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Monoclonal antibody-directed targeting of fluorescent polystyrene microspheres to Peyer's patch M cells

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SUMMARY

The ability to deliver particulates to Peyer's patch M cells for uptake into gut-associated lymphoid tissue was examined by administering simultaneously fluorescent green and red polystyrene microspheres into NZW rabbit intestinal loops containing Peyer's patches. Whereas green and red microspheres were taken up by M cells at equivalent concentrations (120 ± 17 versus 125 ± 18/mm length of dome), particles conjugated to the anti-M-cell monoclonal antibody 5B11 (IgM, κ) were internalized by M cells 3–3·5 times more efficiently than conjugates displaying IgM of unrelated specificity (TEPC 183) or native particles of the reciprocal colour inoculated into the same loop at a comparable load. The microspheres formed a concentration gradient from lumen to subepithelial dome, and localized on M-cell apical membranes, M-cell pockets, and subepithelial domes. The transport rate across M cells of 5B11 or TEPC 183 conjugates was similar to that of untreated microspheres. These observations show that intestinal uptake into Peyer's patches can be up-regulated by targeting M-cell luminal membrane structures.

INTRODUCTION

The epithelium overlying lymphoid follicles in Peyer's patches contains specialized M cells which recognize and take up intraluminal protein antigens (Ag)[1,2] and micro-organisms.[3–5] Binding of Ag to M-cell apical membranes is followed by rapid endocytosis, shuttling across the thin apical cytoplasm, and exocytosis at the basolateral membrane.[6] The M-cell basolateral surface is infiltrated by T cells, B cells and macrophages, forming a pocket subdomain.[7,8] M-cell luminal membranes are highly anionic and display various lectin-binding specificities.[9,10] Although it has been found that lectin–Ag conjugates which bind to M-cell apical membranes are more efficiently transported than non-adherent conjugates,[11] and that particle hydrophobicity and size[12–14] determine the magnitude of localization in Peyer's patches, little is currently known about the surface molecules involved in M-cell recognition of Ag. In this study, we examined M-cell-mediated binding and transport of green and red fluorescent polystyrene microspheres after targeting to M-cell apical membranes. We show that particle conjugates displaying the anti-M cell monoclonal antibody (mAb) 5B11[15] adhered to, and were translocated by, M cells more efficiently than particles displaying Ig molecules of unrelated specificity, or than particles displaying the native surface chemistry. These findings suggest a pathway for specifically targeting molecules to M cells for delivery to mucosal lymphoid tissue.

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MATERIALS AND METHODS

Animals

Five female New Zealand White (NZW) rabbits, from 2·0 to 2·2 kg in weight, were purchased from Rabbitek (Modesto, CA).

Antibodies

The mAb 5B11 (IgM, κ) with specificity for rabbit M cells[15] was prepared from ascites by ammonium sulphate precipitation and purified by HPLC using a Spherogel 3000SW column (Beckmam Instruments Inc., San Ramon, CA). The purification was confirmed by SDS–PAGE,[16] and the M-cell binding specificity analysed by immunohistochemistry as described previously.[15] Purified TEPC 183 myeloma (IgM, κ) of unrelated specificity was obtained from Sigma (St Louis, MO).

Generation of microsphere conjugates

Fluorescent green or red microspheres (0·94 μm or 1·0 μm, respectively; Polysciences Inc., Warrington, PA) were diluted to 5×10^8/ml, and the IgM molecules coupled by adsorption. Briefly, the particles were incubated for 16–18 hr at 4° with 250 μg/ml of mAb 5B11 or TEPC 183 in 0·05 M Tris HCl buffer, pH 9·5, or incubated with Tris buffer alone. The particles were washed extensively with 0·01 M phosphate-buffered saline (PBS). The presence of adsorbed IgM on the particle surface was demonstrated by ELISA using biotinylated rat anti-mouse IgM mAb (clone Bet-2; American Type Culture Collection, Rockville, MD). The concentration of particle-bound IgM was estimated by linear regression analysis ($R^2 = 0{\cdot}963$). Results are

expressed as mean ± standard error of the mean of triplicate determinations in three separate experiments.

Preparation of intestinal loops

Fasted animals were anaesthetized with Nembutal (40 mg/kg), and a laparotomy was performed. A final volume of 250 μl containing paired combinations of equal numbers of green and red microspheres in PBS was injected into ligated intestinal segments containing Peyer's patches. After 30 min, the Peyer's patches were excised, and the non-adherent or loosely adherent particles removed by rinsing with RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES buffer (M.A. Bioproducts, Walkersville, MD).

Immunofluorescence and morphometric analysis

Two tissue blocks from each Peyer's patch, approximately 1 cm in length, were mounted in OCT compound (Miles Scientific, Naperville, IL) and frozen in liquid nitrogen-cooled Freon 22. Fifty cross sections per block were cut (7 μm) with a Hacker cryostat. Three separate Peyer's patch regions were analysed for the presence or absence of particles: (i) the follicle epithelial surface, comprised of the outermost 10 μm of follicle epithelium; (ii) the remaining width of the follicle-associated epithelium (FAE), spanning from the surface to the basement membrane; and (iii) the subepithelial dome.[13] The particle count within each compartment was standardized per unit length of epithelium. Length (1·3 ± 0·1 mm) and width (48 ± 1·6 μm) measurements of the follicle epithelium overlying domes were performed with a Zeiss Videoplan III image analyser. Cryosections were viewed in a Zeiss fluorescence microscope with filters for selective FITC or TRITC excitation. Transport rates were compared using the Students' *t*-test, and significant differences considered at $P < 0.05$.

Specificity of M-cell microsphere associations

M cells were immunolabelled in serial cryosections of Peyer's patches challenged *in vivo* with microspheres using culture supernatants of clone 5D9, as described previously.[13] Control cryosections were incubated with biotinylated secondary antibodies, or with isotype-matched mAb of unrelated specificity.

Table 1. mAb-dependent localization of green or red fluorescent polystyrene microspheres in Peyer's patches

Paired combination	Particle count*	Ratio green/red
5B11-green TEPC 183-red	468 ± 56 160 ± 22	3·01 ± 0·28
TEPC 183-green 5B11-red	160 ± 38 364 ± 77	0·35 ± 0·05
5B11-green —red	363 ± 36 105 ± 14	3·46 ± 0·34
—green —red	120 ± 17 125 ± 18	0·99 ± 0·11

* Mean number of particles ± standard error of the mean of 15 lymphoepithelial domes from $n = 3$ rabbits.

Table 2. Comparison of distribution of targeted and non-targeted microspheres

	Percentage of total		
Paired combination	FAE* surface	FAE	Subepithelial dome
5B11-green TEPC 183-red	82 ± 4† 79 ± 4†	16 ± 3 19 ± 3	2 ± 1 2 ± 1
TEPC 183-green 5B11-red	85 ± 2‡ 82 ± 3†	13 ± 2 15 ± 3	3 ± 1 3 ± 1
—green —red	67 ± 7 62 ± 9	30 ± 6 36 ± 7	3 ± 1 2 ± 1

* Follicle-associated epithelium overlying Peyer's patch domes.

†$P > 0.05$ relative to -green or -red.

‡$P < 0.05$ relative to -green or -red.

RESULTS

Localization of microspheres in Peyer's patches

Green and red fluorescent particles injected simultaneously into intestinal loops containing Peyer's patches localized specifically in the follicle epithelium and dome compartments, but not in the intervening villus epithelium and lamina propria. Equal numbers of native microspheres (120 ± 17 green versus 125 ± 18 red/mm length) were found co-distributed in Peyer's patches (Table 1). The microspheres formed a concentration gradient spanning from the luminal surface of the follicle epithelium (62–67% of the total particles) to the subepithelial dome (2–3%) (Table 2).

Effect of targeting conjugates on M-cell uptake efficiency

The effect of targeting M cells with 5B11 mAb conjugates on luminal uptake and translocation across the follicle epithelium was examined. Paired combinations of particle conjugates displaying mAb 5B11 or TEPC 183 IgM were administered *in vivo* into intestinal loops containing Peyer's patches. While green or red microspheres bound equivalent concentrations, as determined by ELISA, of either 5B11 mAb or TEPC 183 IgM (45 ± 6 versus 50 ± 3 ng/5 × 10^6 beads), 5B11 conjugates were found in Peyer's patch follicle epithelium and domes three times more frequently than TEPC 183 conjugates of the reciprocal colour administered into the same intestinal loop at an equivalent load (Table 1). Furthermore, 3·5-fold greater numbers of 5B11 conjugates were found to accumulate in Peyer's patch tissue in comparison to unconjugated particles (Table 1). Both 5B11 and TEPC 183 conjugates co-localized in M cells as aggregates alternating at the surface and apical region of the follicle epithelium (Fig. 1). This pattern corresponded to the position of M cells immunostained with mAb 5D9 (not shown). Although uptake of 5B11 conjugates by M cells was augmented in comparison to TEPC 183 conjugates, analysis of the particle

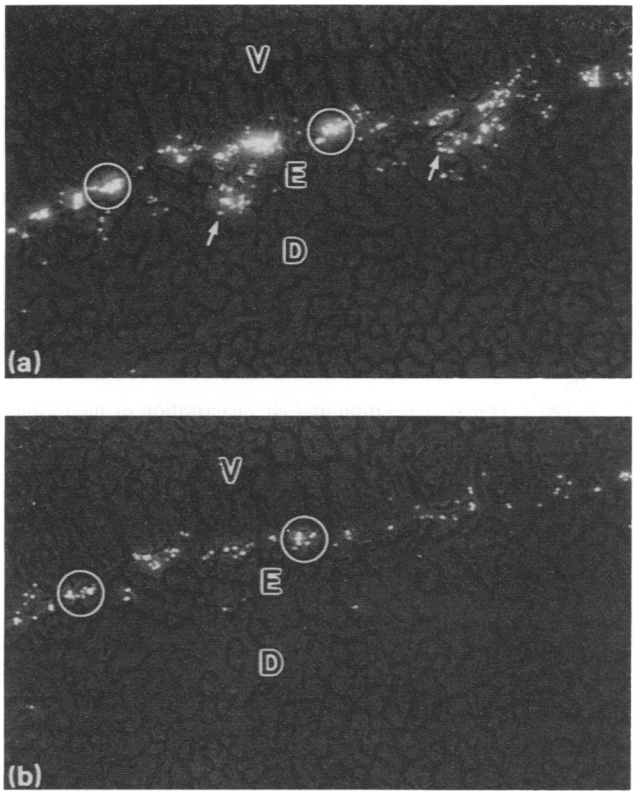

Figure 1. Paired fluorescence micrographs of a Peyer's patch cross-section showing localization and relative magnitude of uptake of particle conjugates injected into the same loop. (a) 5B11–green conjugates. (b) TEPC 183–red conjugates. × 200. The villus (V) epithelium is closely apposed to the follicle epithelium (E) overlying dome (D). Aggregates of microspheres are seen at the surface of the follicle epithelium (E) overlying Peyer's patch dome (D). Clusters of particles within the follicle epithelium itself concentrate in M-cell pockets (arrows). Green and red microspheres co-localize in same regions of follicle epithelium (circles).

distribution did not reveal consistent significant differences in localization after transport. However, a greater proportion of either 5B11 or TEPC IgM-conjugated particles (79–85%) was found at the M-cell apical surface in comparison to unconjugated particles (62–67%) (Table 2).

DISCUSSION

Epithelial M cells populating lymphoid follicle surfaces in Peyer's patches function as specific sites for the localization and transport of intestinal luminal Ag.[1-5] Although polycation and lectin binding studies have partially mapped the binding specificities of M cells, the recognition structures present in M-cell apical membranes have remained poorly defined. In this study, the effect of targeting M cells on uptake into Peyer's patches was examined using a mAb with M-cell specificity conjugated to indicator fluorescent microspheres. Whereas microsphere conjugates displaying TEPC 183 IgM were taken up into Peyer's patches at roughly the same level as native particles, targeted 5B11 conjugates of the reciprocal colour were captured by M cells with much greater efficiency. Because both green and red microspheres were administered simultaneously at equivalent concentrations, these findings indicated that the 5B11 conjugates targeted M-cell apical membranes, and were subsequently translocated across the follicle epithelium into intestinal lymphoid tissue. While binding of *E. coli* strain RDEC-1 to M-cell luminal membranes does not appear to trigger phagocytosis,[17] lectin–Ag conjugates adherent to M cells have been found to be taken up and transported more efficiently than non-adherent conjugates.[11] The finding that bridging the M-cell particle interface with mAb 5B11 resulted in much greater particle deposition in Peyer's patches is consistent with the notion that phagocytosis can be augmented by clustering ligands on the cell surface.[18] That augmentation of M-cell-dependent uptake was specifically mediated by the mAb, and was not a function of differences in particle-bound IgM concentrations, is suggested by observations showing similar concentrations of adsorbed 5B11 mAb or TEPC 183 IgM onto a given load of microspheres.

Previous studies have shown that polystyrene microspheres taken up by M cells are mobilized vectorially across the follicle epithelium, and discharged from M-cell pocket regions into Peyer's patch domes in a time-dependent fashion.[13] The transepithelial traffic of particulates from M cells into Peyer's patches may be aided by fenestrations in the basal lamina supporting the follicle epithelium.[19,20] The observation of conjugate localization in M-cell pockets and Peyer's patch subepithelial domes after 30 min of exposure *in vivo* is consistent with the rapid rate of M-cell-mediated particulate transport.[3,21] Because targeting with 5B11 conjugates was found to increase the number of particles bound to, and transported by, M cells in comparison to TEPC 183 conjugates or unconjugated microspheres, the findings reported here raise the possibility of optimizing strategies for oral delivery. The coupling of Ag or biologically active molecules to probes specific for M cells may similarly result in greater deposition in mucosal lymphoid tissue, and thus generate a more efficacious biological response, i.e. IgA antibody.

Recently, the finding that IgA and IgG antibodies adhere to M-cell apical membranes[22] has suggested the existence of an Ig domain-recognition structure on M cells. In the present study, the binding of TEPC 183 conjugates to M-cell apical surfaces was found to be similar or marginally greater than the binding of microspheres displaying the native surface chemistry, and suggested the absence of an IgM-binding domain on M-cell apical surfaces. Further, the number of TEPC 183 conjugates in Peyer's patches was comparable to unconjugated microspheres. Since M cells lack the polymeric Ig receptor and Fc receptors,[11,23] it is likely that binding of 5B11 conjugates was mediated by the idiotypic specificity of 5B11 mAb for the relevant M-cell-surface structure. This interpretation is also supported by the observation that the anti-M-cell mAb 4G2 (IgM, κ), which recognizes the pocket membrane, does not bind to M-cell luminal membranes *in vivo* (J. Pappo, unpublished data). The Ag recognized by 5B11 mAb is expressed on both apical and pocket membranes of M cells, and apical membranes of adjacent enterocytes.[15] The observation that clusters of conjugates associated with M cells but not enterocytes suggested that the initial binding of IgM conjugates to follicle epithelium may be labile, and must be followed by generation of M-cell apical membrane projections and particle engulfment.

M cells lack a well-developed lysosomal apparatus,[24] and shuttle luminal Ag into subepithelial domes without apparent structural degradation.[3,5] While 5B11 mAb could still be detected immunohistologically in association with microspheres

within M cells and subepithelial domes (J. Pappo, unpublished data), it is not known whether M cells process and present Ag directly and, specifically, whether the 5B11 mAb was processed en route to subepithelial domes. Because a mucosal IgA B-cell response can be generated to Ag which are phagocytosed by M cells,[25] the results presented here raise the possibility of specifically targeting molecules to M cells. Whether an intestinal IgA antibody response is generated against the 5B11 mAb once translocated into mucosal lymphoid tissue is not currently known.

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