Effective immune surveillance of foreign antigens and pathogens on the intestinal mucosal surface requires transepithelial transport across the epithelial barrier to specialized sites containing organized mucosal lymphoid follicles (1). Delivery of antigens, particles, and microorganisms into these “inductive” sites is accomplished by a distinct epithelial cell type, the M cell, that occurs only in the lymphoid follicle-associated epithelium (FAE) (1). M cells are specialized for endocytosis and vesicular transport into sequestered intraepithelial spaces and to the underlying lymphoid tissue (1, 2). Antigen-sensitized, IgA-committed lymphoblasts proliferate in these inductive sites and eventually “seed” local and distant mucosal tissues with IgA plasma cells that produce protective, polymeric IgA for transport into mucosal secretions (3).

The ability of M cells to endocytose samples of luminal contents has been exploited by microbial pathogens that use this cell as an invasion route by selectively adhering to M cell apical surfaces (for a review see reference 4). Selective, efficient M cell transport is also considered a desirable feature of mucosal vaccines, a concept supported by the effectiveness of live, genetically engineered, attenuated vaccine strains of pathogens that enter the mucosa via M cells (5, 6). The M cell surface characteristics that account for this selectivity are unknown, however, and M cell targeting of nonliving vaccines has proven difficult because there is little information available concerning the apical membrane.
components that might serve as potential receptors on this cell type. One approach has been to package antigens in microparticles (7) since this provides protection from intestinal enzymes and takes advantage of the fact that M cells can endocytose particles up to several microns in diameter, whereas enterocytes cannot (8, 9). Although some microparticles and liposomes have been shown to adhere to mucosal surfaces by hydrophobic interactions and to be taken up into mucosal lymphoid tissue (10, 11), uptake is generally inefficient because such particles are readily entrapped in mucus gels and many fail to reach the mucosa. For optimal uptake efficiency, macromolecules or particles should mimic M cell-invasive pathogens: they should be coated with a ligand that allows passage through mucous gels and selective adherence to M cells.

The nontoxic, pentameric binding (B) subunit of cholera toxin (CTB) has been successfully used to target antigens to mucosal surfaces. CTB does not bind to mucins but binds specifically to ganglioside GM1, a glycolipid present in membranes of all cells (12), including apical membranes of intestinal epithelial cells (13). Binding of CTB is not M cell specific: indeed, binding and endocytosis of CT by enterocytes results in the well-known secretory effect of cholera holotoxin (13). Nevertheless, mucosal immune responses to soluble protein antigens can be dramatically altered by conjugation to CTB (14). On this basis it has been suggested that M cell-specific uptake and optimal immune responses might be achieved by coating antigen-containing microparticles with CTB since the particulate carrier would prevent endocytosis by enterocytes and allow endocytosis by M cells. This approach, however, would require that the CTB particle complex maintain GM1 ganglioside binding capacity.

The apical surfaces of M cells and enterocytes differ dramatically. Apical surfaces of enterocytes are highly differentiated structures consisting of rigid, closely packed microvilli (15) whose membranes contain highly glycosylated, stalked glycoprotein enzymes (16). In addition, enterocytes express large, transmembrane mucin like glycoproteins that form a continuous 400–500-nm-thick blanket that covers the tips of the microvilli (17–19). This appears to serve as a size-selective diffusion barrier that excludes particles such as bacteria and viruses, preventing their contact with the enterocyte plasma membrane and impeding access to the small inter-microvillus membrane domains involved in endocytosis (20, 21). The apical surfaces of M cells, in contrast, may allow closer contact of particles and microorganisms because they generally lack densely packed microvilli, have broad membrane microdomains from which endocytosis occurs, are deficient in stalked glycoprotein enzymes (4, 22), and usually lack the thick filamentous glycoprotein coat typical of enterocytes (20). If so, CTB–coated particles would be expected to have relatively free access to GM1 receptors on M cells. On the other hand, M cells do have apical membrane glycoconjugates (23) and some ultrastructural studies have documented thick surface coats on M cells (24). Whether glycolipids are accessible or masked on M cell membranes could be the determining factor in the success or failure of CTB-targeted mucosal vaccine particles.

To test the accessibility of intestinal epithelial cell membranes to particulate antigens, we studied the effect of particle size on the ability of CTB to bind to GM1 on M cells and enterocytes. Ganglioside GM1 has been demonstrated to be the only receptor for cholera toxin in diverse cell types (25) including enterocytes of rabbit small intestine (26) and enterocyte-like intestinal cell lines (27). The carbohydrate head groups of GM1 protrude only 2.5 nm above the surface of the membrane lipid bilayer (28), and the GM1 binding sites in CTB pentamers are 2.3-nm-deep cavities (deduced from its homologue, heat-labile Escherichia coli enterotoxin B subunit; 29). Thus, to bind to GM1, CTB must come into very close contact with the lipid bilayer. Our data show that the accessibility of GM1 to CTB is dramatically altered by immobilization of the ligand on particles, and that particle size determines whether the CTB binding is ubiquitous, restricted to M cells, or abolished.

Materials and Methods

Animals and Cell Lines. Female New Zealand White rabbits weighing 1.4–3.8 kg were purchased from Pine Acres (Norton, MA) or Charles River Laboratories (Wilmington, MA). BALB/c 3T3 fibroblast cell line clone A31 was obtained from the American Type Culture Collection (Rockville, MD) and clone Caco-2/Caco2, derived from the Caco-2 human adenocarcinoma cell line, was a gift from Dr. Mark Mooseker (Yale University, New Haven, CT).

Reagents and Particles. CTB and its FITC and biotin conjugates (CTB–FITC, CTB–biotin) were purchased from List Biological Laboratories Inc. (Campbell, CA). Avidin-coated, carboxymodified, red or green fluorescent latex particles and biocytin were obtained through Molecular Probes, Inc. (Eugene, OR); red fluorescent uncoated latex particles were purchased from Polysciences Inc. (Warrington, PA). BSA was from Boehringer Mannheim (Indianapolis, IN) and avidin was from ICN Pharmaceuticals (Costa Mesa, CA). All lectins were purchased from Vector Laboratories Inc. (Burlingame, CA) except for Lelma fluvius agglutinin which was from EY Laboratories Inc. (San Mateo, CA). The mouse anti-human sucrase-isomaltase mAb Caco 3/73 and the mouse anti-human dipetidylpeptidase IV mAb DAO 7/ 219 were kindly provided by Dr. Andrea Quaroni (Cornell University, Ithaca, NY; 30). TRITC– and FITC-streptavidin were from Molecular Probes, Inc. and peroxidase-labeled streptavidin was from Sigma Chemical Co. (St. Louis, MO). FITC-labeled goat anti–mouse IgG was from Cappel (Durham, NC).

Preparation of the Probes. CTB was coupled to 14 nm colloidal gold sol prepared by the citrate-tannic acid method, and BSA was coupled to 5 nm colloidal gold made by the modified citrate method (31). Colloidal gold sols were adjusted 0.5 pH units above the isoelectric point (pi) of the protein, and protein was added in low ionic strength solution to final concentrations of 5–50 μg protein/ml. After 15–30 min of stirring at 4°C, the colloids were stabilized by addition of BSA to a final concentration of 0.1% (wt/vol), stirred for another 15 min, and washed twice in 6.7 mM Na phosphate buffer, pH 7.3, by centrifugation at 48,000 (14 nm gold) or 60,000 g (5 nm gold) for 60–90 min. The washed colloids were stored at 4°C for up to 1 wk or in 50% (vol/vol) glycerol at −20°C for longer periods.
Table 1. Dynamic Light Scattering Data

|        | D* 10^{-11} m^2/s | D_h nm | M_j kDa |
|--------|-------------------|--------|---------|
| CTB    | 6.34 ± 2.2        | 7.4 ± 2.4 | 70.1 ± 7.2 |
| CTB-FITC | 7.22 ± 26       | 6.4 ± 1.6 | 51.5 ± 4.8 |
| Avidin | 6.62 ± 17         | 7.0 ± 1.4 | 63.5 ± 4.3 |
| BSA    | 6.13 ± 9          | 7.2 ± 1.4 | 66.3 ± 2.2 |

Data are given as mean values ± SD, calculated from 6 to 19 independent experiments.

*Diffusion coefficient at 24°C (297.15 K) (CTB, CTB-FITC, avidin) or at 21.7°C (294.85 K) (BSA) in 40 mM Na phosphate buffer, pH 7.5 (CTB, CTB-FITC, BSA), or PBS (avidin), as determined by dynamic light scattering.

Hydrodynamic diameter, calculated from the Stokes-Einstein equation: $D_h = 2k_BT/6\pi \eta D$, where $k_B$ is the Boltzmann constant, $T$ is the absolute temperature, $\eta$ is the viscosity of water (1.019 × 10^{-3} Nm^{-1}s^{-1}), and $D$ is the diffusion coefficient.

Molecular mass, calculated from $D_h$ by the empirically determined algorithm for globular proteins: $M = 0.7745 \times D_h^{2.46}$.

CTB-P were prepared by coupling biotinylated CTB to avidin-coated, carboxy-modified, red fluorescent latex particles (Av-P) of 1 μm nominal size. To avoid aggregation of Av-P and CTB-biotin, both of which are multivalent, the maximum CTB surface load was calculated according to the model described below and coupling was done with CTB-biotin in eightfold molar excess. Thus, particles and CTB-biotin were mixed to final concentrations of 0.4% (vol/vol) particles and 400 μg/ml CTB-biotin in 1 ml 20 mM Na phosphate buffer, pH 7.5, 30 mM NaCl, and 400 μM NaN3. The particle/protein mixture was rocked at 1.5 rpm for 72 h at 4°C in the dark, centrifuged at 500 g, and the supernatant was removed. Particles were repeatedly washed by resuspension in PBS containing 250 μg/ml gentamicin and centrifugation, until free CTB-biotin was no longer detectable by GmELISA using streptavidin-peroxidase as the detection system (32). The resulting CTB-P were stable for at least 3 mo at 4°C.

*Euonymus europaeus* agglutinin (EEA)-coated microparticles (EEA-P) were prepared by mixing Av-P and an estimated eightfold molar excess of EEA-biotin to final concentrations of 0.4% (vol/vol) particles and 875 μg/ml EEA-biotin in 1.2 ml PBS containing 5 mM Heps, 3 mM NaN3, 50 μM CaCl2, and 5 μM MnCl2. Control biocytin microparticles (BC-P) were prepared by mixing excess biocytin with avidin-coated, carboxy-modified, green fluorescent latex microparticles of 1 μm nominal size at final concentrations of 0.4% (vol/vol) particles and 1 mM biocytin in 1.5 ml PBS. Coupling and washing conditions were the same as described for CTB-P. Microparticles were resuspended in PBS containing 250 μg/ml gentamicin, quantitated in a hemocytometer, adjusted to 5 × 10^6 particles/ml, and stored at 4°C in the dark.

Determination of Probe Diameter and Ligand Load. As the final hydrodynamic diameters of protein–particle conjugates could not be analyzed directly, the diameters of the soluble and solid probe components were measured separately and the final sizes and ligand loads were calculated on the basis of these measurements. The diameters of the solid particles were readily determined by electron microscopy (EM). The sizes of the protein components were derived from their diffusion coefficients in solution, which were used to calculate hydrodynamic diameters and molecular masses. The diffusion coefficients of the protein components were determined by dynamic light scattering in aqueous solution at 780 nm. Since the solubility of CTB-biotin (<1 mg/ml in 20 mM Na phosphate buffer, pH 7.5) was below the detection limit of the light scattering device, CTB was analyzed in place of CTB-biotin. The proteins were dissolved in 40 mM Na phosphate buffer, pH 7.5 (CTB, CTB-FITC, BSA), or PBS (avidin) at concentrations of 2 (CTB, CTB-FITC) or 5 μg/ml (avidin, BSA), centrifuged twice at 13,000 g for 10 min, filtered through a 20-nm Anotop 10 filter (Whatman, Hillsboro, OR.), and injected into a dp-801 molecular size detector (Biotage, Inc., Charlottesville, VA) equipped with a personal computer. Data were retrieved and analyzed using the Biotage data logging software package and the QuatroPro 2.0 program (Borland International, Scotts Valley, CA). The dynamic light scattering data are summarized in Table 1. The calculated hydrodynamic diameters of avidin and CTB pentamers were almost identical to those determined crystallographically (33, 34), and their molecular masses calculated from diffusion coefficients were similar to those reported by others (12, 35). This confirmed the globular shape of avidin and CTB in solution, and allowed us to use a “tight packing on a sphere” model for calculations of ligand number, protein layer thickness, and final particle size of the colloidal gold and fluorescent microparticle probes.

The diameters of the colloidal gold particles were determined on EM photographs of dispersed gold sols and tissue sections as described previously (36). The gold particles used for coating with CTB and BSA were 14.0 ± 1.3 nm and 5 nm in diameter, respectively. The diameters of all latex particle cores, the specific surface area (SSA), and the specific number of biotin binding sites (SNBBS) for Av-P and the parent particles of Be-P were provided by the manufacturers: polystyrene latex microparticles (PLP diameter 0.99 μm ± 4.4%; Av-P (diameter 1.09 μm ± 5.3%, SSA 52,176 cm²/g, SNBBS: 1.39 nmol/mg); and parent particles of BC-P (diameter 0.977 μm ± 2.6%, SSA 58,311 cm²/g, SNBBS 1.79 nmol/mg).

To calculate the final diameters and ligand loads of the particular probes, it was assumed that the probe consists of a central sphere (gold or latex core) covered completely by one or more layers of tightly packed small spheres, i.e., globular protein molecules (avidin, CTB, BSA, or EEA). In case of a multilayer, each protein molecule of the outer layer would be positioned in the triangular gap formed by three occupants of the underlying layer. Assuming an avidin/biotin binding ratio of 1:4, the number of avidin layers ($n_{layer}$) on Av-P and the parent particles of Be-P was calculated by the equation:

$$n_{layer} = \frac{\sqrt[3]{SNBBS \times SSA}}{8 \times N_A \times d_{avidin}^2} \times d_{particle}$$ (for $d_{particle} >> d_{avidin}$) (1)

where $N_A$ is the Avogadro constant and $d_{avidin}$ is the hydrodynamic diameter of avidin. Using equation (1) $n_{layer} = 1.6$ for Av-P (monolayer plus 60% filled second layer) and 1.95 for the parent particles of Be-P (second layer). Since a partially filled avidin layer has the same effective thickness as a completely filled layer, there must be two avidin layers for both particle types. The total thickness (or height) of the avidin coats ($h_{coat}$) was then calculated by the equation:

$$h_{coat} = \left(1 + (n_{layer} - 1)\right) \times \frac{d_{particle}}{\sqrt{3}}$$ (for $n_{layer} > 1$) (2)
To obtain the final probes, Av-P were reacted with excess CTB-biotin or EEA-biotin, whereas the colloidal gold was coated with CTB or BSA. Under the conditions used we assumed that only one layer of these proteins could bind and the thickness of the added monolayer was identical to the diameter of the protein molecule. Thus the total hydrodynamic diameter \( d_h \) of a particular probe is described by the equation:

\[
d_h = d_{\text{particle}} + 2 \times \left( \sum_{i=0}^{m} h_{\text{coat}} \right)
\]

where \( d_{\text{particle}} \) is the particle diameter as measured by EM, \( h_{\text{coat}} \) is the total height of a particular protein coat, \( i \) is the summation index, and \( m \) is the total number of protein coats coupled onto the particle surface. As the particle core diameters and the hydrodynamic diameters of the proteins were measured independently, the Gaussian error propagation rule applies and was used for the calculation of the final standard deviations.

Using the final hydrodynamic diameters of Av-P, colloidal gold, and the protein ligands, the ligand load on the surface of a particle, \( n_{\text{molecules}} \), was calculated using the equation:

\[
n_{\text{molecules}} = \frac{2 \pi}{\sqrt{3}} \left( \frac{d_{\text{particle}}}{d_{\text{protein}}} + 1 \right)^2
\]

where \( d_{\text{particle}} \) is the particle diameter (including the height of the avidin coat in the case of Av-P) and \( d_{\text{protein}} \) is the hydrodynamic diameter of the ligand. Final probe characteristics are summarized in Table 2.

**Application of Probes to Rabbit Intestinal Mucosa.** Rabbits were fasted overnight (water ad libidum), anaesthetized by i.p. injection of 25% (wt/vol) urethane in PBS (10 ml/kg), and the intestines were exposed by laparatomy. For in vivo studies, three to five jejunal/ileal segments 2-3 cm in length and containing a Peyer’s patch were ligated, and 500 \( \mu \)l of probe solution was injected into the lumen. Probes applied in vivo included CTB-FITC at 1 mg/ml, CTB-P, Av-P, and PLP at 10^8 particles/ml, and a 1:1 mixture of CTB-P and Bc-P at 5 \times 10^8 particles/ml each, all in PBS containing 50 \( \mu \)g/ml gentamycin. The ligated loops were returned to the abdominal cavity and excised 60 min later. Loops were opened and mucosal surfaces washed extensively with cold PBS. The entire tissue was immersed in freshly depolymerized 3% (wt/vol) paraformaldehyde in PBS (PFA-PBS), and mucosal samples were dissected in fresh fixative.

Colloidal gold probes aggregated rapidly after injection into ligated loops in vivo. Thus, these probes were applied to mucosal explants ex vivo. Jejunal/ileal segments containing Peyer’s patches were excised from anesthetized rabbits, the mucosal surface was rinsed with PBS, the muscularis externa was stripped off, and the mucosa was cut into pieces of 3 \times 3 \times 1 mm. Mucosal explants were placed in 100 \( \mu \)l of oxygenated HBSS containing 0.5% (wt/vol) BSA and 10 OD_560 of CTB-gold or BSA-gold. After incubation at room temperature (RT) for 1 h, the tissues were rinsed in PBS and fixed in a solution containing 2.5% (wt/vol) glutaraldehyde, 2% (wt/vol) formaldehyde, 4 mM CaCl_2 and 2 mM MgCl_2 in 0.1M Na cacodylate buffer, pH 7.4. For application of CTB-FITC, explants were placed in 200 \( \mu \)l oxygenated, high glucose (25 mM) DME (GIBCO BRL, Gaithersburg, MD) containing 250 \( \mu \)g/ml CTB-FITC and incubated for 30 or 45 min at 15°C in the dark. Explants were then washed five times with 1.5 ml PBS and fixed immediately in PFA-PBS. All procedures involving rabbits were performed in accordance with the Guide-

**Table 2. Physical and Chemical Properties of the Probes**

| Probe     | Size* Mean ± SD | CTB load‡ | Surface property§         |
|-----------|-----------------|-----------|---------------------------|
|           | \( nm \)        | molecules/probe |                       |
| CTB-Probes|                 |           |                           |
| CTB-FITC  | 6.4 ± 1.6       | 1         | Hydrophilic, low positive charge [pI~7.8] |
| CTB-gold  | 28.8 ± 5.0      | ~30       | Hydrophilic, low positive charge [pI~7.8] |
| CTB-P     | 1,130.0 ± 58.0  | ~80,000  | Hydrophilic, low positive charge [pI~7.8] |
| Control Probes |             |           |                           |
| BSA-gold  | 19.4†          | n/a      | Hydrophilic, low negative charge [pI~5.3] |
| Av-P      | 1,115.4 ± 58.0  | n/a      | Hydrophilic, high positive charge [pI~10.5] |
| Bc-P      | 1,002.0 ± 26.0  | n/a      | Hydrophilic, high positive charge [pI~10.5] |
| PLP       | 990.0 ± 44.0    | n/a      | Hydrophobic, not charged   |
| EEA-P     | ~1,130.0 ± 60.0§| n/a      | Hydrophilic, high negative charge [pI~4.5] |

All computations are based on the model of dense packing of globular proteins on the particle surfaces and were calculated as described in Materials and Methods.

*Stokes diameter. Computed from the diameter of the particle core as determined by EM and the hydrodynamic diameter of the proteins of the surface coats as measured by dynamic light scattering (Table 1).

‡Number of CTB molecules per individual particle. Computed from the diameter of the particle core as determined by EM and the hydrodynamic diameter of the proteins of the surface coat as measured by dynamic light scattering (Table 1).

§Hydrophobicity, -philicity, and presumed charge of the probe surface at pH 7.3-7.5. In brackets, pI of the proteins displayed on the probe surface. pLCTB 7.8 (12); pLBSA 3.3 (51); pLbactin 10.5 (52); pLPEA 4.3-4.7 (45).

†No standard deviation was calculated for the size of the gold particle core.

‡Estimated size of the EEA-P which was prepared from the same particle stock as the CTB-P. n/a, not applicable.
lines for Animal Experimentation established by Harvard Medical School and Children's Hospital.

Microscopic Analysis of Probe Binding and Cell Surface Architecture. For analysis of the binding and uptake of fluorescent proteins and microparticles in mucosal tissues, samples were prepared for cryostat sectioning. Small tissue blocks were soaked for 2 h in 15% (wt/vol) sucrose in PBS followed by infiltration for 10 min in OCT compound (Miles Scientific, Naperville, IL). They were mounted in Cryo-Gel embedding compound (Instruments, Hackensack, NJ), frozen rapidly on the cryostat quick freezing holder, and 4-8-μm sections were cut at −18 to −20°C in a Microm cryostat (International Equipment Company, Needham, MA). Sections were mounted on glass slides with Moviol (Calbiochem-Novabiochem Corp., San Diego, CA) containing 2.5% (wt/vol) 1.4-diazabicyclo-[2.2.2]octane (Sigma Chemical Co.) (Moviol-DABCO) and photographed with a Zeiss Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) equipped for epifluorescence using T-Max 400 film (Eastman Kodak, Rochester, NY).

For EM of tissues exposed to colloidal gold probes, glutaraldehyde/formaldehyde-fixed/fixed rabbit Peyer's patch mucosal samples were processed as previously described (20, 21). For EM visualization of cell surface glycoalyx, rabbit Peyer's patch tissue and Caco-2 monolayers were fixed by a simultaneous osmium-glutaraldehyde procedure described by Bye et al. (24). Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a 100CX electron microscope (JEOL, Peabody, MA).

Cell Culture. BALB/c 3T3 fibroblasts were grown in high glucose (25 mM) DME supplemented with 10% (vol/vol) calf serum (Hyclone Laboratories, Inc., Logan, UT), 2 mM glutamine, 100 U/ml penicillin/100 μg/ml streptomycin (GIBCO BRL), 25 mM Hapes, and 3.7 g/liter NaHCO3 (Sigma Chemical Co.) at 37°C in a humidified atmosphere containing 10% (vol/vol) CO2. Caco-2 cells were cultured in the same medium devoid of Hapes but with the addition of 10 μg/ml human transferrin (Boehringer Mannheim) in a humidified atmosphere containing 5% (vol/vol) CO2. For immunocytotoxic and particle binding studies, 3T3 fibroblasts or Caco-2 cells were seeded onto 13-mm diameter glass coverslips (Bellco Glass, Vineland, NJ) in 24-well tissue culture plates (Costar, Cambridge, MA). The fibroblasts were used for experiments at 12–13 d (6–7 d after confluence) and the Caco-2 cells at 24 d (21 d after confluence).

Application of CTB and Lectin Microparticle Probes to Fibroblasts and Intestinal Cells In Vitro. BALB/c 3T3 fibroblasts were washed gently five times with 2–3 ml prewarmed (37°C) PBS containing 0.9 mM CaCl2 and 0.5 mM MgCl2 (CM-PBS), and 500 μl of a probe mixture was added to each well of the 24-well plates. Microparticles were applied as 1:1 mixtures of 5 × 107 particles/ml each of CTB-P (or Av-P) and Bc-P in DME containing 50 μg/ml gentamicin. To test competition of CTB-P binding by free CTB, coverslips were preincubated for 5 min with 250 μl of 2 or 20 μg/ml CTB in DME before addition of 250 μl of 1:1 mixtures of 106 particles/ml each of CTB-P and Bc-P in DME. After incubation for 60 min at 37°C, coverslips were washed three times for 10 min by gently adding and aspirating 2 ml CM-PBS, fixed in 1.5 ml PFA-PBS for 2 h, washed in PBS followed by distilled water, and mounted on glass slides with Moviol-DABCO. Cell monolayers were examined and photographed en face with a Zeiss Axiophot microscope equipped for epifluorescence using Kodak T-Max 400 film.

Caco-2 cells on coverslips in 24-well plates were washed as above. 500 μl of DME containing 106 CTB-P or EEA-P/ml, 50 μg/ml gentamicin, and 10 μg/ml transferrin were added to each well, and plates were incubated for 60 min at 37°C. Coverslips were then washed three times, fixed, washed again, and mounted on glass slides as described above. Some cell monolayers that were exposed to microparticles were subsequently labeled with soluble CTB, lectins, or antibodies as described below. In this case, fixed cells were incubated for 15 min in 2 ml of 50 mM NH4Cl in PBS to quench free aldehydes and washed again before staining. In some experiments with EEA-P, the coverslips were fixed and quenched before exposure to microparticles.

Labeling of Cultured Cells with Soluble Toxin, Lectins, and Antibodies. The microparticle-labeled, fixed and quenched Caco-2 cells were incubated in 0.2% (vol/vol) gelatin (Sigma Chemical Co.) in PBS for 30 min at RT to block nonspecific protein binding sites, and stained with either 10 μg/ml lecin-FITC conjugates, 10 μg/ml CTB-FITC, or 1:100 dilutions of antibodies in PBS/gelatin for 15–16 h at 4°C. Cells labeled with antibodies were washed three times for 10 min in PBS/gelatin and stained with 2 μg/ml FITC-labeled goat anti–mouse IgG for 90 min at RT. Coverslips were washed, mounted, and examined as described above. In some experiments, washed, fixed, and quenched Caco-2 cells not exposed to microparticles were labeled with 10 μg/ml biotinylated lectins, stained with 10 μg/ml streptavidin-FITC or –TRITC, and processed as above.

Analysis of Microparticle Adherence and Uptake. Uptake of fluorescent microparticles by the FAE of rabbit Peyer's patches was quantitated by counting particles in 8-μm cryostat sections of mucosal tissue. To avoid artifactual displacement of free luminal microparticles during sectioning, mucosal surfaces were vigorously washed before fixation so that luminal material and loosely adherent particles were eliminated. Only particles that were in direct contact with the FAE, or located within the epithelium or underlying lymphoid follicle, were counted. For each Peyer's patch, an average of 65 representative follicle sections were quantitated, and microparticle uptake was expressed as the average number of microparticles per section of follicle for a given Peyer's patch. Microparticle binding to cultured fibroblasts and Caco-2 cells was quantitated from photographs of at least three randomly selected nonoverlapping regions of each cell monolayer viewed en face. Microparticle binding was averaged for each experiment and expressed as particles per mm2.

Statistics. Statistical analysis of microparticle binding and uptake studies was performed on a Macintosh Ilii computer (Apple, Cupertino, CA) using the StatView II program (Abacus Concepts, Berkeley, CA). Mixed probes of test and control particles, and differentiated and undifferentiated areas of Caco-2 cells, were considered paired samples. The results of independent experiments were treated as unpaired samples. Differences in binding or uptake among particle types at a significance level of ≥95% were calculated by two-tailed Student’s t test.

Results

Characterization of the Probes. To test the accessibility of intestinal cell membrane glycolipids to particulate ligands in the size ranges of viruses, bacteria, and particulate mucosal vaccines, CTB was used as model ligand in the form of probes of three distinct sizes: CTB-FITC, CTB-colloidal gold and red fluorescent CTB-P. Nonadherent BSA-gold served as negative control for CTB-colloidal gold. As negative control for CTB-P we used the parent red fluorescent avidin microparticles from which the CTB-P were prepared (Av-P), or when a clearly discernible internal control
by the negative effect of large particle size on adherence, as shown below.

**Figure 1.** Binding of CTB-FITC to FAE and villus epithelium of rabbit Peyer's patch mucosa. Mucosal tissues were exposed to 250 μg/ml CTB-FITC for 45 min at 15°C ex vivo (A), or to 1 mg/ml CTB-FITC for 60 min in vivo (B). Cryostat sections were viewed by fluorescence microscopy. (A) Soluble CTB-FITC labeled the entire FAE which contains both M cells and enterocytes. (B) CTB-FITC labeled the surfaces of enterocytes on most villi, although certain areas were devoid of label (tip of right villus). Bar, 50 μm.

for CTB-P binding was required, we used green fluorescent biocytin-quenched avidin microparticles (Bc-P) of approximately the same size as CTB-P. As positive controls we used microparticles coated with EEA-P that were prepared from Av-P, and uncoated, hydrophobic PLP.

The probe properties that were of particular importance for this study were hydrodynamic diameter, which affects access to membrane glycolipids, surface charge/hydrophobicity, which affects nonspecific binding, and ligand load, which affects the avidity of specific binding (Table 2). We assumed that the pI of the protein forming the outermost layer of the probe determined the surface charge, and that protein coats would be hydrophilic and uncoated polystyrene latex would be hydrophobic. The ligand load of each probe is dependent on the probe size and ranged from 1 pentameric CTB molecule/probe for CTB-FITC to ~80,000 CTB molecules/probe for CTB-P. However, the potential positive effect of ligand load on binding avidity was offset by the negative effect of large particle size on adherence, as shown below.

**Soluble CTB Has Access to Apical Plasma Membranes of All Intestinal Epithelial Cells In Vivo.** Rabbit Peyer’s patches were exposed to 1 μg/ml CTB-FITC (diameter 6.4 nm) 1 h in vivo or to 250 μg/ml CTB-FITC ex vivo for 30 or 45 min at 15°C to retard endocytosis. Analysis of ~500 sections from multiple Peyer’s patches from two rabbits showed that randomly distributed areas of mucosal surface were labeled but other areas were unlabeled, presumably due to adherent mucus that was not removed. Within the labeled areas, all epithelial cell surfaces of the FAE showed CTB-FITC binding (Fig. 1 A), although staining intensity varied from cell to cell. Similarly, the brush borders of absorptive enterocytes on villi, both in Peyer’s patches and in other regions, bound soluble CTB-FITC whether exposed in ligated loops at 37°C or in mucosal explants at 15°C (Fig. 1 B). These data indicate that whereas mucus or other factors may have impeded contact of probes with mucosal surfaces, once these luminal diffusion barriers were breached, plasma membrane GM1 of both enterocytes and M cells was accessible to CTB-FITC.

**CTB Coupled to Colloidal Gold Particles Binds Selectively to M Cells.** Rabbit Peyer’s patch explants were exposed to CTB-colloidal gold (total diameter 28.8 nm) for 1 h at R.T ex vivo, and analysed by EM. Many samples showed gold particles caught in adherent mucus but no probe on mucosal surfaces. In areas where adherence of CTB-gold occurred, it was selective for M cells (Fig. 2 A). On these cells, CTB-gold bound to the entire apical plasma membrane including microvilli, microfolds, and coated pits. CTB-gold particles were also present in coated and uncoated vesicles, implying that endocytosis had occurred (Fig. 2 B). In contrast, as shown in previous studies (36), BSA-gold failed to adhere to any epithelial cells on villi or in the FAE in spite of its smaller diameter (data not shown). Thus, when administered as particles <30 nm in diameter, CTB had access to its ganglioside receptor on certain M cells but not on enterocytes.

**CTB Coupled to Microparticles Does Not Have Access to Apical Membrane Glycolipids of Intestinal Epithelial Cells In Vivo.** The latex particle-based probes (such as CTB-P, diameter 1.13 μm) carried fluorescent markers that allowed us to count individual microparticles on or in mucosal tissue. Pilot experiments showed that uptake of CTB-P varied widely among Peyer’s patches, even in the same rabbit. To determine whether this variation was specific to the CTB-P probe, we tested uptake of uncoated, uncharged, hydrophobic latex particles (PLP) that had previously been shown to bind avidly to mucosal surfaces and to be readily transcytosed by M cells (10). These were applied to multiple Peyer’s patches of a single rabbit and to single patches of different rabbits, by injection of 5 × 10⁶ PLP into ligated loops and incubation for 1 h. Washing conditions were stringent enough to remove all loosely adherent particles from the mucosal surface, leaving only those that were tightly attached or that had been taken up into the tissue. Counting of surface-attached and endocytosed particles confirmed that endocytic activity was relatively uniform in all 15–20 FAE of a given Peyer’s patch, but that entire patches varied widely in their endocytotic activity. The “high uptake patches” showed avid binding and significant uptake of PLP in almost all domes, whereas in the “low uptake patches,” the majority of the domes remained unlabeled and uptake was very low, most likely because these patches were coated with mucus.

1050 Barrier Function of Intestinal Cell Glycocalyx
Figure 2. Selective binding of CTB-colloidal gold to rabbit Peyer's patch M cells. Mucosal explants were exposed to CTB-gold for 1 h at RT in vitro. (A) EM analysis revealed that CTB-gold adhered almost exclusively to the apical surfaces of M cells, whereas adjacent enterocytes had few gold particles associated with their microvilli. (B) In M cells, gold particles were present in clathrin-coated pits and vesicles indicating endocytosis and transport. Bar, 400 nm.

To eliminate the confounding factor of variability among Peyer's patches, a 1:1 mixture each of $2.5 \times 10^8$ red fluorescent CTB-P and green fluorescent control Bc-P was applied to ligated loops for 1 h. Examination of cryostat sections revealed that both types of particles adhered in small numbers and were endocytosed, but CTB-P were no better than control Bc-P (Fig. 3). Statistical analysis of particle uptake for a total of 15 patches, normalized to $2.5 \times 10^8$ particles per loop, showed that patches were either high uptake or low uptake for all types of particles analyzed (Fig. 4A). Analysis of the high uptake patches revealed that CTB-P were taken up even less efficiently than Bc-P and that both protein-coated particles were endocytosed less efficiently than the PLP control (Fig. 4B). The preferential uptake of Bc-P over CTB-P was consistent for every patch at a ratio of 1.8 Bc-P:1 CTB-P (correlation coefficient 0.988). These data indicate that the CTB ligand immobilized on particles $\geq 1 \mu m$ in diameter did not have access to glycolipids in the apical plasma membranes of either enterocytes or M cells, and that other factors such as ionic in-
Figure 3. Simultaneous uptake of CTB-coated and control biocytin-quenched microparticles into rabbit Peyer's patch domes. Peyer's patch mucosa was exposed to equal numbers (2.5 × 10⁷ particles) of red fluorescent CTB-P and green fluorescent control Bc-P for 1 h in vivo. Fluorescence microscopy of a representative cryostat section shows that both types of particles were taken up into the dome, but not into adjacent villi. Control Bc-P (green) were taken up in greater numbers than CTB-P (red), suggesting that uptake was due to nonspecific interaction of the cationic particles with FAE cell surfaces. The particles are located primarily within the FAE, presumably associated with M cells. Scale bar, 100 μm.

Figure 4. Quantitative analysis of CTB-P, Bc-P, and PLP uptake by rabbit Peyer's patch domes. Uptake of three types of 1-μm particles by the FAE was analyzed by counting fluorescent particles in 974 domes in cryostat sections of 15 rabbit Peyer's patches that had been exposed to 2.5 × 10⁷ particles each of CTB-P and Bc-P, or 5 × 10⁸ PLP for 1 h in vivo. Counts are expressed as average particles per dome section of a given patch, normalized to application of 2.5 × 10⁸ particles per patch. (A) Particles were taken up uniformly in all domes of a given Peyer's patch, but there was wide variation among patches. In some patches, all domes showed few or no particles (Low uptake), whereas in other patches domes were heavily labeled (high uptake). (B) Peyer's patches showed either low or high uptake activity for all types of particles. Uptake of both interactions in case of the cationic Bc-P, or hydrophobic interactions in case of PLP, influenced binding and uptake of these particles by M cells.

CTB Coupled to Microparticles Binds to G₄₃ on Cultured Fibroblasts. To rule out the possibility that the immobilization of CTB on fluorescent microparticles masked, inactivated, or destroyed the ligand, we tested the ability of CTB-P to bind to a fibroblast cell line known to express high levels of G₄₃ ganglioside (25). Live 3T3 cell monolayers were exposed to a 1:1 mixture of 2.5 × 10⁷ particles each of CTB-P and Bc-P for 1 h at 37°C. Over 38 times more CTB-P than Bc-P adhered. Blocking of G₄₃ sites with free CTB reduced binding of CTB-P to control levels (Fig. 5). This confirmed that CTB-P were capable of binding to G₄₃ on live cells via the CTB immobilized on their surfaces.

CTB-P Bind to Apical Membranes of Undifferentiated but Not Differentiated Caco-2 Cells. Because the fibroblast membrane is not a valid model for the highly specialized apical domain of intestinal epithelial cells, we tested the binding of CTB-P to polarized monolayers of Caco-2BBe2 adenocarcinoma cells. These cells form a columnar enterocyte-like epithelium with well-organized brush borders when cultured on Transwell filters (37). When cultured on glass, however, small “islands” of flat cells that do not develop brush borders or express apical membrane enzymes remain within the monolayer, even long after confluence (38, and Fig. 6 A). When such monolayers were exposed to 5 × 10⁷ CTB-P for 1 h at 37°C, binding of particles was largely restricted to the undifferentiated islands (Fig. 6 C), with types of control particles (Bc-P and PLP) was consistently higher than that of CTB-P (*), with clear significance for CTB-P vs. Bc-P (two-tailed, paired t test, P <0.05) and borderline significance for CTB-P vs. PLP (two-tailed, unpaired t test, P = 0.065).
Figure 5. CTB-mediated binding of 1 μm particles to live BALB/c 3T3 fibroblasts. Fibroblast monolayers were exposed to 2.5 × 10^7 each of CTB-P and Bc-P, or Av-P and Bc-P, for 1 h at 37°C. Particle binding was analyzed by fluorescence microscopy. CTB-P binding was significantly (*) higher than binding of the Bc-P or Av-P controls. In the presence of free CTB, binding of CTB-P was reduced to control levels. Data in A represent the mean values of five independent experiments (two-tailed, paired t test, P <0.05).

2,331 ± 414 particles/mm² on undifferentiated cells versus 10 ± 1 particle/mm on well-differentiated cells. To determine whether this selectivity was due to differences in G_{M1} expression, we exposed the monolayer to CTB-FITC after particle binding and observed that binding of soluble CTB-FITC occurred throughout the monolayer. Indeed, CTB-FITC labeling was equal or higher on the well-differentiated cells than on the cells in the islands (Fig. 6 B). This indicates that selective binding of CTB-P to undifferentiated Caco-2 cells was due to enhanced accessibility of G_{M1} and not simply to the presence of G_{M1} in the apical membrane.

Apical Membrane Components of Undifferentiated and Differentiated Caco-2 Cells. To explore the basis for selective binding of CTB-P to undifferentiated Caco2_{und} cells, we compared the expression of glycoproteins, including stalked brush border enzymes and glycocalyx, on apical plasma membranes of differentiated and undifferentiated Caco-2 cells. As shown previously (38), the undifferentiated islands were completely negative for both dipeptidylpeptidase IV, an enzyme that appears early during enterocyte differentiation (39), and sucrase-isomaltase, an enzyme considered a marker of terminal differentiation (40). All "differentiated" cells were positive for dipeptidylpeptidase IV and ~70% expressed sucrase-isomaltase, but all failed to bind CTB-P. This indicates that membrane surface components of partially differentiated cells were sufficient to block access of CTB-P to G_{M1}.

Enterocyte differentiation is also accompanied by the appearance of other highly glycosylated, membrane-anchored glycoproteins that form the glycocalyx on microvillous membranes (19). To monitor the appearance and oligosaccharide heterogeneity of these components on differentiating Caco-2 cells, we applied a battery of lectins specific for epitopes typical of N- and O-linked complex oligosaccharides. Most of these lectins bound to differentiated Caco-2...
Figure 7. Ligands directed against carbohydrate epitopes of the glyco-
calyx mediate binding of 1 μm particles to differentiated Caco-2 cells.
Caco-2 cell monolayers were briefly fixed, exposed for 60 min at 37°C to
5 × 10^5 red fluorescent EEA-P followed by EEA-biotin and streptavidin-
FITC, and analyzed en face by phase contrast (A) and fluorescence mi-
croscopy (B and C). The islands of undifferentiated cells (A, arrowheads)
lacked the complex carbohydrate epitopes recognized by EEA (B). (C)
Binding of EEA-P (dots) occurred primarily on well-differentiated cells
but not to flat cells in the undifferentiated islands, and indicated both N-linked core glycosylation (Con A;
branched N-linked hexasaccharides [41]; Lycopersicon esculentum agglutinin: oligomeric N-acetylglucosamine [42]) and O-linked glycosylation (Vicia villosa agglutinin: terminal N-acetylglactosamine [43]) as well as heterogeneous, complex oligosaccharides as shown in a previous study from this laboratory (38). Cells in the undifferentiated islands were stained only by Lotus lectin, a probe that shows highest affinity for the N-linked disaccharide fucosyl α(1-6) N-acetylglucosamine, but does not bind to that epitope when the chitobiose core and the carbohydrate antennae of the N-linked oligosaccharide are intact (44). Taken together, the lectin-staining data demonstrated that columnar Caco-2 cells with well-developed brush borders displayed abundant apical membrane glycoconjugates with branched complex carbohydrates and mature oligosaccharide side chains, and that this was associated with inaccessibility of GM1 to CTB-P. In contrast, cells in the undifferentiated islands lacked mature glycoconjugates and displayed only the truncated disaccharide recognized by Lotus lectin. On these cells, GM1 was sufficiently exposed to allow CTB-P binding.

Microparticles Have Access to Terminal Sugars of Membrane
Glycoconjugates of Differentiated Caco-2 Cells In Vitro. The lec-
tin data suggested that a particle-associated ligand directed
against peripheral components of the glycocalyx itself
should be able to adhere to differentiated cells but not to
the poorly glycosylated undifferentiated cells. To test this
hypothesis, we generated 1 μm particles coated with lectin
EEA that recognizes complex carbohydrate epitopes (45)
that would be expected to occupy distal positions in oli-
gosaccharide side chains. These microparticles bound av-
idly to differentiated Caco-2 cells and binding was closely
correlated with the density of EEA receptors, as revealed by
subsequent counterstaining with EEA-FITC (Fig. 7). In
contrast, very low numbers of particles bound to cells in
the undifferentiated islands, and binding was restricted to
the few cells that showed low levels of the EEA receptor.
Cross-linking of the glycocalyx by PFA fixation before ap-
plication of EEA-P exposure did not affect the binding pat-
tern, confirming that the EEA receptor occupied a periph-
eral position on the cell surface. Thus, microparticles of
the same size as CTB-P but bearing a ligand directed against
a peripheral component of the epithelial cell glycocalyx
readily bound to apical surfaces of differentiated Caco-2
cells.

Ultrastructural Features of the Glycocalyx of Intestinal Epithe-
lia Cells In Vivo and In Vitro. On apical brush borders of
intestinal enterocytes, a thick “filamentous brush border
glycocalyx” (FBBG) coats the tips of microvilli (18, 19).
There are conflicting views about the presence and thick-

and correlated well with EEA receptor distribution, demonstrating that
EEA-coated particles had access to EEA receptor sites in the Caco-2 cell
glycocalyx. Scale bar, 200 μm.
ness of the glycocalyx on M cells, and no data on this component in Caco-2BBBe2 cells. We therefore reexamined all of these cell types using a method that renders cell surface coats visible by EM (24), in the hope of defining ultrastructural features that might account for the inaccessibility of GM1 to CTB-P. The FBBG on enterocytes of the FAE was a dense network ~500 nm thick (Fig. 8 A), and was identical to that on villus enterocytes (Fig. 8 B), as previously described by others (17–19, 24). In contrast, the glycocalyx of M cells varied widely, from a homogeneous, continuous layer up to 450 nm thick from the membrane, interspersed with filaments up to 200 nm in length (Fig. 9, A and B). M cells with relatively uniform microvilli had a thick enterocyte-like glycocalyx (Fig. 9 A), whereas M cells with microvilli of variable length and thickness had thin, sparse cell coats (Fig. 9 B). M cells that bound CTB-colloidal gold generally had variable microvilli (Fig. 2) and presumably a thin glycocalyx. Caco-2 cells in the well-differentiated areas of the monolayers had uniform microvilli as previously described (37) but lacked the 500-nm-thick glycocalyx associated with microvillus tips of enterocytes in vivo. A thin glycocalyx extended 10–20 nm from the lateral sides of microvilli and 40–60 nm from the tips (Fig. 9 C). In contrast, the apical surfaces of cells in the undifferentiated islands lacked organized brush borders and had no visible membrane surface coat (Fig. 9 D). Thus, the presence or absence of lectin binding correlated well with the presence or absence of a glycocalyx visible by EM.

Discussion

The parameters that govern selective adherence of macromolecules and microorganisms to M cells are of great importance for understanding the pathogenesis of certain infectious diseases and for the rational design of mucosal vaccination strategies. It has long been recognized that M cell–specific transcytosis is due partly to the distinct architecture and high endocytic activity of these cells. M cells represent only a tiny minority in the intestinal epithelium, however, and thus efficient uptake requires selective adherence to M cell apical surfaces (4, 20). Tropism or target cell selectivity by microorganisms is generally based on the presence of unique host cell surface molecules that are exploited as receptors (46, 47). Thus investigations of M cell targeting have generally attempted to identify and exploit unique M cell apical surface components (5). We have now shown that M cell–specific adherence may also be due to
enhanced accessibility of receptors on M cells and that the glycocalyx on apical membranes of both enterocytes and M cells plays an important role in limiting the adherence of particulate ligands to membrane glycolipids.

To clearly define the role of steric hindrance in cell type–specific binding requires a model system in which receptor accessibility is the only variable. For this reason we chose a monospecific ligand (CTB) whose receptor (GM1) lies in the outer leaflet of the plasma membrane, and we applied CTB in the form of probes of comparable surface charge that varied only in size. The CTB probes used in this study could be considered models for soluble antigens (6.4 nm CTB-FITC), macromolecular complexes or small viruses (28.8 nm CTB-colloidal gold) and bacteria (1.13 μm CTB-P). Whereas soluble CTB-FITC bound to apical plasma membranes of all cell types in the rabbit small intestinal epithelium, CTB-colloidal gold adhered exclusively to Peyer’s patch M cells and CTB-P failed to adhere to any epithelial cell surface. Thus, association of ligand with particles can result in M cell–specific adherence, but only within a restricted size range. As all intestinal epithelial cells express the cholera toxin receptor ganglioside GM1, this size selectivity must be attributed to differences in plasma membrane accessibility of GM1 on enterocytes and M cells.

The Role of the Glycocalyx. The term “glycocalyx” generally refers to the various glycoconjugates that form coats on cell plasma membranes. The apical membranes of intestinal epithelial cells are particularly rich in highly glycosylated proteins, some of which are integral membrane enzymes that function in terminal digestion (17). The unique 500-nm-thick apical coat that blankets the microvillous tips of absorptive enterocytes lining the intestine, the FBBG (13, 19) facilitates local digestion of nutrients by entrapping pancreatic enzymes (48) but may also serve to protect apical cell surfaces against microbial pathogens and foreign materials in the intestinal lumen. Although the ultrastructural features of the FBBG were originally described 30 years ago (17) its major component was only recently shown to be a transmembrane mucin of 400 kD with abundant heterogeneous oligosaccharide chains (18). The ability of CTB and CTB-FITC to bind to GM1 on enterocytes, and the inability of CTB-colloidal gold to penetrate the FBBG, suggest that the functional pore size of the enterocyte FBBG is ≥7.4 nm but <28.8 nm. Thus it is not surprising that the
FBBG was sufficient to completely prevent binding of CTB-coated, 1-μm microparticles to enterocytes on villi and in the FAE. The subpopulation of M cells that displayed an enterocyte-like FBBG would presumably show a similar inaccessibility to small particles. Most M cells lack the FBBG by ultrastructural criteria, and the relatively thin M cell glycocalyx has not generally been considered a significant barrier to ligand-coated particles or microorganisms in vivo. However, M cells do have abundant, terminally glycosylated apical surface glycoconjugates and by EM, M cells display a continuous glycocalyx measuring at least 20–30 nm in height that apparently prevented access of CTB-coated 1-μm particles. This was consistent with the observation that the 10–20-nm-thick filamentous coat on microvillar tips of well-differentiated Caco-2 cells also provided a barrier to these particles.

**Implications for Microbial Attachment.** Our results demonstrate that the presence of an appropriate receptor on intestinal apical membranes is necessary but not sufficient for microbial attachment; the receptor must also be accessible to the ligands or adhesins on the surfaces of viruses or bacteria. For example, galactosylceramide has been proposed as the epithelial cell component that could serve as receptor for binding of HIV to human rectal epithelial cells (49), but this seems an unlikely mechanism in light of the fact that the human rectal enteroctye glycocalyx is comparable in thickness to that of enterocytes of other regions and species (50). Indeed, we previously observed that HIV failed to penetrate the FBBG of rabbit or mouse enterocytes on villi or the FAE (21). On the other hand, HIV did adhere to rabbit and mouse M cells and was transcytosed. It will be important to determine in human rectum whether the relevant glycolipid is present on M cells and if HIV enters via this route. If so, receptor accessibility could account for a cell-selective uptake mechanism that would deliver the virus directly to its target cells in mucosal lymphoid tissues.

Many enteric bacteria exploit carbohydrate epitopes as receptors for initial adherence (47), and such binding may be independent of microbial size when these sites are located in the periphery of the glycocalyx. However, the thick FBBG of enterocytes may mask potential receptors, especially those located near the membrane bilayer, and this could explain in part the predilection of several enteric pathogens for M cells (4). Bacterial pathogens that use glycolipids as receptors would be unlikely to bind to either M cells or enterocytes unless they can enzymatically cleave components of the glycocalyx or use microbial surface extensions to penetrate this barrier.

**Implications for Mucosal Vaccine Design.** Microparticulate antigen carriers 1 μm or larger are considered promising candidates as M cell-directed mucosal vaccines (7, 9). Uptake of particles by M cells is not entirely dependent on specific ligand binding, since adherence to M cells by any mechanism leads to endocytosis and transport (10, 20). In the present study, avidin-coated, biocytin-quenched (Bc-P) and uncoated latex (PLP) microparticles were both taken up better by the FAE than CTB-P. The highly cationic avidin molecules on the outer surface of Bc-P presumably interacted with the anionic surfaces of intestinal epithelial cells, as has been observed with other polycationic probes (20). The polystyrene of PLP is extremely hydrophobic and binds avidly to intestinal cell surfaces (10), mucus, fibroblasts, or even BSA-coated glass cover slips (Frey, A. unpublished observations). Microparticulate oral vaccines that depend on such nonspecific interactions for transport into mucosal inductive sites can be effective in evoking mucosal immune responses (7) but they require very large oral doses, and it seems likely that vaccine efficiency could be enhanced by addition of an M cell–specific ligand. We have now shown that CTB, and probably any other ligand that binds to membrane glycolipids, cannot mediate targeting of vaccines as large as 1 μm to M cells. Such large particles will require ligands directed to unique peripheral components of the M cell glycocalyx; identification of such components is currently underway. Vaccine targeting to M cells could be accomplished using a ligand such as CTB, however, if the size of the final vaccine conjugate is restricted to the nanometer range that allows selective access to M cell membranes.

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References

1. Neutra, M.R., E. Pringault, and J.P. Kraehenbuhl. 1996. Antigent sampling across epithelial barriers and induction of mucosal immune responses. Annu. Rev. Immunol. 14:273–300.

2. Neutra, M.R., and J.P. Kraehenbuhl. 1992. Transepithelial transport and mucosal defense: the role of M cells. Trends Cell Biol. 2:134–138.

3. Kraehenbuhl, J.P., and M.R. Neutra. 1992. Transepithelial transport and mucosal defense: secretion of IgA. Trends Cell Biol. 2:170–174.

4. Neutra, M.R., P.J. Giannasca, K.T. Giannasca, and J.-P. Kraehenbuhl. 1995. M cells and microbial pathogens. In Infections of the Gastrointestinal Tract. M.J. Blaser, P.D. Smith, J.I. Ravdin, H.B. Greenberg, and R.L. Guerrant, editors. Raven Press Ltd., New York. 163–178.

5. Neutra, M.R., and J.P. Kraehenbuhl. 1996. Antigen uptake by M cells for effective mucosal vaccines. In Mucosal Vaccines. H. Kiyono, M. Kagnoff, and J. McGhee, editors. Academic Press, New York NY. In press.

6. Mekalanos, J.J. 1992. Bacterial mucosal vaccines. In Genetically Engineered Vaccines. Advances in Experimental Medicine and Biology. Vol. 237. J.E. Ciardi, J.R. McGhee, and J.M. Kieth, editors. Plenum Press, New York. 43–50.

7. Eldridge, J.H., J.K. Staas, J.A. Meulbroek, J.R. McGhee, J.I. Ravdin, S.S. Davis, and I. Sjoholm. 1989. Microparticles as potentially orally active immunological adjuvants. Vaccine. 7:421–424.

8. Ermak, T.H., E.P. Dougherty, H.R. Bhagat, Z. Kabok, and J. Pappo. 1995. Uptake and transport of copolymer biodegradable microspheres by rabbit Peyer’s patch M cells. Cell Tissue Res. 279:433–436.

9. Pappo, J.H., C.H. Allan, and J.S. Trier. 1984. Structure, distribution and origin of M cells in Peyer’s patches of mouse ileum. Gastroenterology. 86:789–801.

10. Crichtley, D.R., C.H. Streuli, S. Kellie, S. Ansell, and B. Patel. 1982. Characterization of the cholera toxin receptor on Balb/c 3T3 cells as a ganglioside similar to, or identical with, ganglioside GM1. Biochim. J. 204:209–219.

11. Griffiths, S.L., R.A. Finkelstein, and D.R. Crichtley. 1986. Characterization of the receptor for cholera toxin and Escherichia coli heat-labile toxin in rabbit intestinal brush borders. Biochem. J. 238:313–322.

12. Orlandi, P.A., D.R. Crichtley, and P.H. Fishman. 1994. The heat-labile enterotoxin of Escherichia coli binds to polyacatosaminoglycan–containing receptors in Caco-2 human intestinal epithelial cells. Biochemistry. 33:12886–12895.

13. McDaniel, R.V., K. Sharp, D. Brooks, A.C. McLaughlin, A.P. Winiski, D. Cafiso, and S. McLaughlin. 1986. Electrokinetic and electrostatic properties of bilayers containing gangliosides GM1, GDS or GT1. Comparison with a nonlinear theory. Biophys. J. 49:741–752.

14. Szema, T.K., S.E. Prong, K.H. Kalk, E.S. Wartna, B.A.M. van Zanten, B. Witholt, and W.G.J. Hol. 1991. Crystal structure of a cholera toxin-related heat-labile enterotoxin from Escherichia coli, Nature (Lond.). 351:371–377.

15. Quaroni, A., and K.J. Isseibacher. 1985. Study of intestinal differentiation with monoclonal antibodies to intestinal cell surface components. Dev. Biol. 111:267–279.

16. Slot, J.W., and H.J. Geuze. 1984. A new method of preparing gold probes for multiple-labeling cytochemistry. Eur. J. Cell Biol. 38:87–93.

17. Mekalanos, J.J. 1998. Production and purification of cholera toxin. Methods Enzymol. 165:169–175.

18. Pugliese, L., A. Coda, M. Malcovati, and M. Bolognesi. 1993. Three-dimensional structure of the tetragonal crystal form of egg-white avidin in its functional complex with biotin at 2.7 Å resolution. J. Mol. Biol. 231:698–710.

19. Mosser, G., V. Mallouh, and A. Brisson. 1992. A 9 Å two-dimensional projected structure of cholera toxin B-subunit-GM1 complexes determined by electron crystallography. J. Mol. Biol. 226:23–28.

20. Mosser, G., V. Mallouh, and A. Brisson. 1992. A 9 Å two-dimensional projected structure of cholera toxin B-subunit-GM1 complexes determined by electron crystallography. J. Mol. Biol. 226:23–28.
35. Heney, G., and G.A. Orr. 1981. The purification of avidin and its derivatives on 2-iminobiotin-6-aminohexyl-sepharose 4B. Anal. Biochem. 114:92–96.
36. Weltzin, R.L., P. Lucia-Jandris, P. Michetti, B.N. Fields, J.P. Kraehenbuhl, and M.R. Neutra. 1989. Binding and transepithelial transport of immunoglobulins by intestinal M cells: demonstration using monoclonal IgA antibodies against enteric viral proteins. J. Cell Biol. 108:1673–1685.
37. Peterson, M.D., and M.S. Mooseker. 1992. Characterization of the enterocyte-like brush border cytoskeleton of the C2age clones of the human intestinal cell line, Caco-2. J. Cell Sci. 102:581–600.
38. Giannasca, K.T., P.J. Giannasca, and M.R. Neutra. 1996. Adherence of Salmonella typhimurium to Caco-2 cells: identification of a glycoconjugate receptor. Infect. Immun. 64:135–145.
39. Gorvel, J.P., A. Ferrero, L. Chambraud, A. Rigal, J. Bonicel, and S. Maroux. 1991. Expression of sucrase-isomaltase and dipeptidylpeptidase IV in human small intestine and colon. Gastroenterology. 101:618–625.
40. Vachon, P.H., and J.-F. Beaulieu. 1992. Transient mosaic patterns of morphological and functional differentiation in the Caco-2 cell line. Gastroenterology. 103:414–423.
41. Debray, H., D. Decout, G. Strecker, G. Spik, and J. Monfreuil. 1981. Specificity of twelve lectins towards oligosaccharides and glycoproteins related to N-glycosylproteins. Eur. J. Biochem. 117:41–55.
42. Nachbar, M.S., J.D. Oppenheim, and J.O. Thomas. 1980. Lectins in the U.S. diet. Isolation and characterization of a lectin from the tomato (Lycopersicon esculentum). J. Biol. Chem. 255:2056–2061.
43. Tollefsen, S.E., and R. Kornfeld. 1983. The B4 lectin from Vicia villosa seeds interacts with N-acetylgalactosamine residues alpha-linked to serine or threonine residues in cell surface glycoproteins. J. Biol. Chem. 258:5172–5176.
44. Susz, J.P., and G. Dawson. 1979. The affinity of the fucose-binding lectin from Lotus tetragonolobus for glycopeptides and oligosaccharides accumulating in fucosidosis. J. Neurochem. 32:1009–1013.
45. Petryniak, J., and J.J. Goldstein. 1987. Euphorbus europaeus lectin. Methods Enzymol. 138:552–561.
46. Nibert, M.L., D.B. Furlong, and B.N. Fields. 1991. Mechanisms of viral pathogenesis. J. Clin. Invest. 88:727–734.
47. Hultgren, S.J., S. Abraham, M. Caparon, P. Falk, J.W. St. Geme III, and S. Normark. 1993. Pilus and nonpilus adhesins: assembly and function in cell recognition. Cell. 73:887–901.
48. Ugolev, A.M., and P. Delaey. 1973. Membrane digestion. A concept of enzymatic hydrolysis on cell membranes. Biochim. Biophys. Acta. 300:105–128.
49. Fantini, J., D.G. Cook, N. Nathanson, S.L. Spitalnik, and F. Gonzalez-Scarano. 1993. Infection of colonic epithelial cell lines by type 1 human immunodeficiency virus (HIV-1) is associated with cell surface expression of galactosyl ceramide, a potential alternative gp120 receptor. Proc. Natl. Acad. Sci. USA. 90:2700–2704.
50. Neutra, M. 1979. Linear arrays of intramembrane particles in microvilli in primate large intestine. Anat. Rec. 193:367–381.
51. Kaplan, L.J., and J.F. Foster. 1971. Isoelectric focusing behavior of bovine plasma albumin, mercaptalbumin, and β-lactoglobulins A and B. Biochemistry. 10:630–636.
52. Green, N.M. 1975. Avidin. Adv. Protein Chem. 29:85–133.