Engineering of Dendrimer Surfaces to Enhance Transepithelial Transport and Reduce Cytotoxicity

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Purpose. To evaluate the cytotoxicity, permeation, and transport mechanisms of PAMAM dendrimers and surface-modified cationic PAMAM dendrimers using monolayers of the human colon adenocarcinoma cell line, Caco-2.

Methods. Cytotoxicity was determined using the MTT assay. The effect of dendrimers on monolayer integrity was determined from measurements of transepithelial electrical resistance (TEER) and [^{14}C]mannitol apparent permeability coefficient (P_{app}). The P_{app} of dendrimers through monolayers was measured in both the apical (A)-to-basolateral (B) and B→A directions at 4°C and 37°C and also in the presence and absence of ethylenediamine tetraacetic acid (EDTA) and colchicine.

Results. The cytotoxicity and permeation of dendrimers increased with both concentration and generation. The cytotoxicity of cationic dendrimers (G2, G3, G4) was greater than that of anionic dendrimers (G2.5, G3.5) but was reduced by conjugation with lauroyl chloride; the least cytotoxic conjugates were those with six attached lauroyl chains. At 37°C the P_{app} of cationic dendrimers was higher than that of anionic dendrimers and, in general, increased with the number of attached lipid chains. Cationic dendrimers decreased TEER and significantly increased the P_{app} of mannitol. Modified dendrimers also reduced TEER and caused a more marked increase in the P_{app} of mannitol. The P_{app} values of dendrimers and modified dendrimers were higher in the presence of EDTA, lower in the presence of colchicine, and lower at 4°C than at 37°C.

Conclusions. The properties of dendrimers may be significantly modified by surface engineering. Conjugation of cationic PAMAM dendrimers with lauroyl chloride decreased their cytotoxicity and increased their permeation through Caco-2 cell monolayers. Both PAMAM dendrimers and lauroyl-PAMAM dendrimer conjugates can cross epithelial monolayers by paracellular and transcellular pathways.

KEY WORDS: PAMAM dendrimer; cytotoxicity; Caco-2 cells; dendrimer surface modification; engineering of dendrimer surfaces.

INTRODUCTION

Polyamidoamine (PAMAM) dendrimers are a class of hydrophilic polymers, the surfaces of which possess either positively charged primary amine groups (full generation: G1, G2, etc.) or negatively charged carboxyl groups (half generation: G1.5, G2.5, etc.) at physiological pH (1). Dendrimers have found several pharmaceutical applications including the encapsulation/solubilization of drugs, carriers for transepithelial transport, delivery of DNA and oligonucleotides, and platforms for the development of cancer therapeutics (2). Their potential as drug carriers has been investigated, and the surface amine groups of PAMAM dendrimers have been used to attach useful moieties, e.g., peptides and antibodies.

There have been two major reports on the biological properties of dendrimers. Roberts *et al.* (3) reported the concentration- and generation-dependent cytotoxicity of PAMAM dendrimers (G3, G5, G7) toward V79 Chinese hamster cells. *In vivo* cytotoxicity experiments in mice (7 days to 6 months) showed no evidence of behavioral changes, weight loss, or immunogenicity with G3 and G5 dendrimers. It was concluded that the dendrimers did not exhibit properties that would preclude their use in biological applications. Malik *et al.* (4) observed that red blood cell hemolysis by PAMAM dendrimers was charge, concentration, and generation dependent. The hemolysis was thought to be a consequence of aggregation of adsorbed dendrimer molecules on the cells. Similarly, G3 and G4 PAMAM dendrimers were markedly cytotoxic toward the B16F10 mouse melanoma cell line, but the anionic PAMAM dendrimers (G1.5, G2.5, G3.5) showed no cytotoxicity; the observed cytotoxicity of the G3 and G4 dendrimers was attributed to their polycationic charge. However, it should be noted that the toxicity and biological profile of a dendrimer-based delivery system (with surface modifiers and a payload of drug) is likely to be different from that of an unmodified dendrimer (5).

Dendrimers have been shown to cross cell barriers at sufficient rates to act as potential carrier/delivery systems (6,7). Transport studies on rat everted intestinal sacs showed that anionic PAMAM dendrimers exhibited rapid serosal transfer rates with low tissue binding (7). Conversely, cationic PAMAM dendrimers were found to associate strongly with tissue and had low transport rates. The reason for this difference may be the strong interaction between the negatively charged cell membrane and cationic molecules. El-Sayed *et al.* (8) studied the transport of PAMAM dendrimers (G0–G2) across Caco-2 cells and suggested that dendrimer transport was via the paracellular pathway.

The objectives of this study were to investigate the cytotoxicity and transport of PAMAM dendrimers (G2, G2.5, G3, G3.5, and G4) using monolayers of the human intestinal adenocarcinoma cell line Caco-2, which exhibit many characteristics of the human small intestinal epithelium (9,10) and have been extensively used as an *in vitro* model in absorption studies (11–14). Cationic PAMAM dendrimers (G2, G3, and G4) were modified by surface attachment of the biocompatible absorption enhancer lauroyl chloride (C_{12}, a medium-chain fatty acid), and the cytotoxicity and transepithelial transport of the conjugates were compared with unmodified dendrimers. Recent investigations have identified lauroyl chloride as an interesting absorption enhancer with greater absorption-enhancing properties than other medium-chain fatty acids (15–17). The possible mechanisms of enhancement are thought to include regulation of paracellular permeability by an effect on the phospholipase-C (PLC)-dependent pathway and perturbation and change of fluidity of the cell membrane via the transcellular pathway (18). The potential cytotoxicity of both unmodified and conjugated PAMAM dendrimers has

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been assessed by measurement of cell survival using the MTT assay. The influence of the Ca^{2+} chelator ethylenediamine tetraacetic acid (EDTA), which is known to cause disruption of the tight junctions (19), and the endocytosis inhibitor colchicine (20) on the transepithelial transport of dendrimer, and of dendrimers on the permeability of monolayers to the paracellular marker [^{14}C]mannitol have been determined to assist in the elucidation of the transport mechanisms.

MATERIALS AND METHODS

Materials

PAMAM dendrimers (G2, G2.5, G3, G3.5, and G4) with ethylenediamine cores were purchased from Dendritech Inc. (Michigan, USA). 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Lancaster Synthesis (Morecombe, Lancashire, UK). Ethylenediamine (99%), 2,4,5-trinitrobenzenesulfonic acid (TNBS), fluorescein isothiocyanate (FITC), FITC-labeled dextrans 4000 (FD4) and 10,000 (FD10), lauroyl chloride, colchicine, trypan blue, 3-(4,5-dimethythiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethysulfoxide (DMSO), and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma-Aldrich Co. Ltd. (Poole, Dorset, UK). [^{14}C]Mannitol (specific activity 50 mCi/mmol) was purchased from Amersham-Biosciences (Little Chalfort, Bucks, UK). Cell culture materials were from Gibco BRL Life Technologies (Paisley, Scotland); fetal bovine serum was from Labtech International, and polycarbonate cell culture inserts (Transwell® 12 mm diameter) and cluster plates (96 well) were purchased from Corning Costar UK Ltd. (High Wycombe, Bucks, UK).

Synthesis and Characterization of Lauroyl-Dendrimer Conjugates

Conjugates were designated GxLy where x represents the dendrimer generation and y the number of attached lauroyl chains. Triethylamine (coupling agent) was added (molar ratio equivalent to number of lauroyl chains) to a solution of cationic PAMAM dendrimers (G2, G3, or G4) in dried N,N-dimethylformamide (DMF). Lauroyl chloride was slowly added over a period of 10 min, and the reaction mixture was refluxed at 50°C for 4 days and evaporated *in vacuo*. The residue was refluxed with 1 M NaOH, filtered, phase partitioned with diethyl ether for 24 h, and the aqueous layer dialyzed against distilled water to remove salts.

Evidence for the covalent bonding of lauroyl chains to the dendrimer was from ^{1}H-NMR (Bruker Advance 300, Coventry, UK) and RP-HPLC (Hewlett-Packard series II 1090, Germany) using a Luna 5-μm, C18 column (250 × 4.6 mm, Phenomenex, Cheshire, UK), a mobile phase of methanol:0.1% (v/v) acetic acid (90:10), a flow rate of 1 ml/min, and UV detection at λ = 230 nm. HPLC chromatograms of conjugates showed the presence of a lauroyl-dendrimer peak at 2.6–2.7 min for both G2L6 and G2L9, 2.5–3.1 min for G3L6, 2.6–3.5 min for G3L9, and 2.5–2.9 min for both G4L6 and G4L9, compared to 1.9 and 11.8 min for G2 dendrimer and lauroyl chloride, 1.6 min for both G3 and G4 dendrimer, and 11.8 min for lauroyl chloride in mixtures of non–covalently bound components. ^{1}H-NMR (270 MHz, MeOD):

G2L6 δ = 0.87–0.92 (18H, CH_3), 1.25–1.30 [96H, $(CH_2)_8$], 1.59 (12H, CH_2), 1.89–2.58 (68H, $CH_2C=O$ + $CH_2C=ON$), 2.66 (56H, CH_2N), 2.78–2.79 (60H, NCH_2), 3.28–3.31 (56H, $O=CNCH_2$)

G2L9 δ = 0.87–0.92 (27H, CH_3), 1.25–1.29 [144H, $(CH_2)_8$], 1.59 (18H, CH_2), 1.89–2.58 (74H, $CH_2C=O$ + $CH_2C=ON$), 2.79 (56H, CH_2N), 2.99 (60H, NCH_2), 3.26 (56H, $O=CNCH_2$)

G3L6 δ = 0.89 (18H, CH_3), 1.25–1.30 [96H, $(CH_2)_8$], 1.59 (12H, CH_2), 1.88–2.38 (132H, $CH_2C=O$ + $CH_2C=ON$), 2.59 (120H, CH_2N), 2.74–2.80 (124H, NCH_2), 3.26 (120H, $O=CNCH_2$)

G3L9 δ = 0.87–0.89 (27H, CH_3), 1.29–1.36 [144H, $(CH_2)_8$], 1.59 (18H, CH_2), 1.88–2.37 (138H, $CH_2C=O$ + $CH_2C=ON$), 2.60 (120H, CH_2N), 2.79 (124H, NCH_2), 3.28 (120H, $O=CNCH_2$)

G4L6 δ = 0.89 (18H, CH_3), 1.29 [96H, $(CH_2)_8$], 1.59 (12H, CH_2), 1.82–2.38 (260H, $CH_2C=O$ + $CH_2C=ON$), 2.66 (248H, CH_2N), 2.79 (252H, NCH_2), 3.30 (248H, $O=CNCH_2$)

G4L9 δ = 0.89 (27H, CH_3), 1.28 [144H, $(CH_2)_8$], 1.59 (18H, CH_2), 1.88–2.37 (266H, $CH_2C=O$ + $CH_2C=ON$), 2.58 (248H, CH_2N), 2.78 (252H, NCH_2), 3.28 (248H, $O=CNCH_2$)

The average number of covalently bound lauroyl chains per dendrimer was calculated from the relative intensities of the ^{1}H-NMR peaks originating from the lauroyl substituents compared to those from the dendrimer core.

Synthesis of Fluorescein Isothiocyanate-Dendrimer Conjugates

For the quantitative evaluation of dendrimer permeation across Caco-2 cells, the dendrimers were labeled with fluorescein isothiocyanate (FITC). For cationic dendrimers (G2, G3, and G4), the linkage was between the amine group of the dendrimer and the isothiocyanate group of FITC (thiourea bond); anionic dendrimers (G2.5 and G3.5) were first modified with ethylenediamine (7). In brief, anionic dendrimers were mixed with EDC at a molar ratio of 1:1 for 30 min at room temperature. Ethylenediamine (a molar equivalent to EDC) was added slowly, and the reaction incubated for 4 h. The 2,4,5-trinitrobenzenesulfonic acid (TNBS) assay was used to determine the number of added amine groups on the surface of anionic-modified dendrimers (21). FITC linkage of both cationic and anionic-modified dendrimers was achieved as follows. A methanolic solution of FITC was added slowly to the dendrimer in phosphate-buffered saline (PBS) (dendrimer:FITC molar ratio = 1:1.2) at room temperature and incubated for 24 h in the dark with stirring. Separation of unreacted FITC was by dialysis against distilled water through dialysis tubing (molecular weight cutoff 1350 or 3500, Medicell International Ltd., London, UK) until free FITC could not be detected by thin-layer chromatography [mobile phase chloroform, methanol, and ammonia (5:4:1)]. Attachment of FITC to dendrimer was verified by ^{1}H-NMR and spectrofluorimetry (Fluroskan, Ascent FL, Model 374, Finland, excitation wavelength 475 nm, emission wavelength 583 nm); an average molar ratio of 1:1 dendrimer:FITC was ob-

tained. *In vitro* stability tests of FITC-PAMAM dendrimers at 37°C in PBS pH 7.4 revealed no dissociation of FITC from the dendrimer over a period of 5 days, indicating a stable linkage of FITC to dendrimer.

Light-Scattering Measurements

Static and dynamic light-scattering techniques were used to ascertain if aggregation of the dendrimers and dendrimer conjugates occurred under the conditions of the study. Measurements were performed on unlabeled dendrimers and their conjugates. Solutions in PBS were clarified by filtration through Millipore Millex filters (Triton free, 0.22 μm porosity) into scattering cells previously cleaned with condensing acetone vapor. Static light scattering (SLS) intensities were measured at 37°C by means of a Brookhaven BI 200S instrument (Brookhaven, NY, USA) with vertically polarized incident light of wavelength 488 nm supplied by a 2W argon ion laser (Coherent Innova 90, CA, USA). The basis for analysis of SLS was the Debye equation,

$$K^*c/(I\text{-}I_S) = 1/M_W + 2A_2c + \ldots\ldots \quad (1)$$

where I is intensity of light scattering from solution relative to that from the scattering standard benzene, I_s is the corresponding quantity for the solvent, c is the concentration (in g/dm^3), M_w is the mass-average molar mass of the solute, A_2 is the second virial coefficient (higher coefficients being neglected), and K* is the appropriate optical constant, which includes the specific refractive index increment, determined using an Abbé 60/ED precision refractometer (Bellingham and Stanley Ltd., U.K.).

Dynamic light-scattering measurements were made under similar conditions by means of the Brookhaven BI 200 S combined with a Brookhaven BI 9000 AT digital correlator. The correlation functions from DLS were analyzed by the CONTIN method to obtain the apparent diffusion coefficient (D_{app}) and hence the apparent hydrodynamic radius ($r_{h,app}$) through the Stokes-Einstein equation:

$$r_{h,app} = kT/(6\pi\eta D_{app}) \quad (2)$$

where k is the Boltzmann constant and η is the viscosity of water at temperature T.

Cell Culture

Caco-2 cells (passage 41–63) were maintained in Dulbecco's Modified Eagles Medium (DMEM) containing 10% (v/v) fetal bovine serum, 2 mM glutamine, 10 mM nonessential amino acids, 50 IU/ml penicillin, and 50 μg/ml streptomycin at 37°C and in an atmosphere of 5% CO_2. Growth medium was changed on alternate days. Cell number was assessed by trypan blue exclusion analyses.

Monolayer Integrity

Monolayer integrity was determined by measurement of the transepithelial electrical resistance (TEER) using an epithelial tissue voltohmmeter (EVOM, World Precision Instruments, Sarasota, FL, USA) and by assessing permeation of FITC-dextran markers (FD4, FD10) and [^{14}C]mannitol. Only monolayers with a TEER in the range of 800–1000 Ω/cm^2 were used.

Cytotoxity Studies

Approximately 3×10^4 cells were seeded into individual wells of 96-well tissue culture plates and maintained for 96 h. Cells were incubated with dendrimers for 3 h, and cytotoxicity measured by a modification of the MTT assay (22) using a multiplate reader (MRX, Dynatech Laboratories, Guernsey, U.K.). Assays were carried out as five replicates, and IC_{50} values (concentration at which 50% inhibition of mitochondrial dehydrogenase activity was measured) were determined.

Transport Studies of Dendrimers and Lauroyl-Dendrimer Conjugates

Caco-2 cells were seeded onto polycarbonate filters at a density of 1.2×10^5 cells/cm^2, and confluent monolayers (21–28 days) were used for the transepithelial transport studies. Transport was determined in both apical-to-basolateral (A→B) and basolateral-to-apical (B→A) directions. The transport medium was HBSS with 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and was placed in the donor and receiver compartments. Dendrimers (at noncytotoxic concentrations) were placed in the donor compartment, and cells were incubated at 4°C or in a humidified atmosphere at 37°C. Samples were removed from the receiver compartment at time zero and after 60, 120, and 180 min, and from the donor compartment after 180 min and analyzed for fluorescence. The effect of dendrimers on the integrity of the Caco-2 cell monolayer was investigated by monitoring the TEER throughout the experiment and after a recovery period of 24 h. The apparent permeability coefficients (P_{app}) were calculated (9).

Determination of the Effects of Dendrimer and Lauroyl-Dendrimer Conjugates on Caco-2 Monolayer Integrity

The effect of dendrimers on monolayer integrity was assessed by measurement of [^{14}C]mannitol P_{app}. Dendrimer and [^{14}C]mannitol (specific activity 56.0 mCi/mmol) were added to the donor chamber, and permeability in both directions (A→B, B→A) was studied. The permeability of the monolayers to [^{14}C]mannitol was studied in dendrimer-free transport medium as a control. Samples collected from the receiver chamber at 60, 120, and 180 min were analyzed by liquid scintillation counting (Wallac 1409, Turku, Finland). The P_{app} of [^{14}C]mannitol was calculated (9).

Determination of the Effect of EDTA on the Permeation of Dendrimer and Lauroyl-Dendrimer Conjugates

EDTA was used to study the effect of tight junction integrity on the permeation of dendrimers across Caco-2 cell monolayers. Briefly, the culture medium in both apical and basolateral chambers was replaced with transport medium containing 2.5 mM EDTA 30 min before experimentation (TEER values of approximately 150 Ω/cm^2 were achieved). Determinations of dendrimer transport and A→B and B→A permeabilities were carried out as described above.

Determination of the Effect of Colchicine on the Permeation of Dendrimer and Lauroyl-Dendrimer Conjugates

The effect of the endocytosis inhibitor colchicine on transepithelial dendrimer permeation was investigated. Col-

chicine (10 μM) was added to the donor chamber 30 min before the study; the cells were then incubated with the dendrimers, and the permeability study (A→B, B→A) was carried out as described above.

Statistical Analysis of Data

Statistical analysis was carried out using the Student's *t* test. Probability values of $p < 0.05$ were considered to be significant.

RESULTS

Cytotoxity Studies (MTT assay)

The cytotoxicity of the PAMAM dendrimers is concentration, generation, and charge dependent (Fig. 1). Anionic PAMAM dendrimers (G2.5, G3.5) exhibited significantly less cytotoxicity ($p < 0.0001$) than cationic PAMAM dendrimers (G2, G3, G4). Low-generation cationic dendrimer (G2) showed significantly less cytotoxicity ($p < 0.0001$) than higher generations (G3, G4). The cytotoxicity decreased significantly ($p < 0.0001$) when the cationic PAMAM dendrimers were conjugated with six lauroyl chains (Table I). For example, the IC_{50} values of G3L6 and G4L6 dendrimers were more than sevenfold greater than those of the equivalent unmodified dendrimer. Dendrimer-lipid conjugates with fewer or more than six attached chains were less effective at reducing the cytotoxicity of the cationic dendrimers.

Permeability Studies with Dendrimers and Lauroyl-dendrimer Conjugates

Permeability studies were carried out at non-toxic dendrimer concentrations (as determined by the MTT assay), and results are summarized in Table II. The influence of dendrimer generation on permeability is not clear from the limited data available; comparison of P_{app} values of G2 and G3 cationic dendrimers at 10 μM concentration (G4 is toxic at this concentration) showed an increase in permeability with generation, whereas comparison of G2.5 and G3.5 anionic dendrimers at 1000 μM concentration suggests a decrease in permeability with generation. It is clear from Fig. 2, however, that the P_{app} of unmodified cationic PAMAM dendrimers was significantly greater ($p < 0.0001$) than that of anionic PAMAM dendrimers and paracellular marker molecules ([^{14}C]mannitol, and the FITC-labeled dextrans FD4 and FD10). Interestingly, the P_{app} values at 3 h for G2L6 and G3L6 were approximately twice those of the corresponding unmodified PAMAM dendrimers at the same concentrations (Table II). A significant increase ($p < 0.01$) of P_{app} was also noted with G3L9 but not with G2L9. A possible reason for the lower P_{app} of G2L9 compared with G2L6 was seen from an examination of the state of aggregation of the conjugates under the conditions of the measurements.

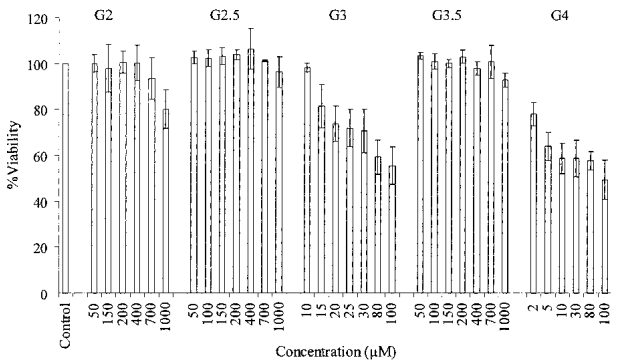

Fig. 1. The effect of PAMAM dendrimer generation and concentration on the viability of Caco-2 cells (mean ± SD, n = 5).

Table I. The Effect of Surface Modification on the Cytotoxicity of PAMAM Dendrimers as Determined by IC_{50} (mean ± SD, n = 5)

Dendrimer	IC_{50} (mM)
G2	>1
G2L6	>1.5
G2L9	1.06 ± 0.03
G3	0.14 ± 0.00
G3L6	>1
G3L9	0.31 ± 0.00
G3L13	0.22 ± 0.00
G4	0.13 ± 0.02
G4L3	0.36 ± 0.04
G4L6	>1
G4L9	0.10 ± 0.00
G4L15	0.04 ± 0.02
G2.5	>1
G3.5	>1

The molecular weights and diameters of the unmodified cationic dendrimers G2, G3, and G4 were in good agreement with reported mean values (2) (see Table III), and no evidence of aggregation was noted over the concentration range of the measurements. The molecular weight and size of all conjugates except G2L9 increased proportionally to the number of lipids attached to dendrimers. However, the molecular weight of the G2L9 conjugate was almost twice the calculated value, and the measured diameter was also larger than expected. Dimerization of this conjugate might be a conse-

Table II. The Effect of Dendrimer Concentration on the Permeability (A → B) and TEER (mean ± SD, n = 4) at 37°C

Dendrimer	Concentration (μM)	A → B $P_{app} \times 10^{-6}$ (cm/s) (3 h)	TEER (% control at 3 h)
G2	10	0.52 ± 0.05	60
G2	700	0.97 ± 0.03	20
G2L6	700	2.38 ± 0.24	50
G2L9	700	0.33 ± 0.29	100
G2.5	1000	0.15 ± 0.02	100
G3	10	1.58 ± 0.14	20
G3L6	10	2.01 ± 0.18	65
G3L6	100	2.31 ± 0.10	65
G3L6	200	2.73 ± 0.03	25
G3L9	10	2.91 ± 0.29	60
G3L9	100	4.94 ± 0.04	35
G3.5	1000	0.02 ± 0.00	100
G4L6	50	3.10 ± 0.32	65
G4L6	200	7.40 ± 0.09	50
G4L9	50	8.03 ± 0.09	60
FD4	250	0.08 ± 0.00	100
FD10	100	0.07 ± 0.02	100
[^{14}C]Mannitol	3.6	0.11 ± 0.00	100

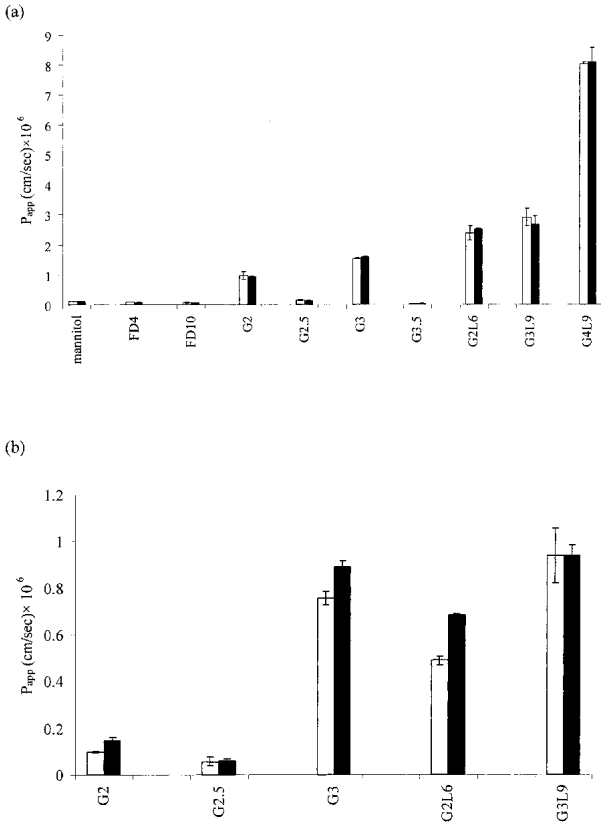

Fig. 2. A→B (□) and B→A (■) permeability across Caco-2 cell monolayers at 3 h of (a) PAMAM dendrimers (700 μM G2, 1000 μM G2.5, 10 μM G3, and 1000 μM G3.5), lauroyl-PAMAM dendrimer conjugates (700 μM G2L6, 10 μM G3L9, and 50 μM G4L9), and paracellular markers (3.6 μM [^{14}C]mannitol, 250 μM FD4 and 100 μM FD10) at 37°C, and (b) PAMAM dendrimers (700 μM G2, 1000 μM G2.5 and 10 μM G3) and lauroyl-PAMAM dendrimer conjugates (700 μM G2L6 and 10 μM G3L9) at 4°C (mean ± SD, n = 4).

quence of the conformational preference of an open/disk-like conformation of G2 PAMAM dendrimers. Higher-generation PAMAM dendrimers have a more globular conformation (2) and are less likely to aggregate. The self-association of G2L9 is the most probable cause of the lower than expected P_{app} value for this conjugate.

Figure 2 compares A→B and B→A permeabilities, at 3 h, for selected dendrimers and marker molecules at 4°C and 37°C. The permeabilities measured at 4°C were all significantly lower ($p < 0.005$) than those obtained at 37°C. At 37°C A→B P_{app} was not significantly different from B→A P_{app}, suggesting that these molecules are not substrates for the intestinal efflux transporter P-glycoprotein (P-gp), which is functionally active in these cells.

Measurements of TEER over a 24-h period at 37°C (Fig. 3) showed that apical incubation with cationic PAMAM dendrimers (G2, G3) resulted in a pronounced decrease in TEER, whereas no change was observed with incubation of anionic PAMAM dendrimers and paracellular markers. The extent of recovery of TEER (reflecting recovery of tight junction integrity) was monitored after dendrimer removal (at 3 h) from monolayers. After 24 h, TEER reverted to 96% and 98% of the initial values for G2 and G3 dendrimers, respectively. The decrease in TEER when cells were exposed to lauroyl-dendrimer conjugates was less than the decrease observed with unmodified dendrimers (e.g., approximately 50% decrease in the initial TEER at 3 h with G2L6 compared with 80% decrease for G2). Only a slight decrease in TEER (not significantly different from control) was found when dendrimers and conjugates were incubated on the basolateral side compared with the apical side (data not shown). The influence of PAMAM dendrimer and modified PAMAM dendrimer concentration on monolayer permeability and TEER are summarized in Table II. In general, an increase in dendrimer concentration results in an increase of P_{app}, the effect being most significant for higher-generation dendrimers.

Analyses of samples from receiver compartments by thin-layer chromatography confirmed that intact FITC-labeled dendrimers passed through Caco-2 cell monolayers.

Table III. Dendrimer Molecular Weight and Hydrodynamic Diameter from Light-Scattering Studies*

Dendrimer	10^{-3} M_w† (g/mol)	Diameter† (nm)
G2	3.73 (3.26)	2.5 (2.9)
G2L6	4.76 (4.35)	2.8
G2L9	10.00 (4.90)	4.1
G3	7.06 (6.91)	3.2 (3.6)
G3L6	8.00 (8.00)	3.4
G3L9	9.09 (8.55)	3.8
G4	15.21 (14.22)	4.3 (4.5)
G4L6	16.13 (15.31)	4.5
G4L9	18.18 (15.85)	4.5

* Values in parentheses for conjugates are calculated/obtained from literature values (2) for unmodified dendrimers.
† Estimated uncertainties: M_w ± 10%; diameter ± 0.4 nm.

The Effect of Dendrimer and Lauroyl-Dendrimer Conjugates on Monolayer Integrity

The effect of dendrimer on monolayer integrity was investigated by measuring the P_{app} of [^{14}C]mannitol in the presence and absence of dendrimers and lauroyl-dendrimer conjugates. The presence of G2 and G3 dendrimers in the donor chamber significantly increased ($p < 0.005$) the P_{app} values of

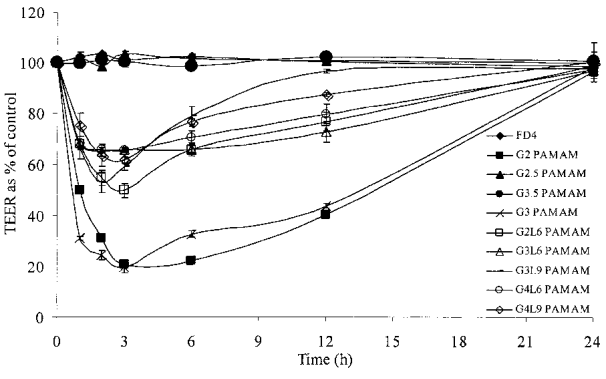

Fig. 3. Effect of PAMAM dendrimers (700 μM G2, 1000 μM G2.5, 10 μM G3 and 1000 μM G3.5), lauroyl-PAMAM dendrimer conjugates (700 μM G2L6, 10 μM G3L6, 10 μM G3L9, 50 μM G4L6 and 50 μM G4L9) and FD4 (250 μM) on TEER of Caco-2 cell monolayers after apical incubation. Dendrimer-containing medium was replaced with growth medium after 3 h, and cells maintained up to 24 h (mean ± SD, n = 5).

mannitol, the effect gradually increasing with time over a period of 3 h (Fig. 4). More pronounced increases in P_{app} were observed on incubation with lauroyl-dendrimer conjugates, G3L6, G3L9, G4L6, and G4L9 ($p < 0.005$), but in these systems the most pronounced increase in P_{app} was between 1 and 2 h. It should be noted that the B→A P_{app} of mannitol with all dendrimer systems at 3 h was significantly ($p < 0.05$) higher than A→B P_{app}.

The Effect of EDTA on Permeation of Dendrimer and Lauroyl-Dendrimer Conjugates

A decrease in TEER was observed following 30 min exposure of the Caco-2 monolayer to EDTA, indicating opening of tight junctions. P_{app} values of both PAMAM dendrimers and lauroyl-dendrimer conjugates with and without EDTA are shown in Fig. 5. Following EDTA treatment (A and B compartments), A→B P_{app} values of dendrimers and conjugates increased appreciably compared to untreated monolayers, indicating permeation via the paracellular pathway. Recovery in TEER values was achieved by 48 h after removal of the chelating agent (data not shown).

The Effect of Colchicine on Permeation of Dendrimer and Lauroyl-Dendrimer Conjugates

The effect of colchicine on dendrimer permeation is shown in Fig. 6. Colchicine caused the A→B P_{app} values at 1 h to decrease to 11, 62, 38, 22, and 43% of the original values for G2, G2.5, G3, G2L6, and G3L9, respectively. The A→B P_{app} values observed at 2 and 3 h in colchicine treated cells were similar to values in cells treated with colchicine for 1 h; however, in the absence of colchicine, A→B P_{app} increased with time. There was no statistically significant difference between B→A and A→B permeabilities in the presence or absence of colchicine.

DISCUSSION

In vitro systems for predicting oral drug absorption should ideally be able to model the physiological transport characteristics of the *in vivo* epithelium and provide a reasonable quantitative prediction of drug translocation. In this study, the cytotoxicity of PAMAM dendrimers and lauroyl-PAMAM dendrimer conjugates, their transepithelial transport across Caco-2 cell monolayers, and their influence on monolayer permeability were investigated.

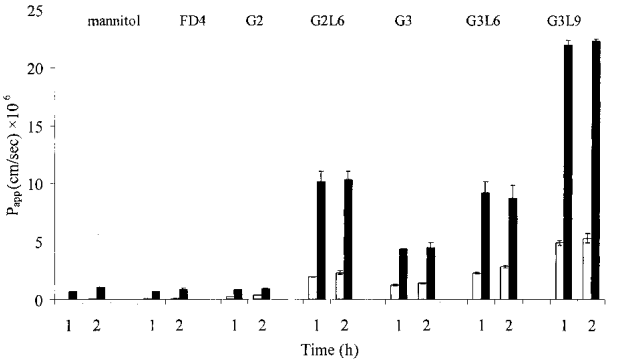

Fig. 5. A→B permeability across Caco-2 cell monolayers of PAMAM dendrimers (700 μM G2 and 10 μM G3), lauroyl-PAMAM dendrimer conjugates (700 μM G2L6, 10 μM G3L6, 10 μM G3L9) and paracellular markers (3.6 μM [^{14}C]mannitol, 250 μM FD4), in the presence (■) and absence (□) of 2.5 mM EDTA (mean ± SD, n = 3).

Assessment of cytotoxicity of PAMAM dendrimers using the MTT assay showed a molecular-weight-, size-, charge-, and concentration-dependent cytotoxicity, in agreement with previous studies (3,4). Anionic PAMAM dendrimers exhibited significantly less cytotoxicity than cationic PAMAM dendrimers. However, a reduction in cytotoxicity of cationic PAMAM dendrimers was shown when lauroyl chains were attached to cationic PAMAM dendrimers, possibly as a consequence of a decrease or shielding of the positive charges of cationic PAMAM dendrimers by the lauroyl chains. Similar decreases of cytotoxicity have been reported when dendrimers were complexed with DNA and oligonucleotides (23,24) and attributed to the reduction of overall positive charges.

The correlation between monolayer permeability and the decrease in transepithelial electrical resistance with cationic PAMAM dendrimers suggests that one possible transport mechanism of dendrimers across epithelial cells is via the paracellular route. Movement of molecules via this route is, in general, size dependent (25); the limited permeability data obtained for the anionic dendrimers are in agreement with this. The higher P_{app} of the larger G3 dendrimer compared to G2 may result from the greater number of positive surface

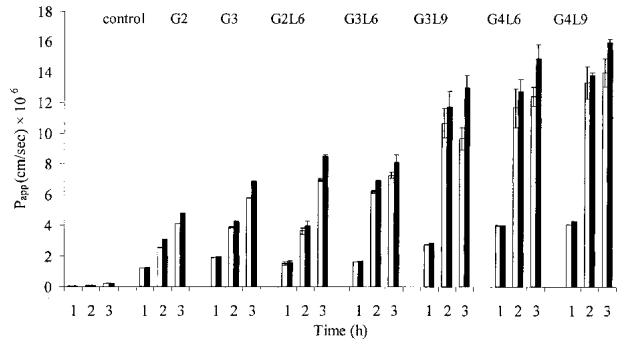

Fig. 4. A→B (□) and B→A (■) permeability across Caco-2 cell monolayers of the paracellular marker [^{14}C]mannitol (3.6 μM) (control) in the presence of PAMAM dendrimers (700 μM G2 and 10 μM G3) and lauroyl-PAMAM dendrimer conjugates (700 μM G2L6, 10 μM G3L6, 10 μM G3L9, 50 μM G4L6, and 50 μM G4L9) (mean ± SD, n = 4).

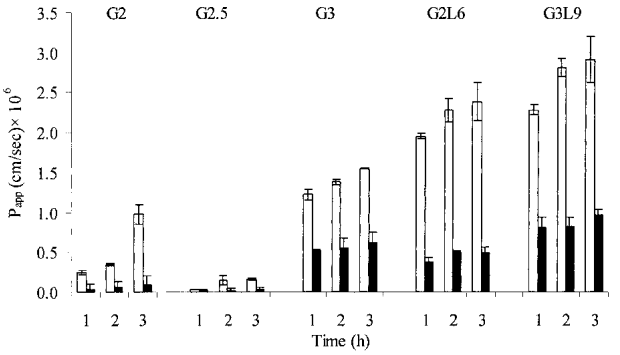

Fig. 6. A→B permeability across Caco-2 cell monolayers of PAMAM dendrimers (700 μM G2, 1000 μM G2.5, and 10 μM G3) and lauroyl-PAMAM dendrimer conjugates (700 μM G2L6, 10 μM G3L9) in the presence (■) and absence (□) of 0.01 mM colchicine (mean ± SD, n = 3).

charges (64 amine groups, compared to 32 on the G2) that may be able to induce a reversible increase in monolayer permeability by interaction with tight junctions. This observation also correlates well with a similar effect reported for other polycationic polymers such as chitosan and polyethyleneimine on tight junction permeability (26,27). Interestingly, lauroyl-PAMAM dendrimer conjugates exhibited a higher P_{app} than unmodified PAMAM dendrimers, possibly because of the permeation-enhancing influence of the lipid chains (15–17).

El-Sayed *et al.* (8) reported higher B→A than A→B permeation of both dendrimers and mannitol. The P_{app} values of unmodified and modified PAMAM dendrimers in our study were similar in both directions. Additionally, mannitol, which is not a P-gp substrate, exhibited the same B→A and A→B P_{app}, an observation also reported in other studies (28,29). Noach *et al.* (30) showed that transport of hydrophilic compounds through tight junctions of the paracellular pathway was enhanced in the presence of EDTA. Our studies indicate that EDTA enhanced the transepithelial transport of dendrimers and, furthermore, that dendrimers themselves, like EDTA, increase the permeability of the Caco-2 cell monolayers toward the paracellular marker [^{14}C]mannitol. Although the transport mechanism of dendrimers and conjugates is not fully understood, the increase in the P_{app} of dendrimers in the presence of EDTA and enhancement of the mannitol P_{app} by dendrimers may be explained by an interaction of dendrimers and conjugates with tight junctions. The slightly higher mannitol P_{app} observed when dendrimers were added to the basolateral domain, compared to apical application, may not be surprising because the addition of EDTA and ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA) to the basolateral side of cells results in a more pronounced effect on the permeability coefficient of mannitol (30,31). Moreover, dendrimer permeation was temperature-sensitive and was significantly reduced by an endocytosis inhibitor. These findings indicate that the transport mechanism of dendrimer and conjugates in Caco-2 cell monolayers is not only via the paracellular pathway but may also involve endocytosis-mediated transepithelial transport via a transcellular route.

In conclusion, our study indicates that the interaction of positively charged dendrimers with Caco-2 cells induces cytotoxicity and a relatively rapid increase in paracellular permeability. Interestingly, PAMAM dendrimers modified with lauroyl moieties showed significantly less cytotoxicity and enhanced permeation compared to unmodified dendrimers. We provide evidence that transepithelial dendrimer and conjugate transport can occur via both a paracellular pathway and a transcellular endocytotic route that is susceptible to inhibition by a microtubule-disrupting agent. These studies also suggest that modification of the surface of PAMAM dendrimers may contribute toward the development of safer and more effective drug delivery systems in the future.

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