Seasonal influenza virus infections cause considerable morbidity and mortality in the world, and there is a serious threat of a pandemic influenza with the potential to cause millions of deaths. Therefore, practical influenza vaccines and vaccination strategies that can confer protection against intranasal infection with influenza viruses are needed. In this study, we demonstrate that using LTK63, a nontoxic mutant of the heat-labile toxin from Escherichia coli, as an adjuvant for both mucosal and systemic immunizations, systemic (intramuscular) immunization or combinations of mucosal (intranasal) and intramuscular immunizations protected mice against intranasal challenge with a lethal dose of live influenza virus at 3.5 months after the second immunization.

Infections with seasonal influenza virus strains are a major cause of morbidity and mortality in the world (23, 64). Therefore, there is an urgent need to establish vaccines and immunization protocols that can prevent disease and death caused by both seasonal and the currently looming pandemic influenza virus strains. Commercially available inactivated whole- and split-virus vaccines have been successful to prevent disease caused by influenza virus infection (22, 45). Although systemically administered vaccines are known to induce serum immunoglobulin G (IgG) antibodies, they are generally poor stimulators of secretory IgA at respiratory mucosal sites (10, 23, 43). Thus, influenza vaccines that can generate significant secretory IgA, as well as maintain high serum IgG titers, by exploiting mucosal immunizations are under investigation (15, 18, 37, 55, 62). A licensed live attenuated intranasal (i.n.) vaccine has been in use that is capable of induction of serum IgG and local IgA responses (1, 8). However, the use of live attenuated vaccines, although appropriate for seasonal influenza vaccines, may not be ideal for pandemic influenza virus strains, due to risks of developments of chimeric strains between the vaccine and wild-type strains. Therefore, in the present study, the focus was on nonreplicating purified hemagglutinin (HA) antigen plus adjuvants that can potentially be safely used for both pandemic and seasonal influenza virus strains. Moreover, the use of adjuvants allows for antigen sparing, which is problematic particularly for pandemic influenza virus strains.

Many murine and human studies support the importance of mucosal IgA responses in protection against influenza virus infection and disease (3), even though in murine models a controversial role for mucosal IgA has also been suggested (65). In this regard, of particular importance have been the findings in both murine and human studies that mucosal IgA induced by i.n. immunization, as opposed to serum IgG induced by systemic immunization, protected against multiple strains of influenza virus (4–6, 12, 30, 34, 49–52, 54). Therefore, recent efforts have focused on i.n. immunization strategies that induce both local IgA and systemic IgG responses (27, 30, 34).

The role of i.n. versus systemic immunization and induction of local IgA as opposed to serum IgG in protection against replication in the nose and lungs or in protection from disease is well established (11, 25, 27, 40, 60). Therefore, i.n. immunizations alone or in combination with intramuscular (i.m.) immunization may show superior local and systemic antibody responses. For optimal induction of immune responses through the i.n. route, effective and safe mucosal adjuvants are required. Mutants of the heat-labile enterotoxin (LT) from enteropathogenic Escherichia coli have been shown to be safe in animal and human studies (38). LTK63 is an effective mucosal adjuvant with no detectable toxic ADP-ribosyltransferase activity (38) and holds promise as a mucosal adjuvant.

Mucosal followed by systemic immunizations with Helicobacter pylori-derived protein antigens induced enhanced local and systemic responses compared to mucosal alone, parenteral alone, or parenteral followed by mucosal immunizations (56). Moreover, data from rhesus macaque studies suggested that i.n. immunizations followed by i.m. immunizations significantly enhanced serum and vaginal antibody responses against simian-human immunodeficiency virus challenge (7, 57). More recently, we tested whether the combination of i.n. immunization, followed by i.m. immunization with cell culture-derived HA from two strains of influenza A virus and a strain of influenza B virus, induced enhanced local and systemic immune responses compared to i.m. immunization followed by i.n. immunization, i.m. immunization alone, or i.n. immunization alone. Using LTK63 as an adjuvant for the i.n. immunizations and no adjuvants or delivery systems for the i.m. immunizations, we found that i.n./i.m. immunizations induced enhanced serum hemagglutination inhibition (HI), as well as local and systemic antibody and TH1- and TH2-type cytokine responses (55).

Although LT or cholera toxin (CT) and their mutants have...
been extensively used as mucosal adjuvants, their use as systemic adjuvants has been more limited. CT has been shown as an effective systemic adjuvant in several studies (16, 26, 48, 53, 58). If combinations of mucosal and systemic vaccinations should be used for humans, it would be more practical to use the same vaccine formulation for both mucosal and systemic vaccination regimens. Therefore, establishment of the systemic adjuvanticity of LTK63 as a viable vaccine candidate is important. Thus, in the present study, we sought to determine whether using the exact same formulation for both i.n. and i.m. immunizations, with LTK63 as the mucosal and systemic adjuvant, enhanced immune responses or protection against i.n. challenge at 3.5 months after the final i.m., i.n., or i.n./i.m. immunizations could be achieved. The kinetics of serum IgG, IgG1, IgG2a, IgA, and cytokines were monitored after immunizations and challenge. Moreover, the protective efficacy was established by measuring weights and survival rates, as well as serum HI titers.

MATERIALS AND METHODS

Animals, immunizations, and challenge. Female BALB/c mice (six or eight animals per group) were immunized with cell culture-derived HA antigen from the New Caledonia H1N1 strain. The animals were immunized via two i.m. immunizations (i.m./i.m.), two i.n. immunizations (i.n./i.n.), one i.m. immunization followed by one i.m. immunization (i.m./i.m.), or one i.n. immunization followed by one i.m. immunization (i.m./i.m.) at a 28-day interval. The HA doses were 1 μg, whereas the LTK63 dose was 5 μg. For both i.n. and i.m. immunizations LTK63 was used as the adjuvant. Sera were collected at 2 days after the second immunization (2dp2), 2 weeks after the second immunization (2wp2), 4wp2, 8wp2, 13wp2, 1 day postchallenge (dpchall), and 3dpchall. Nasal washes were collected by holding the head in an upright position so that one nostril faced upward and applying 0.5 ml of phosphate-buffered saline (PBS) through the nostril pointing upward and collecting the wash from the other nostril. The nasal washes were snap-frozen on dry ice and stored at −80°C until use.

For challenge, 10 mice were used for each immunization group. One week after the final immunization, 10 μl of virus solution containing 150 mouse 50% lethal doses (mLD₅₀) of the A/WS/33 (H1N1) influenza virus strain was inoculated into each nostril (300 mLD₅₀/mouse) without anesthesia. The survival and weight loss of the challenged mice was monitored for 14 days after infection.

Standard colorimetric and Europium-based fluorescent ELISA. HA-specific serum IgG1 and IgG2a titers against H1N1 influenza virus strain were quantified by using a standard enzyme-linked immunosorbent assay (ELISA). Briefly, ELISA plates (96-well U bottom plates; Nunc Maxisorp) were coated with 1 μg and 10 μg of HA/well in PBS overnight at 4°C. After a wash with 1× PBS plus 0.05% Tween 20 (Sigma), the wells were blocked with PBS–2% fetal calf serum for 30 min (min) at 37°C, and serum samples were added in an initial dilution of 1:50, after which 1:3 serial dilutions performed in the blocking reagent. A standard serum was included in each assay as positive control. The samples and standard sera were incubated at 4°C overnight and washed with PBS–0.05% Tween. The plates were washed, and biotinylated anti-mouse IgG, IgG1, IgG2a, and IgA antibodies (Southern Biotechnology Associates, Alabama) were added at 1:8,000 in PBS–2% fetal calf serum. The plates were then incubated for 2 h at room temperature. The plates were then washed, and avidin-horseradish peroxidase (Pharmingen) at 1:1,000 was added, followed by incubation at 37°C for 30 min. The plates were washed and developed with tetramethylbenzidine (Kirkegaard and Perry) for 15 min and then stopped with 2 N HCl. The optical density (OD) was read on a Wallac Victor 1420 Multilabel fluorescence reader at 616 nm. The data were presented as mean titers from two subgroups and two experiments plus the standard deviation.

HI assay. Serum samples collected after the final immunization were assayed for HI titer according to standard protocols published previously (28).

Multiple Lumines assay. Total gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α; TH1), and interleukin-5 (IL-5) and IL-13 (TH2) concentrations were measured simultaneously in serum at various time points as indicated in Results, using the Multiplex Lumines assay, according to manufacturer’s protocol (Upstate/Millipore, Billerica, MA).

Statistical analysis. To determine statistically significant differences between immunization groups, a parametric one-way analysis of variance test was used to measure statistically significant differences between the groups with unpaired samples (Minitab Release 14.20 software; Minitab, Inc., State College, PA). To determine statistically significant differences in the same group before and after immunizations or challenge, a Student t test was used (Microsoft Excel software package).

RESULTS

Systemic immunizations with LTK63 as adjuvant enhance anti-HA serum antibody responses. LTK63 has been widely used as an effective mucosal adjuvant. In the present study, our aim was to use the same vaccine formulation for both mucosal and systemic immunizations. Thus, to determine whether LTK63 could also be used as a systemic adjuvant, mice were immunized i.m. with HA mixed with LTK63. We found that LTK63 significantly enhanced both serum IgG1 (Fig. 1; P < 0.05) and IgG2a antibody (Fig. 1; P < 0.01) responses, compared to immunizations with HA alone, at 2 weeks after the second immunization. These data show that LTK63 was an effective adjuvant for systemic immunizations.

Kinetics of serum IgG, IgG2a, and IgG1 antibody responses after systemic and/or mucosal immunizations. The generation and maintenance of antibody responses is a major goal of vaccination. To determine the generation and maintenance of serum antibody responses after immunizations with HA + LTK63,
mice were by immunized by the i.m./i.m., i.n./i.n., i.n./i.m., or i.m./i.n. protocols described above. We then measured serum IgG, IgG2a, IgG1, and IgA responses beginning 3 days after the second immunization (3dp2), through 13 weeks after the second immunization (13wp2), and at 1dpchall and 3dpchall after i.n. challenge with the homologous mouse adapted H1N1 live influenza virus. i.m./i.m. immunizations induced significantly higher levels of serum IgG responses than all other immunization groups (except for the i.m./i.n. group at 3dp2), beginning at 3dp2 and through 13wp2 (Fig. 2A; \( P < 0.001 \)).

The i.n./i.n. group induced the lowest serum antibody responses throughout the same period. The i.n./i.m. and i.m./i.n. immunizations induced intermediate serum IgG responses, which were lower than for i.m./i.m. immunizations and higher than for i.n./i.n. immunizations (Fig. 2A; \( P < 0.01 \)). However, whereas at 3dp2, i.m./i.n. immunizations induced significantly higher serum IgG responses compared to i.n./i.m. immunizations, the responses were similar at 2wp2, 4wp2, and 13wp2 (Fig. 2A). At 8wp2, however, i.n./i.m. immunizations induced higher serum IgG responses than i.m./i.n. immunizations (Fig. 2A; \( P < 0.05 \)). Importantly, at 1dpchall and 3dpchall i.m./i.m., i.n./i.m., and i.m./i.n. immunizations induced similar serum IgG responses, which were higher than those seen with the i.n./i.n. immunizations (Fig. 2A; \( P < 0.001 \)). Serum IgG1 responses were similar or identical to serum IgG responses throughout the studied period (Fig. 2B). The differences in serum IgG2a (Fig. 2C) and IgG1 (Fig. 2B) responses were generally similar between the immunization groups.

These data show that when identical vaccines were used for both systemic and combinations of mucosal and systemic immunizations, systemic immunizations induced significantly higher serum IgG responses.

**Kinetics of serum IgA antibody responses after systemic and/or mucosal immunizations.** The kinetics and dynamics of the serum IgA responses were different than those of serum IgG, IgG1, and IgG2a. Although serum IgA responses were generally low and similar between the immunization groups at 3dp2, i.n./i.m. immunizations induced lower responses com-

**FIG. 2.** Kinetics of serum anti-HA IgG, IgG1, IgG2a, and IgA. Mice were immunized i.m./i.m., i.n./i.n., i.n./i.m., or i.m./i.n., as described in the text, at a 3-week interval. Serum antibody titers were measured by ELISA. Sera were collected at 2dp2, 2wp2, 4wp2, 8wp2, 13wp2, 1dpchall, and 3dpchall. The data are presented as mean endpoint titers of IgG (A), IgG1 (B), IgG2a (C), and IgA (D) of each group ± the standard deviation.
pared to i.m./i.m. (P < 0.01), i.m./i.n. (P < 0.05), and even i.n./i.n. (P < 0.05) immunizations at 2wp2 (Fig. 2D). At 8wp2, i.m./i.m. immunizations induced higher serum IgA responses than i.n./i.n. immunizations (P < 0.01). No other significant differences were detected at 8wp2 for other groups or at 13wp2 for all groups. However, at 1dpchal and 3dpchal, i.m./i.m. immunizations induced higher serum IgA responses than i.n./i.n. immunizations (P < 0.05) but not i.m./i.n. or i.n./i.m. immunizations (Fig. 2D). These data show that, in general, mucosal, systemic, or combinations of mucosal and systemic immunizations using the same vaccine, i.e., HA/H11001LTK63, induced similar serum IgA responses.

Because antibody responses in both the upper and the lower respiratory tracts are known to play a role in protection against nasally transmitted influenza virus infections, we next measured the percentage of HA-specific IgA of total IgA, respectively, in nasal washes at 3 days after challenge. Nasal IgA responses were similar in the i.m./i.m., i.n./i.n., and i.n./i.m. immunization groups. However, i.n./i.n. or i.m./i.m. immunizations induced higher IgA responses than i.n./i.m. immunizations (P < 0.05) but not i.m./i.n. or i.n./i.m. immunizations (Fig. 2D). These data suggest that, except for the lowest nasal wash responses in the i.n./i.m. group, application of LTK63 as both mucosal and systemic adjuvant induced similar serum and nasal wash IgA responses in all immunization groups.

**i.n. challenge with live influenza virus and protective responses.** To measure the protective efficacy of the various routes of vaccination with the same vaccine used for mucosal and systemic immunizations, the mice were challenged i.n. at 3.5 months after the second and final immunization. The survival of individual mice and their weight loss was then measured as a correlate of protection. We found that 100% of the mice in all immunization groups, including the unvaccinated/challenged group, survived the i.n. challenge during the first 4dpchal (Fig. 4A). However, by 7dpchal, none of the mice in the unvaccinated or i.n./i.n. immunization groups survived the i.n. challenge (Fig. 4A). The most protected immunization groups were the i.m./i.m. group, followed by the i.n./i.m. group at 7dpchal, 8dpchal, 9dpchal, and 10dpchal, and 11dpchal (Fig. 4A). From 9dpchal to 11dpchal, only 15% of the mice survived in the i.m./i.n. group, whereas the survival rates were 65 and 50%, respectively, for the i.m./i.m. and i.n./i.m. groups (Fig. 4A). The body weights of the mice correlated well with the survival rates for all of the immunization groups (Fig. 4B). These data show that i.m./i.m., followed by i.n./i.m. immunizations, induced the most protective responses.

**Serum HI responses against homologous influenza virus strain.** Serum HI titers are an accepted correlate of protection against seasonal influenza viruses. To determine whether HI titers were induced and maintained over a long time, we measured serum HI titers against the homologous H1N1 strain at short-term (4wp2) and relatively long-term (13wp2) intervals after the second immunization. Serum HI titers were induced after i.m./i.m., i.n./i.m., and i.m./i.n. immunizations at 4wp2 and were maintained at the same level until 13wp2, before the nasal challenge (Fig. 5). No significant differences were observable in serum HI titers between the i.m./i.m., i.n./i.m., and i.m./i.n. immunizations at 4wp2 and were maintained at the same level until 13wp2, before the nasal challenge (Fig. 5). No significant differences were observable in serum HI titers between the i.m./i.m., i.n./i.m., and i.m./i.n. groups at either 4wp2 or 13wp2. Serum HI titers were undetectable after i.n./i.n. immunizations at both the 4wp2 and the 13wp2 time points (Fig. 5). These data show that the induction of serum HI responses correlated with protection against death or weight loss.

**Pre- versus postchallenge total serum TH1 and TH2 cytokines.** Immunostimulating adjuvants, such as LTK63, are known to induce both antigen-specific and unspecific responses. To determine whether a correlation could be established between total serum cytokines and protection following various immunization routes, Th1 and Th2 type cytokines were
measured in sera at 2wp2, 13wp2, 1dpchall, and 3dpchall. Serum IL-5 levels were relatively low and similar in all immunization groups at 2wp2 and 13wp2. However, by 3dpchall the serum IL-5 levels were significantly increased in all immunization groups compared to the prechallenge levels (Fig. 6A). Moreover, at 3dpchall, serum IL-5 levels were significantly higher in the i.m./i.m. group than in the i.n./i.n. and i.m./i.n. groups (P < 0.001 for both groups). Also, serum IL-5 levels were significantly higher in the i.m./i.m. group than in the i.n./i.m. (P < 0.001) group. Thus, the enhanced postchallenge total serum IL-5 levels correlated with the better protection in the i.m./i.m. and i.n./i.m. immunization groups.

Similar to serum IL-5 levels, serum IL-13 levels were also low to undetectable before challenge. However, after challenge, the IL-13 levels were significantly enhanced only in the i.m./i.m. group compared to prechallenge levels (Fig. 6B). Moreover, serum IL-13 levels were significantly enhanced in the i.m./i.m. compared to the i.n./i.n. (P < 0.01) and i.m./i.n. (P < 0.01) groups but similar to the i.n./i.m. group. The enhanced postchallenge total serum IL-13 levels, together with the enhanced serum IL-5 levels, correlated with protection in the i.m./i.m. and i.n./i.m. immunization groups.

At 2wp2, the total serum TNF-α levels were similar between i.m./i.m. and i.m./i.n. groups but significantly lower in each group than in the i.n./i.m. or i.n./i.n. group (Fig. 6C). At 13wp2, the total serum TNF-α levels were similar in all immunization groups. However, at 3dpchall, the i.m./i.m. and i.n./i.m. total serum TNF-α levels were similar, but in the i.m./i.m. group they were significantly higher than the i.n./i.n. (P < 0.05) or i.m./i.n. (P < 0.001) group, suggesting a possible correlation with protection. Total serum IFN-γ levels were low to undetectable in all immunization groups at all pre- and postchallenge time points, and no differences were discernible between any groups at any time points or for any group pre- and postchallenge (data not shown). Of note, no correlation was found between serum IgE titers and serum IL-5 or IL-13 levels at 4wp2, 13wp2, or 3dpchall (data not shown).

Taken together, these data suggest a correlation between total serum TH2 cytokine levels (IL-5 and IL-13) levels at 3dpchall and protection from death or weight loss after i.n. challenge with live influenza virus.

**DISCUSSION**

In a series of previous studies, we documented that mucosal immunizations followed by systemic immunizations enhanced serum antibody responses, as well as induced mucosal antibody responses, compared to systemic immunization alone, mucosal immunization alone, or systemic immunization followed by mucosal immunization with protein- or DNA-based vaccines (7, 24, 55–57). In the studies with protein-based vaccines, however, the mucosal and systemic vaccine formulations were different. In a previous study, using influenza virus HA, while LTK63 was used for mucosal immunizations, no adjuvants or delivery systems were used for systemic immunizations (55). Moreover, in another study, using *H. pylori*-based proteins Cag-A and NAP, LTK63 was used for mucosal immunizations, while MF59 was used for the systemic (i.m.) immunizations (56). Thus, our goal in the present study was to determine
whether using the exact same vaccine formulations for both mucosal and systemic immunizations could enhance immune responses if mucosal followed by systemic immunizations were performed. This is important since using the same vaccine formulation for both mucosal and systemic immunization has important development and clinical implications. It is also important to note that, to date, studies on the use of HA as a protein-based vaccine have not shown any efficacy through i.n. vaccinations, except when CT was used as an adjuvant. Because the aim of the present study was to compare various routes of immunization using the exact same formulation, we used LTK63 as an adjuvant for both mucosal and systemic vaccination routes. Moreover, most studies have used sublethal influenza virus challenge doses, but here we selected a more vigorous lethal challenge dose to better determine the protective efficacy of the vaccines and the vaccination routes. Although it would have been better to include control vaccination groups with HA alone used for i.n. or i.m. immunizations, our immunogenicity data (Fig. 1) clearly demonstrated that two i.n. immunizations with HA, without LTK63, failed to induce any detectable serum antibody responses, and thus we deemed it highly unlikely that i.n. immunizations with HA but without LTK63 would induce any protective responses, particularly 3.5 months after the vaccinations.

We showed that using the same formulations for both mucosal and systemic immunizations, i.n./i.m. immunizations did not enhance serum antibody responses of any isotype or isotype subtypes. Nonetheless, i.n./i.m. immunizations were as effective as i.m./i.m. immunizations, but better than i.m./i.n. or i.n./i.n. immunizations, for protection against death or weight loss after a 3.5-month resting period and nasal challenge with homologous live influenza virus. It has been demonstrated that mucosal immunizations are more protective than systemic immunizations against mucosal challenge after a long-term resting period (21). Whether a longer resting period would have proved the i.n./i.m. immunization strategy more efficacious seems unlikely but remains to be seen. Another limitation of the present study was that no dose response was included, which would have been important in light of the protective efficacy of the vaccine against a relatively high challenge dose. Measurement of serum cytokines can be used to determine the overall effect of infection or vaccination on the host. Thus, hepatitis C virus or *Schistosoma mansoni* infections can differentially regulate TH1 and TH2 cytokine levels in serum of patients (19, 20). Moreover, respiratory syncytial virus infection of infants regulates serum TH1 and TH2 cytokine balances (14). Because immunopotentiating adjuvants can change the total serum balance of TH1 or TH2 cytokines, we set out to determine the effect of LTK63 on serum IL-5, IL-13, IFN-γ, and TNF-α. Interestingly, while prechallenge no significant difference in serum IL-5 was discernible, the enhanced post-challenge total serum IL-5 levels correlated with the better protection in the i.m./i.m. and i.n./i.m. immunization groups. Although inclusion of IL-4 would have provided additional informative data, we focused on IL-5 and IL-13 because of their potential for inducing eosinophilia and asthma and because of their roles as downstream events after IL-4 induction of TH2 responses (42).

In the present study, we demonstrated that LTK63 could act as a systemic adjuvant. Interestingly, compared to CpG, which is a well-known TH1-inducing immunopotentiating adjuvant, LTK63 induced similar levels of serum IgG2a responses, which is an indirect measure of TH1-type cytokine responses. While CpG also suppressed IgG1 responses, this was not as evident.

**FIG. 6.** Kinetics of total serum IL-5, IL-13, and TNF-α. Mice were immunized twice i.m./i.m., i.n./i.n., i.n./i.m., or i.m./i.n. at a 3-week interval. Sera were collected at 2wp2, 13wp2, 1dpchall, and 3dpchall. Total serum IL-5 (A), IL-13 (B), and TNF-α (C) levels were measured by a Luminex assay. The data are presented as the total serum cytokines in pg/ml from each group of mice ± the standard deviation.
for LTK63. This is consistent with previous studies in which LTK63 was shown to induce a balanced TH1- and TH2-type response (46, 47). Moreover, LTK63 has been implicated to induce TH1 type responses after low-dose parenteral immunization (47). In immunizations against influenza virus with live attenuated influenza virus have been shown to be generally safe and to protect against disease (30, 34). Furthermore, LTK63, which in many animal studies has been shown to be safe, was also safe in humans after i.n. immunization against influenza virus (38). Interestingly, it has been shown that the mere i.n. administration of LTK63, in the absence of specific antigen, induced innate immune responses and protected against respiratory infection (63). Thus, while previous studies suggest that LTK63 used as an i.n. adjuvant may be potentially safe and effective in a human i.n. vaccine against influenza, our current data show that it may potentially also be used as a systemic adjuvant as well.

While TH2-type responses may be desired to protect against helminths and parasites, TH1-type responses are more desirable for antiviral and anti-intracellular bacterial pathogens. Although LT or CT and their mutants have been extensively used as mucosal adjuvants, their use as systemic adjuvants has been far more limited. CT has been shown as an effective systemic adjuvant in several studies (16, 26, 48, 53, 58). Interestingly, it was shown that, whereas the systemic administration of CT induced both TH1- and TH2-type responses, the non-toxic moiety of CT, CTB, induced TH1-type responses (2). In this regard, CpG is known to strongly induce TH1-type responses in both mice (33) and primates (59). Of particular importance in the present study was the finding that LTK63, used as a systemic adjuvant, induced similar serum IgG2a responses to CpG. However, whereas CpG appeared to concomitantly suppress IgG1 responses, LTK63 maintained the IgG1 responses. Thus, while more rigid cytokine response analysis is needed, it appears that LTK63 as a systemic adjuvant can enhance both TH1- and TH2-type responses.

It is generally accepted that i.m. or other routes of systemic immunizations induce relatively poor mucosal IgA responses compared to mucosal routes of immunization. In the present study, however, our data showed that i.m. immunizations were equal or better than i.n. immunizations alone, or combinations of i.n. and i.m. immunizations, for the induction of antigen-specific serum IgA or IgG in nasal washes. It is possible that LTK63 given i.m. induced the IgA responses. It is well established that CT, which closely resembles LT in structure and function, induced isotype class switching to IgA (31, 39).

Protection of the upper respiratory tract (nasal mucosa and trachea) by IgA and of the lower respiratory tract (lungs) by IgG against influenza virus infection has been shown in animal models (41, 44) and clinical trials (9, 36). Interestingly, it was shown in humans that IgG antibodies in the nasal washes of volunteers immunized i.n. or systemically were serum derived (61). Moreover, it was shown in human volunteers after secondary infection with influenza virus that, while the main portion of serum IgA was of the IgA1 subtype, both IgA1 and IgA2 were detected in nasal secretions and appeared to be secretory IgA (13). Direct inhibition of virus attachment to bronchial epithelia and direct virus neutralization through binding are only two effector mechanisms of IgA. It has been demonstrated that both NK cells and alveolar macrophages express Fce receptors which mediate target cell killing and opsonization, respectively (17, 35). Moreover, dimeric IgA can neutralize intracellular viruses (29, 32). Thus, the combination of i.n. and i.m. immunization, used in our study, holds promise to induce local IgA with its many effector functions.

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