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A novel bioadhesive intranasal delivery system for inactivated influenza vaccines

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Abstract

The aim of the current studies was to evaluate a bioadhesive delivery system for intranasal administration of a flu vaccine, in combination with a mucosal adjuvant (LTK63). A commercially available influenza vaccine, containing hemagglutinin (HA) from influenza/A Johannesberg H1N1 1996, and LTK63 or LTR72 adjuvants, which are genetically detoxified derivatives of heat labile enterotoxin from *Escherichia coli,* were administered IN in a bioadhesive delivery system, which comprised esterified hyaluronic acid (HYAFF) microspheres, to mice, rabbits and micro-pigs at days 0 and 28. For comparison, additional groups of animals were immunized intranasally with the HA vaccine alone, with soluble HA+ LTK63, or IM with HA. In all three species, the groups of animals receiving IN immunization with the bioadhesive microsphere formulations, including LT mutants, showed significantly enhanced serum IgG responses ($P<0.05$) and higher hemagglutination inhibition (HI) titers in comparison to the other groups. In addition, the bioadhesive formulation also showed a significantly enhanced nasal wash IgA response ($P<0.05$). Most encouragingly, in pigs, the bioadhesive microsphere vaccine delivery system induced serum immune responses following IN immunization, which were significantly more potent than those induced by traditional IM immunization at the same vaccine dose ($P<0.05$).

Keywords: Bioadhesive; HYAFF microparticles; Flu antigen; Immunogenicity

1. Introduction

Since the majority of pathogens initially infect their hosts through mucosal surfaces, the induction of mucosal immunity is likely to make an important contribution to protective immunity. In addition, mucosal administration, which avoids the use of needles, is becoming an increasingly attractive approach for the development of new generation vaccines. Although a number of vaccines are commercially available which control the spread of influenza [1–5], these vaccines induce serum immunity, but do not induce mucosal immunity at the site of infection in the nasal cavity. In addition, commercially available vaccines are ineffective for the induction of cytotoxic T lymphocyte (CTL) responses, which are responsible for killing virally infected cells. Therefore, the currently available vaccines are not considered to be optimal [6,7]. As a consequence, work is currently underway to develop more effective influenza vaccines that induce mucos-

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al IgA responses through local administration, and also induce more potent systemic responses [8–10]. An early study in mice showed that IN immunization with a potent adjuvant induced superior cross-protective immunity than parenteral immunization, supporting the use of the IN route for flu vaccine development [11]. The most potent mucosal adjuvants which are available for local immunization are heat labile enterotoxin from *Escherichia coli* (LT) and cholera toxin (CT) from *Vibrio cholerae,* and these molecules and their subunits have shown some promise as intranasal adjuvants for flu [9–15]. However, since the native toxins CT and LT are the causative agents, respectively for cholera and traveler's diarrhea, they are considered to be too toxic for use in humans. Therefore, several groups have focused on the development of detoxified mutants of LT and CT as mucosal adjuvants. Colleagues within Chiron have focused on the development of LT mutants with reduced, or eliminated enzymatic activity, since it is the ADP-ribosylating enzymatic activity of LT and CT which causes abnormal intracellular accumulation of cAMP and excess fluid secretion from intestinal cells. We have used site-directed mutagenesis to replace single amino acids within the enzymatic A subunit of LT and have developed mutants (LTK63 and LTR72) with reduced or eliminated enzymatic activity [16]. LTK63 is completely devoid of ADP-ribosyltransferase activity and appears to be non-toxic both in vivo and in vitro, while LTR72 has residual enzymatic activity ($<$1% of the native LT) and has significantly reduced, but detectable toxicity [16]. Both of these mutants have been previously shown to be potent mucosal adjuvants for antibody and CTL induction in a number of studies in mice [9,15–17].

Since the early 1980s several groups have focused on bioadhesion as a concept to improve local and systemic drug delivery [18,19]. In general, bioadhesive delivery systems are designed to adhere to various tissue surfaces, mainly the mucosal epithelium. An alternative term, mucoadhesion is also used often to describe the interaction of a polymer delivery system and a mucosal site. Mucoadhesion appears to require a highly expanded and hydrated polymer network, which promotes an intimate molecular contact between the delivery system and the mucus layer [20–24]. The mechanisms of bio- or mucoadhesion can involve physical or chemical interactions, including electrostatic or hydrophobic bonding, van der Waal's forces or hydrogen bonding. Irrespective of the mechanisms involved, the main advantages of bioadhesive delivery systems include extended residence time at the site of action, local delivery to a selected site and enhanced interaction with the mucosal epithelium [20]. In a range of studies in recent years, several bioadhesive polymers have been described, including chitosans, methacrylic acids, starch, gelatin, hyaluronic acid and cellulose derivatives to enhance the absorption of co-administered protein drugs [22–25]. Hyaluronic acid is a naturally occurring mucopolysaccharide consisting of residues of D-glucuronic acid and *N*-acetyl-D-glucosamine. Through the esterification of the carboxyl groups of hyaluronic acid with alcohols, biodegradable polymers have been developed, called HYAFF [22]. The HYAFF™ polymers can be used to make microspheres using a coacervation phase-separation process [26]. The HYAFF microspheres have strong bioadhesive properties and have been used for delivery of calcitonin and insulin following mucosal administration [23,24]. However, HYAFF microspheres have not previously been used for mucosal delivery of vaccines.

In the current studies, we report the use of an influenza hemagglutinin (HA) vaccine administered IN to mice, rabbits and micro-pigs along with LT mutants and a bioadhesive delivery system, comprising HYAFF microspheres. The IN route has been used previously to administer vaccines and adjuvants to animal models [8]. The responses to the bioadhesive formulations were compared to HA combined with LT mutants by the IN route, and also HA administered alone by the IN and IM routes. For IM administration, we used a commercially available vaccine which like most other flu vaccines, is unadjuvanted.

2. Materials and methods

2.1. Materials

A monovalent A/Johannesberg split vaccine preparation of purified influenza HA was provided by

Chiron Vaccines, Siena, Italy. Dosing of the vaccine was based on HA content as assayed by single radial immunodiffusion (SRID) as described previously [9]. The mucosal adjuvants, LTK63 and LTR72 were fermented and purified at Chiron, Emeryville, USA. HYAFF microspheres were prepared by a coacervation phase separation technique as previously described [23,24,26] and were provided by Fidia Advanced Biopolymers, Padova, Italy. The bioadhesive properties of HYAFF microspheres has previously been described both in rat [24] and sheep [23] models.

2.2. Methods

The combined bioadhesive vaccine formulations were prepared as follows; HA and LT mutants at the doses described in the text were incubated with HYAFF microspheres in PBS. The suspension was kept at 4°C for 6 h and then freeze dried overnight. Prior to administration to the animal models, the microsphere formulation was re-suspended in PBS, to allow easy administration of the dose as a suspension. The HYAFF dose was 5 mg for mice (25 μl volume of administration) and 20 mg of microspheres per animal for rabbits (200 μl) and pigs (250 μl). The HA and LT mutant doses was changed according to the animal species being tested and details are included in the text.

2.3. Microparticle characterization

The HYAFF microspheres were sized on a Malvern Mastersizer both before and after combination with the antigen and adjuvant. The HYAFF microspheres as provided by the manufacturer had a mean size of 8.4 μm, as previously described [25]. The size following hydration in vitro during association with the the antigen/adjuvant was 32 ± 2.3 μm. After freeze drying, the size of the microspheres in the final formulation was determined to be 8.2 ± 0.6 μm.

2.4. Antigen and adjuvant integrity

Both the HA and the LT mutants were evaluated by an ELISA [9] to evaluate antigenic integrity following formulation with the HYAFF microspheres. The rate of in vitro release of HA and the LT mutants in PBS from the surface of the HYAFF microspheres was also estimated by ELISA [9]

2.5. Immunization protocols

2.5.1. Mice

The first study was a preliminary evaluation in mice of the bioadhesive vaccine delivery system using HYAFF microspheres. The antigen and adjuvant doses used were 10 μg HA and 25 μg LTK63. Four groups of Balb/C mice (10 per group) were immunized IN with either the HYAFF-HA-LTK63 formulation, HYAFF-HA, HA+LTK63 soluble proteins or HA alone. The animals were boosted at day 28 with the same formulations and blood was collected at day 42.

2.5.2. Rabbits

In the first study in rabbits, three groups of New Zealand whites (five or six per group) were immunized IN with either a HYAFF-HA-LTR72 formulation, HA+LTR72 soluble proteins or HA alone. The HA dose in all groups was 25 μg and the LTR72 dose was 50 μg. The animals were boosted at day 28 and blood was collected at days 28 and 42. In a second smaller study, two groups of rabbits (five per group), were immunized IN with a HYAFF-HA-LTK63 combination, with doses of HA at 10 μg and LTK63 at 25 μg. For comparison, a second group of rabbits were immunized IM with HA 25 μg. Blood samples were collected at days 14 and 42.

2.5.3. Micro-pigs

In the pig study, three groups of four Yucatan micro-pigs (8–10 kg) were used and were housed in pairs. In this study, the responses induced by IN immunization were compared to IM immunization. The doses selected for pigs were 25 μg of HA for all groups, and 100 μg of LTK63 for the two IN groups. One group was immunized IN at weeks 0 and 4 with the HYAFF-HA-LTK63 bioadhesive microsphere formulation. A second group was immunized IN with soluble HA+LTK63 at the same dose. For comparison, a third group of pigs were immunized IM with 25 μg HA.

2.6. Immunoassays

Serum total anti-HA IgG were measured by ELISA as previously described [16]. Briefly, ELISA plates (Immulon-1, 96 well, U-bottom, obtained from Dynatech Laboratories, Chantilly, VA) were coated with HA (10 μg/ml) overnight. After blocking (1% goat serum, 0.3% Tween 20 in phosphate-buffered saline), plates were coated with 1:3 serially diluted serum samples. After washing (blocking buffer), the plates were coated with 1:4000 goat anti-pig IgG horseradish peroxidase conjugate (Gibco, Grand Island, NY) and developed using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Absorbances were measured at A_{490} using a standard ELISA reader. The titers represent reciprocal serum dilutions giving an A_{490} of 0.5 and were normalized to a serum standard assayed in parallel.

Nasal wash samples from pigs were assayed for IgA using a bioluminescent immunosorbant assay (BIA) as previously described [9,16]. Briefly, ELISA plates (MicroLite obtained from Dynatech) were first coated with the HA antigen (5 μg/ml) overnight. After blocking (5% goat serum, 25 mM Tris, 10 mM EGTA, 150 mM KCl, 2 mg/ml BSA, 0.3% Tween-20, pH 7.5), plates were coated with 1:3 serially diluted nasal wash samples in blocking buffer. The plates were developed using 1:1000 diluted goat anti-pig IgA biotin conjugate (EY Labs, San Mateo, CA) pre-saturated with purified pig IgG (1 mg/ml, Sigma Chemical Company, St Louis, MO) to reduce IgG cross-reactivity. Plates were then incubated with 1:500 diluted streptavidin–jellyfish aequorin conjugate (SeaLite Sciences, Bogart, GA). Luminescence was triggered with 10 mM calcium acetate and measured using a luminometer (Dynatech ML3000). Quantitation was based on relative light units (RLU) representing total luminescence integrated over 3 s (arbitrary units). Titers represent log dilution values linearly extrapolated from the log RLU data to a cut-off value at least two standard deviations above mean background.

Serum samples for each animal were assayed for hemagglutination inhibition (HI) titers by the Viral and Rickettsial Disease Laboratory (Department of Health Services, Berkeley, CA) using a standard assay based on the ability of sera to inhibit the agglutination of goat red blood cells (RBC) in the presence of HA antigen [28–30]. Fresh RBC were diluted to 0.4% cell suspension using OD_{540} against a cyanmethemoglobin reference standard. A/Johannesberg HA antigen stock was titered to 4 HA units defined as the highest concentration required to agglutinate a 0.2% RBC suspension. Serum samples were serially diluted two-fold into an ELISA plate then the HA antigen at a final concentration of 1 HA unit and 0.2% RBC was added. The HI titer was then defined as the reciprocal dilution of the serum required to completely inhibit agglutination. HI titers are normally determined to reflect the potency of influenza vaccines and these titers have been shown to correlate closely with protective efficacy of vaccines.

2.7. Statistics

Analysis of variance was calculated by using the StatView program for Macintosh computers. Differences among groups of animals at significance levels of 95% were calculated by analysis using Fisher's protected least-significant-difference test.

3. Results

3.1. Antigen and adjuvant integrity and release rates in vitro

The HA antigen and the LT mutants both remained largely intact after formulation and release from HYAFF microspheres in vitro, with no significant changes in antibody binding characteristics (data not shown). The rate of release of both HA and the LT mutants indicated a fairly large burst release, with 32% of LTK63 and 24% of HA released in day 1, followed by a more slow release phase, with 75% of both HA and LTK63 released by day 10 in vitro. We do not consider that the in vitro release rate of the formulations has any relevance for the likely rate of release of antigen and adjuvant in vivo. However, these studies were performed to determine if the antigen and adjuvant were actually released from the formulation and to allow an evaluation of integrity of both following release.

3.2. In vivo immunogenicity

3.2.1. Mice

The group of mice receiving the bioadhesive microsphere formulation (HYAFF-HA-LTK63), showed significantly enhanced serum IgG antibody responses in comparison to the other groups immunized IN with soluble antigen alone, with HA-HYAFF, or with HA+LTK63 ($P<0.05$) (Fig. 1). The HI titers were also highest in the group of mice immunized with the bioadhesive microsphere formulation (Fig. 2). Mouse sera needed to be pooled to obtain sufficient serum to allow the HI titer to be obtained. Therefore, mean values are shown in Fig. 2.

3.2.2. Rabbits

The group of rabbits immunized with the bioadhesive HYAFF-HA-LTR72 formulation had significantly higher serum IgG titers than the groups immunized IN with soluble HA alone, or soluble HA+LTR72 at day 42 ($P<0.05$) (Fig. 3). In addition, in the second rabbit study, the group immunized IN with HYAFF-HA-LTK63 showed a higher mean serum IgG antibody responses than the group immunized IM, although there was no significant difference ($P>0.05$) (Fig. 4). The IN immunized group in this study also showed a clear trend for enhanced HI titers over the IM immunized group (Table 1).

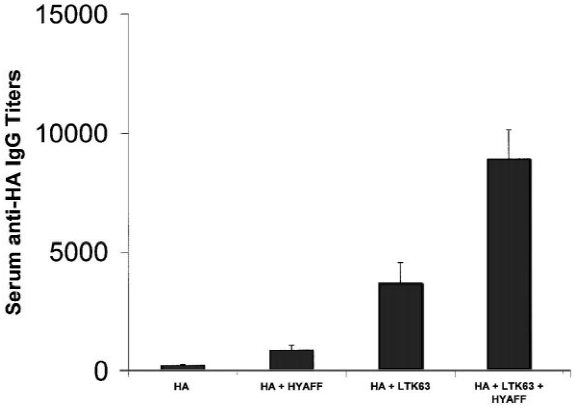

Fig. 1. Anti-HA serum IgG antibody titers in groups of mice ($n=10$) immunized with either HA alone IN, HA+HYAFF IN, HA+LTK63 IN or HA+LTK63+HYAFF IN. The graph represents the geometric mean titers±S.E.M. for each group at day 42. The HYAFF+LTK63+HA formulation was significantly better than all other groups ($P<0.05$).

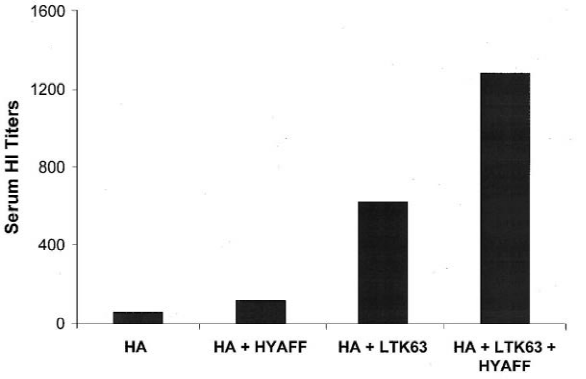

Fig. 2. Serum hemagglutination inhibition titers (HI) in groups of mice ($n=10$) immunized with either HA alone IN, HA+HYAFF IN, HA+LTK63 IN or HA+LTK63+HYAFF IN. The graph shows a single value obtained from pooled sera at day 42.

3.2.3. Micro-pigs

In the pig study, the group receiving the bioadhesive formulation (HYAFF-HA-LTK63) had a significantly higher antibody response than the groups immunized IN with soluble HA+LTK63 and the group immunized IM with HA at day 56 ($P<0.05$) (Fig. 5). The nasal IgA titers were also significantly

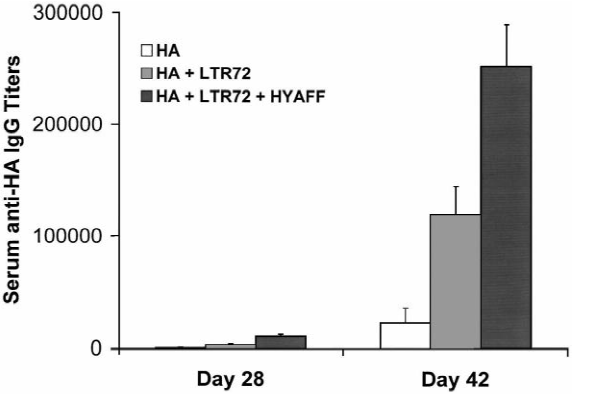

Fig. 3. Serum anti-HA serum IgG antibody titers in groups of New Zealand white rabbits ($n=5$ or 6) immunized with either HA alone IN, HA+R72 IN or HA+R72+HYAFF IN. The graph represents the geometric mean titers±S.E.M. for each group at days 28 and 42. The HYAFF+LTR72+HA formulation was significantly better than soluble HA and HA+LTR72 ($P<0.05$) at day 42.

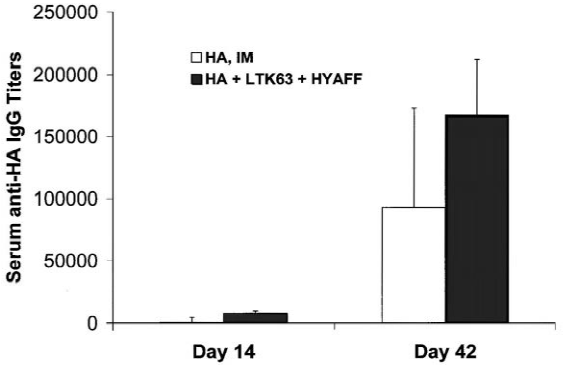

Fig. 4. Anti-HA serum IgG antibody titers in two groups of rabbits (n=5) immunized with either HA alone IM, or HA+LTK63+HYAFF IN. The graph represents the geometric mean titers±S.E.M. for each group at days 14 and 42. The groups were not significantly different from each other at days 14 and 42 (P>0.05).

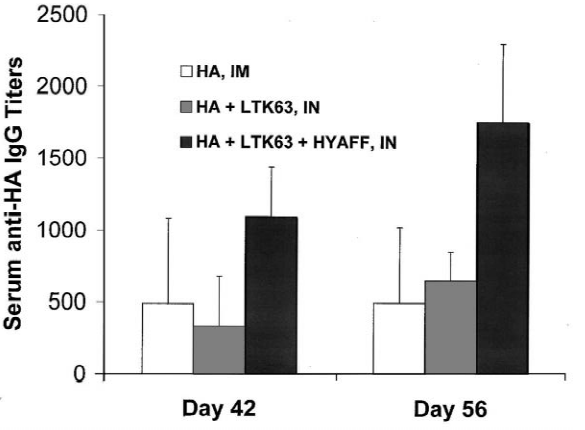

Fig. 5. Anti-HA serum IgG antibody titers in three groups of micro-pigs (n=4 per group) immunized with either HA alone IM, HA+LTK63 IN or HA+LTK63+HYAFF IN. The graph represents the geometric mean titers±S.E.M. for each group at days 42 and 56. The HYAFF+LTK63+HA formulation was significantly better than the HA (IM) and HA+LTK63 (IN) at day 56 (P< 0.05).

higher in the group of animals immunized with bioadhesive microspheres (P<0.05) (Fig. 6). In addition, the HI titers also tended to be higher in the group of animals immunized IN with the bioadhesive microsphere formulation (Table 2).

4. Discussion

The initial observations in mice offered significant encouragement that the bioadhesive microsphere delivery system may offer some benefit over administration of soluble HA with the potent adjuvant LTK63. However, the limitations of small animal models and their inability to accurately predict responses in human subjects is well known and widely acknowledged. This is particularly true in relation to mucosal delivery, since mice have very small nasal cavities which can accommodate only very low volumes of fluid (20–25 μl). Therefore, during drug or vaccine delivery studies, the mouse nasal cavity is often completely filled following

Table 1
Hemagglutination-inhibition (HI) titers and serum IgG titers at day 42 from individual animals in two groups of rabbits immunized with either HA alone IM, or HA-LTK63-HYAFF IN

Formulation	Route	Serum IgG ELISA titers (Day 42)	Hemagglutination inhibition titers (Day 42)
HA alone		463112	1280
	IM	77087	160
		68812	320
		28274	640
		99682	160
HA+LTK63+HYAFF		85838	640
	IN	n.d.	n.d.
		255575	1280
		263413	1280
		136493	640

n.d.=not done.

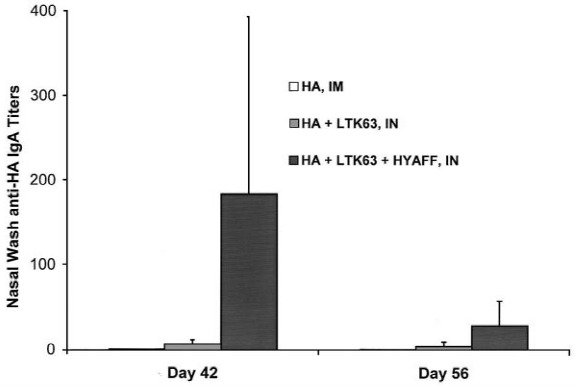

Fig. 6. Anti-HA nasal IgA antibody titers in three groups of micro-pigs ($n=4$) immunized with either HA alone IM, HA+LTK63 IN or HA+LTK63+HYAFF IN. The graph represents the geometric mean titers±S.E.M. for each group at days 42 and 56. The HYAFF+LTK63+HA formulation was significantly better than the HA (IM) and HA+LTK63 (IN) at days 42 and 56 ($P<0.05$).

formulation administration, a situation that does not accurately reflect an approach that would prove acceptable in humans. Hence it was considered necessary to further evaluate the bioadhesive formulations in more rigorous studies in larger animal models. In the mouse study, the bioadhesive microspheres alone did not provide an adjuvant effect and the presence of the LT mutant was necessary to achieve potent immune responses following intranasal administration. Therefore, the bioadhesive formulation alone with HA, without the addition of LT mutants, was not evaluated further. The poor response to the bioadhesive formulation alone delivered mucosally is consistent with observations from an earlier study, in which only a marginal enhancement was seen with mucosal immunization with bioadhesive microspheres and mucosal delivery was not comparable with IM immunization [31]. In contrast, an alternative bioadhesive polymer, chitosan, which is a widely used pharmaceutical excipient [32], has shown encouraging results following IN immunization in a small animal model [33]. Nevertheless, chitosan and related molecules have previously been described as potent adjuvants or immunomodulatory compounds following parenteral immunization [34]. Therefore, the adjuvant effect with IN chitosan may not come solely from the bioadhesive properties of this polymer. It remains to be seen if simple 'bioadhesion' is enough to impart a potent adjuvant effect following mucosal delivery, but our data would seem to indicate that inclusion of an adjuvant active molecule may also be necessary to induce a potent response.

In the rabbit studies, the observations from the mouse model were extended to a larger animal and the second LT mutant, LTR72 was also evaluated. In previous studies, LTR72 was shown to be a more effective mucosal adjuvant in mice than LTK63 for a model protein [15]. In addition, studies in mice with HA have shown that IN HA+LTR72 was capable of

Table 2
Hemagglutination-inhibition (HI) titers and serum IgG titers at day 42 from individual animals in three groups of micro-pigs immunized with either HA alone IM, HA+LTK63 IN or HA+LTK63+HYAFF IN

Formulation	Route	Serum IgG ELISA titers (Day 42)	Hemagglutination inhibition titers (Day 42)
HA alone	IM	137	80
		2668	640
		273	160
		589	160
HA+LTK63	IN	97	40
		1499	1280
		86	320
		984	640
HA+LTK63+HYAFF	IN	1908	1280
		889	320
		1764	640
		485	2560

inducing more potent serum immune responses than IM immunization with HA at similar dose [9]. Therefore, LTR72 was evaluated in the first rabbit study, which showed a clear demonstration of the benefit of delivering the HA-LTR72 combination in a bioadhesive microsphere delivery system. This study also served to confirm the significant potential of LTR72 as an IN adjuvant for HA, both with and without the HYAFF. Since the LTR72 bioadhesive formulation was very potent in rabbits, we also decided to evaluate the LTK63 bioadhesive formulation that had previously performed so well in the mouse study. In addition, we also decided to compare the IN bioadhesive formulation with the traditional approach to immunization, using HA alone by the IM route. In a small study, involving two groups of rabbits, the HYAFF-HA-LTK63 combination formulation was compared to IM immunization with a similar dose of HA. Both the serum IgG and the serum HI titers induced by IN immunization in this study were comparable, or greater than those induced by IM immunization. These observations encouraged us to continue with this formulation approach into a larger animal model, the pig. The omnivorous pig has all the components of the ring of lymphoid tissue, the Waldeyer's ring, which are found in humans and therefore represents a good animal model to evaluate intranasal immunization approaches [27].

For the pig study, we continued to use the LTK63 adjuvant, since it is completely non-toxic both in vitro and in vivo [16] and therefore, is the preferred candidate for subsequent human studies. In the pig study, we undertook a rigorous evaluation of the combination bioadhesive formulation and compared the responses obtained IN, with those obtained after IM immunization with the same dose of HA. Very encouragingly, IN immunization with soluble HA+LTK63 induced comparable serum IgG antibody responses to IM immunization with an equivalent dose of HA. This observation further confirmed the significant potential of LTK63 as a mucosal adjuvant and extended our earlier observations in mice [9,14–17] to a larger animal. Furthermore, the bioadhesive HYAFF-HA-LTK63 formulation induced a significantly enhanced serum IgG antibody response in comparison to IM immunization with HA. The bioadhesive formulation also induced a significantly enhanced nasal wash IgA antibody response. In addition, despite significant variability amongst individual pigs, there was a clear trend for the bioadhesive formulation to also induce enhanced HI titers over IM immunization.

The observations from these studies are notable in several ways. Firstly, they serve to illustrate the potency of the LT mutants as IN adjuvants for HA in three animal models. Studies already published [9,14–17] and many unpublished observations (O'Hagan et al., in press) have shown that these mutants are potent mucosal adjuvants for a wide range of immunogens, to include recombinant proteins, protein polysaccharide conjugates, peptides and DNA, when delivered by several different routes. In a previous study, we compared a range of different antigen delivery systems and adjuvants, including microparticles and Iscom's, for IN delivery of a recombinant protein and showed that LTK63 was the most potent adjuvant for induction of serum immunity [16]. In addition, the results described here show that the potency of the HA+LT mutant combination can be enhanced by formulation into a bioadhesive microsphere delivery system. IN immunization with the bioadhesive microsphere formulation in pigs induced a significantly enhanced serum immune response in comparison to traditional IM immunization. Since several flu vaccines are already commercially available, if they are eventually to be replaced by new vaccines administered by the IN route, then the new vaccine must induce at least a similar level of serum immunity. This was achieved in the current studies in both rabbits and pigs. In addition, in the pig study, the IN approach also induced a significant IgA response in the nasal cavity, which might help to protect against initial infection. Although not evaluated in the current studies, IN immunization with LT mutants has previously been shown to induce potent CTL responses, which should also help with viral clearance mechanisms ([17] and unpublished data). It was also notable that in the current studies, for easy administration to all animal models, the microsphere formulations were used as suspensions in saline. The bioadhesive properties would be expected to be enhanced if the formulations were administered as dry powders, and this may have increased their potency further.

Although extensively investigated for many years, the mechanism of action of most vaccine adjuvants, including bacterial toxins remains poorly defined [35], although the toxins have been shown to induce a wide range of potent changes in immune cells [36]. Nevertheless, it appears likely that mutant toxins such as LTK63 may exert some of their adjuvant effects intracellularly due to their interaction with vesicular transport systems [36]. However, extensive in vitro work both on the adjuvant and on the delivery system will be required to accurately determine the mechanism of action of the combined formulation. The bioadhesive microspheres may contribute to the immune response obtained due to one or more of the following reasons; (a) increased duration of retention in the nasal cavity, (b) greater interaction with the epithelium, (c) enhanced absorption, or (d) sustained release from the microspheres. Each of these effects may act upon the antigen, the adjuvant, or both. Although further studies are necessary to determine the mechanism of action of the formulation, it is notable that the microspheres alone were ineffective and the presence of a mucosal adjuvant was necessary for potent responses. It is planned that the bioadhesive formulations described in the current studies will be evaluated in human clinical trials in the near future.

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References

[1] Y. Ghendon, The immune response of humans to live and inactivated influenza vaccines, Adv. Exp. Med. Biol. 257 (1989) 37–45.
[2] M.A. Riddiough, J.E. Sisk, J.C. Bell, Influenza vaccination: cost-effectiveness and public policy, J. Am. Med. Assoc. 249 (1983) 3189–3195.
[3] P.W. Glezen, Serious morbidity and mortality associated with influenza epidemics, Epidemiol. Rev. 4 (1982) 25–44.
[4] D.C. Powers, S.D. Sears, B.R. Murphy, B. Thumar, M.L. Clements, Systemic and local antibody responses in elderly subjects given live or inactivated influenza A virus vaccines, J. Clin. Microbiol. 27 (1989) 2666–2671.
[5] J. Treanor, G. Dumyati, D. O'Brien, M.A. Riley, G. Riley, S. Erb, R. Betts, Evaluation of cold-adapted, reassortant influenza B virus vaccines in elderly and chronically ill adults, J. Infect. Dis. 169 (1994) 402–407.
[6] M.L. Clements, I. Stephens, New and improved vaccines against influenza, in: M.M. Levine, G.C. Woodrow, J.B. Kaper, G.S. Cobon (Eds.), New Generation Vaccines, 2nd Edition, Marcel Dekker Inc, New York, 1997, pp. 545–570.
[7] B.S. Bender, M.P. Johnson, P.A. Small, Influenza in senescent mice: impaired cytotoxic T-lymphocyte activity is correlated with prolonged infection, Immunology 72 (1991) 514–519.
[8] S.M. Michalek, D.T. O'Hagan, S. Gould-Fogerite, G.F. Rimmelzwaan, A.D.M.E. Osterhaus, Antigen delivery systems: Nonliving microparticles, liposomes, cochleates and iscans, in: P.L. Ogra, J. Mesteacy, M.E. Lamm, W. Strober, J. Bienenstock, J.R. McGhee (Eds.), Mucosal Immunology, 2nd Edition, Academic Press, San Diego, 1999, 759–778.
[9] J.D. Barackman, G. Ott, D.T. O'Hagan, Intranasal immunization in mice with influenza vaccine in combination with the adjuvant LTR72 induces potent mucosal and serum immunity, which is stronger than that with traditional intramuscular immunization, Infect. Immun. 67 (1999) 4276–4279.
[10] C.O. Elson, Cholera toxin as a mucosal adjuvant, in: C.O. Elson, H. Kiyono, P.L. Ogra, J.R. McGhee (Eds.), Mucosal Vaccines, Academic Press, New York, 1996, pp. 59–72.
[11] S. Tamura, H. Asanuma, Y. Ito, Y. Hirabayashi, Y. Suzuki, T. Nagamine, C. Aizawa, T. Kurata, A. Oya, Superior cross-protective effect of nasal vaccination to subcutaneous inoculation with influenza haemagglutinin vaccine, Eur. J. Immunol. 22 (1992) 477–481.
[12] S. Tamura, Y. Ito, H. Asanuma, Y. Hirabayashi, Y. Suzuki, T. Nagamine, C. Aizawa, T. Kurata, Cross-protection against influenza virus infection afforded by trivalent inactivated vaccines inoculated intranasally with cholera toxin B subunit, J. Immunol. 149 (1992) 981–988.
[13] S. Tamura, H. Asanuma, T. Tomita, K. Komase, K. Kawahara, H. Danbara, N. Hattori, K. Watanabe, Y. Suzuki, T. Nagamine, C. Aizawa, A. Oya, T. Kurata, Escherichia coli heat-labile enterotoxin B subunit supplemented with a trace amount of the holotoxin as an adjuvant for nasal influenza vaccine, Vaccine 12 (1994) 1083–1089.
[14] G. Douce, M. Fontana, M. Pizza, R. Rappuoli, G. Dougan, Intranasal immunogenicity and adjuvanticity of site-directed mutant derivatives of cholera toxin, Infect. Immun. 65 (1997) 2821–2828.
[15] M.M. Giuliani, G. Del Giudice, V. Giannelli, G. Dougan, G. Douce, R. Rappuoli, M. Pizza, Mucosal adjuvanticity and immunogenicity of LTR72, a novel mutant of *Escherichia coli* heat-labile enterotoxin with partial knock-out of ADP-

ribosyltransferase activity, J. Exp. Med. 187 (1998) 1123–1132.
[16] M. Ugozzoli, D.T. O'Hagan, G.S. Ott, Intranasal immunization of mice with herpes simplex virus type 2 recombinant gD2: the effect of adjuvants on mucosal and serum antibody responses, Immunology 93 (1998) 563–571.
[17] C.D. Parlidos, M. Pizza, R. Rappuoli, M.W. Steward, The adjuvant effect of a non-toxic mutant of heat-labile enterotoxin of *Escherichia coli* for the induction of measles virus-specific CTL responses after intranasal co-immunization with a synthetic peptide, Immunology 89 (1996) 483–487.
[18] J.D. Smart, I.W. Kellaway, H.E. Worthington, An in-vitro investigation of mucosa-adhesive materials for use in controlled drug delivery, J. Pharm. Pharmacol. 36 (1984) 295–299.
[19] D. Duchene, F. Touchard, N.A. Peppas, Pharmaceutical and medical aspects of bioadhesive systems for drug administration, Drug Dev. Ind. Pharm. 14 (1988) 283–318.
[20] C.-M. Lehr, From sticky stuff to sweet receptors — achievements, limits and novel approaches to bioadhesion, Euro. J. Drug Metab. Pharmac. 21 (1996) 139–148.
[21] J.M. Gu, J.R. Robinson, H.S. Leung, Binding of acrylic polymers to mucin/epithelial surfaces: structure–property relationships, Crit. Rev. Ther. Drug. Carrier Sys. 5 (1988) 21–67.
[22] C. Davide, D. Patrick, M. Radice, B. Paola, A. Giovanni, D.F. Williams, Semisynthetic resorbable materials from hyaluronan esterification, Biomaterials 19 (1998) 2101–2127.
[23] L. Illum, N.F. Farraj, A.N. Fisher, I. Gill, M. Miglietta, L. Benedetti, Hyaluronic acid ester microspheres as a nasal delivery system for insulin, J. Control. Release 29 (1994) 133–141.
[24] J. Richardson, P.A. Ramires, M.R. Miglietta, M. Rochira, L. Bacella, L. Callegaro, L. Benedetti, Novel vaginal delivery systems for calcitonin: I Evaluation of HYAFF/calcitonin microspheres in rat, Int. J. Pharmaceutics 115 (1995) 9–15.
[25] L. Illum, N.F. Farraj, B.R. Johansen, S.S. Davis, D.T. O'Hagan, Preliminary investigations on the nasal absorption of biosynthetic human growth hormone-use of a bioadhesive microsphere delivery system, Int. J. Pharm. 63 (1990) 207–211.
[26] L. Benedetti, E.M. Topp, V.J. Stella, Microspheres of hyaluronic acid esters — fabrication methods and in vitro hydrocortisone release, J.Control. Release 13 (1990) 33–41.
[27] M.E. Perry, Y. Mustafa, S.T. Licence, D. Smith, A. Whyte, Pig palatine tonsil as a functional model for human, Clin. Anat. 10 (1997) 358a.
[28] R. Johannsen, H. Moser, J. Hinz, H.J. Freisen, H. Gruschkau, Quantitation of haemagglutinin of influenza tween-ether split vaccines by immunodiffusion, Vaccine 3 (1985) 235–240.
[29] J.C. Hierholzer, M.T. Suggs, Standardized viral hemagglutination and hemagglutination-inhibition tests: I Standardization of erythrocyte suspensions, Appl. Microbiol. 18 (1969) 816–823.
[30] J.C. Hierholzer, M.T. Suggs, E.C. Hall, Standardized viral hemagglutination and hemagglutination-inhibition tests: II Description and statistical evaluation, Appl. Microbiol. 18 (1969) 824–833.
[31] D.T. O'Hagan, D. Rafferty, S. Wharton, L. Illum, Intravaginal immunization in sheep using a bioadhesive microsphere antigen delivery system, Vaccine 11 (1993) 660–664.
[32] L. Illum, Chitosan and its use as a pharmaceutical excipient, Pharm. Res. 15 (1998) 1326–1331.
[33] I. Jabbal-Gill, A.N. Fisher, R. Rappuoli, S.S. Davis, L. Illum, Stimulation of mucosal and systemic antibody responses against Bordetella pertussis filamentous hemagglutinin and recombinant pertussis toxin after nasal administration with chitosan in mice, Vaccine 16 (1998) 2039–2046.
[34] I. Azuma, Synthetic immunoadjuvants: application to nonspecific host stimulation and potentiation of vaccine immunogenicity, Vaccine 10 (1992) 1000–1006.
[35] M. Singh, D.T. O'Hagan, Advances in vaccine adjuvants, Nat. Biotech. 17 (1999) 1075–1081.
[36] R. Rappuoli, M. Pizza, G. Douce, G. Dougan, Structure and mucosal adjuvanticity of cholera and *Escherichia coli* heat-labile enterotoxins, Immunol. Today 20 (1999) 493–500.