± 8 146 730	2 454 270 ± 2 294 540	5 217 440 ± 2 164 170	4 240 660 ± 2 939 990	77 000 ± 38 559

Results are presented as the number of particles (g tissue)$^{-1}$ (mean ± s.e.m.).

lium along the entire length of the small and large intestine of the rat within 60 min of a gavage dose. The particles entered the subepithelial compartment via both Peyer's patches and the villous absorptive regions of the small intestine. The number of particles detected in the distal region seemed larger than in the proximal part of the small intestine, although the difference was not statistically significant.

Recent publications have reported conflicting findings about the absorptive capacity for particles of each region of the intestine. Hodges et al (1995) and Gonnella & Walker (1987) stated that the proximal intestine was the preferential site of uptake, whereas the experimental evidence of Michel et al (1991) suggested that the transepithelial transport of nanoparticles was greater across the distal intestine than across the jejunum or duodenum. It has also been claimed that in aged rats a greater number of large particles was transported across the gut wall via distal Peyer's patches than via proximal Peyer's patches (Simon et al 1994). These discrepant results might have been partly a result of the different ages of the animals studied or because of variation in the chemical and physical properties of the particles, including variation of the particle doses used in the various studies. It is also possible that endocytic activity, which has been proposed as the major mechanism of macromolecule absorption, might vary along the length of the gastrointestinal tract (Aungst & Shen 1986). However, the results in this work indicated that the uptake of particles is no different in any intestinal tissue samples taken from different regions of the intestine other than as a result of the dose administered. Because no morphological analysis was performed in this study, knowledge about the uptake of latex particles by specific cells within the intestinal tract has not been enhanced. Moreover, this work does not provide further information about the route of particle uptake.

Previous investigations have stated that Peyer's patches are important as a major site of uptake and transport of a wide range of macromolecules, including inert particles and pathogenic organisms, from the intestinal lumen across the intestinal barrier into the systemic organs via the lymphatic circulation and bloodstream (LeFevre et al 1978, 1989; Wolf & Bye 1984; Jani et al 1989, 1990; Sass et al 1990; Amerogen et al 1994; Clark et al 1994; Regoli et al 1995). However, the results of the current study support earlier findings that the transmucosal passage of large particles in the intestine occurs via both the villous epithelium and the Peyer's patches (Matsumo et al 1983; Gonnella & Walker 1987; Kataoka et al 1989; Michel et al

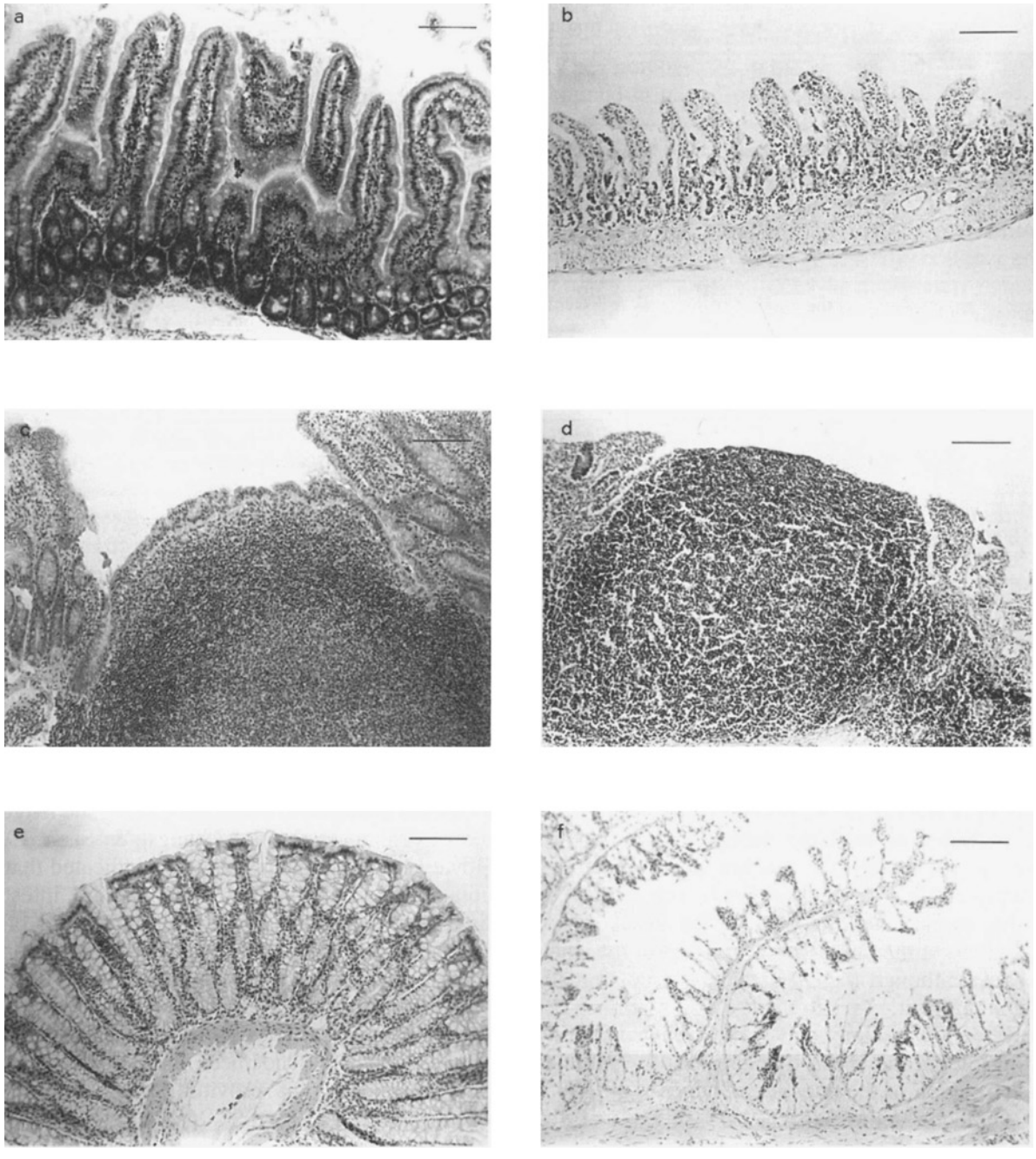

Figure 1. Histological sections showing intact and epithelium-stripped gut tissues: a, intact intestinal villi; b, the epithelium-stripped villus cores; c, intact dome of Peyer's patch; d, dome of epithelium-stripped Peyer's patch; e, intact colon; f, the epithelium-stripped colon. b, d and f are shown completely denuded of superficial epithelial cells. Haematoxylin and eosin stain. Bar = 100 μm.

1991; Hodges et al 1995). The current study also revealed that a large number of non-translocated particles adhered to the mucosal surface of intact intestinal tissues. For comparison, particularly after the largest dose administered, the number of particles detected in each region of the intact intestinal tissue was approximately 100-fold larger than the number of particles detected in each region of the corresponding epithelium-stripped intestinal tissue. Quantitative studies using digestion of intact intestinal tissues can, therefore, lead to erroneous results. The experiments described here provide accurate data from studies in which contamination by particles only superficially adherent to the mucous layer has been prevented by removal of the surface layer of mucus and the mucosal epithelium. It is true that stripping off the cells will also have removed particles in the process of being trans-

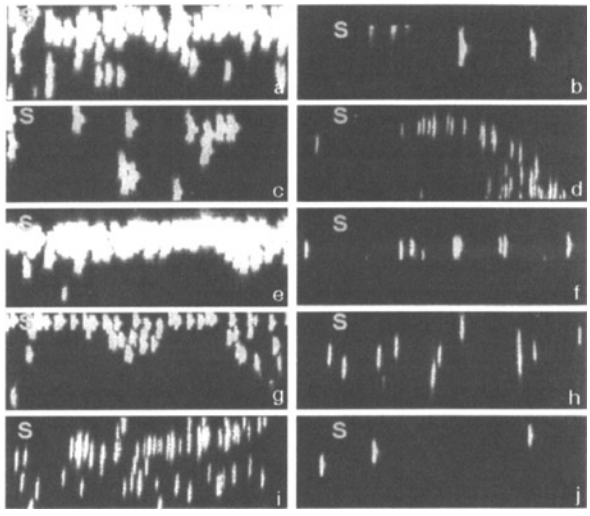

Figure 2. Confocal scanner images showing cross-sections (162 μm depth) of the surfaces of intact and epithelium-stripped intestinal tissues from rats dosed orally with $2{\cdot}33 \times 10^9$ particles for 60 min: a, intact proximal Peyer's patches; b, dome of epithelium-stripped proximal Peyer's patches; c, intact distal Peyer's patches; d, dome of epithelium-stripped distal Peyer's patches; e, intact proximal intestine; f, the epithelium-stripped proximal intestine; g, intact distal intestine; h, the epithelium-stripped distal intestine; i, intact colon; j, the epithelium-stripped colon tissues. Fluorescent particles within the submucosal tissues are clearly seen in both intact and epithelium-stripped intestinal tissue. A large number of particles is seen on the epithelial layer of the intact intestinal tissues. S = luminal surface of the intestinal tissue.

ported through the epithelium, but it is not known what proportion of these particles completely traverse the epithelial layer and enter the subepithelial compartment. It is also unclear what fraction of particles within the epithelial cells remain in this compartment or are returned back into the gut lumen (Bockman & Stevens 1977; Joel et al 1978).

We have described a novel model for the quantitative study of particle uptake across the intestinal epithelium into the subepithelial compartment. The particles detected within the remaining epithelium-stripped gut tissues can be regarded as the absolute number transferred from the gut lumen into underlying tissues and in this study was found to be less than 1% of the administered dose. In this study contamination was negligible because no particles were found in control tissues. A small number of particles was found in the technique-control tissues but the number did not exceed 1% of the count in particle-treated tissues. It is apparent, therefore, that very few of the superficial particles, after removal in the EDTA solution, become adherent to the newly exposed submucosa.

In addition, particles within the submucosa of epithelium-stripped tissue were detected by confocal microscopic examination. These findings support our quantitative data by showing the presence of particles in the submucosal regions of Peyer's patches and Peyer's patch-free intestinal tissues. The method is proposed as an accurate quantitative means of studying the uptake of large particles across the mucosal barrier of the intestine.

Several studies using microsphere carriers have shown that effective oral delivery of drugs and vaccines is possible (Eldridge et al 1990; Amidon & Lee 1994). However, further work is needed to understand the largely unknown processes involved in the epithelial transport of large particles and the dynamics of uptake and route of conveyance.

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