The Mechanism of Uptake of Biodegradable Microparticles in Caco-2 Cells Is Size Dependent

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Received June 10, 1997; accepted August 5, 1997

Purpose. To study the uptake of biodegradable microparticles in Caco-2 cells.

Methods. Biodegradable microparticles of polylactic polyglycolic acid co-polymer (PLGA 50:50) of mean diameters 0.1 μm, 1 μm, and 10 μm containing bovine serum albumin as a model protein and 6-coumarin as a fluorescent marker were formulated by a multiple emulsion technique. The Caco-2 cell monolayers were incubated with each diameter microparticles (100 μg/ml) for two hours. The microparticle uptake in Caco-2 cells was studied by confocal microscopy and also by quantitating the 6-coumarin content of the microparticles taken up by the cells. The effects of microparticle concentration, and incubation time and temperature on microparticle cell uptake were also studied.

Results. The study demonstrated that the Caco-2 cell microparticle uptake significantly depends upon the microparticle diameter. The 0.1 μm diameter microparticles had 2.5 fold greater uptake on the weight basis than the 1 μm and 6 fold greater than the 10 μm diameter microparticles. Similarly in terms of number the uptake of 0.1 μm diameter microparticles was 2.7×10^3 fold greater than the 1 μm and 6.7×10^6 greater than the 10 μm diameter microparticles. The efficiency of uptake of 0.1 μm diameter microparticles at 100 μg/ml concentration was 41% compared to 15% and 6% for the 1 μm and the 10 μm diameter microparticles, respectively. The Caco-2 cell microparticle (0.1 μm) uptake increased with concentration in the range of 100 μg/ml to 500 μg/ml which then reached a plateau at higher concentration. The uptake of microparticles increased with incubation time, reaching a steady state at two hours. The uptake was greater at an incubation temperature of 37°C compared to at 4°C.

Conclusions. The Caco-2 cell microparticle uptake was microparticle diameter, concentration, and incubation time and temperature dependent. The small diameter microparticles (0.1 μm) had significantly greater uptake compared to larger diameter microparticles. The results thus suggest that the mechanism of uptake of microparticles in Caco-2 cell is particle diameter dependent. Caco-2 cells are used as an *in vitro* model for gastrointestinal uptake, and therefore the results obtained in these studies could be of significant importance in optimizing the microparticle-based oral drug delivery systems.

KEY WORDS: drug carrier; oral drug delivery; vaccine; absorption; bioavailability; endocytosis.

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INTRODUCTION

Biodegradable microparticles have been investigated as a drug carrier system for gastrointestinal delivery of therapeutic agents (1–5) to enhance drug absorption, improve bioavailability (6), target therapeutic agents to particular organs (7), and for sustained drug action. The orally administered microparticles also gain entry into the gut-associated lymphoid tissue (GALT), mainly the Peyer's patch tissue through the follicle-associated epithelium (M-cells), thus inducing an immune response against the encapsulated vaccine (8–10).

However, the oral administration of microparticles in general results in their inefficient uptake by the gut-associated tissue. The physical properties of microparticles, such as particle size, surface charge, and hydrophobicity are some of the factors which affect the gastrointestinal (GI) uptake of microparticles (10,11). Therefore, it is important to investigate the factors influencing the GI uptake of microparticles in order to obtain an efficient microparticle based oral drug delivery system including for oral vaccine.

The human colon adenocarcinoma cell line, Caco-2 has features similar to the absorptive intestinal cells, such as microvilli, the carrier mediated transport systems, and paracellular transport through the tight junctions. Therefore the Caco-2 cell monolayers have been used as an *in vitro* model to study drug absorption (12), binding characteristics (13), and metabolism (14). In the present study we have used Caco-2 monolayers as an *in vitro* model to investigate the uptake of microparticles.

MATERIALS AND METHODS

Materials

The Caco-2 cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), non essential amino acids, L-glutamine, sodium pyruvate, trypsin (0.25%), EDTA, penicillin-streptomycin solution (Gibco Laboratories, Lenexa, KS), 6-Coumarin (Polyscience Inc., Warrington, PA), sulforhodamine 101, polyvinyl alcohol (PVA, MW: 30,000–70,000), and bovine serum albumin, Fraction V (Sigma Chemical Co. St. Louis, MO) were purchased. Polylactic polyglycolic acid copolymer (PLGA, 50:50, MW-100,000, inherent viscosity: 1.07 measured in hexafluoroisopropanol) was obtained from Birmingham Polymers, Inc. (Birmingham, AL).

Formulation of Various Diameter Microparticles

Microparticles of the diameter 0.1 μm, 1 μm, and 10 μm were formulated by the methods described in our earlier publication (15). In brief, an aqueous BSA (10% w/v) solution was emulsified in a PLGA solution (3–15% w/v in methylene chloride) containing 0.05% w/v of 6-coumarin, using sonication to form a primary water-in-oil (w/o) emulsion. The w/o emulsion was further emulsified into an aqueous PVA solution (0.5–2.5% w/v) to form a multiple water-in-oil-in-water (w/o/w) emulsion using various energy sources to obtain microparticles of the desired dimensions.

0724-8741/97/1100-1568$12.50/0

Cell Culture

Caco-2 cells were grown and routinely maintained at 37°C in DMEM supplemented with 25 mM D-glucose containing 10% FBS, 1% non-essential amino acids, 1% L-glutamine, 1mM sodium-pyruvate and penicillin (100 U/ml)—streptomycin (100 μg/ml) in an atmosphere of 5% CO_2 and 90% relative humidity. The cells were harvested with trypsin-EDTA and seeded on the polycarbonate filters (3.0 μm pores, 4.71 cm^2 growth area, Costar, Cambridge, MA) inside the transwell cell culture chambers at a cell density of 300,000 per filter. The culture medium (1.5 ml in the filter and 2.5 ml in the well) was replaced at every 48 hrs. The monolayers were used between the passage 42 and 72 at 21 days post seeding.

Transepithelial Electrical Resistance (TEER) Measurements

The TEER was measured prior to and at the end of each experiment to assess the integrity of the Caco-2 monolayers (EVOM epithelial Voltohmmeter with Endohm™ electrode, World Precision Instruments, Inc., Sarasota, FL). The monolayer resistance obtained by subtracting the intrinsic resistance (membrane alone) from the total resistance (membrane + monolayer) was corrected for the surface area and expressed as Ω cm^2.

Permeability Measurements and Uptake of Microparticles

The transport buffer (145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1mM NaH_2PO_4, supplemented with 5 mM D-glucose), and adjusted to pH 6.5 with 25 mM 2-(N-morpholino) ethanesulfonic acid (MES) was used as an apical buffer, and to pH 7.4 with 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) as a basolateral buffer. The mucosal (apical) side of the chamber was filled with 1.5 ml and the serosal (basolateral) side with 2.5 ml of respective transport buffers. After equilibration at 37°C for 30 min., the apical side buffer was replaced with a 1.5 ml microparticle suspension (100 μg/ml in apical buffer) containing sulforhodamine (0.001% w/v) as a permeability marker. The monolayers were incubated with microparticles for two hrs during which aliquots of 1 ml from basolateral side were taken at every 30 min. to determine the permeability of sulforhodamine through the tight junctions of the Caco-2 monolayers. The volume of the basolateral solution was maintained with the fresh buffer. All the experiments were carried out under temperature controlled conditions at 37°C.

The apical-to-basolateral permeability coefficient (P_{eff} in cm/sec) was calculated according to the following equation:

$$P_{eff} = \frac{dc \cdot V}{dt \cdot 60 \cdot A \cdot C_0} \ (cm/sec) \qquad (1)$$

where (dc/dt) = flux across the monolayer (μg/min.), V the volume in the basal chamber, A the surface area of the monolayer (cm^2) and C_0 is the initial concentration (μg/ml) of sulforhodamine in the apical chamber. The flux across the monolayer describes the amount transported versus time and was calculated from the slope of the regression line.

In vitro Release of 6-coumarin from Microparticles

The control experiments were carried out to ensure that the 6-coumarin estimated in the Caco-2 cell monolayers was due to the uptake of microparticles and not due to the uptake of 6-coumarin that might have been released from the microparticles during the experiment. Therefore, *in vitro* release of 6-coumarin from the microparticles (0.1 μm) was studied under identical physiologic conditions. In a second set of experiment, the *in vitro* release of dye was carried out in the presence of olive oil to create a perfect sink condition as the dye has limited solubility in buffer. For this study 1 ml nanoparticle suspension (100 μg/ml) was layered with 200 μl olive oil and was incubated at 37°C for 2 hours on a shaker. The oil phase was centrifuged at 14,000 rpm to remove the microparticles that migrated into the oil phase during incubation. The supernant oil phase (100 μl) was extracted five times with methanol (200 μl each extract) and the combined extract was evaporated. The residue was reconstitued in mobile phase for HPLC analysis of 6-coumarin as described below. For each control experiments, supernatant from a suspension of microparticles incubated *in vitro* under identical conditions in buffer was exposed to the Caco-2 cell monolayers.

Effect of Microparticle Concentration and Time of Incubation on Uptake

These experiments were carried out with only 0.1 μm size microparticles. Caco-2 cells were incubated with microparticles in the concentration range of 100 to 4,000 μg/ml for two hrs. In another set of experiments microparticles were incubated at 100 μg/ml concentration for 0 to 4 hrs.

Fluorescence Microscopy

The Caco-2 cell monolayers were incubated for two hours with a suspension of various size microparticles (100 μg/ml) at 37° and in a separate set of experiments at 4°C to study the effect of incubation temperature on uptake. The monolayers were washed three times with fresh transport buffer to remove excess microparticles and the cells were fixed on a glass slide with 3% paraformaldehyde solution in PBS for 30 min. The monolayers were then mounted in a mixture of glycerol:PBS (90:10) and the cells were viewed under a fluorescence microscope (Model Orthoplan, Leitz, W. Germany) using FITC filter (wavelength 450–490 nm).

Analytical Methods

The Caco-2 cell monolayers, either from the microparticle or control groups were lyophilized with 1 ml of transport buffer (pH 6.5) and extracted for 6-coumarin with ethyl acetate (5 × 3 ml). The combined extracts was evaporated separately for each sample using SpeedVac evaporator (SpeedVac® Plus SC110A, Savant Instruments, Inc., Farmingdale, NY) and the residue was reconstituted in 400 μl acetonitrile for HPLC analysis.

The Waters HPLC system consisted of a pump (model 501), an autosampler (Wisp model 712), a Data Module (model 740), and a μBondapak C_{18} column with 10 μm packing (Waters, Milford, MA). A mixture of acetonitrile and water (65:35, v/v) containing 5 mM 1-heptane sulfonic acid sodium

salt was used as a mobile phase. The eluents were monitored using a scanning fluorescence detector (Waters, model 470, Milford, MA) set at 450 nm excitation and 490 nm emission wavelengths.

A standard curve for 6-coumarin was constructed for each batch and size of microparticles by spiking different weight concentrations of microparticles (10 μg to 80 μg) in transport buffer followed by lyophilization and extraction of 6-coumarin as above. The amount of microparticles (μg) taken up by the Caco-2 monolayers was calculated from the standard curves for each size microparticles.

Sulforhodamine was analyzed using a μBondapak C_8 column (Waters, Milford, MA) with 4 μm packing using mobile phase consisting of mixture of acetonitrile:phosphate buffer (10 mM, pH 7.4) (70:30, v/v) with fluorescence detector set at 586 nm excitation and 610 nm emission wavelengths.

Calculation of Number of Microparticles From Mass and Efficiency of Uptake

The number of microparticles was calculated from their mass as estimated above using the following equation:

$$\text{Number of particles} = \frac{w \times k \times 10^9}{d^3} \qquad (2)$$

where, w is the amount of particles in μg taken up by sq. mm area of the tissue and d is the diameter of particles in μm (16). The factor k takes into account the density (g/cm^3) of the polymer used.

The efficiency of microparticle uptake was calculated from the theoretical dose of microparticles exposed to the Caco-2 cell monolayers and the actual uptake. The theoretical dose, calculated from the total area of the Caco-2 monolayer exposed to the microparticles (471 mm^2) and the total amount of microparticles added. At 100 μg/ml microparticles concentration theoretical dose would be 0.32 μg/mm^2.

Statistical Analysis

Results are presented as mean ± s.e.m. Statistical comparisons were made with Student's t-test at a 95% confidence level. Difference between the uptake of various size microparticles by the Caco-2 monolayers was considered significant at $p < 0.05$.

RESULTS

Our study demonstrated that the microparticle uptake in Caco-2 is microparticle diameter, concentration, and incubation time and temperature dependent. The transepithelial electrical resistance of the Caco-2 monolayers indicates that the microparticles do not affect the integrity of the monolayer.

Effect of Microparticle Diameter on Uptake

Figure 1 demonstrates that the smaller diameter microparticles, 0.1 μm had 2.5 fold greater uptake compared to the 1 μm diameter microparticles and about 6 fold greater than the 10 μm diameter microparticles on the weight basis. In terms of the number of microparticles, the 0.1 μm diameter microparticles had 2.6 × 10^3 times greater number of microparticles than the 1 μm and 5.9 × 10^6 times greater than the 10 μm diameter microparticles. The efficiency of uptake of 0.1 μm

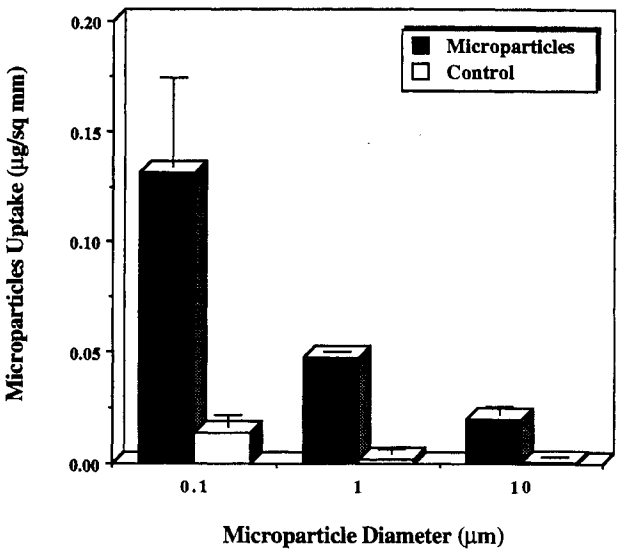

Fig. 1. Effect of microparticle diameter on uptake in Caco-2 cells. The Control is 6-coumarin released under *in vitro* conditions from various diameter microparticles and incubated with Caco-2 cells (n = 3).

microparticles calculated from the theoretical dose at 100 μg/ml microparticle concentration was 41%, whereas for 1 μm and 10 μm size microparticles these were 15% and 6%, respectively (Table I).

The control experiments carried out by incubating Caco-2 cells with the dye released from the microparticles did not show any significant uptake (Figure 1). The amount of dye released *in vitro* at 100 μg/ml concentration from the 0.1 μm diameter microparticles was 1.2 ± 0.2 ng/ml (n = 3) which was 40 fold lower than the solubility of the dye under similar conditions (49 ± 6 ng/ml, n = 3), representing a perfect sink condition for *in vitro* release. More than 99.4% w/w of the dye was associated with the microparticles following over 48 hr incubation, indicating that the dye is mostly associated with the microparticles (Figure 2). The *in vitro* experiment carried out in the presence of olive oil also demonstrated that 98.8% dye was retained inside the particles after 2 hours of incubation. Therefore, the fluorescent activity measured in the Caco-2 cells was mainly due to the uptake of microparticles.

Table I. The Effect of Microparticle Diameter on Uptake in Caco-2 Cells

Particle Diameter[c] (μm)	Microparticles[a] (μg/mm^2)	Microparticles[b] (Number/mm^2)	% Efficiency[d]
0.1	0.131 ± 0.040	186.8 × 10^9	41.07
1.0	0.048 ± 0.000	6.8 × 10^7	15.04
10.0	0.020 ± 0.003	2.8 × 10^4	6.27

[a,b]= mean of 3 samples. Theoretical dose = 0.32 μg/mm^2.
[c] Nominal diameters; Actual mean diameters were 0.12 ± 0.005 μm, 1.1 ± 0.1 μm and 9.4 ± 0.2 μm (through ref 15).
[d] % Efficiency = [Uptake by cells (μg/mm^2)/Microparticle mass added to the cell culture (μg/mm^2)] × 100.

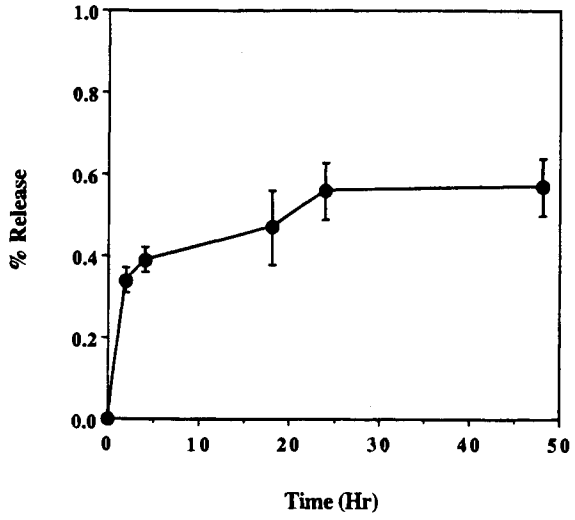

Fig. 2. *In vitro* release of 6-coumarin from 0.1 μm diameter microparticles (n = 3).

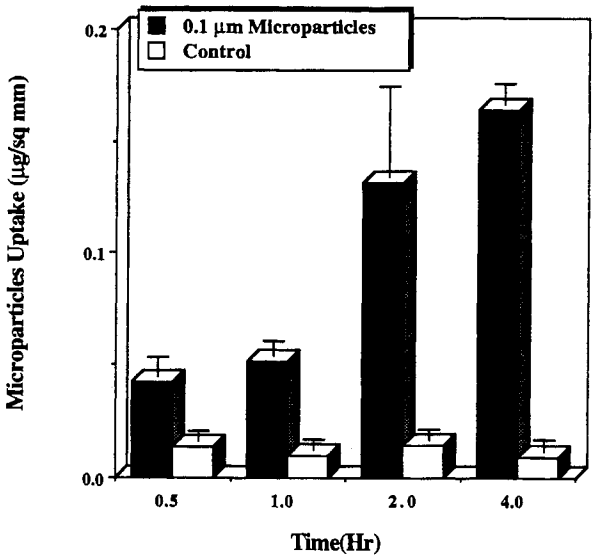

Fig. 4. Effect of time of incubation on uptake of 0.1 μm diameter microparticles in Caco-2 cells. The Control is 6-coumarin released under *in vitro* conditions for various time periods and incubated with Caco-2 cells (n = 3).

Concentration and Incubation Time Dependent Uptake of Microparticles

The uptake of 0.1 μm diameter microparticles in Caco-2 cell increased with increase in concentration of microparticles in the incubation medium within 100 to 500 μg/ml range (Figure 3). However, further increase in concentration to 4 mg/ml had no significant increase in cell uptake, indicating a saturation limit. In another series of experiments, the Caco-2 cell microparticle uptake increased with the incubation time up to two hrs which then showed a plateau effect ($p > 0.1$) (Figure 4).

Confocal Fluorescence Microscopy

The confocal microscopic examination of the Caco-2 cell monolayers demonstrated a significantly greater intracellular accumulation of 0.1 μm microparticles as compared to larger size microparticles. Microparticles of 0.1 μm and 1 μm diameters were seen throughout the cytoplasm, most likely in the endosomes. Microparticles of 10 μm diameter were taken up to a lesser extent and were mainly seen adsorbed on to the cell surface (Figure 5). The uptake of microparticles was significantly reduced for all size microparticles when the incubation temperature was reduced to 4°C (Figure 5).

The transepithelial electrical resistance of the Caco-2 cell monolayers was in the range of 375 to 475 Ω cm^2 and sulforhodamine flux was less than 0.7×10^{-5} cm/sec, indicating the integrity of the Caco-2 monolayers throughout the experiments and microparticles had no effect on the tight junction.

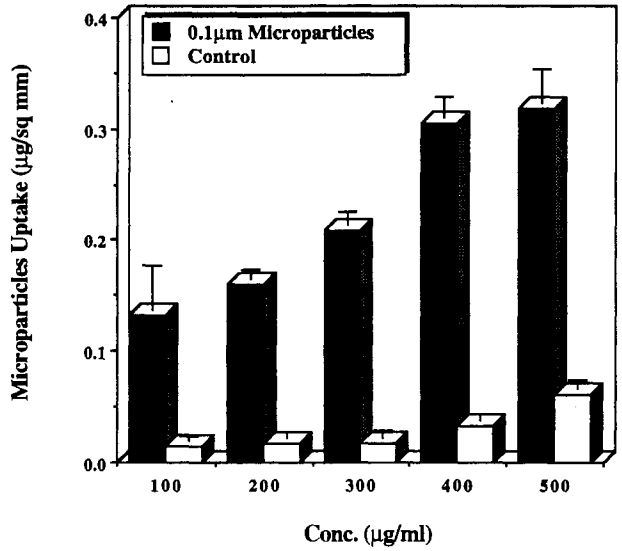

Fig. 3. Effect of concentration on uptake of 0.1 μm diametermicroparticles in Caco-2 cells. The Control is 6-coumarin released under *in vitro* conditions at various microparticle cocentrations and incubated with Caco-2 cells (n = 3).

DISCUSSION

Our studies demonstrated that the Caco-2 cell microparticle uptake is dependent upon the microparticles diameter with smaller diameter particles having significantly greater uptake. The smaller size microparticles (0.1 μm) were mostly localized in the endosomes as evident from the confocal microscopy studies. The lowering the incubation temperature reduces the microparticle uptake, demonstrating that the uptake is an active process.

The smaller diameter microparticles seems to have efficient interfacial interaction with the cell membrane compared to larger diameter microparticles. Probably the larger diameter microparticles (>1 μm) are taken up by mechanism other than endocytosis, such as fluid-phase pericytosis and should be the subject for further investigation. Thus, the small diameter (<0.1 μm) microparticles have better intracellular targeting. In our recent studies we have demonstrated gene expression in the arterial wall of the rabbit iliac artery that was infused with PLGA nanoparticles containing alkaline phosphatase as a marker gene (17).

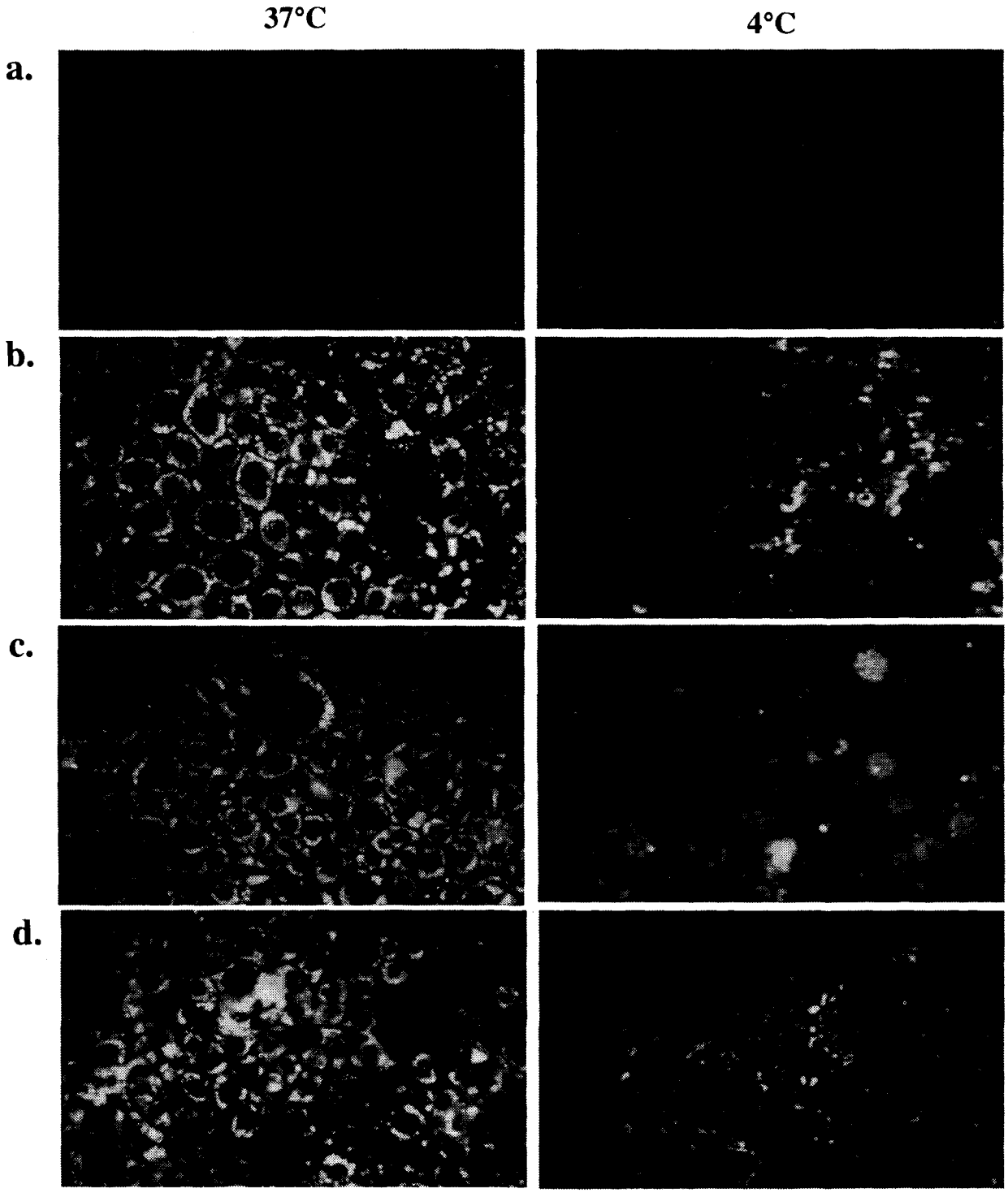

Fig. 5. Confocal microscopy: Uptake of different diameter microparticles in Caco-2 cells at A) 37°C and B) 4°C. a = control; b = 0.1 μm; c = 1 μm and d = 10 μm.

The human colon adenocarcinoma cell line, Caco-2 has been investigated as a useful alternative to animal models to study intestinal absorption of therapeutic agents including proteins, peptides, and oligonucleotides (18–21). Recently Walter *et al.* (22) co-cultured Caco-2 with a mucin producing goblet cell clone, HT-29-MTX to introduce a mucin barrier so as to obtain a good correlation between the permeabilities observed in Caco-2 cells with that in humans. The microparticle uptake studies observed in Caco-2 cells probably be correlated with the *in vivo* studies. In our recent studies using an *in situ* rat intestinal model we observed greater uptake of smaller diameter (0.1 μm) microparticles compared to the large diameter micro-

particles (0.5, 1, and 10 μm) by the gut-associated tissue, including Peyer's patch tissue (15). Hillery *et al.* (23) also noticed a significant uptake of 60 nm size polystyrene particles by the Peyer's patch tissue of the small intestine in rats.

The small diameter microparticles could improve efficacy of the microparticle-based oral drug delivery systems (24–26). In oral immunization, poor immune response could be due to an inefficient uptake of the vaccine by the gut-associated lymphoidal tissue (27,28). Thus by using smaller size microparticles, the efficiency of uptake of vaccine could be improved (15). In addition, smaller size particles could have an easy access from the Peyer's patch tissue to the mesenteric lymph nodes (29–31).

Transmucosal passage of nanoparticles (isobutyl-2-cyanoacrylate, 100–200 nm) from the intestinal lumen to the vascular compartment through a paracellular pathway has been reported (24). The oral bioavailibility of the poorly absorbable or agents which are unstable in the environment of gastric mucosa could thus be improved using small size microparticles. In fact, Leroux *et al.* (32) have demonstrated a significant improved bioavailability of HIV-1 protease inhibitors using pH sensitive nanoparticles. Hillery *at al.* (33) also demonstrated similar results for the Luteinizing Hormone Releasing Hormone (LHRH) using co-polymeric nanoparticles.

The Caco-2 cell monolayers, because it provides a good model for intestinal epithelium, could be used prior to *in vivo* studies for a rapid assessment of the factors influencing the microparticle uptake, such as particle hydrophobicity (9), surface charge (11), or any receptor mediated uptake (34). It would be of further interest to investigate if the efflux mechanism of the gut cells could be bypassed using microparticles as a carrier system to enhance absorption of the agents which are otherwise poorly absorbed because of the efflux action (12).

CONCLUSIONS

In this study we have demonstrated the size dependency of uptake of biodegradable PLGA microparticles by Caco-2 cells, with the smallest diameter microparticles, 0.1 μm showing significantly greater uptake compared to larger diameter microparticles. It seems that the mechanism of microparticle uptake in Caco-2 cells is size dependent. The uptake was also dependent upon the concentration of particles, and the time and temperature of incubation, indicating energy dependent process of uptake. The increased uptake of smaller particles by the epithelial cells indicates the possibility of using biodegradable nanoparticles as a drug carrier system for oral delivery of therapeutic agents, including proteins, peptides, and nucleic acids. Smaller diameter microparticles with enhanced intestinal epithelial uptake could be ideal carriers for intestinal administration of sustained release vaccines.

ACKNOWLEDGMENTS

This work was partially supported by TSRL Inc., Ann Arbor, under a subcontract from the U.S. Army SBIR program (VL) (Contract # DAMD 17-95-C-5060) and Matrigen Inc., Ann Arbor, MI. Authors wish to thank Iris Templin and Ardith Bates for their secretarial assistance.

REFERENCES

1. J. Kreuter. *J. Anat.* **189**:503–505, 1996.
2. R. Bodmeier, H. G. Chem, and O. Paeratakul. *Pharm. Res.* **6**:413–417 (1989).
3. C. Michel, M. Aprahamain, L. Defontaine, P. Couvreur, and C. Damge. *J. Pharm. Pharmacol.* **43**:1–5 (1991).
4. A. T. Florence, A. M. Hillery, N. Hussain, and P. U. Jani. *J. Controlled Release* **36**:39–46 (1995).
5. D. Shah and W. C. Shen. *J. Pharm. Sci.* **85**:1306–1311 (1996).
6. P. Maincent, R. Le Verge, P. A. Sado, P. Couvreur, and J. P. Devissaguet. *J. Pharm. Sci.* **75**:955–958 (1986).
7. P. A. Kramer and T. Burnstein. *Life Sci.* **19**:515–520 (1976).
8. O. Strannegard and A. Yurchison. *Int. Arch. Allergy* **35**:579–590 (1969).
9. J. H. Eldridge, C. J. Hammond, J. A. Muelbroek, J. K. Staas, R. M. Gilley, and T. R. Tice. *J. Controlled Release* **11**:205–214 (1990).
10. C. A. Gilligan and A. Li Wan Po. *Int. J. Pharm.* **75**:1–24 (1991).
11. A. Li Wan Po, E. Rogers, M. Shepphard, and E. M. Scott. *Adv. Drug. Del. Rev.* **18**:101–109 (1995).
12. I. J. Hidalgo and J. B. Li. *Adv. Drug. Del. Rev.* **22**:53–66 (1996).
13. C.-M. Lehr and V. H. L. Lee. *Pharm. Res.* **10(12)**:1796–1799 (1993).
14. K. L. Audus, R. L. Bartel, I. J. Hidalgo, and R. T. Borchardt. *Pharm. Res.* **7(5)**:435–451 (1990).
15. M. P. Desai, V. Labhasetwar, G. L. Amidon, and R. J. Levy. *Pharm. Res.* **13**:1838–1845 (1996).
16. R. H. Muller. *Colloidal Carriers for Controlled Drug Delivery and Targeting* CRC Press, Boston, 1991.
17. V. Labhasetwar, B. Chen, D. W. M. Muller, J. Bonadio, K. Ciftci, K. March, and R. J. Levy. *Adv Drug Del Rev.* **24**:109–120 (1997).
18. P. Artursson. *J. Pharm. Sci.* **79(6)**:476–482 (1990).
19. D. C. Kim, P. S. Burton, and R. T. Borchardt. *Pharm. Res.* **10(12)**:1710–1714 (1993).
20. P. Artursson and J. Karlsson. *Biochem. Biophys. Res. Comm.* **175(3)**:880–885 (1991).
21. G. F. Beck, W. J. Irwin, P. L. Nicklin, and S. Akhtar. *Pharm. Res.* **13(7)**:1028–1036 (1996).
22. E. Walter, S. Janich, B. J. Roessler, J. M. Hilfinger, and G. L. Amidon. *J. Pharm. Sci.* **85**:1070–1076 (1996).
23. A. M. Hillery, P. U. Jani, and A. T. Florence. *J. Drug Targetting* **2**:151–156 (1994).
24. M. Aprahamian, C. Michel, W. Humbert, J. Devissaguet, and C. Damge. *Biol. Cell* **61**:69–76 (1987).
25. P. Couvreur and F. Puisieux. *Adv. Drug. Del. Rev.* **10**:141–162 (1993).
26. J. Kreuter. *Adv. Drug. Del. Rev.* **7**:71–86 (1991).
27. J. Mastecky, Z. Moldoveanu, M. Novak, W.-Q Huang, R. M. Gilley, J. K. Staas, D. Schafer, and R. W. Compans. *J. Controlled Release* **28**:131–141 (1994).
28. M. Manganaro, P. L. Ogra, and P. B. Ernst. *Int. Arch. Allerg. Immuno.* **103**:223–233 (1994).
29. K. E. Carr, R. A. Hazzard, S. Reid, and G. M. Hodges. *Pharm. Res.* **13**:1205–1209 (1996).
30. T. Uchida and S. Goto. *Bio. Pharm. Bull.* **17**:1272–1276 (1994).
31. T. H. Ermak, E. P. Dougherty, H. R. Bhagat, Z. Kabok, and J. Pappo. *Cell Tissue Res.* **279**:433–436 (1995).
32. J. C. Leroux, R. M. Cozens, J. L. Roesel, B. Galli, E. Doelker, and R. Gurny. *Pharm. Res.* **13**:485–487 (1996).
33. A. M. Hillery, I. Toth, and A. T. Florence. *J. Controlled Release* **42**:65–73 (1996).
34. H. Sato, Y. Sugiyama, A. Tsuji, and I. Horikoshai. *Adv. Drug. Del. Rev.* **19**:445–467 (1996).