

Oral Administration of Influenza Vaccine in Combination with the Adjuvants LT-K63 and LT-R72 Induces Potent Immune Responses Comparable to or Stronger than Traditional Intramuscular Immunization

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Mucosal immunization strategies are actively being pursued in the hopes of improving the efficacy of vaccines against the influenza virus. Our group investigated the oral immunization of mice via intragastric gavage with influenza hemagglutinin (HA) combined with mutant *Escherichia coli* heat-labile enterotoxins K63 (LT-K63) and R72 (LT-R72). These oral immunizations resulted in potent serum antibody and HA inhibition titers, in some cases stronger than those obtained with traditional intramuscular administration, in addition to HA-specific immunoglobulin A in the saliva and nasal secretions. This study demonstrates that it may be possible to develop effective oral influenza vaccines.

Influenza is a serious human disease exhibiting high mortality in vulnerable populations such as the very young and the very old, as well as causing significant morbidity in the general population (17). The social and economic costs associated with yearly influenza outbreaks are high (7). Formalin-inactivated whole-virus and split-virus vaccines administered intramuscularly (i.m.) are commercially available to control the spread and severity of influenza (15, 38). These prophylactic vaccines, although important agents in controlling influenza, suffer from a number of shortcomings that limit their efficacy and acceptability. Notably, inactivated whole-virus and split-virus vaccines are known to activate CD8⁺ cytotoxic T-lymphocyte responses only sporadically, have poor cross-reactivity to antigenic variants, and produce poor secretory immunoglobulin A (IgA) responses (4, 7, 17, 24, 34, 36). In addition, injection site reactogenicity and weak immune responses can be a problem in very young children (18, 19). Significant efforts are currently being pursued to improve the vaccines' efficacy and tolerability primarily through the development of mucosally active influenza vaccines (2, 7, 10, 33, 40). Oral immunization is considered by many to be a highly desirable form of vaccination, although numerous obstacles make oral immunization using subunit antigens a significant challenge (3, 6, 11). Many approaches have been investigated to develop viable orally active influenza vaccines (3, 21, 29, 30). Mucosal adjuvants, primarily *Escherichia coli* heat-labile enterotoxin (LT) and cholera toxin (CT), are the most commonly employed vaccine enhancers (11, 12). Although potent mucosal adjuvants, LT and CT are toxic in humans at doses useful for adjuvanticity due to their ADP-ribosyltransferase activity (28). The nontoxic B subunit of CT (CTB) has also been investigated; however, studies have indicated that small amounts of the whole CT are required for sufficient adjuvant potency, inhibiting the potential of CTB in

humans (44, 45, 46). Our group has investigated the mutant LT toxins LT-K63 and LT-R72, which demonstrate extremely low (LT-R72) to undetectable (LT-K63) levels of ADP-ribosyltransferase activity yet maintain potent mucosal adjuvant activity, demonstrating that ADP-ribosyltransferase activity may not be linked to the adjuvant activity (2, 13, 16). In this study, the influenza hemagglutinin (HA) antigens A/Beijing8-9/93 HA and A/Johannesburg/97 HA were administered orally in mice with LT-K63 and LT-R72 and the results were compared to those obtained with i.m. immunization for induction of serum antibody and mucosal IgA responses as well as serum HA inhibition titers. Dosing studies were conducted to determine the optimum dose levels of both antigen and adjuvant.

Vaccines used. Purified monovalent A/Beijing8-9/93 (H3N2) and A/Johannesburg/97 (H1N1) split-virus influenza antigens were provided by Chiron Vaccines, Siena, Italy. Dosing was based on HA content as assayed by single radial immunodiffusion as described previously (25). LT-K63 and LT-R72 were prepared as described previously (35). Wild-type LT (wtLT) was obtained from Sigma (*Escherichia coli* heat-labile enterotoxin, lyophilized powder; Sigma-Aldrich, St. Louis, Mo.). All immunogen preparations were formulated in phosphate-buffered saline. Immunogens prepared for intragastric gavage (i.g.) administration included 1.5% (wt/vol) sodium bicarbonate.

Immunization and sample collection. Groups of 10 female BALB/c mice (Charles River Labs, Wilmington, Mass.), 6 to 10 weeks old, were i.m. or i.g. immunized at days 0, 21, and 35 using immunogen preparations as described below. Mice were fasted 12 h prior to each immunization to minimize the possibility of lectins (or other agents) in the feed from inhibiting uptake of the orally delivered immunogens (9). Immunizations were made either by i.m. injection (50 μ l) into the posterior thigh muscle or by direct i.g. (200 μ l) into the stomach using a 20-gauge stainless steel feeding needle attached to a 1-ml syringe. Animals were not anesthetized during immunizations. Serum, saliva wash (SW), and nasal wash (NW) samples were collected from individual animals 2 weeks after the final immunization (day 49) using methods described previously (47).

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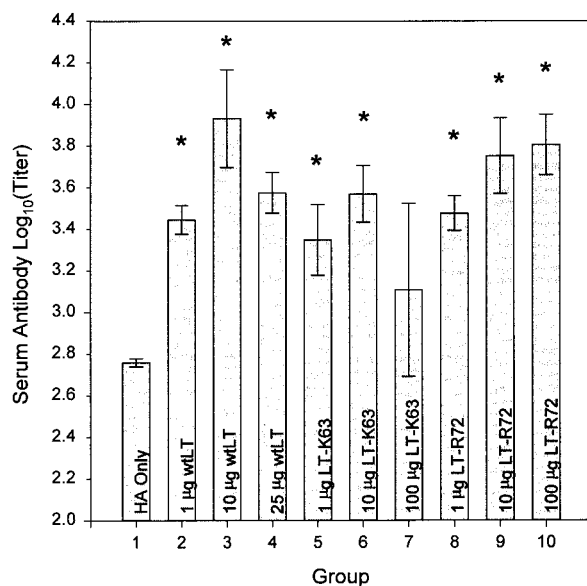


FIG. 1. Comparison of the effects of enterotoxin doses on antigen-specific serum antibody responses after i.g. administration. Shown are means \pm SE of anti-A/Beijing8-9/93 HA antibody titers in the sera of mice immunized with 20- μ g doses of A/Beijing8-9/93 HA antigen either alone (HA only) or in combination with wtLT, LT-K63, and LT-R72 as indicated. Asterisks indicate groups whose values are significantly greater than that of the HA only group ($P \leq 0.05$).

Antibody ELISA. Serum samples from individual animals were assayed for total anti-HA Ig (IgG plus IgA plus IgM) titers by a 3,3',5,5'-tetramethylbenzidine-based colorimetric enzyme-linked immunosorbent assay (ELISA) as previously described, with A/Beijing8-9/93 or A/Johannesburg/97 as appropriate as coating antigen (20). A_{490} was measured 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. SW and NW samples from individual animals were assayed for HA-specific IgA titers using a bioluminescence immunosorbent assay as previously described, with A/Beijing8-9/93 or A/Johannesburg/97 as appropriate as coating antigen (47). The goat anti-mouse IgA biotin conjugate (EY Labs, San Mateo, Calif.) used was presaturated with purified mouse IgG (Sigma Chemical Company, St. Louis, Mo.) to reduce cross-reactivity. Quantitation was based on the number of relative light units representing total luminescence integrated over 3 s (arbitrary units). Titers represent log dilution values linearly extrapolated from the log of the relative light units to a cutoff value at least 2 standard deviations above mean background.

HI assay. Serum samples pooled by group were assayed for hemagglutination inhibition (HI) titer by the Viral and Rickettsial Disease Laboratory (Department of Health Services, Berkeley, Calif.). The HI assay is based on the ability of sample sera to inhibit the agglutination of goat erythrocytes in the presence of HA antigen. The resulting titers are expressed as the reciprocal dilution required for complete inhibition (22, 23).

Statistics. Log anti-A/Beijing8-9/93 and anti-A/Johannesburg/97 HA serum Ig, saliva IgA, and nasal IgA titers from individual animals were analyzed for differences between test

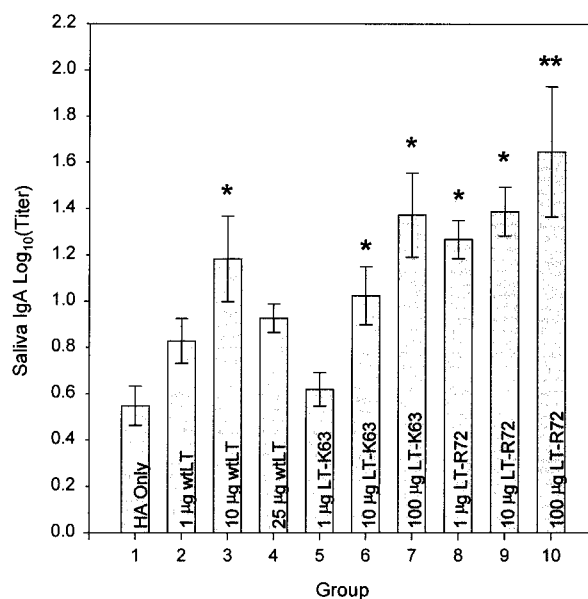


FIG. 2. Comparison of the effects of enterotoxin doses on antigen-specific SW IgA responses after i.g. administration. Shown are means \pm SE of anti-A/Beijing8-9/93 HA SW IgA antibody titers of groups of mice immunized with 20- μ g doses of A/Beijing8-9/93 HA antigen either alone (HA only) or in combination with enterotoxins as indicated. Asterisks indicate groups whose values are significantly greater than that of the HA only group ($P \leq 0.05$). Double asterisks indicate a value that is significantly greater than that of the groups immunized with 10 and 25 μ g of wtLT (groups 3 and 4), in addition to that of the HA only group ($P \leq 0.05$).

groups using a Fisher least-significant-difference procedure using a statistical significance of $>5\%$ ($P \leq 0.05$) as the cutoff interval (1). Additionally, the resulting data were graphically represented as mean titers \pm standard errors (SE) in the usual manner.

Effects of enterotoxin types and doses on antibody responses after i.g. immunization. A dose-ranging study was conducted to determine the dose-response relationship for LT-K63 and LT-R72 for i.g. immunization with A/Beijing8-9/93 HA. Groups of 10 mice were immunized by the i.g. route with 20 μ g of A/Beijing8-9/93 HA in combination with three dose levels of wtLT (1, 10, and 25 μ g), LT-K63 (1, 10, and 100 μ g), and LT-R72 (1, 10, and 100 μ g). Significant mortality in the mice subjected to high doses of wtLT limited the upper dose of wtLT to 25 μ g. An unadjuvanted A/Beijing8-9/93 HA control group (HA only) was immunized by the i.g. route at the highest dose level (20 μ g) for comparison purposes.

Serum antibody responses (Fig. 1) were significantly higher in most cases in animals that received A/Beijing8-9/93 HA in combination with either of the enterotoxins tested than in animals that received unadjuvanted A/Beijing8-9/93 HA. A dose response was not clearly demonstrated, although, in general, groups that received A/Beijing8-9/93 HA in combination with LT-R72 showed serum antibody responses comparable to those of groups receiving A/Beijing8-9/93 HA in combination with wtLT.

A clearer adjuvant dose response was found in the antigen-specific saliva IgA responses (Fig. 2). Significantly stronger saliva IgA responses were demonstrated for all but one of the

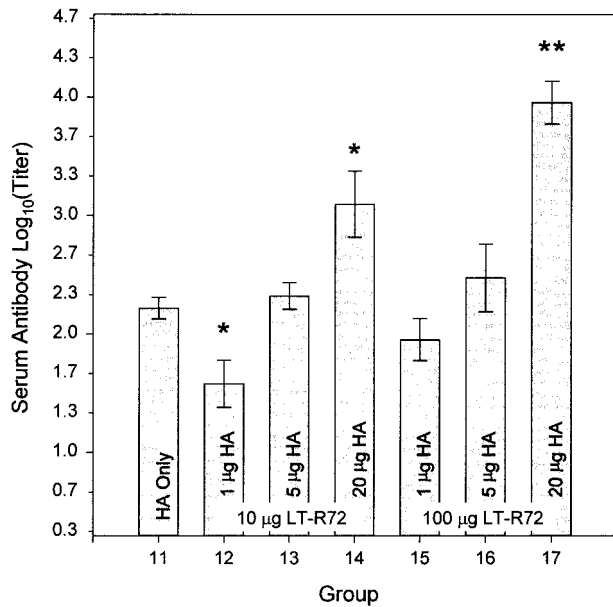


FIG. 3. Comparison of the effects of HA doses on antigen-specific serum antibody responses after i.g. administration. Shown are means \pm SE of anti-A/Beijing8-9/93 HA antibody titers in the sera of mice immunized either with 20 μ g of HA administered alone (HA only) or with 1-, 5-, or 20- μ g doses of A/Beijing8-9/93 HA in combination with either 10 or 100 μ g of LT-R72 as indicated. Asterisks indicate groups whose values are significantly different from that of the HA only group ($P \leq 0.05$). Double asterisks indicate a value that is significantly greater than that of all other groups shown ($P \leq 0.05$).

LT-K63- and LT-R72-adjuvanted groups than for animals that received A/Beijing8-9/93 HA alone. Additionally, animals dosed i.g. with 20 μ g of A/Beijing8-9/93 HA in combination with 100 μ g of LT-R72 were found to have an antigen-specific saliva IgA response significantly higher ($P \leq 0.05$) than that of animals dosed i.g. with either 10 or 25 μ g of wtLT.

Effects of A/Beijing8-9/93 HA dose on antibody responses at two dose levels of LT-R72. A second dose-ranging study was conducted to determine the optimum dose of A/Beijing8-9/93 HA for i.g. immunization when adjuvanted with LT-R72. Groups of 10 mice were immunized by the i.g. route with three dose levels of A/Beijing8-9/93 HA (1, 5, and 20 μ g) in combination with either 10 or 100 μ g of LT-R72. An unadjuvanted A/Beijing8-9/93 HA control group (HA only) was immunized at the highest dose level (20 μ g) for comparison purposes.

The antigen-specific serum antibody responses (Fig. 3) demonstrated a dose-response trend with respect to the dose level of A/Beijing8-9/93 and the dose level of LT-R72 with which the animals were immunized. Serum antibody responses were significantly higher ($P \leq 0.05$) in animals immunized i.g. with 20- μ g doses of A/Beijing8-9/93 HA in combination with either 10 or 100 μ g of LT-R72 than in the unadjuvanted HA control group. The group that received the highest dose level tested (20 μ g of A/Beijing8-9/93 in combination with 100 μ g of LT-R72) had a significantly higher ($P \leq 0.05$) antigen-specific serum antibody response than those of all other groups tested.

The antigen-specific saliva IgA responses (Fig. 4) matched the trend seen with the serum antibody responses with the exception of the group that received 1 μ g of A/Beijing8-9/93 in

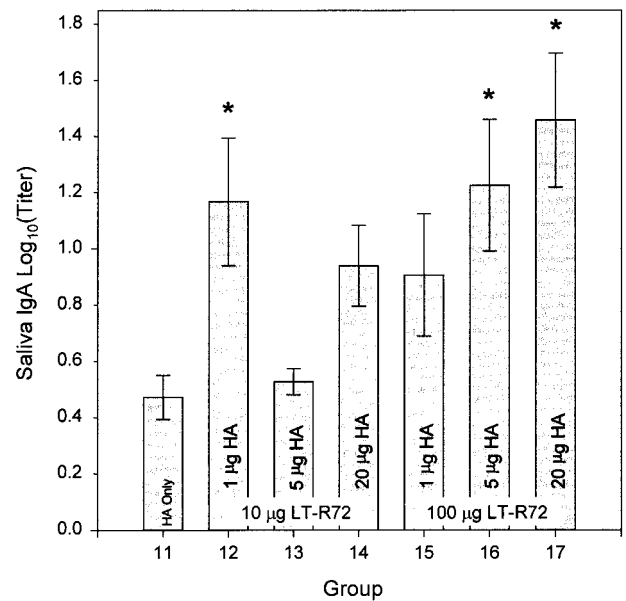


FIG. 4. Comparison of the effects of HA doses on antigen-specific SW IgA responses after i.g. administration. Shown are means \pm SE of anti-A/Beijing8-9/93 HA SW IgA antibody titers of groups of mice immunized either with 20 μ g of HA administered alone (HA only) or with 1-, 5-, or 20- μ g doses of A/Beijing8-9/93 HA in combination with either 10 or 100 μ g of LT-R72 as indicated. Asterisks indicate groups whose values are significantly greater than that of the HA only group ($P \leq 0.05$).

combination with 10 μ g of LT-R72 (group 12). Animals that received either 5 or 20 μ g of A/Beijing8-9/93 HA in combination with 100 μ g of LT-R72 demonstrated a significantly higher ($P \leq 0.05$) antigen-specific saliva IgA response than that of animals that received unadjuvanted A/Beijing8-9/93 HA.

Comparison of i.g. and i.m. immunizations. The serum antibody responses of mice i.g. immunized with A/Johannesburg/97 HA either alone or in combination with an LT were compared to those of mice immunized with A/Johannesburg/97 HA by the i.m. route. Groups of 10 mice were immunized by the i.g. route with 20 μ g of A/Johannesburg/97 HA either alone or in combination with two dose levels of wtLT (1 and 10 μ g), LT-K63 (10 and 100 μ g), or LT-R72 (10 and 100 μ g). A group receiving 1 μ g of A/Johannesburg/97 HA by the i.m. route was included. The 1- μ g HA i.m. dose level was chosen such that, based on data from previous experiments, a strong, protective, immunogenic response in mice would result (data not shown), and it was used here in order to make comparisons with the i.g. responses.

Serum antigen-specific antibody responses (Fig. 5) for mice immunized i.g. with 20 μ g of A/Johannesburg/97 HA adjuvanted with an LT were equivalent to or higher than those for i.m. immunized mice. Mice i.g. immunized with 20 μ g of A/Johannesburg/97 HA either unadjuvanted (HA only) or in combination with 10 μ g of LT-K63 showed serum antigen-specific antibody responses significantly lower ($P \leq 0.05$) than those of mice immunized by the i.m. route; nevertheless, i.g. immunization in the presence of 10 μ g of LT-K63 resulted in antibody responses 1 log higher than those obtained with i.g. immunization with unadjuvanted A/Johannesburg/97 HA. Mice i.g.

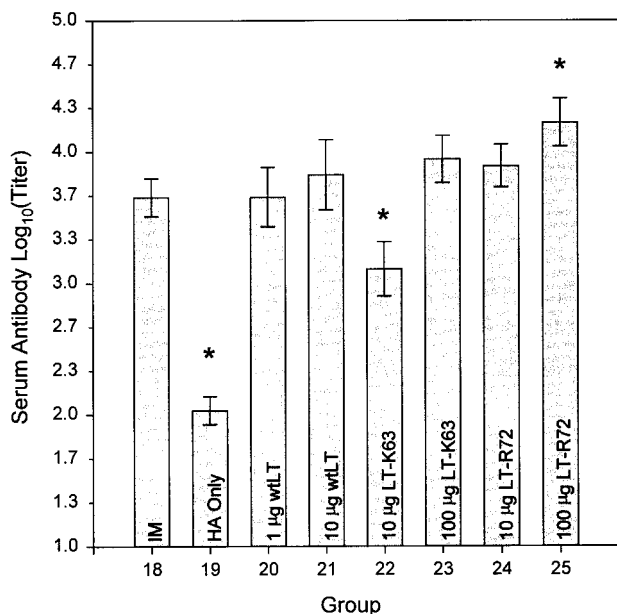


FIG. 5. Comparison of the effects of i.m. and i.g. administrations of A/Johannesburg/97 HA on antigen-specific serum antibody responses. Shown are means ± SE of anti-A/Johannesburg/97 HA antibody titers in the sera of mice immunized i.m. with 1 µg of A/Johannesburg/97 HA unadjuvanted (IM) or immunized i.g. with 20-µg doses of A/Johannesburg/97 HA alone (HA Only) and in combination with either 1 or 10 µg of wtLT, 10 or 100 µg of LT-K63, or 10 or 100 µg of LT-R72 as indicated. Asterisks indicate groups whose values are significantly different from that of the i.m. immunized group ($P \leq 0.05$).

immunized with 20 µg of A/Johannesburg/97 HA in combination with 100 µg of LT-R72 showed serum antigen-specific antibody responses that were significantly ($P \leq 0.05$) higher than those found with i.m. immunization.

Serum HI titers (Fig. 6) for mice i.g. immunized with 20 µg of A/Johannesburg/97 HA in combination with either 10 µg of wtLT or 100 µg of LT-R72 were comparable in potency to those for i.m. immunized mice. Mice that were i.g. immunized with 20 µg of A/Johannesburg/97 HA in combination with either 1 µg of wtLT, 10 µg of LT-R72, or 100 µg of LT-K63 showed modest HI titer levels. Significant HI titers were not demonstrated for mice i.g. immunized with 20 µg of A/Johannesburg/97 HA either alone or in combination with 10 µg of LT-K63.

Antigen-specific NW IgA responses (Fig. 7) were found to be significant only in those mice immunized i.g. with 20 µg of A/Johannesburg/97 HA in combination with either wtLT, LT-K63, or LT-R72. Mice receiving i.m. immunization or immunized i.g. with 20 µg of A/Johannesburg/97 HA alone did not show significant antigen-specific NW IgA responses. No significant differences were seen in the antigen-specific IgA responses as a consequence of enterotoxin dose level or type.

Current commercial influenza vaccines are shown to induce in healthy adult humans serum antibody responses that are protective against viral challenge, but this protective immunity tends to be variable in potency and is relatively short lived, particularly in the elderly and infant populations (7, 15, 24, 34, 38). Mucosal immunization strategies have been extensively investigated as a means to improve the efficacy and duration of influenza vaccination by providing a broader immune response

than that afforded by i.m. immunization (14, 31, 32, 33, 42). Like intranasal immunization, oral immunization has been shown to induce strong secretory IgA responses, improve protective cellular immune responses, and result in significant serum antibody responses as well (5, 14, 26, 29, 31, 32, 43). The secretory IgA responses for oral immunization have been shown for human subjects to be strongest in the urogenital and rectal tracts, and when compared to intranasal immunization, oral immunization has resulted in somewhat muted upper respiratory, nasopharyngeal, and salivary secretory IgA responses (39). These relatively weak upper respiratory IgA responses would seem to be a problem with respect to achieving effective protection against viral challenge against viruses whose primary mode of entry is via the upper respiratory tract (such as influenza). Other studies, however, have shown that there are sufficient local secretory IgA responses, and more importantly, there is evidence of antigen-primed B- and T-cell migration to the upper respiratory sites to induce potent protective immunity (26, 43). Furthermore, oral immunization has been shown to promote memory B-cell maintenance in the bone marrow, a factor that may be important in the development of the persistence of immunity against viral challenge (5). However, to obtain strong immune responses from many antigens, a potent mucosal adjuvant, usually an enterotoxin, must be coadministered (9).

Studies have shown that immune responses to orally immunized antigens were significantly stronger if the antigen by itself had mucosal binding properties or could be made to have mucosal binding properties by chemically coupling to agents with mucoadhesive, lectin, or receptor-binding properties (8, 9, 21, 30). CTB has been used for these purposes with some

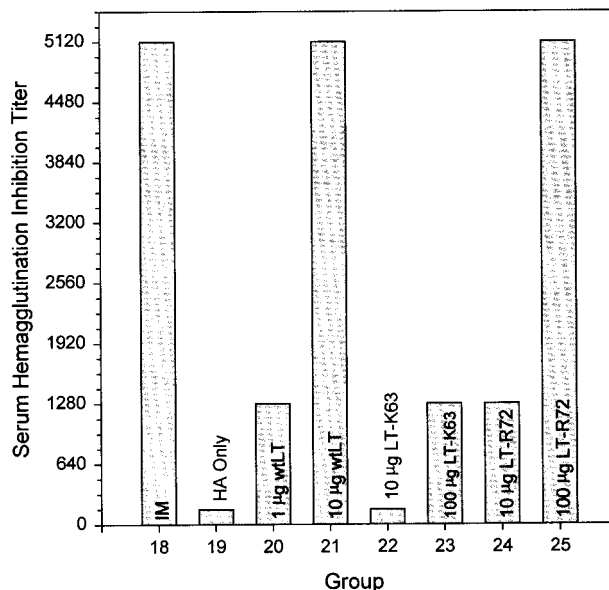


FIG. 6. Comparison of the effects of i.m. and i.g. administrations of A/Johannesburg/97 HA on serum HI titers. The data shown are for pooled sera from groups of mice immunized i.m. with 1 µg of A/Johannesburg/97 HA unadjuvanted (IM), or immunized i.g. with 20-µg doses of A/Johannesburg/97 HA alone (HA Only), and in combination with either 1 or 10 µg of wtLT, 10 or 100 µg of LT-K63, or 10 or 100 µg of LT-R72 as indicated.

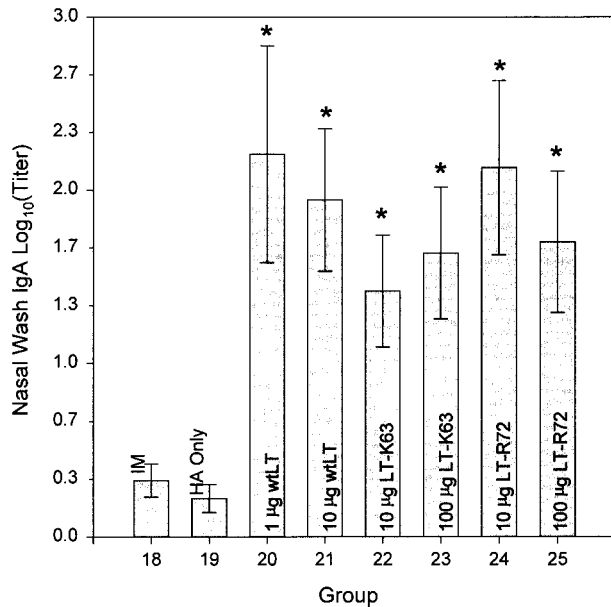


FIG. 7. Comparison of the effects of i.m. and i.g. administrations of A/Johannesburg/97 HA on antigen-specific NW IgA antibody responses. Shown are means \pm SE of anti-A/Johannesburg/97 HA NW IgA antibody titers of mice immunized i.m. with 1 μ g of A/Johannesburg/97 HA unadjuvanted (IM) or immunized i.g. with 20- μ g doses of A/Johannesburg/97 HA alone (HA Only) and in combination with either 1 or 10 μ g of wtLT, 10 or 100 μ g of LT-K63, or 10 or 100 μ g of LT-R72 as indicated. Asterisks indicate groups whose values are significantly greater than that of the i.m. immunized group ($P \leq 0.05$).

success (8, 9). Influenza HA binds neuraminic acid-rich glycoproteins, while LT-R72 and LT-K63 bind GM₁ ganglioside, as well as galactose containing glycoproteins and lipopolysaccharides, all of which ligands are found ubiquitously in the gut (27, 37, 41). Our group has found that antigens that do not have any mucoadhesive or gut-associated binding properties have minimal immunogenicity when delivered orally in mice, other than at very high dose levels, either in the absence of LTs or as mixtures of soluble antigen with soluble LT (unpublished data). With influenza antigens, however, our group and others have shown that modest immune responses occur when reasonable dose levels are delivered orally but substantial and broad immune responses result when the antigens are adjuvanted with LT or CT (26).

Our group has demonstrated here that potent antigen-specific serum antibody titers that are comparable to or stronger than i.m. immunization, as well as modest salivary and nasal IgA responses, can be induced in mice with influenza HA antigens using i.g. immunization by adjuvating with mutant LTs that demonstrate significantly reduced (LT-R72) and unmeasurable (LT-K63) levels of ADP-ribosyltransferase activity (16). The dose level of influenza HA antigen (20 μ g) and mutant LTs (10 to 100 μ g) found necessary for the strongest immune responses may be relatively high. Further formulation efforts are under way in our group to improve the efficiency of oral immunization and, if possible, lower the dose requirements.

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