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# Nasal Lymphoid Tissue, Intranasal Immunization, and Compartmentalization of the Common Mucosal Immune System

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## Abstract

Mucosal application of vaccines with an appropriate adjuvant can induce immune responses at both systemic and mucosal sites, and therefore may prevent not only infectious disease, but also colonization at mucosal surfaces. Intranasal is more effective than intragastric immunization at generating earlier and stronger mucosal immune responses. Nasal lymphoid tissue (NALT) and its local draining lymph nodes may retain long-term immune memory. IgA isotype switching, and the differentiation and maturation of IgA antibody-secreting cells (ASC) may occur before these cells migrate out of NALT, whereas IgG ASC responses require passage of the cells through draining lymph nodes of the NALT. Knowledge of whether immune memory cells can recirculate to and reside in the inductive sites other than their origin after encountering antigen will be helpful for understanding the compartmentalization of the common mucosal immune system as well as for determining the best route for delivering a mucosal vaccine against a particular pathogen.

## Key Words

NALT
T-cells
B-cells
Draining lymph nodes
Mucosal vaccine
IgA

## Introduction

Vaccine research has advanced rapidly in the past few years, because of the urgent need to cope with new emerging pathogens and multi-antibiotic-resistant strains of bacteria, as well as to improve existing vaccines. Vaccines are conventionally administered parenterally and show good efficacy in protection against various pathogens. However, parenteral vaccines can usually only prevent systemic diseases by invasive pathogens, but are not very effective in preventing infection of mucosal surfaces *(1–5)*. This is because parenteral vaccines do not induce immune

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responses at mucosal sites. Mucosally applied vaccines are gaining more attention lately because most common human pathogens invade through or cause infections at mucosal surfaces, and because of the progress of knowledge on the common mucosal-immune system (CMIS) in both animals and humans *(1,3,6)*. It is clear that appropriate stimulation of mucosal-inductive sites can generate immune responses at mucosal effector sites featured by either secretory IgA or cell-mediated immunity *(7–10)*. Based on this knowledge, vaccines designed to generate mucosal responses have been administered intragastrically (i.g.) or intranasally (i.n.) to stimulate Peyer's patches (PP) or nasal lymphoid tissue (NALT), although other mucosal routes have also been explored *(6,11–15)*. The i.g. delivery of mucosal vaccines has been most widely studied, because of the convenience of vaccination by this route *(16)*. The characterization and function of PP as a mucosal-inductive site have been extensively investigated *(3)*. It is well accepted that PP can disseminate immune sensitized cells to mucosal-effector sites *(16,17)*. However, it has been found only recently that, at least in rodents, i.n. delivery of a vaccine is more effective in inducing both stronger systemic and generalized mucosal-immune responses *(12,18–20)*. Furthermore, knowledge on the immune function of NALT in rodents and Waldeyer's ring in humans is limited *(21,22)*, because of the difficulties in obtaining immune cells from these tissues, but progress has been made toward understanding these lymphoid tissues *(21,23–25)*.

The mucosal response to a vaccine can be affected by many factors (e.g., the form of an antigen, the vector employed, and the adjuvant used *[6]*), but the route for delivering a vaccine may be particularly important, since it was realized lately that the CMIS is compartmentalized, so that immune responses at mucosal-effector sites are not uniform *(11–13,15,24)*. The concept of compartmentalization of CMIS not only further defines the common mucosal-immune system, but establishes a theoretic base for considering the best route to deliver a mucosally applied vaccine *(13,15)*. This article focuses on i.n. immunization and recent developments in the study of nasal immune-inductive sites in the context of CMIS compartmentalization, and its implications in determining optimal mucosal immunization routes.

## Compartmentalization of the Common Mucosal-Immune System

The concept of the CMIS and its compartmentalization is summarized in Fig. 1. According to this concept, proper stimulation of a mucosal-inductive site can induce immune responses at remote mucosal-effector sites *(1,3)*. However, stimulation of different mucosal-inductive sites induces immune responses that are distributed unevenly at different effector sites. In general, immune responses are stronger at nearby mucosal-effector sites, or those related in terms of lymph drainage *(11–13,15,24)*.

Compartmentalization of the CMIS is supported by several studies. The i.n. immunization of mice with a protein antigen from *Streptococcus mutans* plus cholera toxin (CT) B-subunit induced responses in saliva, trachea, and gut. However, the IgA response was particularly strong in saliva. In contrast, i.g. immunization with the same antigen plus CT also induced immune responses in the aforementioned mucosal sites, but the strongest IgA response was in the gut *(12)*. Similar findings were also reported in immunization with CT *(13)*. Furthermore, intrarectal immunization with CT induced stronger responses in the rectum and also in the vagina, but little response was detected in saliva *(13,15)*. The i.n. immunization induced better protection from trachea and lung infection after i.n. challenge

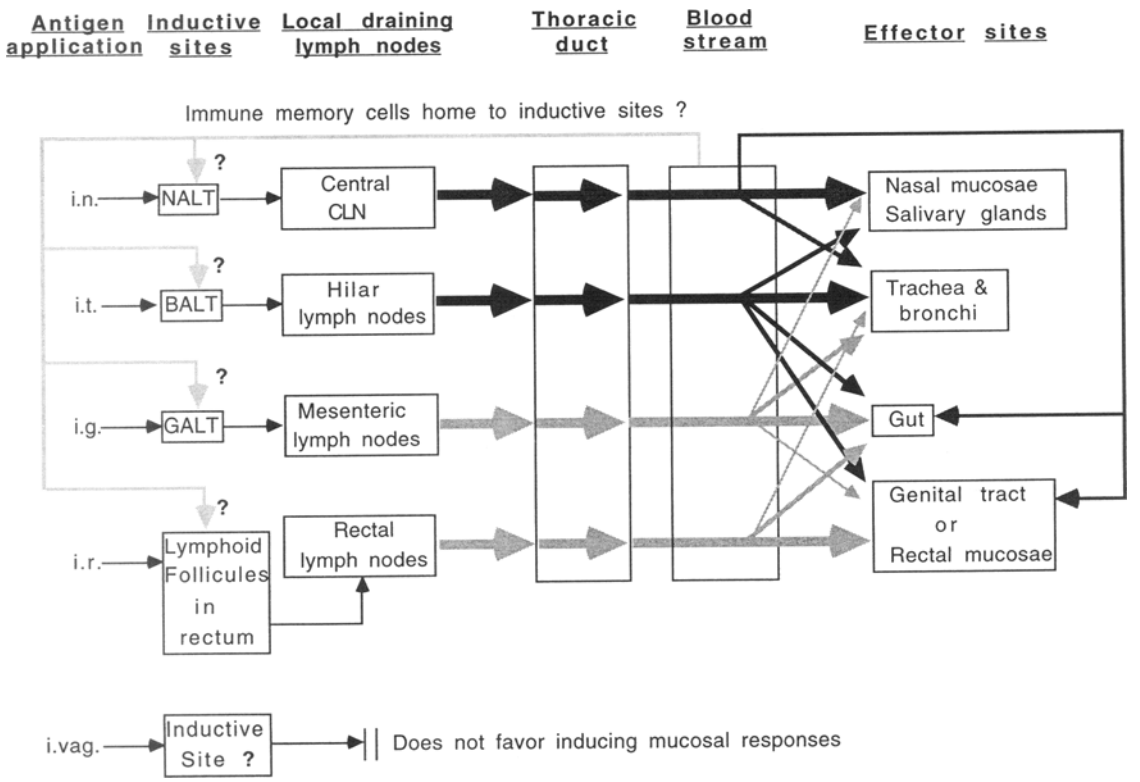


**Fig. 1.** The common mucosal-immune system and relative compartmentalization within the system. Mucosal-inductive sites mainly consist of NALT (adenoids and tonsils in the human nasopharynx), BALT (probably not present in normal human bronchi), GALT (PP), and lymphoid follicules in the rectum. T- and B-lymphocytes stimulated in these tissues preferentially home to related mucosal-effector sites more than distant sites (shown on the right of the graph). The vagina is an exception as it does not have organized lymphoid inductive tissue, but receives cells homing from other inductive sites. The intensity of the arrows represents strength of the immune responses; the width of the arrows represents the relative number of cells migrating to the effector sites. The lightest arrows with question marks represent the unknown process of migration of immune memory cells to inductive sites other than their origin. i.n., intranasal; i.t., intratracheal; i.g., intragastric; i.r., intrarectal; i.vag., intravaginal (*see* refs. *11–13,15*), and Dr. John H. Eldridge, personal communication).

with live *Bordetella pertussis* or respiratory syncytial virus than was provided by immunization with the same antigens by the i.g. route *(2,26)*. Intravaginal (i.vag.) immunization was generally less effective in inducing a significant mucosal-immune response at any mucosal-effector site including itself, although there are some different findings *(13,27–32)*, and the vagina does not contain an organized mucosal-inductive site. Human studies show that although IgA antibody was detected in saliva or nasal wash, antibody-secreting cells (ASC) induced by i.n. or intratonsillar immunizations were not detected in duodenal-cell suspensions, giving a clue that relatively compartmentalized mucosal-immune responses may also occur in humans *(24)*. Furthermore, i.n. immunization of humans with CTB induces high responses in adenoids but less in palatine tonsils, indicating that fewer ASC migrate to other mucosal-inductive sites, even if they are closely related ana-

tomically *(24)*. Although NALT T-cells were reported to adhere better to high endothelial venules of NALT than to those of PP on frozen tissue sections from rats *(23)*, it is not clear however whether immune memory cells home to other mucosal-inductive sites. This information indicates that:

1. Generalized mucosal immune responses can be induced by stimulating a proper mucosal-inductive site;
2. To induce effective and optimal immune responses at a particular effector site, the correct mucosal-inductive site must be stimulated; and
3. The responses at mucosal effector sites do depend on organized immune-inductive sites.

An explanation for the differences in generating nonuniform responses by stimulating different mucosal sites is not clear, but it may be because of such factors as anatomical location of the mucosal-inductive sites and their physiological environment, which may affect the ability of antigen to reach the inductive sites; differences in the dissemination of immune cells; and limitations in the migration of immune memory cells to other mucosal-inductive sites after antigen stimulation.

Although the gut lumen contains a huge quantity and variety of antigens and digestive enzymes, the threshold for the generation of immune responses may be quite high, as a result of digestion and mutual competition. In contrast, antigen uptake in NALT may be greater, where the total antigen load is less and the physiological environment (pH, digestive enzymes) is less severe. The vagina seems an unlikely location in which to initiate a strong mucosal-immune response, because of its reproductive function; additionally, it lacks organized lymphoid follicles equivalent to PP.

Thus, for the induction of strong and generalized mucosal-immune responses, i.n. immunization is usually more efficient than i.g., and the i.vag. route is the least effective *(12,13,15,19,33)*, although the properties of the antigens may play a role. We have observed that *S. mutans* protein AgI/II (an oral bacterial protein) conjugated to CTB plus CT as an adjuvant induced stronger ASC responses to itself than to CT in the salivary glands after either i.n. or i.g. immunization, although i.n. immunization resulted in more ASC to AgI/II in salivary glands than the i.g. route. In contrast, ASC to CT (an enterotoxin) in the lamina propria of the small intestine were more numerous after i.g. or i.n. immunization, although i.g. immunization induced more ASC to CT in lamina propria than i.n. immunization *(12)*. The explanation for these findings is not clear, but similar results have also been noticed by others who induced immune responses by i.vag. or intrarectal immunization of mice with HIV-1 *gag* and envelope antigens expressed in poliovirus or vaccinia virus *(15,34)*, but no responses were obtained by these routes after immunization with influenza virus (Dr. Zina Moldoveanu, personal communication).

The mechanisms of the CMIS and its compartmentalization undoubtedly arose during evolution as a result of interaction between pathogens and their hosts. Although most pathogens cause infection or invade the human body through certain mucosae, a few can cause infections at more than one mucosal site, e.g., *Escherichia coli*, *Chlamydia trachomatis*, adenovirus, cytomegalovirus, coxsackieviruses, Echo virus, and herpes simplex virus (HSV). For such pathogens, a generalized mucosal-immune response may help establishing immunity at different sites to prevent infection. However, as the majority of human pathogens cause infection at or invade through one restricted mucosal site, unwanted responses may be harmful or a waste of energy.

## Intranasal Immunization Against Respiratory and Oral Pathogens

The i.n. immunization has a long history, having been used as a route of variolation in ancient times. More recently it has been intermittently used since at least the 1970s *(35)*, and has now attracted increased attention because of its apparently greater efficacy in inducing a mucosal-immune response than the more conventional regimes of enteric immunization. I.n. immunization has been successfully used against respiratory syncytial virus and HSV-2 infections in respiratory tract, whereas other routes of immunization were not as effective *(2,36)*. The effect of blocking carriage at the nasopharyngeal mucosa by i.n. immunization was seen with group A streptococcus *(37)*. We have recently shown that i.n. immunization of mice with surface protein PspA from pneumococci can effectively block colonization by the bacteria in nasal cavities of mice, while subcutaneous immunization could not achieve this *(5)*. A similar conclusion was drawn from a study of human parainfluenza type 3 virus *(38)*. Furthermore, protection against i.n. inoculation of influenza A was attributed to IgA antibody to the virus *(39)*. It has been reported that i.n. immunization induces strong secretory IgA antibody responses not only in the respiratory tract and salivary glands, but also in the genital tract, as well as IgG antibodies in the circulation *(12,29,40)*. Protective salivary IgA antibodies induced by i.n. immunization with a *S. mutans* protein, or peptides derived from it, inhibited colonization of the bacteria on tooth surfaces *(41,42)*. A recent study showed that vaginal responses induced by i.n. immunization could establish long-term protection against *Chlamydia trachomatis* challenge in mouse vagina *(43)*. We have found that mice and monkeys immunized i.n. with a bacterial protein antigen (*S. mutans* AgI/II) coupled to CTB respond more strongly, and to lower doses, than with the same immunogen administered i.g. *(12,40)*. Recent studies in our laboratory have also shown that commercial CTB (contaminated with CT) or pure recombinant CTB (devoid of any toxic activity) can serve as an adjuvant when mixed with AgI/II and administered i.n., but neither were effective adjuvants when given i.g. *(12)* (Wu and Russell, unpublished data). It is not clear if these differences in the requirements of i.n. and i.g. immunization represent a basic difference between the NALT and gut-associated lymphoid tissue in their ability to respond to these immunogens, or if it is simply the result of greater sensitivity of the NALT. We have been able to induce protective levels of mucosal IgA and circulating IgG antibodies to pneumococcal PspA given i.n. in doses of <1 μg, together with CTB, whereas doses of at least 15–50 μg of AgI/II coupled to CTB appear to be necessary by the i.g. route to induce immune responses *(5,11)*. In this connection, it is important to realize that in i.n. immunization, the immunogen is placed in immediate contact with the inductive site, the NALT, and is not exposed to the harsh environment of gastric acid and gastrointestinal protease before reaching the GALT in the small intestine. However, although in our experiments the immunogen was applied in small volumes (<10 μL/nostril) in unanesthetized animals in order to limit the spread of immunogen into the trachea, transmission to this site of potential induction cannot be ruled out.

## Characterization of NALT and Its Draining Lymph Nodes

Human tonsils form a ring in the nasopharynx called Waldeyer's ring *(22)*. Recent studies on human palatine tonsils provided more information as to how an immune response is generated at this mucosal-inductive site *(22,44–49)*. Cell morphology, populations, and pathological features in the organ

are similar to other mucosal lymphoid tissues *(50)*. Immunization directly into the palatine tonsil or i.n. induced ASC responses in the same tonsil or adenoids as well as IgA and IgG antibodies in nasal wash, saliva, and blood circulation, indicating that the role of Waldeyer's ring is similar to other known mucosal-inductive sites in generating an immune response. Furthermore, i.n. immunization of humans with KLH alone could induce systemic tolerance to the antigen *(51)*, indicating this lymphoid tissue probably has similar function to other mucosal-inductive sites as seen in oral tolerance *(52)*. The phenomenon of nasal tolerance is similar to oral tolerance and may be able to suppress T-cell-mediated autoimmune diseases *(53–55)*. Direct study of normal human tonsillar tissues and its functions, especially the nasal tonsils is difficult for reasons of accessibility, but information on rodent NALT could be helpful for understanding the role of the tonsils.

NALT in rodents is considered to be the equivalent to Waldeyer's ring in humans *(21)*. It is a bilateral strip of nonencapsulated lymphoid tissue underlying the epithelium on the ventral aspect of the posterior nasal passage. NALT-cell morphology, phenotype, development, and structure as well as lymphocyte adherence to this tissue have been studied in rats *(23,56,57)*. Effective immunization of rodents by the i.n. route was considered to be the result of the stimulation of NALT, but the exact process of generating immune responses when NALT is exposed to antigen is not clear *(12,21)*. Recently we have isolated the NALT from mice and studied its immune function as well as cell populations *(25)*.

Lymphoid cell populations in the murine NALT were slightly different from PP. NALT contained a higher proportion of T-cells and lower proportion of B-cells relative to PP. Of $CD4^+$ T-cells, NALT included more $CD45RB^{hi}$ and fewer $CD45RB^{lo}$ cells compared to PP, indicating that NALT T-helper cells contained more naive and fewer memory cells than PP T-helper cells. This may be the result of a smaller antigen load in the nasal cavities than in the gut. Similar to PP, NALT contained a high proportion of unswitched B-cells with IgM–IgD double-positive phenotype and a low proportion of switched B-cells, including surface $IgM^+/IgA^+$ and $IgA^+$, and $IgM^+/IgG^+$ and $IgG^+$ B-cells. IgA-secreting cells were dominant in NALT of normal unimmunized mice, although the numbers were lower than in PP. Furthermore, as early as 2 or 3 d after i.n. immunization, antigen-specific ASC of all isotypes appeared in the NALT, but IgA cells were particularly elevated. This indicated that isotype switching, differentiation, and maturation of B-cells might have occurred in the NALT on exposure to antigen. Another difference between murine NALT and PP is that NALT may have a role in strictly local immunity, since more antigen-specific ASC appeared here after i.n. immunization than appeared in the PP after i.g. immunization. More importantly, NALT-cells proliferated in response to antigen restimulation in vitro 6 mo after the initial immunizations, indicating that NALT can retain long-term memory *(25)* (*see* Table 1 for a summary of the properties of the NALT in comparison with PP).

I.n. immunization with the same amount of antigen as given i.g. induced earlier and stronger mucosal IgA responses in almost all mucosal sites, including saliva, vaginal wash, gut wash and tracheal wash *(12)*, indicating that NALT can more efficiently disseminate immune cells than PP, although other factors may play a role. Indeed, one dose of i.n. immunization with a protein (AgI/II) conjugated to CTB induced mucosal IgA as well as serum IgG responses, which were not detectable after one dose of i.g. immunization even with twice the amount of antigen plus CT as an adjuvant,

**Table 1.** Comparison of cell populations in NALT and PP of unimmunized Balb/c mice and their function after i.n. or i.g. immunization[a]

| Cells | Surface marker | NALT | PP |
|---|---|---|---|
| Population | | | |
| T-cells | $CD3^+$ | 45[b] | 22 |
| T-helper cells | $CD4^+CD8^-$ | 32 | 17 |
| Naive Th cells/$CD4^+$ | $CD4^+CD45RB^{hi}$ | 75 | 55 |
| Memory Th cells/$CD4^+$ | $CD4^+CD45RB^{lo}$ | 16 | 20 |
| Tcs cells | $CD8^+$ | 11 | 4 |
| B-cells | $B220^+$ | 46 | 62 |
| Nonswitched B-cells | $IgM^+IgD^+$[c] | 42 | 61 |
| sIgA switched B-cells | $IgM^+IgA^+$ | 1 | 3 |
| sIgG switched B-cells | $IgM^+IgG^+$ | 5 | 5 |
| Function | | | |
| T-cell memory (proliferation in vitro) | | Yes | Yes |
| IgA B-cell switching | | Indicated | Yes |
| IgA B-cell differentiation | | Indicated | Yes |
| IgA ASC development | | Yes | Yes |
| IgG switching | | Indicated | Indicated |
| IgG differentiation | | Indicated | Indicated |
| IgG ASC development | | Yes | Yes |
| Ig-secreting cells in unimmunized | | Fewer | More |

[a]Refs. *2* and *25*.
[b]% of total gated lymphocytes.
[c]May include cells also expressing surface IgA or IgG.

showing evidence that i.n. immunization could induce earlier mucosal and systemic responses than i.g. immunization (Wu and Russell, unpublished data).

Although ASC in NALT were predominantly of IgA isotype, the systemic immune response was much enriched with the IgG class after i.n. immunization *(12,58)*. The exact mechanism for enhancing the IgG response is not clear, but recent studies suggested that switched IgG B-cells may receive additional help after migrating out of the NALT. Indeed, studies on draining lymph nodes of NALT showed that the superficial and central cervical lymph nodes are locations where the augmentation of IgG responses is strongly suggested *(58)*. Antigen induced interferon (IFN)-γ and interleukin (IL)-4 have been detected in the superficial cervical lymph nodes, and INF-γ also in central cervical lymph nodes after restimulation in vitro with the same antigen *(58)*. These cytokines are known to favor different subclasses of IgG responses *(59–62)*. Supporting this was the finding that IgG ASC, which were less numerous in NALT, were greatly increased in these draining lymph nodes, indicating that systemic IgG responses might be boosted when activated, switched IgG B-cells pass through them. Moreover, lymphocytes from the central cervical lymph node could proliferate in vitro in response to antigen restimulation 6 mo after

**Table 2.** Comparison of cell populations of draining lymph nodes of NALT and PP and their function after i.n. or i.g. immunization[a]

| | Draining lymph nodes of | | |
|---|---|---|---|
| | NALT | | PP |
| Cells | CCLN[b] | SCLN[c] | MLN[d] |
| Population | | | |
| T-cells ($CD3^+$) | 83[e] | 80 | 74 |
| T helper cells($CD4^+CD8^-$) | 63 | 60 | 57 |
| Naive Th cells($CD4^+$ $CD45RB^{hi}$)/$CD4^+$ | 73 | 73 | 73 |
| Memory Th cells($CD4^+$ $CD45RB^{lo}$)/$CD4^+$ | 17 | 15 | 16 |
| Tcs cells ($CD8^+CD4^-$) | 24 | 25 | 19 |
| B cells ($B220^+$) | 15 | 19 | 25 |
| Function | | | |
| T-cell memory (proliferation in vitro) | Yes | No | Yes |
| IgG B cell proliferation and differentiation | Indicated | Indicated | Less likely |
| IgG ASC development | Yes | Yes | Few |

[a]Ref. *12* and *58*, and unpublished observation.
[b]Central cervical lymph nodes.
[c]Superficial cervical lymph nodes.
[d]Mesenteric lymph nodes.
[e]% of total gated lymphocytes.

immunization, indicating that memory cells can reside in these lymph nodes for a long time *(58)* (*see* Table 2 for comparison of the draining lymph nodes of NALT and PP).

The postulated process of generating IgA and IgG responses after i.n. immunization is summarized in Fig. 2. Mucosal IgA responses are generated in NALT by antigen plus appropriate adjuvant. IgA B-cell isotype switching, differentiation, and maturation to IgA ASC might occur in this inductive site, as supported by the findings that transforming growth factor (TGF)-β and dominant type 2-cytokine mRNAs (including IL-5, IL-6, and IL-10) were detected after in vitro restimulation with the antigen, as well as dominant IgA ASC in NALT *(25)* (Wu and Russell, unpublished data). IgG isotype switching might occur in NALT also, although to a much less extent. This was supported by the findings that mRNAs of IL-4 and IFN-γ were also detected after in vitro restimulation with the antigen, as well as the occurrence of IgG1 and IgG2a ASC in NALT *(25)*. However IgG B-cell responses were not enhanced until passing through the draining lymph nodes of NALT with the help of IL-4 and IFN-γ *(58)*. The IgA ASC mainly home to mucosal-effector sites whereas IgG ASC mainly home to systemic lymphoid tissues.

Since i.n. immunization induced stronger, earlier mucosal- and systemic-immune responses than i.g. immunization, is the i.n. route of immunization preferable to the i.g. route for mucosally applied vaccines? The choice of route to deliver a mucosal vaccine may depend on where one wants to establish immune memory to a particular pathogen. However, there is insufficient experimental information to determine the answer at present. As shown in Fig. 1, it is known that

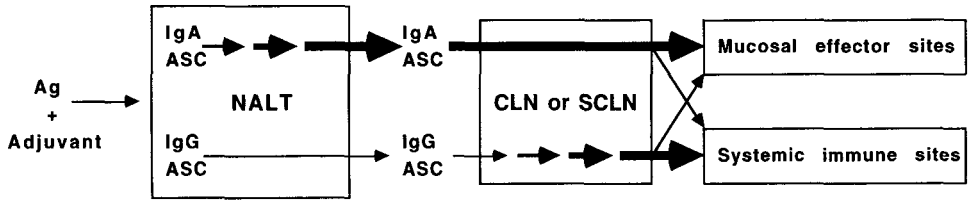


**Fig. 2.** Generation and enhancement of IgA and IgG responses to a protein antigen in NALT and draining lymph nodes. Both IgA and IgG isotypes can be switched in NALT, but IgA ASC increase greatly within the NALT with help from T-cells dominated by type 2 cytokines (IL-5, IL-6, and IL-10). However, a relatively small number of IgG ASC multiply in the draining lymph nodes of NALT under the influence of IFN-γ and IL-4. Thinner arrows indicate smaller numbers and thicker arrows indicate larger numbers of differentiating cells. ASC, antibody-secreting cells; CCLN, central cervical lymph nodes; SCLN, superficial cervical lymph nodes (*see* refs. *25* and *58*).

mucosal-inductive sites can generate mucosal responses by disseminating immune activated cells to mucosal effector sites when properly stimulated by antigens, and that they respond on restimulation and retain memory for a long time *(25)*. However, it is not clear whether memory cells can reside in inductive sites other than the one originally stimulated by antigen, or to what extent they can respond to restimulation and generate recall responses if they migrate to mucosal-inductive sites other than the original. Findings that few ASC occur in palatine tonsils after i.n. immunization in humans *(24)*, that fewer T-cells from NALT bind to high endothelial venules of PP than to those of NALT in rats *(23)*, and that i.g. immunization is less effective than i.n. immunization in protecting the trachea and lung against i.n. challenge with live *B. pertussis* or respiratory syncytial virus *(2,26)*, as well as the phenomenon of compartmentalization of the CMIS *(11–13,15,24)* do not favor the migration of immune cells between inductive sites. However, direct evidence of interaction between mucosal-inductive sites is currently not available, although this is amenable to study in mice where two major inductive sites, NALT and PP, are accessible *(25)*. This information should be helpful in determining the optimal route for a mucosal vaccine against a particular pathogen, and will provide evidence and an explanation for a compartmentalized CMIS. If few memory T-cells emigrate to PP after stimulation of NALT by i.n. immunization, a vaccine for an intestinal pathogen would be best given i.g. rather than i.n. in order to induce long-term immunity. The converse should also be true.

Most studies on mucosal vaccines concentrate on short-term effects, including challenge studies by using pathogens *(2,63)*. However, long-term immune memory can be established by i.n. immmunization, as has been shown by inhibition of pneumococcal colonization in the nasal cavities about 5 mo after i.n. immunization with PspA and CTB *(5)*, and by protection of mice from vaginal challenge with *C. trachomatis* 6 mo after i.n. immunization *(43)*. It will be interesting to see whether other routes of immunization with the same antigens can have the same long-term protective effects as i.n. immunization.

## Special Situations Related to i.n. Immunization

### Mucosal vs Parenteral Immunization

It has been reported that systemic immunization with conjugated *Haemophilus influenzae* vaccine can reduce *H. influenzae* b colonization in the upper respiratory tract from 6.3–1.5% in infants *(64)*, although

mucosal immunization with formalin-killed *H. influenzae* showed better blockage of colonization in the nasopharynx than s.c. immunization in mice *(65)*. These data raise the question whether mucosal vaccines are needed. Many factors may contribute to the observation, but local invasiveness of a pathogen is very likely an important factor to consider. Since most vaccines currently used parenterally in humans show efficacy in protection against invading mucosal pathogens, the key is whether they can be effective for reducing mucosal carriage of the pathogens. Blocking colonization is largely dependent on mucosal antibodies in the case of normal intact mucosal surfaces. However, if a pathogen is locally invasive, it may cause an inflammatory response at the mucosal surface and allow systemic humoral and cellular immune factors to inhibit colonization. Supporting evidence was observed in the nonspecific inhibition of the carriage of pneumococci and group A streptococci carriage at the mouse nasal and pharyngeal mucosae by the inflammation induced by CTB (contaminated with CT) *(66)* (Wu et al., unpublished data). It is probable that antigen-specific IgG leaking from plasma may block colonization, since passive application of antigen-specific IgG blocked colonization of tooth surfaces by *S. mutans (67)*.

The murine nasal cavity is apparently a mucosal-effector site with IgA predominantly of polymeric form (Wu and Russell, unpublished data). With noninvasive pathogens, blocking mucosal carriage probably depends on effective mucosal immunity, as shown by the evidence that colonization of a nonvirulent strain of pneumococcus can be blocked at the nasal mucosae by immunization with PspA only i.n. but not s.c. *(5)*, and blocking *H. influenzae* from carriage at the nasopharynx by oral immunization but not by s.c. immunization *(65)*. In the case of locally invasive pathogens, IgG leakage likely occurs after infection and the resulting damage to the mucosal surface, whereas secretory IgA may be effective before colonization starts, thereby preventing infection and damage. Although the nasal cavity is a mucosal effector site, human nasal secretions also contain IgG *(68)*, which might account in part for the difference between observations in humans and mice. Furthermore, it has been reported that IgA does show better advantage in blocking bacterial adherence to the mucosal surface than IgG does *(69–71)*.

Ideally, vaccines will not only prevent disease but also prevent colonization and carriage at mucosal surfaces, and further prevent spreading and protecting high risk and immune compromised populations. Therefore, mucosal immunization may have an advantage in this regard, since it can generate IgA at mucosal surfaces and facilitate the clearance of organisms, or block their colonization.

## Vaginal Immunity

It has been reported that a mucosal-immune response in the vagina can be induced by i.n., i.g., intrarectal, as well as intramuscular immunizations *(12,13,15,29,31,40)*, and that i.n. immunization was particularly efficient in inducing IgA responses in the vagina of mice *(12,30,40)*. Immunity in the female genital tract is regulated by hormones, and IgA secretion and antigen presentation vary with the estrous cycle *(72,73)*. However, immune responses in the vagina are not efficiently induced by i.vag. immunization, nor are responses at other mucosal sites, although some serum antibody responses may be generated *(13,15,27–30,60,74)*, probably owing to the lack of organized mucosal-inductive sites. The vagina is probably a special place in the CMIS—where immune responses are carefully regulated—because of its reproductive physiologic role. Indeed, if the immune

responses are easily induced by i.vag. immunization, fertilization could be inhibited. However, immunity at this site may be achieved by other mucosal routes of immunization, especially i.n. *(12,30,40,43)*.

The murine vagina is apparently a mucosal-effector site; analysis of antibody in vaginal wash showed predominantly IgA of polymeric form, although IgG isotype was also detectable (Wu and Russell, unpublished observation). Most IgG in the vagina of rats was thought to come from the uterus, since ligation at the cervix resulted in accumulation of antigen-specific IgA and IgG in the uterus but IgG was not then found in the vagina *(72)*. In humans, the vagina is greatly affected by systemic immunity. Menstrual blood contamination and serum transudation may account for most of the IgG, and therefore, human vaginal wash samples taken on different days of the menstrual cycle may reflect accumulated IgG from menses. Nevertheless, it may be beneficial to generate IgA responses in the vagina because IgA has a greater ability to inhibit mucosal colonization of pathogens and, also because IgA remains present in the vagina even after menopause.

## Nasal Adjuvants

Effective mucosal immunization depends on appropriate adjuvants. Protein antigen alone applied i.n. may not induce effective mucosal- and systemic-immune responses *(5,75–77)*. In contrast, it may induce systemic tolerance *(51)* as seen with feeding protein antigens *(52,79)*. Oral tolerance is suppression of the respond to an antigen injected systemically after feeding with the antigen, especially as revealed by suppression of delayed-type hypersensitivity *(80)*. Nasal tolerance is similar to oral tolerance; it is now realized that tolerance in the systemic response can be cell-mediated (Th1 type) *(78)*, humoral (Th2 type) *(81)*, and or both *(81)*, depending on the type of response induced at the mucosal site. Cell-mediated autoimmune reactions have been shown to be suppressed by nasal application or oral feeding with protein antigens *(53,82)*. These suppressive effects involve either anergy in T-cells or the release of IL-4, IL-10, and maybe TGF-β in systemic-lymphoid tissues *(78,82–84)*, indicating a similar principle in nasal and oral tolerance *(82,84)*. However, since mucosal-immune responses are not confined to one type of T-cell response, suppression of humoral responses at systemic sites was also seen when type 1 responses were induced at mucosal sites *(81)*. To avoid the development of systemic tolerance of either type, an appropriate mucosal adjuvant should be used, otherwise, these routes of immunization against invasive pathogens may be more harmful than beneficial. An appropriate nasal adjuvant should be able to enhance mucosal-immune responses, and break nasal tolerance as well as enhance systemic immune responses. Currently, several mucosal adjuvants and carriers are being tested for nasal application in animals, including CTB *(12)*, heat-labile toxin B subunit from *E. coli (85)*, immunostimulatory complexes *(86)*, bacillus calmette—Guerin (BCG) *(20)*, liposomes *(87)*, and biodegradable microspheres *(75)*. Some of these may have prospects for use in humans.

Some adjuvants which cannot be used parenterally route may still be acceptable for mucosal application. Furthermore, an adjuvant which may not work by the oral route may work i.n., because very different physiological environments are in the gut and in the nasal cavities; e.g., CTB is effective i.n. but not i.g. *(11,12,76)*.

## Allergic Reaction

Atopic allergy is commonly revealed in the human respiratory tract as a result of sensitization by certain inhaled antigens. Chronic inflammation is thought to be one major cause

of allergic sensitization *(88)*. Because i.n. immunization may entail a local inflammatory reaction, there is some concern that it could lead to the induction of an allergic response. However, there is little information available and further studies are needed to investigate this important area.

## Concluding Remarks

The route of delivery for a mucosal vaccine may affect the development of protective immunity to a particular pathogen, according to its route of invasion, the different properties of antigens and adjuvants, differences in mucosal-inductive sites, and the compartmentalization of the CMIS. Understanding all these factors will be helpful for selecting appropriate routes for inducing optimal mucosal-immune responses as well as to establish long-term immunity against a particular pathogen.

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