Bilayer Films for Mucosal (Genetic) Immunization via the Buccal Route in Rabbits

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Purpose. The oral buccal mucosa may be an ideal site for mucosal immunization, allowing for the needle-free administration of cost-effective vaccines. A novel mucoadhesive bilayer film was developed to test the feasibility of this route of immunization in rabbits.

Methods. Bilayer films were developed using different ratios of Noveon and Eudragit S-100 as the mucoadhesive layer and a pharmaceutical wax as the impermeable backing layer. Optimal 3/8-inch films were post-loaded with 100 μg of plasmid DNA (CMV-β-gal) or β-galactosidase protein. The *in vitro* release rates and stability of the postloaded antigens were determined. The films were applied to the buccal pouch of rabbits on days 0, 7, and 14, and the humoral and splenocyte proliferative immune responses to β-gal were determined through day 28 and compared to those responses after conventional subcutaneous injection of adjuvanted protein.

Results. The weight ratio of Noveon and Eudragit S-100 had a significant effect on adhesion time of bilayer films. Postloaded plasmid DNA and β-gal remained stable after being released from bilayer films (release of ~60–80% in 2 h for both). Buccal immunization using novel bilayer films (109 ± 6-μm thickness) containing plasmid DNA led to comparable antigen-specific IgG titer to that of subcutaneous protein injection. All rabbits immunized with plasmid DNA via the buccal route but none by the subcutaneous route with protein antigen demonstrated splenocyte proliferative immune responses.

Conclusion. The feasibility of buccal (genetic) immunization with these novel bilayer films was demonstrated.

KEY WORDS: plasmid DNA; β-galactosidase; splenocyte; cholera toxin; vaccine.

INTRODUCTION

It is thought that the most effective vaccines for important viral and bacterial pathogens will require mucosal immunity because these pathogens infect the body primarily at mucosal sites (1,2). Moreover, the feasibility of immunization at mucosal sites, i.e., intestinal (3), nasal (4), and vaginal (5) tissues, has been demonstrated. However, all marketed vaccines except one (oral polio vaccine) are now administered by systemic routes and are ineffective at inducing mucosal immunity. A new promising intranasal flu vaccine (Flumist™) is currently awaiting final regulatory approval. However, the one mucosal route that has been largely overlooked by researchers is the oral mucosal region of the mouth. This may be due to the lack of suitable delivery systems to retain and protect antigen in the mouth for an extended period of time.

The oral mucosa may be an ideal site for vaccination for the following reasons: 1) the oral mucosa is accessible; 2) the oral mucosa is a preferred site for antigen presentation; and 3) oral mucosa immunization is a largely untested vaccination strategy. The buccal mucosa is covered by a network of dendritic cells analogous to Langerhans's cells, which are the most potent professional antigen-presenting cells (6). Langerhans's cells represent the major antigen-presenting cells in human buccal mucosal epithelium, as demonstrated by the presence of Birkeck granule and the expression of CD1a and HLA-DR (7). In addition, a high density of T lymphocytes (40-fold greater than in the skin) and mucosal-associated lymphoid tissue, such as tonsils, salivary glands, Waldeyer's ring, and pharyngeal lymphoid tissue, are present in the buccal mucosa (8). Thus, immunization via the buccal mucosa may provide for both cellular and mucosal (humoral) immune responses. For example, Etchart *et al.* (9) reported that a single immunization with recombinant DNA injected transepithelially in the buccal mucosa of mice induced a measles virus haemagglutimin-specific class I-restricted cytotoxic lymphocyte response in the spleen. Lundholm *et al.* (10) found, using jet injection immunization, that pDNA administered in the oral cheek of mice elicited very high IgA mucosal responses specific to HIV-1 proteins (gp160, p24, or TAT). Also, the immune response was more Th1 biased, evidenced by the high ratio of IgG2a to IgG1 (10). More recently, Wang *et al.* (11) reported that gene gun-mediated oral mucosal transfer of interleukin 12 (IL-12) cDNA coupled with an irradiated melanoma vaccine in a hamster model led to the successful treatment of oral melanoma and distant skin lesions. The feasibility to deliver protein antigen via the buccal mucosa was also demonstrated by Etchart *et al.* (12), who showed that a single buccal immunization with measles virus nucleoprotein, by either topical application onto or intradermal injection in the buccal mucosa, induced *in vivo* priming of protective class I-restricted specific CD8+ cytotoxic lymphocyte. As a whole, these previous studies demonstrate that immunization via the buccal mucosa is feasible. However, more pharmaceutically acceptable and cost-effective delivery systems are still needed to fully explore the potential of this immunization method.

Conceivably, an effective buccal delivery system will provide for easy administration, thereby increasing patient compliance. Several different types of buccal delivery systems, such as sprays, solutions, mono- or multi-layer adhesive films, buccal tablets, and lollipops, have been developed (13). In addition to factors relating to safety and cost, ideal delivery systems for buccal immunization should provide for prolonged exposure (i.e., 2–4 h) of the antigen (protein or plasmid DNA) to the mucosal tissue while at the same time ensuring the retention of its immunogenicity.

The overall goal of the present study was to develop a mucoadhesive bilayer film containing a model protein antigen (β-galactosidase) or plasmid DNA-expressing β-galactosidase and to test the feasibility of buccal immunization in rabbits. The bilayer film was composed of a thin wax layer bonded to a mucoadhesive layer containing Noveon AA-1, a cross-linked mucoadhesive polyacrylate polymer, and Eudragit S-100, an anionic pH-sensitive co-polymer of polymethacrylic acid-co-methyl methacrylate. The use of plasmid DNA as a potential genetic vaccine was emphasized in the present studies because it is well known that such vaccines are able to

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induce both humoral and cellular immune responses in animals (14).

MATERIALS AND METHODS

Materials

Polycarbophil (Noveon AA1) was a gift from BF Goodrich (Charlotte, North Carolina). Eudragit® S-100 was a gift from Rohm America, Inc. (Piscataway, New Jersey). Dentsply® Utility Wax was purchased from DENTSPLY International Inc. (York, Pennsylvania). Ethanol, β-galactosidase, tragacanth, and 2-mercaptoethanol were from Spectrum Laboratory Products, Inc. (New Brunswick, New Jersey). Plasmid DNA containing a cytomegalovirus (CMV) promoter and β-galactosidase reporter gene (CMV-β-gal) was a gift from Valentis, Inc. (The Woodlands, Texas). *O*-Nitrophenyl-β-D-galactopyranoside (ONPG) and MicroBCA protein quantification kit were purchased from Pierce (Rockford, Illinois). PicoGreen DNA Quantification Kit was purchased from Molecular Probes (Eugene, Oregon). Glass microscope cover slips were purchased from Fisher Scientific Inc. Cholera Toxin (CT, #101D) was purchased from List Biologic Laboratories, Inc. (Campbell, California).

Preparation of Mucoadhesive Bilayer Films

Five different ethanol-based gels were prepared comprising the following final Noveon/Eudragit ratios (% w/w): 1:2, 2:2, 2:1, 3:1, and 2:0.5. These gels and subsequent films were designated by the final ratio of Noveon (N) and Eudragit (S) as 0.5N:1S, 1N:1S, 2N:1S, 3N:1S, and 4N:1S, respectively. Briefly, the required amount of Noveon was dispersed into absolute ethanol. The dispersion was then vigorously stirred (Caframo Mixer BDC1850, Ontario, Canada) until a homogenous viscous opaque gel was formed. Then, the required amount of Eudragit S-100 was slowly added into the Noveon gel while stirring. High-speed stirring (1,000 rpm) was used until the Endragit was dispersed in a homogenous gel. After the addition of ethanol to the required weight, the viscous gel was slowly stirred overnight in a closed container. The gel was then sonicated for 30–60 min in an Ultrasonic Cleaner (Branson Untrasonic Co., Danbury, Connecticut) to remove air bubbles before film casting. The required weight of gel was poured into a plastic hollow ring (diameter = 6.2 cm; area = 30.175 cm^2) glued onto Mylar film. The total weight of polymers (Noveon and Eudragit) in the casted gels was held constant by pouring a corresponding volume of gel that would result in final 3/8-inch films containing 10 ± 0.5 mg of total polymer, which was confirmed for all five ethanol-based gels. The casted gels were then left at room temperature overnight to form the mucoadhesive layer and to ensure evaporation of ethanol. The complete removal of ethanol was verified by confirming that the dried film reached a uniform weight. The mucoadhesive films (diameter = 6.2 cm) were coated with melted DENTSPLY® Utility Wax containing 1% (w/w) tragacanth by carefully dipping one side of the mucoadhesive film into the melted wax at 88°C for 1–2 s. The thin wax coating on the films was allowed to cool to dryness at ambient condition. The coating hardened within 5 s and cooled to room temperature within 30 s. This process resulted in a homogenous and permanent thin wax coating on the mucoadhesive layer. Circular bilayer films (3/8-inch diameter) were punched from the larger films using Arch punches (C.S. Osborne and Co., Harrison, New Jersey) and stored in ambient conditions away from light.

Effect of Noveon/Eudragit Ratio on the Adhesion Time of the Bilayer Films to Glass

The adhesion time of bilayer films was determined using glass as a simple model of the negatively charged buccal mucosa. The mucoadhesive side of the films (n = 5–9) was prewetted with 2 μL 10 mM phosphate-buffered saline (PBS, pH 6.0) and attached to a glass microscope coverslip with slight pressure. The films were left at room temperature to dry for at least 2.5 h. Subsequently, the glass coverslips with attached bilayer films were then submerged into 200 mL of 10 mM PBS buffer, pH 6.0, at 37°C that was maintained in a rotating waterbath at 100 rpm. The detachment time of the films from the glass was recorded and reported as the adhesion time. Statistical analysis was completed using a two-sample *t* test, assuming equal variances. A P value ≤ 0.05 was considered to be significant.

Loading of Plasmid DNA or β-Galactosidase on/in the Mucoadhesive Bilayer Film

Plasmid DNA (CMV-β-gal) was loaded on/in the mucoadhesive layer of the bilayer film using either a preloading or postloading method. For preloading, a required volume pDNA was added into the ethanol-based Noveon/Eudragit gels (3N:1S) and stirred for 30 min before casting. The volume of pDNA added was controlled so that a resulting 3/8-inch bilayer film contained 10 μg of pDNA. For postloading, 3/8-inch bilayer films (3N:1S) were made as described above, and then a required volume of pDNA was added to the mucoadhesive layer. Briefly, 5 μL of 0.2 M NaOH was first applied to the mucoadhesive side of the film. The NaOH was required to ensure that plasmid DNA was not affected by the low pH environment of the mucoadhesive layer. Without NaOH neutralization, the pH of the release media (10 mM PBS, pH 7.4) under the conditions used described below decreased to between pH 4–5 within 2 h. After the film was dried, 10 μg of pDNA (4.4 μL of 2.3 μg/μL in 10 mM PBS buffer, pH 7.4) was applied onto the mucoadhesive layer of the bilayer film. The films were then left at room temperature for 2 h to dry. To ascertain whether preloading or postloading of pDNA in/on the bilayer films affected the adhesion time of the bilayer films, glass adhesion studies were performed as described above.

For postloading β-galactosidase protein, 500 μg of β-galactosidase dissolved into 10 mM PBS buffer, pH 7.4, was applied to the mucoadhesive layer of the bilayer films and then left at room temperature for 2 h to dry. Preloading of β-galactosidase in the bilayer films was not investigated.

Release of pDNA or β-Galactosidase from Bilayer Films

The release of preloaded or postloaded pDNA from bilayer films was investigated by submerging 3/8-inch bilayer films (n = 5) into 1 mL of 10 mM PBS buffer (pH 7.4) in a glass scintillation vial. The vial was kept at 37°C in a C76 Water Bath Shaker rotating at 100 rpm. One hundred microliters of solution was withdrawn at specified times for pDNA

quantification using the PicoGreen DNA Quantification Kit (Molecular Probes). One hundred microliters of fresh 10 mM PBS buffer, pH 7.4, at 37°C was added to maintain a constant total volume of 1 mL. Placebo films without pDNA loading were used as negative control. The stability of the pDNA released from postloaded bilayer films was measured using gel electrophoresis with 1% SeaKem Gold agarose gel (Bio-Whittaker Molecular Applications, Rockland, Maine) in 1X TAE buffer (pH 7.4).

The release of β-galactosidase from bilayer films was determined in a similar manner as described above. The concentration of released β-galactosidase protein was measured using a MicroBCA protein quantification kit (Pierce). The enzymatic activity of released β-galactosidase was also measured to evaluate the stability of the protein using a protocol from Edge BioSystems (Gaithersburg, Maryland) with modification. Briefly, 30 μL of release solution was placed into a 7-mL glass vial. Two hundred microliters of "Z" buffer [60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, and 1 mM $MgSO_4$, (pH 7.0) with 50 mM of β-mercaptoethanol added freshly before use] was added into the sample and incubated at 37°C for 5 min. Seventy microliters of of ONPG was then added. After mixing, the sample was monitored until it developed a faint yellow color. The reaction was stopped by adding 0.5 mL of Na_2CO_3 (1 M). The OD420 was measured using an ELX 800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, Vermont). A standard curve of β-galactosidase concentration vs. OD420 was constructed to convert the OD420 from release samples to enzyme activity.

Rabbit Immunization Studies

Female New Zealand White rabbits (2.5–3.0 kg) from Myrtle's Rabbitry (Thompson's Station, Tennessee) were used in the present studies. National Institutes of Health guidelines for the care and use of laboratory animals were observed. All animal studies began after an acclamation period of 1 week. Rabbits were anesthetized before dosing and blood collection by intramuscular injection of ketamine HCl (40 mg/kg) and xylazine (5 mg/kg). All rabbits remained anesthetized for at least 2 h. In the first preliminary study, rabbits (n = 2) were immunized with either pDNA (100 μg) or β-galactosidase protein (100 μg), both adjuvanted with 100 μg of cholera toxin (CT), via the buccal mucosa by applying 3/8-inch postloaded bilayer films on days 0, 7, and 14. As a positive control, two rabbits were immunized with β-galactosidase (100 μg) adjuvanted with CT (100 μg) by subcutaneous injection. Naive rabbits received no treatment other than anesthesia. In a second rabbit study, four rabbits were immunized with pDNA (100 μg) adjuvanted with CT (100 μg) via the buccal mucosa by applying the pDNA-postloaded films. Three rabbits (n = 3) were immunized with β-galactosidase (100 μg) adjuvanted with "Alum" (100 μg) by subcutaneous injection.

In both studies, rabbit blood was withdrawn via the ear vein on days 0, 7, 14, 21, and 28. Sera were separated and stored as previously described (15). β-Galactosidase-specific total IgG tier in the sera was determined by ELISA (15). In addition, in the second study, rabbit spleens were collected at day 28, and splenocytes were prepared as described previously (16). A splenocyte proliferation assay was completed as described elsewhere (16) except that 1.6×10^5 cells/well were co-incubated with 0 or 10 μg/well β-galactosidase for 4 days before assay using the CellTiter 96® Aqueous Non-radioactive Cell Proliferation Assay Kit (Promega). Proliferation results were reported as the percent increase in the splenocyte number four days after stimulation. The proliferation index was calculated as the ratio of splenocyte number with and without stimulation after 4 days. Statistical analysis was completed with a two-sample *t* test assuming unequal variances. A P value ≤ 0.05 was considered statistically significant.

RESULTS

Effect of Noveon/Eudragit Ratio on the Adhesion Time of the Bilayer Films to Glass

Figure 1 shows the glass adhesion time of the 3/8-inch bilayer films (109 ± 6-μm thickness) having different ratios of Noveon and Eudragit (0.5N:1S, 1N:1S, 2N:1S, 3N:1S, and 4N:1S). Increasing the proportion of Noveon to Eudragit from 0.5:1 to 3:1 (w/w) significantly enhanced the adhesion time of the bilayer film from an average of only a few minutes to up to 175 min. Bilayer films composed of a Noveon/Eudragit ratio of 3:1 (w/w) appeared to be optimal because the 4N:1S films adhered for only 120 min. Further, the adhesion times of the bilayer films (3N:1S) were statistically greater than all of other films ($P < 0.05$). Therefore, bilayer films comprised of a Noveon/Eudragit ratio of 3:1 (w/w) were used for all further studies.

Release of Plasmid DNA and β-Galactosidase from Bilayer Films

The release of plasmid DNA preloaded in bilayer films was very slow and incomplete. For example, only 4% of the total preloaded pDNA was release in 10 h (data not shown). However, as shown in Fig. 2, the release of pDNA postloaded on bilayer films was greater and more extensive. Over 60% of

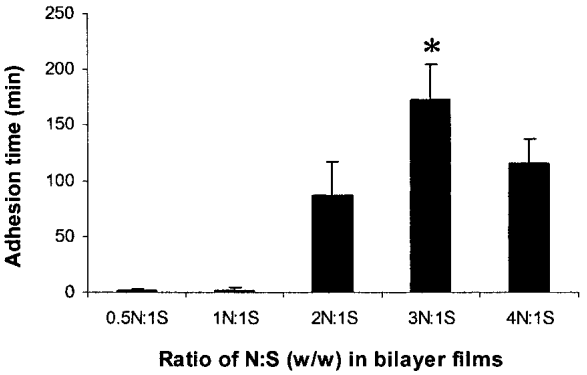

Fig. 1. The weight ratio of Noveon (N) and Eudragit S-100 (S) affected the adhesion time of the bilayer films to a glass matrix. The 3/8-inch bilayer films were prepared as mentioned in Materials and Methods. Films (n = 5–9) were wet with 2 μL of PBS, attached to microscope cover glass, and then submerged into 10 mM PBS, pH 6.0, maintained in a 37°C waterbath rotating at 100 rpm. The detachment time of the bilayer films from the cover glass was recorded and reported as the adhesion time (mean ± standard deviation). *Indicates that the adhesion time for the 3N:1S film was significantly longer than that of the others.

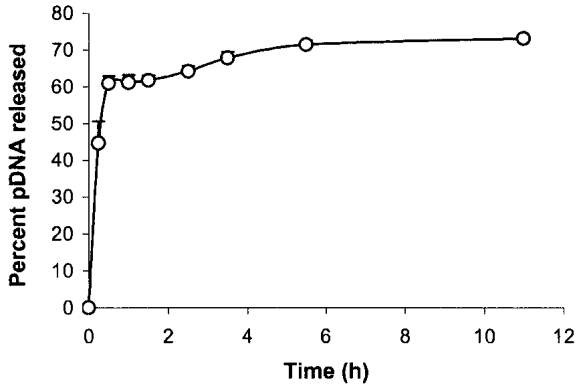

Fig. 2. The release profile of pDNA from postloaded 3/8-inch bilayer films. Bilayer films (n = 5) with pDNA (10 μg/film) postloaded on the mucoadhesive layer were submerged in 10 mM PBS, pH 7.4, maintained in a 37°C waterbath rotating at 100 rpm.

the postloaded pDNA was released in 2 h, in contrast to only about 2% from the preloaded films. Gel electrophoresis showed that the pDNA released from the postloaded films was mostly in supercoiled form (data not shown). Further, as shown in Fig. 3, postloading pDNA had no significant effect on the adhesion time of the bilayer films as compared to both placebo and preloaded bilayer films ($P = 0.43$ by ANOVA). Based on the desirable release profile and the stability of pDNA, the postloading process for pDNA was used to prepare bilayer films for subsequent rabbit studies.

β-Galactosidase was successfully postloaded on the mucoadhesive layer of the bilayer films, and the loading did not affect the cosmetic properties of the films. Fig. 4 shows that about 80% of the postloaded β-galactosidase was released within 2 h. In addition, the released β-galactosidase retained its enzymatic activity as determined by its ability to hydrolyze ONPG to *o*-nitrophenol and D-galactose.

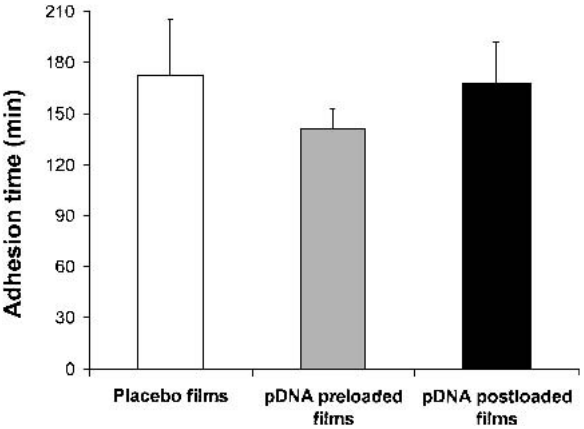

Fig. 3. The effect of pDNA loading methods on the adhesion time of the bilayer films to glass matrix. Placebo 3/8-inch films and bilayer films, preloaded or postloaded with pDNA (10 μg/film), were attached to glass microscope coverslips and submerged in 10 mM PBS buffer, pH. 6.0, maintained in a 37°C waterbath rotating at 100 rpm. The detachment time of the bilayer films from the cover glass was recorded and reported as the adhesion time (mean ± standard deviation, n = 5). ANOVA test did not demonstrate any significant difference between the adhesion times of the three kinds of films ($P = 0.43$).

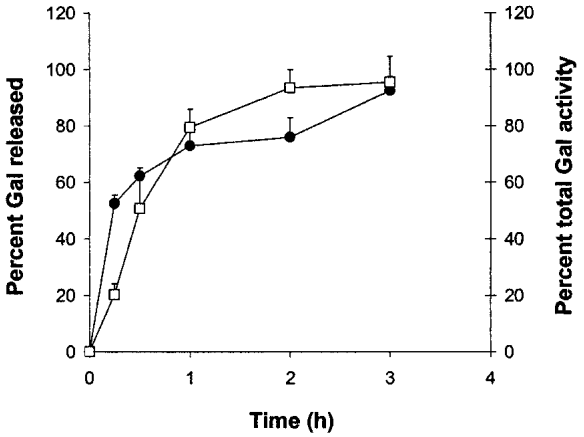

Fig. 4. The release profile of postloaded β-galactosidase (Gal) protein (●) from bilayer films. Five hundred micorgrams of β-galactosidase was postloaded on the mucoadhesive layer of the films (n = 5). β-Galactosidase release was monitored in 10 mM PBS, pH 7.4, maintained in a 37°C waterbath rotating at 100 rpm. β-galactosidase (Gal) enzymatic activity (□) is reported as the percent of the control β-galactosidase. Data were reported as mean ± standard deviation (n = 5).

Immune Responses in Rabbits

The total β-galactosidase-specific IgG titer profile in sera of immunized rabbits for the first rabbit immunization study is shown in Fig. 5. Because only two rabbits were used for each group, statistical analyses were not completed. However, the results did demonstrate that both β-galactosidase protein and pDNA (CMV-β-gal) induced antigen-specific IgG when applied to the buccal mucosa of the rabbits via the mucoadhesive bilayer films. In addition, the level of the IgG titers in rabbits immunized via the buccal route for at least one rabbit in each group was comparable to that of rabbits immunized

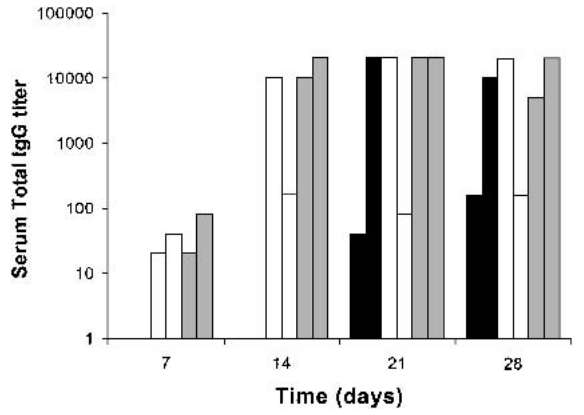

Fig. 5. Rabbit total β-galactosidase-specific IgG titer in sera after buccal immunization with bilayer films. Rabbits (n = 2) were dosed on days 0, 7, and 14 with either pDNA (100 μg; black bars) or β-galactosidase protein (100 μg; white bars), both adjuvanted with 100 μg of CT via the buccal mucosa using postloaded bilayer films. As a positive control, two rabbits were also immunized with β-galactosidase (100 μg) adjuvanted with CT (100 μg) by subcutaneous injection (gray nars). Naive rabbits received no treatment other than anesthesia. Blood was withdrawn at days 0, 7, 14, 21, and 28 via the ear vein. IgG titer in the sera was determined by ELISA and reported for individual rabbits.

by subcutaneous injection of adjuvanted protein. Genetic immunization using plasmid DNA resulted in a delay of the onset of detectable titer until day 21. All treated rabbits demonstrated positive CT-specific IgG titers in sera (results not shown). Furthermore, all immunized rabbits demonstrated positive IgG by ELISA (O.D. 450 nm) in nasal swabs. Rabbits immunized by the buccal route with bilayer films containing plasmid DNA showed O.D. at 450 nm values from nasal swabs that were generally greater than those after immunization with protein either by the buccal route or by subcutaneous injection. For example, for nasal swabs, the increase in O.D. at 450 nm in the ELISA for rabbits immunized by the buccal route with plasmid DNA was generally 4- to 10-fold greater than naive rabbits over the 28-day study, whereas rabbits immunized with protein by either route were only 1- to 3-fold greater than naive rabbits.

In a second rabbit immunization study, the number of rabbits in each group were increased to n = 3–4. The total β-galactosidase-specific IgG titer profile in sera of immunized rabbits is shown in Fig. 6. At day 7, a low level of IgG was detected in one of the four rabbits immunized via the buccal route, and thus the overall IgG titer at day 7 was significantly lower than that of rabbits immunized by subcutaneous injection ($P = 0.04$). However, by day 14, all four rabbits immunized by the buccal route responded with titers that were comparable to those after subcutaneous injection. By day 28, the titer for the rabbits immunized by the buccal route was statistically greater than those rabbits immunized by subcutaneous injection ($P = 0.03$). As shown in Table I, all rabbits immunized via the buccal route with bilayer films containing 100 μg of plasmid DNA but none by the subcutaneous route with 100 μg protein adjuvanted with "Alum" demonstrated splenocyte proliferative immune responses.

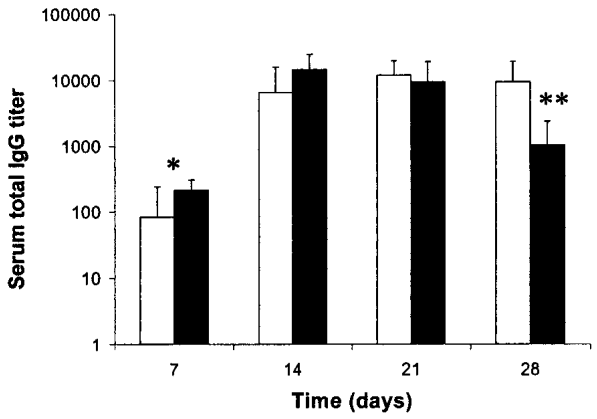

Fig. 6. Rabbit total β-galactosidase specific IgG titer in sera after buccal immunization with bilayer films. In a second rabbit study, rabbits (n = 4) were immunized with pDNA (100 μg) adjuvanted with CT (100 μg) via the buccal mucosa (white bars). Three rabbits (n = 3) were immunized with β-galactosidase protein (100 μg) adjuvanted with "Alum" (100 μg) by subcutaneous injection (black bars). Naive rabbits (n = 3) received no treatment other than anesthesia. Rabbits were dosed on days 0, 7, and 14. IgG titer in the sera for individual rabbits was determined by ELISA and reported as mean ± standard deviation (n = 3–4). *Indicates that the mean titer after both routes of immunization were significantly lower those at all other time points by both routes of immunization. **Indicates that the IgG titer in rabbits after subcutaneous injection was significantly lower than that after buccal immunization on day 28.

Finally, although the two rabbit studies were completed at different times, the results suggested that the titers after buccal immunization using bilayer films containing 100 μg of pDNA were comparable. Further, the results also demonstrated that for rabbits immunized by subcutaneous injection of protein, both "Alum" and CT adjuvanted the immune response to a similar extent.

DISCUSSION

Vaccination without the use of needles would provide a distinct advantage in terms of both cost and safety over conventional vaccines that must be given with needles. In addition, needle-free vaccinations would make the prospects of widespread vaccination more practical and cost-effective. Although the intestinal and nasal mucosa routes have been explored extensively to delivery (genetic) vaccines, the oral mucosa route remains a largely untested vaccination strategy (3,4). This may be due to the lack of suitable delivery systems to retain and protect antigen in the mouth for an extended period of time.

The oral buccal mucosa has all the necessary cells (high density of Langerhan's cells and T lymphocytes) and the mucosal-associated lymphoid tissues for the development of immune responses (6–8). Further, several recent publications have confirmed that it is feasible to elicit both humoral and cellular immune responses by applying (genetic) vaccines to the oral buccal mucosa using either transepithelial needle injection, needle-free jet injection, or gene gun-mediated injection (9–12). However, these reports also underscore the need for improved, cost-effective, and more pharmaceutically acceptable and patient friendly delivery systems.

The rabbit model was chosen in this present study because the rabbit is the only laboratory rodent that has nonkeratinized mucosal lining similar to human tissue (17). However, one drawback associated with using rabbits for immunization studies is that unlike other rodent models such as the mouse and rat, most of the necessary antibodies and cytokine ELISA kits are not commercially available. Thus, for this present study, we could not readily assess IgA titer or Th1- vs. Th2-biased immune responses via the detection of cytokines such as IL-2, interferon-γ, IL-4, etc.

To determine the feasibility of buccal (genetic) immunization in rabbits, novel mucoadhesive bilayer films were developed. The mucoadhesive layer of the bilayer film is comprised of Noveon and Eudragit S-100. Noveon, or polycarbophil, is a homopolymer of acrylic acid cross-linked with divinyl glycol. Noveon has USP designation and is generally regarded as safe. It has been extensively formulated in a variety of drug-delivery systems and commonly is used as a bioadhesive (18). The high molecular weight polycarbophils readily swell in water, providing a large adhesive surface for maximum contact with the mucosal tissue. The exact mechanism of mucoadhesion of these polymers to the buccal tissue is unknown, but is thought to involve extensive hydrogen bonding between the carboxylic acid moieties of the polymers to the sialic acid residues of mucin, the primary substance of the viscous mucous layer on epithelial cells (18). The viscosity of the mucous layer is largely determined by the type and amount of the glycoproteins present in the mucin. Usually, the mucous layer in the oral mucosa is about 500- to 600-μm thick and has a pH range of 5.8–7.1. Eudragit S-100 is a co-

Table I. Proliferation of Isolated Rabbit Sphenocytes after *in Vitro* Stimulation

	Naive			Bilayer film with postloaded pDNA				β-Gal protein via subcutaneous injection		
Rabbit No.	1	2	3	4	5	6	7	8	9	10
Percent increase in cell number	<0	<0	<0	168	277	52	148	<0	<0	<0
Proliferation index	<1	<1	<1	2.68	3.77	1.52	2.48	<1	<1	<1

Note. Rabbits were immunized as in Figure 6 with either pDNA (100 μg) adjuvanted with CT (100 μg) via buccal mucosa or β-galactosidase protein (100 μg) adjuvanted with "Alum" (100 μg) subcutaneously on days 0, 7, and 14. On day 28, rabbits were sacrificed and their spleens were collected. Splenocytes (1.6×10^5) (n = 2) were seeded into 48-well plates and incubated together with β-galactosidase (0 or 10 μg/well) for 4 days at 37°C with 5% CO_2. Cell proliferation was quantified with a CellTiter 96® Aqueous Non-radioactive Cell Proliferation Assay Kit (Promega). The data reported are the average of two values for each sample.

polymer of methacrylic acid and methyl methacrylate (1:2). Polymethacrylates are primarily used as film-coating agents for oral capsule and tablet formulations (19). Although Eudragit has some mucoadhesive properties, we mainly took advantage of its film-forming properties by combining it with the Noveon to provide some rigidity to the mucoadhesive layer. Eudragit S-100 is a pH-sensitive polymer that has solubility in water above pH 7 but is insoluble below pH 7. This insolubility below pH 7 is ideal for use in the oral mucosa to promote longer adhesion time of the bilayer films resulting from retardation of hydration and swelling of the polycarbophil polymer. Thus, the optimal combinations of Noveon and Eudragit in the mucoadhesive layer provided for a bilayer film with excellent and prolonged adhesive properties. The presence of the wax-backing layer greatly enhanced the adhesion time of the mucoadhesive layer by retarding the diffusion of (simulated) saliva (data not reported). Further, the presence of the hydrophobic wax-backing layer presumably helps to retard the diffusion of plasmid DNA and β-galactosidase into the mouth, although this was not investigated.

The results of this present study demonstrated that two large biomolecules, β-galactosidase (256 kDa) and plasmid DNA, could be postloaded on optimal bilayer films and that the majority of the postloaded material was released within 2 h. Further, the stability of the released biomolecules was retained as measured by retained enzymatic activity of the protein and the retention of supercoiled topology for plasmid DNA. The application of these agents to premade and quality-controlled bilayer films may have several advantages, including cost-effectiveness. For example, in contrast to the preloading process, the postloading process creates no waste of the protein or plasmid DNA because they are added directly to the delivery system to be applied.

Taken together, the two rabbit immunization studies demonstrated that buccal (genetic) immunization with these novel mucoadhesive bilayer films is feasible. In addition, the results showed that the buccal immunization was quite reproducible in that all rabbits responded, eliciting titer that was equivalent, if not greater, than those rabbits immunized by conventional subcutaneous injection of adjuvanted protein. Moreover, it was demonstrated that all rabbits immunized by the buccal route with bilayer films containing plasmid DNA showed positive splenocyte proliferative responses, which may be indicative of cellular-based immunity. As expected, no proliferative immune responses were observed with conventional subunit protein immunization, which is well known to produce more humoral-based immune responses. CT was included into the bilayer film because it is known to be a strong mucosal adjuvant (20). The use of 100 μg of CT as an adjuvant by either the buccal or subcutaneous routes did not cause any discernable toxic effects in the rabbits. Additional buccal immunization studies are planned to determine the importance of CT or other adjuvants, such as "Alum," or cationic lipids, in terms of the breadth and depth of the immune responses.

These results suggest that immunization with mucoadhesive bilayer films containing plasmid DNA via the buccal mucosa may elicit systemic humoral and cellular-based immune responses. Although mucosal IgA was not determined in these studies, it is believed based on the presence of nasal IgG and previous literature reports that it is likely that IgA antibodies are also produced after buccal immunization. Methods to quantitatively determine IgA antibodies in rabbits are being pursued actively in our laboratories. The elicitation of systemic humoral, cellular, and mucosal responses via buccal immunization may warrant additional testing with important pathogens.

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