Nonclinical safety evaluation of *Escherichia coli* heat-labile toxin mucosal adjuvant as a component of a nasal influenza vaccine

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Conventional influenza vaccines currently in use are administered parenterally and generally confer good protection against systemic disease through the induction of high titers of serum virus-neutralizing antibodies. Parenteral vaccines are suboptimal in that they fail to induce a local mucosal response that may prevent the early stages of virus infection. Thus, the intranasal administration of a vaccine may provide a viable alternative to the parenteral route. Indeed, intranasal administration of vaccine antigens when formulated with an appropriate mucosal adjuvant (e.g., bacterial toxins), results in a vigorous local and systemic immune response. This review discusses the nonclinical safety evaluation of *Escherichia coli* heat-labile toxin as a mucosal adjuvant for an intranasally administered influenza vaccine.

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It is well accepted that immunization via the mucosal route induces a vigorous local immune response in addition to the systemic immune response evoked by most parenteral vaccines [1,2]. Importantly, and in contrast to systemic immunity, the induction of an immune response at the mucosal surface may prevent the initial infection of epithelial cells. A blockade of replication at the initial stage is likely to result in dramatically reduced shedding of the pathogen and, thereby, in a reduced spreading of the latter among the population at risk. However, innate immunity and the clearing mechanisms of the human body make it very difficult to induce an effective immune response after mucosal application of the antigen alone. In fact, the effectiveness of a mucosal vaccine largely depends on the development of an appropriate antigen formulation that may include a strong immunostimulatory adjuvant. For instance, great care must be taken to achieve the optimal immune response and avoid mucosally induced tolerance [3,4].

Certain bacterial toxins, such as cholera toxin (CT) and the structurally related heat-labile toxin of *Escherichia coli* (LT), have the ability to bind directly to mucosal surfaces and are known to be strong immunogens and also to function as potent mucosal adjuvants enhancing vaccine effectiveness [5,6]. Although LT and CT are closely related, they present distinct features in terms of their biochemical and immunomodulating properties, at least in animal models [7].

Structurally, *E. coli* LT holotoxin is a heterohexameric protein consisting of two different subunits, A and B, that alone are nontoxic. The A subunit, containing the catalytic activity, is anchored in a ring of five smaller identical B subunits, which act as a mediator. Such B pentamers bind to ganglioside GM_1, asialo-GM_1 and GM_2 receptors on epithelial cells resulting in the internalization of the toxin [8].

Subsequently, the A subunit is activated by a trypsin-like protease activity leading to the formation of two peptides, A1 and A2, which are 192 and 47 amino acid residues long,

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respectively. The enzymatically active A1 peptide enters the cytosol where it binds to NAD and catalyzes ADP-ribosylation of $G_{s\alpha}$ protein, a stimulatory component of the adenylyl cyclase complex, and results in elevated levels of cAMP. Upon natural infection, this causes the unbalanced secretion by epithelial cells of water and chloride ions (H_2O and Cl^-) into the small intestine, resulting in the typical diarrheal symptoms. Once released into the cytosol, the free A1 peptide cannot penetrate into another cell in the absence of a functional B pentamer [2,6].

LT has been demonstrated to act as a potent mucosal adjuvant. The administration of both CT and LT enhances serum and mucosal antibody responses to coadministered bystander antigens that are by themselves poor immunogens, provided that they are delivered simultaneously to the same mucosal surface [9–11]. The degree of immunopotentiation as well as the amplitude and localization of the immune response depend on various factors, such as the nature of the bystander antigen, the host immunized and the route of immunization.

The development of effective mucosal vaccines faces specific safety issues. Concerns have recently been voiced over the intranasal use of CT or LT. Indeed, a recent report indicates that intranasally administered CT may reach proximal neural tissues, such as the olfactory bulb of immunized mice, and may promote the translocation of a coadministered antigen to the same tissues [12].

In an attempt to produce a safe and effective influenza vaccine to be administered nasally, a vaccine formulation was developed based on virosomes [13]. Virosomes are spherical, unilamellar vesicles with a mean diameter of approximately 150 nm that harbor influenza virus surface glycoproteins hemagglutinin and neuraminidase anchored in the phospholipid membrane. The unique potential of virosomes as an antigen-presenting system and adjuvant is described in an accompanying publication [14].

In order to achieve high levels of mucosal immunity, LT was also included in the vaccine formulation. A full immunization scheme is comprised of two human doses (HD) administered 1 week apart, one HD corresponding to 7.5 µg of each of the three influenza hemagglutinins recommended by the WHO and 2 µg of a natural LT variant. The HD is administered in a total volume of 200 µl (100 µl per nostril) using a specially designed bi-dose spray applicator.

In addition to the nonclinical safety evaluation program detailed in the present report, the vaccine underwent full clinical development that documented the safety and immunogenicity of the vaccine both at the mucosal and systemic levels [15–18] (high titers of influenza-specific serum antibodies complying with EMEA requirements for the immunogenicity of influenza vaccines). The vaccine was registered in Switzerland and marketed for the influenza season 2000–2001.

In the course of the development of this intranasal trivalent influenza vaccine and in view of concerns of LT toxicity, safety studies were conducted in different animal models to assess the toxicology of intranasally administered LT. The general toxicological program was developed according to European CPMP/SWP/465/95 guidelines on preclinical, pharmacological and toxicological testing of vaccines and after discussion with key European regulatory agencies. Furthermore, pharmacokinetic and biodistribution studies of LT alone or as a component of the influenza vaccine were carried out. Special care was given to the tracking of administered LT to neural tissues, with particular attention to the olfactory bulb.

Animal studies of LT as a mucosal adjuvant

As summarized in TABLE 1, extensive nonclinical pharmacodynamic, toxicological and pharmacokinetic studies involving various animal models have been conducted at different stages of the assessment in order to achieve optimal safety evaluation. In particular:

- The ferret model is well-established and constitutes a useful pharmacodynamic model for influenza infection and shedding as ferrets are sensitive to human influenza strains and vaccine efficacy can be tested through challenge experiments [19–21]
- Rats and mice are approved species that form the basis of many toxicological programs
- Due to the close degree of relatedness between LT of human and porcine origin [6], Göttinger miniature pigs are a suitable model for the assessment of the potential of LT to induce diarrhea
- New Zealand white rabbits are a widely accepted model, recommended by Health Authorities, for the assessment of local tolerance in eye irritation studies as used in the present context for the evaluation of misplaced application of the nasal spray
- Baboons (*Papio p. ursinus*) represent a useful model to reflect humans with remarkable proximity in terms of many physiological, pharmacological and toxicological properties

Pharmacodynamic study in ferrets

Ferrets were used as an appropriate model to quantify virulence and pathogenicity of human influenza viruses and, hence, to investigate vaccine efficacy, based on the comparability of ferrets and humans in terms of sensitivity to infection and clinical response. The study was aimed at demonstrating the immunogenicity of the LT-adjuvanted intranasal influenza vaccine (based on the level of hemagglutinin inhibition antibodies in the serum) in comparison with conventional parenteral immunization. Furthermore, this study was used to determine the efficacy of the vaccine in reducing systemic (fever and body weight loss) and local (inflammatory cell counts, virus shedding in nasal washes) clinical parameters following challenge with infectious homologous virus.

At day 0, ferrets were either mock treated or immunized intramuscularly (im.), intranasally (in.), followed by reapplication of the vaccine at day 7 in the intranasal group. Both intramuscular and intranasal vaccination schemes corresponded to the intended applications for human use. Blood samples were taken prior to all immunizations and also on day 21 prior to challenge. Challenge with the homologous influenza strain A/Sydney/5/97-like (H3N2) was performed at day 21 and clinical parameters were investigated daily up to day 28, at which time the ferrets were sacrificed and blood samples taken.

Table 1. Summary of toxicological program in LT adjuvant administered alone or as a component of nasal influenza vaccine.

Toxicological study	Animal model/cell line	Dosage (route of administration[§])	Major findings
LT adjuvant alone			
Acute toxicological testing	CD-1 Mouse	Up to 1000-fold HD* [§§] (po. and iv.)	No mortality or clinical abnormalities
	Crl:CD(SD) Rat	Up to 1000-fold HD* (po. and iv.)	No mortality or clinical abnormalities
	Göttinger Minipig	15, 50, 150, 450 and 1000 µg LT/200 µl (in. and po.)	No mortality or clinical abnormalities
Subacute (repeat) toxicological testing	CD-1 Mouse	10 mg LT/kg (50-fold HD*) once a week for 4 weeks (iv.)	No mortality or clinical abnormalities
	Baboon	2 mg LT once a week for 4 weeks (in.)	No mortality or clinical abnormalities
Local tolerance	Göttinger Minipig	15, 50, 150, 450 and 1000 µg LT/200 µl (in.)	No clinical abnormalities
	Baboons	2 µg LT in 200 µl (100 µl per nostril) once a week for 4 weeks (in.)	No clinical abnormalities
Biodistribution	Baboon	2 µg ^{125}I–LT in 200 µl (100 µl per nostril) (in.) (10^7 cpm/mg LT)	^{125}I–LT confined to the nasal mucosa. No translocation to the CNS
Mutagenic potential	Mouse lymphoma cell line L5178Y	1.25, 2.5, 5 and 10 µg/ml (with and without prior incubation with rat liver S9 fraction) (*in vitro* assay)	No sign of any mutagenic potential *in vitro*
LT-adjuvanted vaccine			
Acute toxicological testing	CD-1 Mouse	200-, 400-, 700-, 1000-fold HD* (po. and iv.)	po.: no mortality or clinical abnormalities. iv.: LD_{50} = 630 HD*
Subacute (repeat) toxicological testing	CD-1 Mouse	0.1 ml/kg, 1 ml/kg (5- and 50-fold HD*) once a week for 4 weeks (iv.)	No mortality or clinical abnormalities
	Baboon	200 µl (100 µl per nostril) once a week for 4 weeks (in.)	No mortality or clinical abnormalities
Local tolerance	New Zealand White Rabbit	0.1 ml per eye	Devoid of any irritating potential
	Baboon	200 µl once a week for 4 weeks (in.)	No clinical abnormalities
Biodistribution	Balb/c Mouse	2–4 × 10^3 cpm ^{125}I–LT in 20 µl vaccine (in.)(1–2 × 10^4 cpm/µg LT)	Radioactivity eliminated via urine and feces within 24 h. No accumulation of radioactivity detected in any organ
	Balb/c Mouse	2.0 × 10^4 cpm ^{125}I–LT in 500 ml vaccine (iv.) (1–2 × 10^4 cpm/µg LT)	No radioactivity found in the brain. Radioactivity due to free ^{125}I in thyroid gland
	Baboon	2 mg ^{125}I–LT in 200 µl vaccine (100 µl per nostril) (in.) (10^7 cpm/µg LT)	^{125}I–LT confined to the nasal mucosa. No translocation to the central nervous system
Pharmacodynamic study	Ferret	Intranasal and intramuscular doses corresponding to intended application in humans	Efficient prevention of clinical symptoms

[§]Route of administration: in.: Intranasal; iv.: Intravenous; po.: Oral. [§§]HD*, Human dose equivalent calculated on the basis of a child's weight of 10 kg.

The study results demonstrated that vaccination by either route of administration led to an efficient prevention of the clinical symptoms associated with influenza infection. The intranasal route of administration was most effective at preventing both fever and weight loss, two important parameters in monitoring disease. In addition, animals vaccinated via the intranasal route showed reduced inflammatory cell numbers in the respiratory tract and, most importantly, minimal levels of virus shedding [15].

Toxicology testing of LT

The aim of the toxicological program was to determine the potential effects of the LT-adjuvanted vaccine or the LT adjuvant administered alone on the nervous, respiratory and cardiovascular systems and the gastrointestinal tract (TABLE 1).

Mouse & rat studies: toxicity

In acute toxicity studies in CD-1 mice and Crl:CD(SD)BR rats the test compound, either LT alone or in vaccine formulation, was administered orally and intravenously. Findings were unremarkable for the oral route at up to 1000 times the human daily dose equivalent (HD*, see TABLE 1), calculated on the basis of a child's weight of 10 kg, with no mortality or clinical abnormalities. Intravenous treatment with the complete vaccine formulation resulted in some deaths within the first week of dosing in mice only, with the first cases observed in the highest dose group within 48 h; the median lethal dose (LD_{50}) calculated for this extremely challenging administration route was 630 HD*. Depression in body weight was also found in some animals of the intravenous route groups at day 3. Such effects may be expected due to the nature of the compound when administered at very high doses via the intravenous route allowing maximum exposure. Oral administration of equivalent dosages did not induce either mortality or clinical changes in the same animal species.

Intravenous subacute (repeat) dose toxicity studies were also conducted in the mouse model. In total, 80 CD-1 mice were randomized to receive either the complete influenza vaccine formulation or the LT adjuvant alone. Following intravenous treatment once a week for 4 weeks, hematological examinations, blood chemistry tests and urine analyses were performed. In addition, the animals were observed daily for clinical signs and the body weight of all the animals was recorded prior to the start of the study, weekly before each treatment and before sacrifice. This study concluded that the vaccine and LT alone, when repeatedly administered intravenously to CD-1 mice, showed no signs of toxicity.

Göttinger minipigs: toxicity

The maximum tolerated dose (MTD) following intranasal administration of the LT adjuvant alone was investigated in Göttinger minipigs. The observation period consisted of 8 days during which the dose of LT was escalated from 15 to 1125 µg/animal/day, corresponding to over 500 times the daily human dose. The results showed that even these excessive doses did not cause any abnormal signs with regard to body weight and other clinical symptoms. Based on these findings and similar results from an orally administered toxicity test in the same species it was deemed unnecessary to perform a repeat dose toxicity test in the minipig model.

Rabbits: local tolerance

Local tolerance testing was performed with New Zealand white rabbits. Ocular administration of 100 µl/animal of the test formulation, consisting of the complete vaccine, to one of the eyes of each animal was followed by 3 days of observation. The results were conclusive in that no clinical signs, either general or local, were noted in any rabbit.

Baboon study: safety

To further evaluate the safety of LT, tests were performed on LT administered intranasally, either alone or as formulated influenza vaccine, in a baboon model. Among other parameters, local inflammatory responses in the nasal cavities and the spread of any inflammatory response to the meninges and CNS were closely observed. In a repeat dose toxicity study, nine baboons (2–3 years old, two females and one male per group) were given LT alone (group 1) or LT as a constituent of the virosomal vaccine (group 2) by the intranasal route once per week for 4 weeks. Vaccine administration was performed using the same bi-dose applicator (1 µg LT/100 µl aerosol per nostril) as for human immunization. A third group of animals received PBS as a control. No clinical signs, in particular no nasal discharge, bleeding or sneezing, were noted during the entire study in any group. The procedure of taking nasal swabs caused occasional bleeding in single animals of all experimental groups. Body weight development was normal and the hematological values were in the normal range. There was no evidence of any systemic adverse reaction, especially no indication of sensitization to the test articles after the fourth administration. Liver and renal chemical parameters as well as C-reactive protein were analyzed at days 0, 10, 30 and 50 and were in the normal range, showing no evidence of a systemic inflammatory response related to vaccine administration. Microscopic investigations, were performed postmortem on the nasal mucosa and adjacent structures of the brain, several distal regions of the brain, the mucosa of the gastrointestinal tract, lungs and other major organs. There was only minimal local inflammatory reaction of the nasal mucosa, which may have been related to the diagnostic procedure (nasal swab and lavage). Importantly, there was no inflammatory change of the adjacent or distant structures of the brain including the olfactory bulb, optical nerve, hippocampus, middle and inner ear, or any other brain structure. Nonspecific changes, such as mononuclear cell infiltrates were recorded in several organs that represent minor and most likely spontaneous pathology. Lungs and bronchial lymph nodes contained slightly increased numbers of macrophages. The number of medullary macrophages in bronchial lymph nodes was slightly higher in the animals that received LT than in controls. However, this change is not indicative of a chronic inflammatory state.

Biodistribution studies

In order to assess the biodistribution of LT, several studies were conducted in mice, Chacma baboons and other animal species using ^{125}I-labeled LT, either administered alone or in vaccine formulation via intravenous, oral, or intranasal routes.

In a number of cases, a significant accumulation of radioactive label in the thyroid gland was observed. A control experiment in guinea-pigs revealed that this effect can be attributed

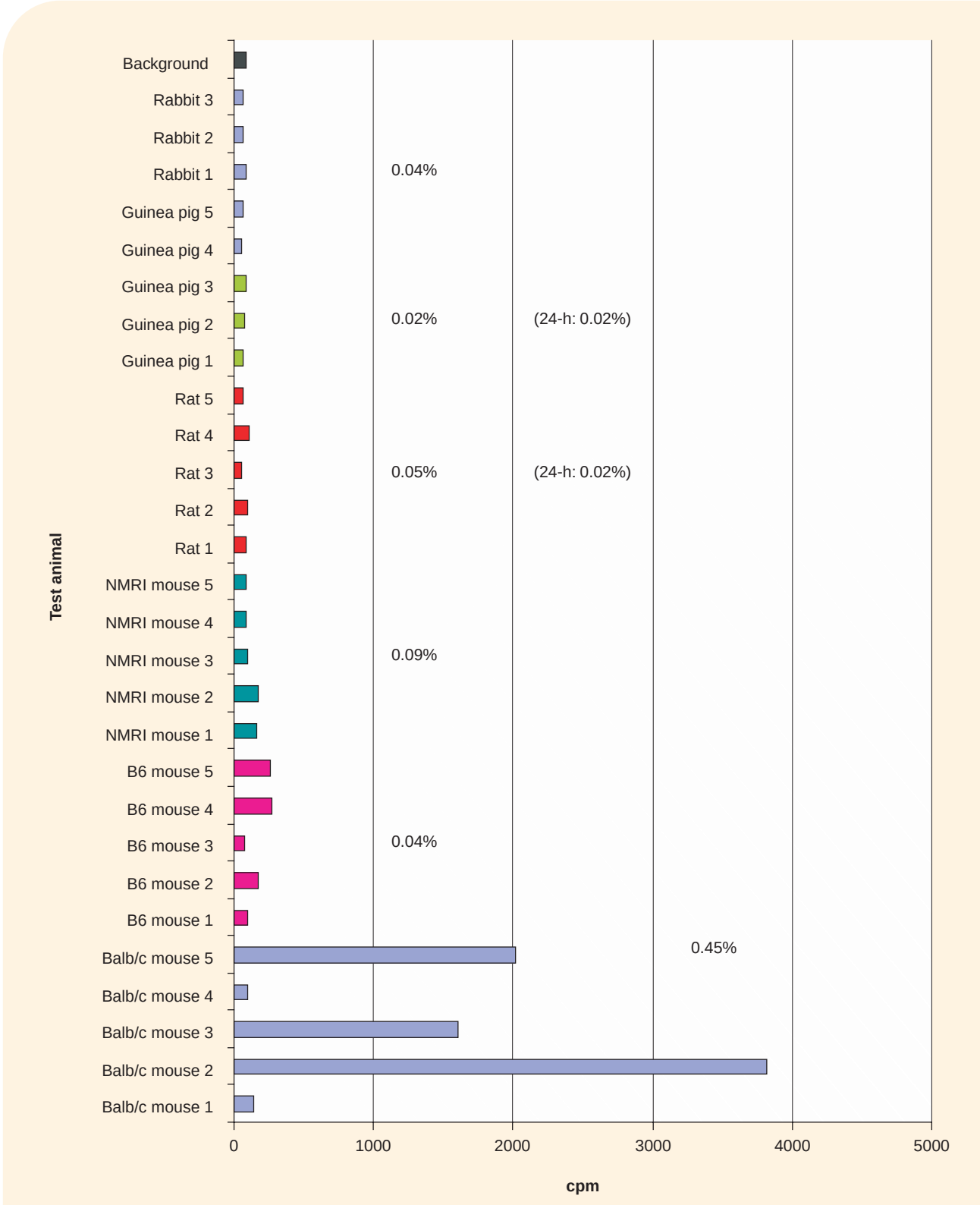

Figure 1. Radioactivity associated with the olfactory bulb of different animal species 6 h after intranasal administration of ^{125}I-labeled LT. Percent values equate to the maximal percentage of administered ^{125}I dose detectable after 6 h (24 h where indicated) in the corresponding species.

to free or *in vivo* released ^{125}I, as accumulation of radioactivity could be totally prevented by intragastric administration of potassium iodide prior to immunization. In subsequent studies involving intranasal administration of ^{125}I-labeled LT at a high specific activity, the labeled vaccine preparation was dialyzed just before nasal administration in order to keep the amount of free ^{125}I spontaneously released from labeled LT as low as possible. However, this procedure did not allow full prevention of metabolization of ^{125}I-labeled LT *in vivo* or the accumulation of released ^{125}I in the thyroid gland.

Intranasal administration in mice

Following intranasal application of ^{125}I–LT in combination with a virosomal influenza vaccine formulation, Balb/C mice were sacrificed and the biodistribution of the radioactivity was measured at 1, 6 or 24 h or 7 days post administration. The bulk of radioactivity was rapidly eliminated through the gastrointestinal and urinary tract upon intranasal delivery of the ^{125}I-labeled LT as a component of the vaccine. After 1 hour, most of the radioactivity was detected in the stomach and in the intestine. 5 hours later (time-point 6 h) a smaller proportion of the radioactivity was still found in the stomach but most of the radioactivity had already been secreted via the urine and feces. After 6 and 24 h and 7 days, significant amounts of radioactivity were found in the thyroid gland, again corresponding to ^{125}I spontaneously released from the labeled toxin.

Intravenous administration in mice

Three days after intravenous application of ^{125}I–LT containing vaccine, mice were sacrificed and the biodistribution of the radioactivity was assessed in different organs. Again, most of the radioactivity was found in the thyroid gland. In one out of five mice, some radioactivity was associated with the head but none in the thyroid gland. This case was explained with an incorrect dissection procedure.

Intranasal administration in baboons

Four female baboons received one human dose of ^{125}I-labeled LT either alone or in combination with the virosomal vaccine, using the clinical spray applicator. Blood was obtained from the femoral vein before the application and 0.5, 1, 4, 24, 48 and 72 h after the application. Urine was collected at the same time points. Feces was sampled before and 24 and 72 h after the intranasal administration. Cerebrospinal fluid (CSF) was obtained 4 h after application by lumbar puncture under anesthesia. Probes of the nasal cavity were taken by cotton swab before application and at 4, 24 and 72 h after application of the labeled LT. The swabs from the nasal cavity and, to a much lesser extent, the urine samples at 24, 48 and 72 h contained detectable radioactivity (three- to tenfold increase over background counts). Importantly, no radioactivity was detected in the samples from the CSF and very low levels in the 24-h blood samples and 24-h feces samples (TABLE 1).

The animals were euthanased 72 h after administration of the test substance and a full necropsy was performed. No macroscopic abnormalities were detected in any animal. The subsequent microscopic investigation included: nasal cavity and eustachian tube, optical nerve, olfactory bulb, the limbic system including the hippocampus and other parts of the brain, lungs (both lobes), heart, stomach, small and large intestine, kidneys, adrenal glands, thyroid gland, spleen, mesenteric and cervical lymph nodes, thymus, skin and skeletal muscle. Biodistribution of the ^{125}I-labeled LT in various tissues and body fluids was determined. The radioactivity measured in blood (1, 4, 24 and 72 h), CSF and stool (1 ml and 1 g, respectively) obtained 24, 48 and 72 h after administration were not above background levels. Most importantly, no radioactivity was detected in the olfactory bulb, optical nerve and brain, as well as in most other organs tested. There were small but detectable amounts of radioactivity localized in the nasal mucosa and larger amounts in the thyroid, the latter ascribed to free iodine spontaneously released from labeled LT. In order to achieve maximal sensitivity of detection, specimens of the olfactory bulb and adjacent structures were exposed to autoradiographic films for up to 3 months. The total absence of signals on the films revealed that no detectable radioactivity had accumulated in these organs.

Studies on the migration of LT in the olfactory bulb of different animal species

Recent data published by van Ginkel and colleagues demonstrated migration of CT in the olfactory nerves, olfactory bulb and the brain of C57/Bl6 mice within 24 h after administration [12]. In order to assess whether LT would show a similar biodistribution as CT and whether the choice of a specific animal strain or species may influence the results, a vaccine formulation containing ^{125}I-labeled LT with a high specific activity (7×10^5 cpm/µg LT) was administered via the intranasal route to three different mouse strains (Balb/C, C57/Bl6, NMRI), Wistar rats, Gohi guinea-pigs and New Zealand White rabbits. Test groups consisted of five animals each, except for rabbits (three animals). After the animals were sacrificed, the olfactory bulbs were collected and associated radioactivity was measured. The 6 h time point was tested in each animal species; an additional 24 h time point was studied in rats and guinea-pigs.

As shown in FIGURE 1, low but significant amount of radioactivity, corresponding to less than 0.5% of total administered radioactivity, was observed in the olfactory bulb of three out of five Balb/c mice, as opposed to amounts close or equal to baseline level in olfactory bulbs of the remaining two Balb/c mice as well as in all five C57/B6 and five NMRI mice. No radioactivity above background level was detected in the olfactory bulbs of any of the rats, guinea-pigs and rabbits.

Bell's palsy models

Following the introduction of the nasal influenza vaccine on the Swiss market, rare cases of Bell's palsy were reported in a temporal association with vaccine administration. Idiopathic facial paresis, also described as Bell's palsy, occurs at a natural

incidence rate of 10–50 per 100,000 and has only recently been shown to be associated with a reactivation of a latent herpes simplex virus (HSV) infection [22–27] resulting in an inflammation of the facial nerve, which in turn can cause compression and temporary loss of function and, ultimately, leads to facial paresis which is often unilateral. In order to reveal a potential causal relationship between immunization with nasal influenza vaccine and the onset of Bell's palsy, a series of animal experiments were performed. The goal was to address the question of whether a nasally applied LT-adjuvanted influenza vaccine can influence the outcome of a viral infection present in the respiratory tract or the associated neural tissue at the time of immunization.

HSV-1 infection in IFN-AR-1$^{-/-}$ mice

Interferon (IFN) receptor knockout mice are known to be more susceptible to viral infection [28]. Mice deficient in IFN-α and -β receptor (IFNAR-1$^{-/-}$) were infected intranasally with a wide dose range (10^2–10^5 pfu) of HSV-1 (McIntyre strain) and the survival was monitored over time. Control groups given 20 µl PBS intranasally were compared with mice that received 20 µl LT-adjuvanted influenza vaccine twice, 1 week before and at the time of infection. The number of surviving mice was determined over time. No mouse survived infection at any dose, the average survival time varying between 2–3 days (highest dose) to 6–7 days (lowest dose). No difference in average survival time was observed between treated and control groups in any of the HSV-1 dose groups. In a second series of experiments, seven doses of nasal vaccine or LT adjuvant alone (2 µg in 20 µl) were administered within the first 36 h after infection with a low infectious dose (10^2 pfu), again without any effect on the kinetics of survival. Thus, neither the nasal vaccine nor LT alone had a detectable effect on the survival time of mice infected with HSV-1, at least in this experimental setting [MÖLLING AND PAVLOVIC, UNPUBLISHED DATA].

Sendai virus infection in CD1 mice

Local and systemic spreading of Sendai virus strain F1-R [29,30] has been assessed after nasal inoculation in CD1 mice followed by treatment with either LT alone, LT-adjuvanted influenza vaccine, or PBS only at 12, 24 and 48 h after infection. Five days after infection, virus loads were determined in the lung, representing local replication and in the liver, reflecting the systemic virus spread, using a hemadsorption assay. No significant differences in viral loads between treated and control groups were observed in any of the experiments. In order to achieve systemic virus spreading and detect viruses in the liver, the dose had to be increased to 10^7 pfu, resulting in a lethal outcome in over 50% of test animals. However, the mortality rate was similar for the treated and control groups [BITZER, UNPUBLISHED DATA].

Facial paresis in latently HSV-1 infected mice

A mouse model for Bell's palsy was recently described in the literature by Hato and coworkers [31–33], in which by reactivation of a latent HSV-1 infection an incidence of 20% of unilateral facial paresis was reproducibly observed. After the initial cutaneous infection with HSV-1, 50% of the animals develop a transient unilateral facial paresis that lasts 10 days on average. The subsequent viral latency in the facial nerve can be reactivated 2 months later by a combination of systemic immunosuppression and a local trauma. The temporary immunosuppression is achieved by administration of anti-CD3 antibody to deplete the leukocyte population dramatically. The applied local trauma consists of a skin irritation at the site of the original infection, at the ear base, which is innervated by the facial nerve.

In order to reveal a potential for HSV-1 reactivation in this model, a group of 18 persistently infected animals were treated with two doses of intranasal LT-adjuvanted influenza vaccine in combination with systemic immunosuppression. None of these animals developed facial paresis whereas in the positive control group treated according to the original protocol, two out of ten animals developed a clear facial paresis, as expected. In both groups, some animals died of a generalized HSV-1 infection dominated by encephalitis: 2 of 18 in the vaccine group and one of ten in the control group.

Conclusions & expert opinion

Overall, the extensive nonclinical safety evaluation program performed during the development of the LT-adjuvanted, virosome-based, intranasal trivalent influenza vaccine provided no evidence of a potential risk associated with the use of the vaccine in humans.

Acute and subacute (repeat) toxicity tests of the LT adjuvant alone or the complete vaccine formulation in mice and rats indicated a large safety margin. Only after intravenous administration of the complete vaccine in mice, acute toxicity was observed with a LD_{50} corresponding to 630 HD equivalents.

Oral and intranasal dose-escalation studies in mice and Göttinger minipigs did not reveal significant effects, even when 500 human doses were applied. Similarly, ocular application in rabbits did not result in detectable local irritations or systemic symptoms.

In repeat dose toxicity studies in baboons, it was shown that weekly intranasal administration of LT was well-tolerated and did not cause any clinical adverse effects. Importantly, the last administration was equally well-tolerated as was the first, suggesting that no local sensitization occurred to the vaccine components under the experimental conditions. In conclusion, repeat dose toxicity tests did not show any adverse effect related to 4 weekly intranasal administrations of LT in primates.

In order to evaluate the biodistribution kinetics of LT adjuvant for nasal immunization, ^{125}I-labeled LT was administered via intranasal and intravenous route and the distribution was assessed over time in mice and in baboons. An important goal was to address the question whether the LT would be translocated to the neural tissues associated with the nasal epithelia and, ultimately, to the brain. Indeed, in the course of vaccine development, van Ginkel and colleagues reported data suggesting that ^{125}I-labeled CT administered via the nasal route could translocate to the olfactory bulb of C57BL/6 mice [12].

The data of the biodistribution study in mice showed that most of the intranasally applied ^{125}I–LT is eliminated via urine and feces within 24 h. The most prominent accumulation of radioactivity aside from the site of application, the nasal mucosa, was observed in the thyroid gland, particularly after intravenous application of radioactive-labeled LT. Since this uptake could be blocked in a control experiment by the excess of free, unlabeled iodine, the measured radioactivity in the thyroid gland can be ascribed to free radioactive iodine resulting from metabolized ^{125}I–LT. Importantly, after intravenous administration of ^{125}I–LT, no radioactivity was found in the brain showing that LT did not cross the blood–brain barrier.

The biodistribution of intranasally administered ^{125}I–LT in baboons was similar to that found in mice. Importantly, the baboon distribution study demonstrated that LT was confined to the nasal mucosa and did not migrate to the adjacent neural structures (e.g., the olfactory bulb, optical nerve, the cortex, hippocampus, or cerebellum); nor did it enter the CSF after the intranasal administration. Thus, in contrast to the observations of van Ginkel and coworkers in the C57BL/6 mouse, no evidence of translocation of the toxin adjuvant was found in a more relevant species. In addition, inflammatory reactions reported by von Ginkel and colleagues in the mouse were not detected in the baboon, neither by biochemical nor by microscopic investigations.

In order to further evaluate the potential risk of translocation of the toxin adjuvant to adjacent neural tissues, a multispecies study was performed, in which LT labeled at a higher specific activity was administered intranasally to test animals. Results demonstrated that the translocation of ^{125}I–LT to the olfactory bulb after intranasal application is highly dependent on the tested species and even varies between different mouse strains. Surprisingly, and in contrast to a very similar study performed with CT [12], no significant translocation of ^{125}I-labeled LT into the olfactory bulb was detected, neither in the C57BL/6, the strain used by van Ginkel and workers in the CT study, nor in NMRI mice. However, a significant, albeit low, amount of radioactivity was observed in the olfactory bulbs of three out of five Balb/c mice. These observations strongly pose the question of the relevance of a given animal model, here the intranasal mouse model, in the anticipation of potential side effects in humans, particularly when the model has not yet been validated for the specific route of administration. Accordingly, more than one animal model should be used when attempting to draw conclusions on safety.

As stressed above, rare cases of Bell's palsy were reported in a temporal association with vaccine administration after introduction of the nasal influenza vaccine on the Swiss market. Due to the very low natural incidence of Bell's palsy in untreated subjects (10–50 per 100,000), comparable to the one reported for vaccinees, it proved extremely difficult to obtain solid statistical evidence in either retrospective or prospective clinical studies for or against the hypothesis of a causal relationship between intranasal influenza vaccination and the occurrence of Bell's palsy. Therefore, possible indirect side effects of nasal immunization on pre-existing viral infections were tentatively addressed in different mouse models, in particular regarding HSV-1 replication and reactivation. The most relevant animal model for Bell's palsy achieved a much higher incidence (20%) and the infection models yielded a quantitative readout, thereby providing a higher probability to detect a potential effect of nasal immunization. However, within the limitations of the test systems, none of the results from the animal studies supported the hypothesis that the nasal influenza vaccine or components thereof, such as the LT, can enhance viral replication or reactivate a latent infection.

In conclusion, the comprehensive nonclinical evaluation program conducted in the course of the development of an intranasal LT-adjuvanted virosomal trivalent influenza vaccine allowed the documentation of the safety, immunogenicity and efficacy of the vaccine in a variety of animal models. In particular, dedicated studies performed with the vaccine and the LT adjuvant alone do not support a possible association, if any, between nasal vaccination and an increased incidence of Bell's palsy in vaccinees.

Five-year view

Most conventional viral and bacterial vaccines are administered via the parenteral route and promote significant protection against disease by inducing a strong systemic immune response. However, such vaccines do not induce a good local immunity; an immunity capable of preventing systemic spread of the pathogen and also conferring a strong mucosal immunity able to block the agent at the time of the first contact with the host. In addition to an optimal protection of the vaccinee, such a double (local and systemic) protection mechanism would conceptually have a positive impact on the epidemiology of the infectious agent (possibility of herd immunity). Thus, one of the challenges of modern vaccinology is to develop vaccines able to prevent both infection and symptoms, rather than disease only.

Mucosal immunization is generally accepted as being the best strategy for priming of the mucosal immune system. However, in spite of the apparent ease of administration, such an approach is facing a number of difficulties related to the need to guarantee a safe delivery of sufficient and controlled amounts of the immunogen to the inductive sites of the mucosal immune system. Furthermore, the addition of adjuvant compounds may be requested in order to achieve the highest protective local response to inactivated vaccine antigens.

The LT-adjuvanted virosomal influenza vaccine described in the present contribution was the very first nasal vaccine to be approved for human use after extensive nonclinical and clinical evaluation of safety and immunogenicity. After introduction on the market, a rare temporal association between nasal vaccine administration and the onset of facial paralysis symptoms in vaccinees was observed. Due to the low incidence of reported cases and the ensuing practical difficulty in achieving statistical power to differentiate between a potential causal link and mere

temporal coincidence between vaccination and the onset of Bell's palsy, the vaccine was withdrawn from the market. As stated above, results reported here of the comprehensive toxicological program designed after consulting regulatory agencies do not support such a link and therefore provide no definite clue to that issue.

At a meeting on the 'Safety Evaluation of Toxin Adjuvants Delivered Intranasally' convened by the National Institute of Allergy and Infectious Diseases (NIAID) on July 9th, 2001 in Gaithersburg, most of the discussions dealt with the choice of appropriate animal models and the harmonization of assays to assess the safety of enterotoxin-based adjuvants [101]. Clearly, one of the major challenges for the development of future adjuvanted nasal vaccines will be the selection and development of relevant standard nonclinical models.

In spite of the above remaining uncertainty linked to the nasal administration of LT-based mucosal adjuvants, the extensive toxicological program conducted with the natural LT variant incorporated in the nasal influenza vaccine establishes the safety of the adjuvant when administered via different routes, for example its use as a key adjuvant to potentiate the immune response to antigens coadministered via the novel transcutaneous immunization (TCI) approach [34].

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Key issues

- In addition to the systemic immune response evoked by most parenteral vaccines, immunization via the mucosal route induces a vigorous local immune response that may prevent the initial infection of epithelial cells. A block of pathogen replication at the initial stage is likely to result in dramatically reduced shedding of the pathogen and thereby in a reduced spreading of the pathogen among the population at risk.
- The effectiveness of a mucosal vaccine largely depends on the development of an appropriate antigen formulation that may include a strong immunostimulating adjuvant.
- The administration of heat-labile toxin of *Escherichia coli* (LT) or cholera toxin (CT) enterotoxin enhances serum and mucosal antibody responses to coadministered bystander antigens that are by themselves poor immunogens, provided they are delivered simultaneously to the same mucosal surface.
- In the aim of producing a safe and effective influenza vaccine to be administered by the nasal route a vaccine formulation was developed based on a virosome adjuvant concept supplemented with LT in order to achieve high levels of mucosal immunity.
- In the course of development of such intranasal trivalent influenza vaccine and in view of potential concerns of LT toxicity, extensive nonclinical pharmacodynamic, toxocological and pharmacokinetic studies involving various animal models were conducted in order to achieve optimal safety evaluation.
- The comprehensive nonclinical evaluation program conducted in the course of development of the virosome/LT-adjuvanted intranasal trivalent influenza vaccine allowed documentation of the safety, immunogenicity and efficacy of the vaccine in a variety of animal models and provided no evidence of a potential risk associated with its use in humans.

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Website

101 www.niaid.nih.gov/dmid/enteric/intranasal.htm
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