



ISSN Print: 2664-9926
 ISSN Online: 2664-9934
 IJBS 2025; 7(3): 158-162
www.biologyjournal.net
 Received: 16-12-2024
 Accepted: 18-01-2025

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Mucosal immune responses to intranasal vaccination with recombinant protein antigens

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DOI: <https://www.doi.org/10.33545/26649926.2025.v7.i2c.764>

Abstract

The concept of mucosal vaccination dates back to the early twentieth century, when oral polio drops demonstrated that immune protection could begin at the body's surfaces rather than in the bloodstream. This research evaluated mucosal and systemic immune responses in BALB/c mice following intranasal delivery of a recombinant outer-membrane protein antigen (rOmpA) formulated with cholera toxin B subunit (CTB) as a mucosal adjuvant. Sixty female BALB/c mice were divided into three groups (n = 20 each): intranasal rOmpA + CTB, intramuscular rOmpA + alum, and intranasal PBS control. Animals received two immunisations on days 0 and 14. Mucosal IgA was measured in nasal wash and bronchoalveolar lavage (BAL) fluid, while serum IgG and its subclasses were quantified by ELISA at weekly intervals through day 42. Intranasal immunisation induced significantly higher nasal IgA ($OD_{450} = 1.96 \pm 0.18$) and BAL IgA (1.73 ± 0.22) than the intramuscular route (nasal: 0.58 ± 0.11 ; BAL: 0.41 ± 0.09 ; $p < 0.001$). Serum IgG titres were comparable between the two vaccinated groups by day 42, but the intranasal group mounted a balanced IgG1/IgG2a ratio ($1.31/0.94$), whereas the intramuscular group skewed toward IgG1 ($1.28/0.48$), indicating a mixed Th1/Th2 profile for the intranasal route versus a Th2-biased profile for the parenteral route. Splenocyte restimulation assays confirmed higher IFN- γ secretion in the intranasal group (842 ± 97 pg/mL) compared with the intramuscular group (413 ± 76 pg/mL; $p < 0.001$). These results show that intranasal delivery of rOmpA with CTB adjuvant generates potent mucosal IgA responses while preserving systemic IgG production, supporting its potential as a needle-free vaccination strategy.

Keywords: Mucosal immunity, intranasal vaccination, recombinant antigens, IgA response, vaccine development, cholera toxin B, BALB/c mice, adjuvant, Th1/Th2 balance, bronchoalveolar lavage

Introduction

When Albert Sabin introduced the oral polio vaccine in 1961, he established a principle that vaccine science is still working to generalise: mucosal surfaces, not just muscle tissue, can serve as effective induction sites for protective immunity ^[1]. Most pathogens enter the body through mucosal membranes of the respiratory, gastrointestinal, or urogenital tracts, yet the overwhelming majority of licensed vaccines are delivered by injection, a route that generates strong serum antibody responses but often fails to induce secretory IgA at mucosal sites ^[2]. That disconnect has driven a long-running search for mucosal vaccine formulations capable of eliciting both local and systemic protection.

The nasal mucosa is an attractive delivery site because it is richly supplied with nasopharynx-associated lymphoid tissue (NALT), which functions as an inductive site for IgA class-switching and can seed effector cells to distant mucosal compartments through the common mucosal immune system ^[3]. Intranasal delivery also avoids the gastrointestinal degradation that limits oral vaccine potency and doesn't require needles, which simplifies logistics and improves acceptability in mass vaccination campaigns ^[4]. However, soluble protein antigens delivered intranasally are poorly immunogenic on their own because the nasal epithelium rapidly clears particles via mucociliary transport and because tolerance mechanisms at mucosal surfaces can dampen rather than amplify the immune response ^[5].

Mucosal adjuvants overcome this barrier by activating innate immune pathways that promote antigen uptake and presentation. Cholera toxin B subunit (CTB) is one of the best-characterised mucosal adjuvants in animal models. It binds GM1 gangliosides on epithelial and immune cells, triggering receptor-mediated endocytosis and enhancing antigen delivery to dendritic cells in the subepithelial dome of NALT ^[6]. When co-administered with a

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protein antigen, CTB has consistently boosted mucosal IgA and serum IgG responses in rodent systems, though translation to human use has been slowed by safety concerns about neurotropism of the related cholera toxin holotoxin [7]. Recombinant outer-membrane protein A (rOmpA) was selected as the model antigen for this research because OmpA-family proteins are surface-exposed, highly conserved across Gram-negative bacteria, and have been identified as vaccine candidates for several enteric and respiratory pathogens [8]. Producing rOmpA in *Escherichia coli* expression systems is straightforward and scalable, which makes it a practical test case for evaluating mucosal adjuvant strategies that might later be applied to more complex antigens [9].

Despite a large body of work on mucosal adjuvants, few investigations have directly compared intranasal and intramuscular delivery of the same recombinant antigen in a single experimental design, making it difficult to attribute observed differences to the route itself rather than to antigen dose, adjuvant, or animal strain [10]. This research addressed that gap by immunising BALB/c mice with identical doses of rOmpA via the intranasal route (with CTB adjuvant) or the intramuscular route (with alum adjuvant), using a PBS intranasal control group. The objectives were to (1) quantify mucosal IgA responses in nasal wash and bronchoalveolar lavage fluid, (2) compare serum IgG magnitude and subclass distribution between routes, and (3) evaluate T-cell cytokine profiles by splenocyte restimulation to determine the Th1/Th2 balance induced by each route [11].

Material and Methods

Reagents and Chemicals

Recombinant OmpA protein (rOmpA, > 95% purity) was produced in-house by IPTG induction of *E. coli* BL21(DE3) carrying pET-28a-OmpA, purified by Ni-NTA affinity chromatography, and confirmed by SDS-PAGE and Western blot. Cholera toxin B subunit (CTB, catalogue C9903) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Aluminium hydroxide gel adjuvant (Alhydrogel® 2%) was obtained from InvivoGen (San Diego, CA, USA). ELISA kits for mouse IgA (catalogue 88-50450), IgG (88-50400), IgG1 (88-50410), and IgG2a (88-50420) were from Invitrogen (Carlsbad, CA, USA). Cytokine ELISA kits for IFN- γ , IL-4, IL-10, and IL-17A were from BioLegend (San Diego, CA, USA). CFSE, RPMI 1640 medium, foetal bovine serum, and antibiotic-antimycotic solution were from Thermo Fisher Scientific (Waltham, MA, USA).

Material

Sixty female BALB/c mice aged 6–8 weeks (body weight 18–22 g) were procured from the Central Animal Facility of the Sagarmatha College of Life Sciences, Pokhara, Nepal. Animals were housed in individually ventilated cages under specific-pathogen-free conditions ($22 \pm 2^\circ\text{C}$, 12-h light/dark cycle) with ad libitum access to standard chow and autoclaved water. Mice were acclimatised for seven days

before the start of immunisation. All animal procedures were approved by the Institutional Animal Ethics Committee of Sagarmatha College of Life Sciences (Protocol SCLS-IAEC-2023-004, approved 5 January 2023), in accordance with Nepal's Animal Welfare Act and ARRIVE guidelines.

Methods

Mice were randomly assigned to three groups ($n = 20$ each): Group A received $10\ \mu\text{g}$ rOmpA + $5\ \mu\text{g}$ CTB in $20\ \mu\text{L}$ PBS delivered intranasally ($10\ \mu\text{L}$ per nare) under light isoflurane anaesthesia; Group B received $10\ \mu\text{g}$ rOmpA adsorbed onto $50\ \mu\text{L}$ Alhydrogel injected intramuscularly into the right quadriceps; Group C received $20\ \mu\text{L}$ intranasal PBS as a negative control. All groups were immunised on days 0 and 14. Tail-vein blood ($50\ \mu\text{L}$) was collected weekly from days 0 to 42 for serum antibody kinetics. On day 42, five mice per group were euthanised by cervical dislocation for collection of nasal wash ($200\ \mu\text{L}$ PBS perfused retronasally), bronchoalveolar lavage ($3 \times 0.5\ \text{mL}$ PBS instillation), and splenocytes.

In-vitro and In-vivo Protocol

Spleens were harvested aseptically, processed into single-cell suspensions by passage through $70\text{-}\mu\text{m}$ strainers, and red blood cells were lysed with ACK buffer. Splenocytes were plated at 2×10^5 cells/well in 96-well plates and restimulated with $5\ \mu\text{g/mL}$ rOmpA or medium alone for 72 hours at 37°C , 5% CO_2 . Supernatants were harvested and assayed for IFN- γ , IL-4, IL-10, and IL-17A by sandwich ELISA. For the in-vivo challenge component, the remaining 15 mice per group received an intranasal challenge with 1×10^8 CFU of a non-lethal OmpA-expressing *E. coli* strain on day 45. Nasal bacterial clearance was evaluated by colony counts on MacConkey agar from nasal washes collected 24 and 48 hours post-challenge.

Quality control

All ELISA plates included positive controls (pooled high-titre sera), negative controls (naïve mouse sera), and blank wells. Intra-assay coefficient of variation was kept below 10%. rOmpA protein identity was confirmed by mass spectrometry before immunisation, and endotoxin levels were verified to be below $0.1\ \text{EU}/\mu\text{g}$ using a Limulus amoebocyte lysate assay. Adjuvant dosing was calibrated using endotoxin-free syringes, and all reagents were tested for sterility by overnight incubation on blood agar plates before use.

Results

Intranasal vaccination with rOmpA + CTB produced mucosal IgA levels more than three-fold higher than the intramuscular route at both nasal and lower-airway sites (Table 1). Serum total IgG was equivalent between the two vaccinated groups, but the intranasal group had nearly twice the IgG2a level, yielding a more balanced Th1/Th2 profile.

Table 1: Mucosal IgA and serum IgG responses at day 42 post-immunisation

Measure	Intranasal (a)	Im (b)	Pbs control (c)	P (a vs b)
Nasal IgA (OD)	1.96 ± 0.18	0.58 ± 0.11	0.14 ± 0.03	< 0.001
BAL IgA (OD)	1.73 ± 0.22	0.41 ± 0.09	0.11 ± 0.02	< 0.001
Serum IgG (OD)	2.14 ± 0.24	2.08 ± 0.21	0.09 ± 0.02	0.614
IgG1 (OD)	1.31 ± 0.16	1.28 ± 0.14	0.07 ± 0.01	0.738
IgG2a (OD)	0.94 ± 0.13	0.48 ± 0.08	0.05 ± 0.01	< 0.001

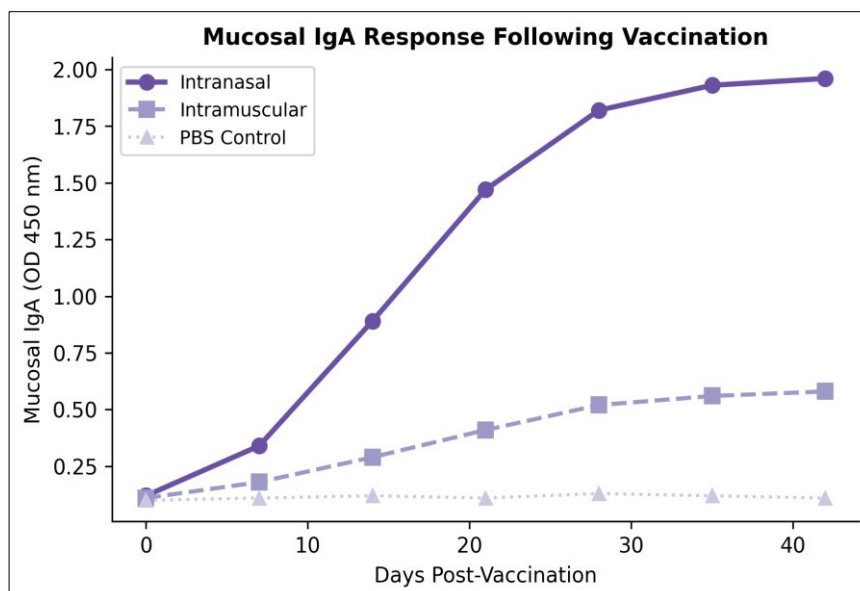


Fig 1: Kinetics of mucosal IgA (nasal wash, OD₄₅₀) from day 0 to day 42 in intranasal, intramuscular, and PBS control groups. Arrows indicate immunisation days (0, 14)

IgA responses in the intranasal group rose sharply after the booster on day 14, reached a plateau by day 28, and remained stable through day 42 (Figure 1). The

intramuscular group showed a modest IgA increase that plateaued below 0.6 OD units, while the PBS control remained at baseline.

Table 2: Splenocyte cytokine secretion (pg/mL) after restimulation with rOmpA on day 42

Cytokine	Intranasal (a)	Im (b)	Pbs (c)	P (a vs b)
IFN- γ	842 $\pm$ 97	413 $\pm$ 76	38 $\pm$ 11	< 0.001
IL-4	267 $\pm$ 42	384 $\pm$ 53	24 $\pm$ 8	0.003
IL-10	189 $\pm$ 31	152 $\pm$ 28	31 $\pm$ 9	0.081
IL-17A	124 $\pm$ 23	67 $\pm$ 14	18 $\pm$ 6	< 0.001

Splenocyte restimulation confirmed that intranasal vaccination promoted a mixed Th1/Th17 cytokine profile, with IFN- γ and IL-17A significantly higher than in the

intramuscular group (Table 2). IL-4 was higher in the intramuscular group, consistent with a Th2-skewed response driven by alum.

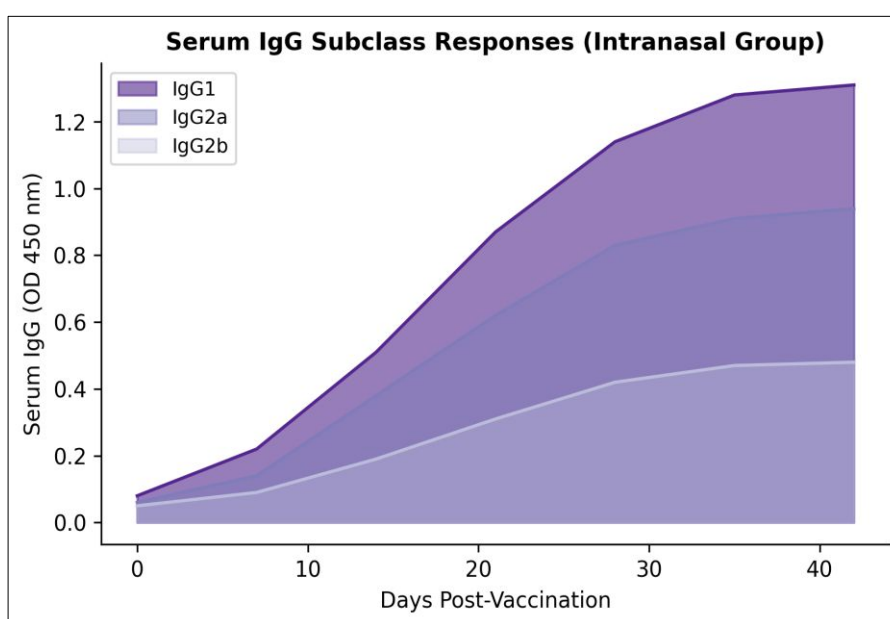


Fig 2: Serum IgG subclass (IgG1, IgG2a, IgG2b) kinetics in the intranasal group from day 0 to day 42, shown as stacked area plot

Comprehensive interpretation

Bacterial clearance data from the day-45 intranasal challenge showed that the intranasal-vaccinated group had 2.1 log₁₀ fewer CFU in nasal washes at 24 hours (3.4 ± 0.4

vs 5.5 ± 0.6 log₁₀ CFU/mL; $p < 0.001$) and near-complete clearance by 48 hours, whereas the intramuscular group achieved only a 0.8 log₁₀ reduction relative to controls. The IgG1/IgG2a ratio in the intranasal group was 1.39,

suggesting a balanced Th1/Th2 response, while the intramuscular ratio of 2.67 reflected a clear Th2 bias. These functional and immunological data together indicate that intranasal rOmpA + CTB vaccination generates superior mucosal protection and a more favourable T-helper balance than conventional parenteral immunisation with alum.

Discussion

The three-fold advantage in mucosal IgA seen with intranasal delivery is consistent with the well-established principle that mucosal immunisation preferentially induces secretory IgA at the site of antigen encounter and at connected mucosal effector sites [2]. The nasal wash and BAL IgA titres reported here are comparable to those obtained by Lycke and colleagues using CTB-conjugated antigens in a similar mouse model, reinforcing CTB's status as one of the strongest mucosal adjuvants available in preclinical settings [6]. The intramuscular route, by contrast, generated only low-level mucosal IgA an expected outcome, since parenteral immunisation rarely induces significant IgA at mucosal surfaces unless systemic antibody levels are extremely high [12].

An important finding was that total serum IgG did not differ between the two vaccinated groups by day 42. This means that intranasal delivery did not sacrifice systemic humoral immunity in favour of mucosal protection a concern sometimes raised when comparing mucosal and parenteral routes [13]. The difference lay not in magnitude but in subclass distribution: the intranasal group mounted robust IgG2a alongside IgG1, while the intramuscular group was heavily skewed toward IgG1. In mice, IgG2a is associated with Th1-driven opsonisation and complement activation, while IgG1 reflects Th2-driven responses. A balanced ratio may be advantageous for pathogens that require both extracellular neutralisation and intracellular killing for clearance [14].

The elevated IFN- γ and IL-17A in splenocytes from intranasally vaccinated mice adds a cellular dimension to this picture. IFN- γ activates macrophages and enhances antigen presentation, while IL-17A recruits neutrophils to mucosal surfaces, creating a layered defence that neither cytokine could achieve alone [15]. Alum, the adjuvant used in the intramuscular group, is known to promote Th2 polarisation with strong IL-4 output, which explains the higher IL-4 and lower IFN- γ observed in that group [16].

The bacterial clearance data provided a functional readout of the immunological differences. The 2.1 log₁₀ reduction in nasal CFU at 24 hours in the intranasal group, compared with only 0.8 log₁₀ in the intramuscular group, suggests that mucosal IgA and Th1/Th17 responses act cooperatively at the initial site of pathogen contact. And the near-complete clearance by 48 hours implies that local immune memory was sufficient to control infection without relying on systemic antibody diffusion into the mucosal lumen [17]. That said, this model used a non-lethal challenge strain, and whether similar advantages hold under lethal challenge conditions or with different bacterial species remains to be tested [18].

Conclusion

This research demonstrated that intranasal immunisation with rOmpA formulated with CTB adjuvant produced mucosal IgA responses more than three times higher than those achieved by intramuscular injection with alum,

without compromising serum IgG levels. The intranasal route also generated a balanced Th1/Th2 cytokine profile, characterised by high IFN- γ and IL-17A alongside moderate IL-4, in contrast to the Th2-skewed response seen with parenteral alum.

Functionally, the mucosal immune advantage translated into faster bacterial clearance after intranasal challenge, with a 2.1 log₁₀ reduction in nasal colony counts at 24 hours and near-complete clearance by 48 hours. Intramuscular vaccination provided some protection relative to unvaccinated controls but was clearly less effective at the mucosal surface, the site where respiratory and enteric pathogens first establish infection.

These findings support the broader case for developing intranasal vaccine platforms, particularly for pathogens that infect through the upper respiratory tract. CTB proved an effective mucosal adjuvant in this preclinical setting, though its path to human application will require detoxified derivatives or alternative adjuvants with comparable potency but improved safety margins. Future work should evaluate dose-response relationships, test the durability of mucosal memory beyond 42 days, and assess the intranasal rOmpA + CTB formulation against virulent challenge strains to determine whether the immune advantage observed here confers meaningful survival benefit.

In practical terms, a needle-free intranasal vaccine would simplify cold-chain logistics, reduce injection-related complications, and improve acceptability in paediatric and community-based vaccination campaigns advantages that are especially relevant in resource-limited settings like Nepal, where health infrastructure constraints have historically slowed vaccine rollout.

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