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Visual Evaluation of Binding to Mucosal Cells of a Medical Device Against the Common Cold

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The objective of this study was to investigate the possibility of visualizing the ability of hydroxypropylmethylcellulose (HPMC) and a nasal spray (First Defense), in which the bioadhesive is HPMC, to bind to human mucosal cells using inorganic (black carbon particles and Congo red dye) and organic markers (*Escherichia coli*). A significant reduction in the bacterial adhesiveness has been observed. Our findings indicate the possibility of counteracting the lock-and-key mechanism of microorganism adhesion using the bioadhesive properties of polymers, such as HPMC, in First Defense to prevent a possible contact between adhesins and complementary receptors.

Keywords hydroxypropylmethylcellulose; first defense; bioadhesives; carbon black; congo red; *escherichia coli*

INTRODUCTION

Increasing pharmacological and pharmaceutical attention is being given to natural or synthetic polymeric materials that have the ability to adhere, generally in thin films, to nasal, buccal, vaginal, and gastrointestinal mucosal tissue to control drug absorption or prolong local drug delivery, both of which can be advantageous in the treatment of local conditions.

Adhesion is defined as the state in which two surfaces are held together by interfacial forces (ASTM, 1984; Park & Park, 1990), with the term bioadhesion being used if one or both of the adherents are biological. A bioadhesive can therefore be defined as a biocompatible substance that is capable of interacting with biological materials and being retained on them, or holding them together, for an extended period of time (Park, Cooper, & Robinson, 1987; Park & Park, 1990).

The interactions between polymeric materials and mucosal tissue surfaces are complex and have not yet been fully elucidated. However, the most widely accepted theories include the molecular adsorption theory, which states that adhesion is due to combined result of the secondary forces such as van der Waals dispersion forces, hydrogen binding, and related forces (Good, 1977; Tabor, 1977); the wetting theory, which states that the intimate contact between the adherents depends on their wetting equilibrium and interface tensions (Wang & Bazos, 1983); the electronic theory, based on the fact that electron transport across the interface induces the formation of a double layer of electric charge at the bioadhesive interface (Derjaguin, Toporov, Muller, & Aleinikova, 1977); the diffusion/interpenetration theory, which states that, because of the concentration gradient, bioadhesive polymer chains penetrate at rates that depend on the diffusion coefficient of a macromolecule through a cross-linked network and the chemical potential gradient (Mikos & Peppas, 1990; Voyutskii, 1963); and fracture theory, which relates the difficulty in separating two surfaces (after adhesion) to the adhesive bond strength, which is equivalent to tensile fracture strength (Mikos & Peppas, 1990).

Given the variety of bioadhesion phenomena and bioadhesives, the final result is probably attributable to the combinations of the different mechanisms that contribute to the formation of adequately strong interactions, to different extents, between bioadhesives and biological surfaces.

The ability of bioadhesives to bind to and be retained by mucosal surfaces also means that they prevent the mucosal surfaces from coming into direct contact with particulate matter in the environment. A protective effect is obtained if this covering ability is used to prevent their contact with airborne microorganisms, such as viruses, bacteria, fungi, or pollens present in the air and flowing through the nose during inspiration.

Human rhinoviruses, a genus of the picornavirus family, are the major cause of common colds in humans (Xing, Casasnovas, & Cheng, 2003). They gain entrance to nasal epithelial cells by binding to a specific cell receptor that has been identified as a membrane protein called intercellular adhesion molecule-1, a member of the immunoglobulin superfamily (Casasnovas & Springer, 1995; Winther et al., 2002). A similar situation underlies bacterial and fungal infections. The adhesion of these microorganisms to human mucosal cells (and the consequent colonization) is based on the binding of specialized molecules

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(adhesins) on the microorganism surface to complementary molecules (receptors) on the mucosal epithelial cells (Braga, 2000). It is this "lock-and-key" mechanism that provides the rationale underlying the therapeutic approach to preventing the infectious process by covering the adhesion molecules and receptors with bioadhesives, and thus blocking their access and binding to each other.

This concept has recently been applied in the formulation of a nasal spray called First Defense, a medical device to be used against common cold. The bioadhesive in First Defense is hydroxypropylmethylcellulose (HPMC), which when sprayed on the nasal mucosa has the dual action of covering the receptor surfaces entrapping the inhaled rhinoviruses, thus preventing the adhesins from reaching their complementary receptors. Furthermore, the HPMC in First Defense is acidified to a pH of 3.5–4.0 with pyroglutamic and succinic acids, thus creating a hostile environment for rhinoviruses, which lose their vitality at acidic pH (Balasingam et al., 2005; Hughes, Hamparian, & Cramblett, 1973). The presence of active nasal mucociliary clearance is helpful in removing all materials toward the pharynx and stomach, and the usefulness of this new preventive approach has been confirmed by the positive results of clinical trials (Brown & Turner, 2003; Hull et al., 2005; Rennie, Wright, Williams, & England, 2005).

The aim of this study was to investigate the possibility of visualizing the ability of HPMC and First Defense to bind to mucosal surfaces using human buccal cells as a model for mucosal adhesion (Gibbons & Daukers, 1983; Nantwi, Cook, Rogers, & Smart, 1997; Patel, Smith, Grist, Barnett, & Smart, 1999), and both inorganic and organic visual markers to facilitate visual detection, instead of the commonly used methods that are more devoted to measure the tensile adhesive strength (peak detachment force). The first inorganic marker chosen was a suspension of colloidal carbon black in water, which did not stain human buccal cells but deposited on HPMC and First Defense, thus revealing their adhesion to the cells. The second inorganic marker was Congo red, a dye having a great affinity for cellulose (Mirza, Iqbal, & Huma, 1996). *Escherichia coli* cells were used as the organic marker because their adhesion or nonadhesion to mucosal cells can be clearly observed under a microscope.

MATERIALS AND METHODS

Chemicals

HPMC (E4M, 4,000 mPa s; Fluka Chemie, Switzerland) was dissolved in distilled water by gentle heating and stirring to obtain a 2% (wt/vol) solution, which was stored at 30°C for 24 h to allow the polymer chains to hydrate fully before use. The effects of HPMC were determined at a final concentration of 1% (the same as that used in First Defense), which was obtained by mixing HPMC with vehicle (distilled water or phosphate-buffered saline [PBS]) or the medium used in the tests.

First Defense (Procter & Gamble, Vicks Division, Rome, Italy) was purchased in a chemist's shop and used undiluted. It is a nasal spray, classified as a medical device, and consists of HPMC (1%), succinic acid, disodium succinate, pyroglutamic acid, phenylethyl alcohol, zinc ethylenediaminetetraacetic acid (EDTA), zinc acetate dihydrate, Tween 80, sensates, and sodium saccharin. As First Defense has a pH of 3.5–4.0, HPMC was also studied after adjusting its pH to 3.5–4.0 using pyroglutamic and succinic acids solutions, the same as those used in First Defense. The effect of HPMC was also investigated at pH 7.

Commercial Indian ink (Pelikan, Milan, Italy) is a suspension of colloidal carbon black in water. Congo red (Sigma, Milan, Italy) is a dye that has a great affinity for cellulose and can thus dye HPMC alone or in solution in the First Defense. Congo red was used at a concentration of 0.8%.

Collection of Human Buccal Cells

Healthy nonsmoking volunteers took part in the study. They were required not to eat or drink for at least 60 min before the mucosa of each cheek was gently scraped with a sterile plastic spatula, which was subsequently twirled in 2 mL of PBS (0.02 M phosphate and 0.15 M NaCl, pH 7.3) to dislodge the buccal cells. The cell suspension obtained by pooling the cells from three or four participants was washed three times to free it from debris and nonadherent bacteria by centrifugation (260 *g*, 10 min, 21°C) at low-speed. PBS was added to the washed epithelial suspension to reach a concentration of 3×10^5 cells/mL, as determined by direct microscopic counts in a Bürker chamber (Passoni, Milan, Italy).

Inspection of Binding Properties Using Inorganic Markers

In the control test, 50 μL of Indian ink was incubated, under rotation at 8 rpm, for 15 min at 30°C with 170 μL of human buccal cells and 500 μL of vehicle (distilled water, pH 7 or 3.5–4.0). At the end of the incubation period, 3 mL of distilled water was added to obtain an appropriate volume for filtration. The suspension of buccal cells in Indian ink was filtered on a cellulose nitrate membrane filter (pores 8 μm, diameter 25 mm; Schleicher & Schuell, Dassel, Germany), which was pressed on a microscope slide; retention of the ink by the buccal cells was observed using Normaski interference contrast microscopy. To investigate whether the binding of HPMC and First Defense to the surface of buccal cells can be visualized by variations in the degree of Indian ink retention, the same procedure was followed by first incubating the cells with 1% HPMC solution at pH 7 or 3.5–4.0 (500 μL) or First Defense (500 μL).

To visualize the presence of transparent HPMC directly, Congo red was used. A 170 μL suspension of buccal cells was incubated, under rotation at 8 rpm, for 15 min at 30°C, with 500 μL of vehicle (distilled water, pH 7 or 3.5–4.0) or HPMC (pH 7 or 3.5–4.0) or First Defense. The suspension was centrifuged at 260 *g* for 10 min, the pellet was resuspended in 1 mL distilled water, and then 50 μL of Congo red was added and mixed. One drop was placed on a slide and observed using Normaski interference contrast microscopy.

Inspection of Binding Properties Using Bacteria as Organic Markers

We used one strain of *E. coli* ATCC 25922 and two strains of *E. coli* isolated from human urinary infections to test the binding of HPMC and First Defense to buccal cells. In this case, the bacteria were used as organic markers to explore the interference of HPMC and First Defense with the bacterial lock-and-key (adhesion-receptor) mechanism. Suspensions of each organism were prepared from overnight cultures in tryptic soy broth (Sigma) under static conditions at 37°C. The organisms were harvested, washed three times in PBS, and adjusted to 3×10^8 organisms/mL, as determined by direct microscopic counts in a Petroff-Hausser chamber (Thomas Scientific, Swedesboro, NJ, USA). The buccal cells were incubated under rotation at 8 rpm at 37°C for 1.5 h with 1.5 mL of vehicle (PBS at pH 7 or 3.5–4.0), HPMC (pH 3.5–4.0), or First Defense (pH 3.5–4.0).

The ability of the bacteria to adhere to the buccal cells was investigated by resuspending the pellet in 1 mL of PBS (3×10^5 cells/mL) and mixing it with 1 mL of a bacteria suspension (3×10^8 bacteria/mL) in polystyrene tubes, which were rotated end over end at 8 rpm for 1 h at 37°C. The buccal cells were separated from the nonadherent bacteria by centrifugation; the final cell pellet was resuspended in 2 mL of PBS, filtered on a cellulose nitrate membrane filter (Schleicher & Schuell) with pores of 8 μm in size with diameter of 25 mm. The filter was pressed on a microscope slide stained with Gram stain and observed using Normaski interference contrast microscopy.

As differences in the bacterial strains and the cell surface characteristics (different participants and different oral exposure conditions) lead to variations in the number of bacteria attached to individual buccal cells in any given sample, bacterial adhesion was determined by counting the total number of bacteria adhering to 50 randomly chosen cells in each sample (Gibbons & Van Houte, 1971). Buccal cell suspensions incubated with PBS alone were always included to establish the number of bacteria already attached (natural adhesion) at the time of cell collection. The results are given as the mean ± *SEM* of nine separate experiments, three for each strain and each tested compound. The differences from controls were calculated using Student's *t* test for paired data and were considered statistically significant when the $p \leq 0.05$.

To have a better visualization of the phenomenon, the interference of HPMC and First Defense with bacterial adhesion was also performed using scanning electron microscopy.

The cells and bacteria under the different test conditions were placed on round coverslips, fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.1, for 6 h. After dehydration, the coverslips were coated with 200 Å of gold and observed under a scanning electron microscope.

RESULTS

As the aim of this study was to highlight the ability of HPMC and First Defense to bind to mucosal cells, the results are mainly confined to the related images, and only summary numerical data are given concerning the bacterial adhesion test. Figure 1A shows buccal cells after incubation with Indian ink. No Indian ink particles were deposited on the cells at pH 7. When the Indian ink challenge was performed after the cells had been incubated with HPMC at pH 7, ink was clearly visible on the cell surfaces (Figure 1B), and the same was observed after incubation with HPMC at pH 4, although the deposition was patchier (Figure 1C). Figure 1D shows similar patchy ink deposition after the First Defense challenge test. There was a certain degree of variability in the different samples, with about 48–56% of the cells being bound.

Figure 2A shows an example buccal cell incubated with Congo red; no clear staining is seen. When Congo red was added to cells incubated with HPMC at pH 7, the dye revealed red HPMC masses of different size in contact with the cells (Figure 2B). The same result was observed at pH 3.5–4.0, but as Congo red is metachromatic, the color of the HPMC masses was blue-violet (Figure 2C); the same blue-violet color was observed in the masses attached to buccal cells after the incubation with First Defense (pH 3.5) (Figure 2D). The changes in the solvent environment at pH 3.5 tended to thicken the structure of HPMC and First Defense, thus giving rise to a sort of mirage of superimposition. However, the fact that this is not

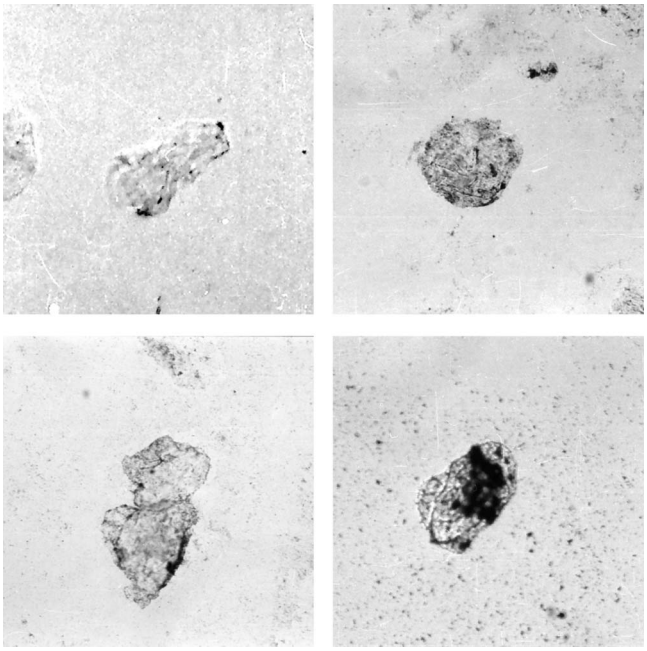

FIGURE 1. Light microscopy showing the deposition of the carbon black particles of Indian ink on buccal cells before and after incubation with hydroxypropylmethylcellulose (HPMC) and First Defense. (A) Control cell (without HPMC incubation) showing no deposition. (B) Diffuse ink retention after incubation with HPMC at pH 7. (C) Patchy Indian ink retention after incubation with HPMC at pH 3.5–4.0. (D) Patchy Indian ink retention after incubation with First Defense (pH 4).

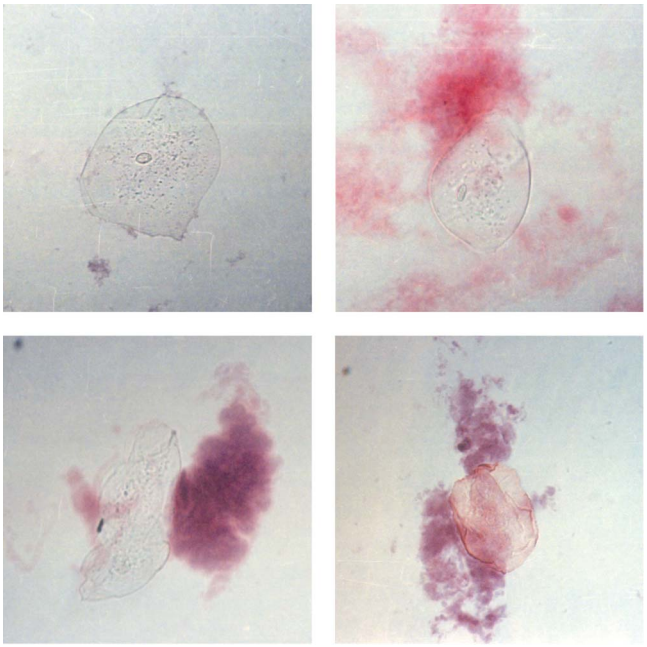

FIGURE 2. Light microscopy showing Congo red staining of buccal cells before and after incubation with hydroxypropylmethylcellulose (HPMC) and First Defense. (A) Control cell (without HPMC incubation) showing no staining. (B) Red staining of HPMC-bound cells after incubation with HPMC at pH 7. (C) Blue-violet staining of HPMC-bound cells after incubation with HPMC at pH 4. (D) Blue-violet staining of First Defense-bound cells after incubation with First Defense.

real can be clearly seen under a microscope: if the marker simply overlaps the cell, tapping the slide will separate cell and marker; this does not occur when the two are "glued" together as in our case. The findings in the case of Indian ink and Congo red also show that the area of HPMC or First Defense binding can vary from total binding to different degree of partial binding or no binding.

The second part of the study using bacteria as organic markers showed no statistical differences in adhesiveness when the bacteria were incubated with vehicle at pH 7 or 3.5–4.0 (Table 1), but there was a statistically significant reduction in bacterial adhesion after the buccal cells were incubated with HPMC at pH 3.5–4.0 in comparison with the control at pH 3.5–4.0. The same inhibitory results were obtained using First Defense (Table 1), although the effect of HPMC was significantly greater (Table 1).

The scanning electron microscopy observations confirmed the above findings (Figure 3).

DISCUSSION

The bioadhesive ability of HPMC is used in First Defense to bind the surface of mucosal cells and prevent their direct contact with potentially pathogenic microorganisms. This is a particular application of the classic activity of bioadhesives in binding two surfaces, which cannot be suitably investigated by classic tensile and shear testing methods but requires inspection with the aid of different markers to reveal the presence of bound polymers on the cell surface. Such approaches have previously been used to evaluate the mucoadhesion of polymers following aqueous dispersion (Kockisch, Rees, Young, Tsibouklis, & Smart, 2001) and the activity of bioadhesive agents in the oral cavity (Patel et al., 1999). By adopting the same rationale, we found that HPMC, owing to its viscosity, sticks the colloidal suspension of the carbon black particles of Indian ink, thus revealing the presence of bound polymers on the cell surface. HPMC alone and First Defense clearly bind to the surface of human buccal cells, well-known and widely accepted examples of mucosal cells. The presence of a pH 3.5–4.0 environment in First Defense led to a different appearance of this marker in comparison with that induced by a pH 7 environment. The masses of the Indian ink particles on the surface of the cells incubated with First Defense at pH of 3.5–4.0 is

TABLE 1
Effects of Hydroxypropylmethylcellulose (HPMC) and First Defense on the Adhesiveness of *Escherichia coli* to Human Buccal Cells

Strain (*E. coli*)	Control (pH 7)	Control (pH 4)	HPMC (pH 4)	First Defense (pH 4)
ATCC 25922	1,606	1,690	990	1,303
	1,264	1,211	574	765
	1,261	1,242	742	1,015
Clinical isolate	1,566	1,611	y	1,278
	1,048	1,037	505	606
	944	895	470	515
Clinical isolate	1,024	1054	615	748
	943	946	660	734
	989	994	573	739
Mean ± *SEM*	1,182.78 ± 86.13	1,186.67 ± 95.54	659.89[a,b] ± 54.68	855.89[a] ± 93.58

[a]$p \leq 0.01$ vs. control pH 4. [b]$p \leq 0.01$ vs. First Defense.

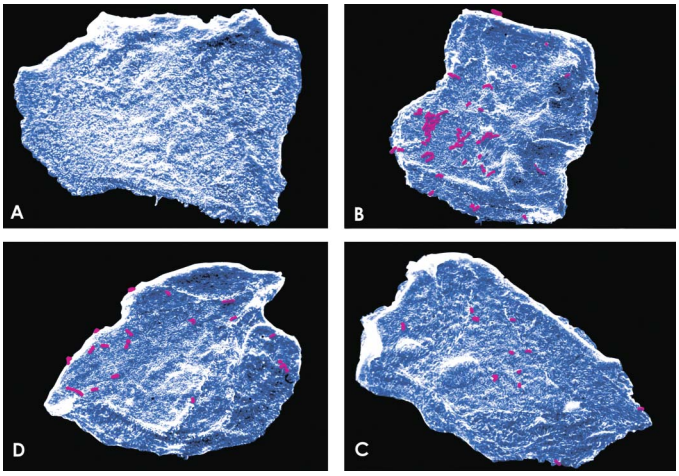

FIGURE 3. Scanning electron micrographs showing the effects of hydroxypropylmethylcellulose (HPMC) and First Defense on *Escherichia coli* adhesion to human buccal cells. (A) Control cell without *E. coli* incubation. (B) *E. coli* adhesion without HPMC incubation. (C) After incubation with HPMC. (D) After incubation with First Defense (×1,600).

probably due to the coaggregation of the colloidal particles of carbon black as a result of a reduction in their Stern layer due to the pH conditions.

Cell retention of the HPMC present in First Defense was confirmed using Congo red, a dye having great affinity for cellulose that directly revealed the presence of the cell-bound polymer.

As the HPMC in First Defense masks the surface receptors of buccal cells, the adhesins of microorganisms as such cannot reach them. This reduces bacterial adhesiveness and thus reveals the extent of the nonspecific anti-bacterial adhesiveness effects of bioadhesives. This approach has previously been used to investigate the antibacterial adhesiveness effect of HPMC (Steinberg, Rozen, Klausner, Zachs, & Friedman, 2002) and poloxamers (Veyries, Fourisson, Joly-Guillou, & Rouveix, 2000). The same masking phenomenon as above can have potential benefits also against the adhesion of viruses, particularly cold viruses.

Our findings indicate that the adherence of the three *E. coli* strains to human buccal cells was clearly inhibited. The HPMC challenge at pH 3.5–4.0 reduced the adhesiveness of *E. coli* by 44%, although the reduction of First Defense was 27%. Both these reductions are statistically significant. One possible reason for this difference could be the different viscosity (spinnability) of the two solutions: First Defense is less viscous than the HPMC solution although both contain 1% concentration of HPMC. First Defense has a complex formula with low pH and a relatively high ionic strength vs. a simple aqueous solution. There may also be some process effects on the polymer chain length because of the shearing action of the homogenizer used during manufacture.

The inspection tests using either inorganic or organic markers revealed that the binding of buccal cells may be complete, patchy, or totally absent. This different behavior is probably because the observed cells are generally separated from each other, thus leading to a discontinuity in the binding surface; the distribution of the polymer should be more homogeneous in a continuous mucosal layer such as that existing under in vivo conditions. The ability of HPMC to bind to buccal cells has been visually confirmed by other authors using atomic force microscopy (Patel et al., 2000), which allows a view from the top with high resolution and enlargement of the cell surface; untreated cells have relatively smooth surfaces covered with small “crater-like” pits and indentations, whereas treated cells appeared to have lost the craters and indentations (Patel et al., 2000), thus indicating the presence of a covering effect.

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