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kiyono@ims.u-tokyo.ac.jp

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t.t.macdonald@qmul.ac.uk
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vineyj@amgen.com

Executive Director:
Mark H Epstein, ScD
mepstein@paimgmt.com

Office:
Society for Mucosal Immunology
5272 River Road, Suite 630
Bethesda, MD 20816
Tel: (301) 718-6516
Fax: (301) 656-0989
E-mail: smi@paimgmt.com

Cover Art: BALB/c mice were adoptively transferred with CFSE-labelled, DO11.10 T cells and fed OVA. Inguinal lymph nodes were taken 20hrs after feeding and imaged by multiphoton excitation microscopy. The location of cells within the intact lymph node was measured in 21 planes per three-dimensional stack of 222 x 178 µm optical sections collected at 2.5 µm intervals between each plane progressing deeper into the lymph node from an initial depth of approximately 100 µm below the surface. The time interval between each stack was 18s. The image shown is a single plane 150 µm within an intact lymph node.

Image provided by: Bernd H. Zinselmeyer1,2, John Dempster3, Alison M. Gurney4, David Wokosin2, James M. Brewer1 and Paul Garside1. 1Division of Immunology, Infection and Inflammation, University of Glasgow; 2Centre for Biophotonics, University of Strathclyde; 3Department of Physiology and Pharmacology, University of Strathclyde.

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Table of Contents

Introduction .......................... 3

Bacterial enterotoxins as mucosal vaccines and immunomodulators .......................... 3-6

Horses for courses: Probiotic organisms as mucosal adjuvants .......................... 6-9

Mast cells and Mucosal Vaccines .......................... 9-12

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Introduction

Numerous studies have established “proof-of-principle” for mucosally applied vaccines to elicit protective immunity. However, many such studies have relied upon experimental adjuvants often unsuitable for human application. A major limitation to advancing mucosal immunization in humans has been the absence of acceptable mucosal adjuvants. To help highlight this pressing need, we would like to begin this discussion with three short articles giving a status of where we are and what possibilities may lie ahead. First, we begin with the work by Jan Holmgren, Ali M. Harandi, and Cecil Czerkinsky that describes the future of mutant enterotoxins as human mucosal adjuvants. Second, Paul Forsythe and John Bienenstock illustrate the use of protobiotics as natural adjuvants that can be adapted to use, and it is widely recognized that for both purposes there is a need for developing effective mucosal adjuvants. In this brief overview, we will summarize our perspectives on the development of combined mucosal antigen-delivery and adjuvant systems based on cholera toxin (CT) and heat-labile Escherichia coli enterotoxin (LT).

Cholera toxin and cholera vaccine

CT was the first toxin dissected in its molecular structure and function (1). The AB5 subunit structure was identified, with a B pentamer responsible for binding to target cells and a toxic-active A subunit mediating cell toxicity; the specific cell receptor was identified as the GM1 ganglioside; the A subunit (A1 fragment) was shown to intracellularly, by an enzymatic process, ADP-ribosylate the Gs protein leading to adenylate cyclase activation and increased cyclic AMP formation; and these effects were functionally linked to explain the excessive salt and fluid losses characteristic of the cholera disease. Subsequent work showed that E. coli LT is strikingly similar to CT in its structure and function, the main difference being that LT can use GM1 and a few structurally related glycoproteins and complex glycolipids as receptors.

Early work also identified the non-toxic B subunit pentamer (CTB) as a rational safe oral vaccine immunogen against cholera (1). This finding and studies showing that intestinal antibacterial cholera immunity cooperates synergistically with intestinal antitoxic immunity, led to the development of the oral CTB-whole cell cholera vaccine. This vaccine is now registered under the trade name Dukoral® in more than 50 countries world-wide, and represents one of the still few mucosal vaccines available for human use. It has in several large field trials in Asia, Latin-America and Africa been shown to provide 80-90% protection against cholera and improve adaptive immunity against viral and other antigens.

Finally, as an alternative to exogenously administered adjuvants is the potential to stimulate innate cells, possibly mast cells, to serve as a source of mucosal adjuvants (cytokines), addressed by Herman F. Staats, James B. McAlachlan, Christopher P. Shelburne, Justin P. Hart, Salvatore V. Pizzo, and Soman N. Abraham. From these three articles, it is evident that novel approaches are warranted to develop the next generation of mucosal adjuvants that will be suitable for human use.
additionally 50-70% short-term cross-protection against diarrhea caused by enterotoxigenic *E. coli* (ETEC).

For further details of the molecular properties and cellular actions of CT and LT and cholera vaccine see refs 3 & 4.

**Toxins as tools for studying mucosal immune responses**

The oral B subunit-whole cell cholera vaccine and its isolated CTB component have also been widely used tools for defining interlinked mucosal inductive and expression sites in humans after vaccination (Fig. 1). Whereas oral immunization may induce substantial antibody responses in the small intestine, in the ascending colon and in the mammary and salivary glands, it is relatively inefficient at evoking an IgA antibody response in the distal segments of the large intestine, in the tonsils, or in the female genital tract mucosa. Conversely, rectal immunization evokes strong local antibody responses in the rectum, but little if any response in the small intestine and in the proximal colon.

Nasal or tonsillar immunization in humans results in antibody responses in the upper airway mucosa and regional secretions (saliva, nasal secretions), without evoking much of an immune response in the gut; however, of special interest for possible vaccination against HIV and other sexually transmitted infections, nasal immunization can give rise to substantial IgA and IgG antibody responses in the human cervico-vaginal mucosa, similar to the genital response to intravaginal vaccination. Intriguingly, at least in mice transcutaneous immunization may also induce a mucosal immune response in the female genital tract.

In addition to providing fundamental knowledge about lymphocyte homing after mucosal vaccination in humans, these findings give promise that oral, nasal, vaginal or rectal immunization in humans with other vaccine-relevant immunogens linked to CTB may in a similar way elicit site-directed mucosal IgA and IgG immune responses and mucosal protection against pathogens in humans.

**Adjuvant action of CT and LT**

CT and LT can affect several steps in the induction of a mucosal immune response to a co-administered antigen. These effects, which alone or in combination might explain their strong adjuvant action, include: 1) increased permeability of the epithelium leading to enhanced uptake of co-administered antigen; 2) enhanced antigen presentation by various APCs; 3) promotion of isotype differentiation in B cells leading to increased IgA formation; and 4) complex stimulatory as well as inhibitory effects on T cell proliferation and cytokine production. Further, CT and LT have been shown to not only avoid inducing oral tolerance but also to abrogate otherwise efficient regimens for mucosal tolerance induction and as shown recently in connection with this to also abrogate the development of regulatory/suppressor T cells associated with oral tolerance induction, both CD25+ and CD25- T cells (J-B Sun et al., unpublished). It may also be relevant that oral administration of CT has been found to lead to a transient depletion of CD8+ intraepithelial lymphocytes (C-F Flach et al., unpublished).

Among these effects, those leading to enhanced antigen presentation by various APCs are probably of the greatest importance for the adjuvant activity (6). CT markedly increases antigen presentation by dendritic cells (DCs), macrophages and B cells and has also been found, at least *in vitro*, to make intestinal epithelial cells become effective APCs. Consistent with this, CT up-regulates the expression of MHC/HLA-DR molecules, CD80/B7.1 and CD86/B7.2 co-stimulatory molecules, as well as chemokine receptors such as CCR7 and CXCR4 on both murine and human DCs and other APCs. Importantly, CT also induces the secretion of IL-1β from DCs, thus supporting similar observations made earlier for macrophages. IL-1 not only induces the maturation of DCs, but is also by itself an efficient mucosal adjuvant when co-administered with protein antigens and might mediate a significant part of CT’s adjuvant activity.
The precise cellular mechanism by which CT and LT exert their adjuvant action remains incompletely known (2,5,7). It is clear that the adjuvant activity of CT and LT is critically dependent on retained receptor binding activity of the B subunit and that it is quantitatively closely associated with the ADP ribosylating activity of the A subunit; the extent to which the adjuvant function of ADP-ribosylation is mediated through cAMP and/or through some as yet undefined signal has not been conclusively defined.

It has been claimed that CT primarily induces a Th2 type of immune response characterized by CD4+ T cells producing IL-4, IL-5, IL-6 and IL-10 and by the production of IgA, IgG1 and IgE antibodies. LT, on the other hand, has been reported to induce a mixed Th1- and Th2-type immune response. However, our own and other studies have shown that also CT can induce mixed Th1- and Th2-types of immune responses, in contrast to CTB, which appears to induce a more restricted Th2 type of immune response. Thus DCs, which had been pre-treated in vitro with a protein antigen (ovalbumin, OVA) linked to or admixed with CT and then injected into mice, induced both Th1 and CTL antigen-specific responses, in addition to a Th2 response. In contrast, DC pulsed in vitro with OVA linked to CTB only gave rise to a Th2 type of immune response (5).

Mucosal adjuvants based on engineered enterotoxin derivatives

Since the toxicity of CT and LT preclude their use in humans, much effort has been made to generate toxicologically acceptable derivatives with retained adjuvant activity (Fig. 2). The products include, e.g.:

- Single mutations in ADPR site e.g. LTK63
- Single mutations to block "nicking" e.g. LTG192
- Peptide extension to block ADPR site e.g. 23-CT
- CTA1 linked to non-CTB APC binding protein e.g. CTA1-DD
- Cpg ODNs linked to CTB

![Figure 2: Examples of mucosal adjuvant molecules based on detoxified CT or LT. For references see (5, 7).](image)

b) Mutant LT or CT proteins have also been generated in which the toxic-active A (A1) subunit has been modified in various ways to remove the ADP-ribosylating activity/toxicity. In general, a loss in toxicity has been matched with a corresponding loss in adjuvanticity, but a few proteins are available with significant adjuvanticity in the absence of detectable toxicity when given intranasally.

c) Yet another approach has been to instead prepare hybrid molecules in which the fully active CTA1 subunit has been linked to an engineered specific APC binding protein derived from S. aureus protein A (CTA1-DD). This specifically targets the molecule to B cells and has in experimental systems proved to be a very efficient and safe adjuvant for co-administered antigens when given intranasally. The incorporation of CTA1-DD and antigen into ISCOM particles may render the adjuvant effective for oral use.

d) Bacterial DNA or synthetic oligodeoxynucleotides containing unmethylated “CpG-motifs” (CpG ODN) linked to CTB represents another promising type of mucosal adjuvant. CpG ODN stimulate cells that express Toll-like receptor 9, thereby initiating an immunomodulating cascade. Although as yet mainly considered for systemic use, CpG ODN has been found to markedly enhance both innate and adaptive mucosal immunity in animal models after nasal, oral or vaginal administration, effects which were strongly enhanced when CpG ODN was linked to CTB (J.Adamsson et al., unpublished). Of special interest, is the finding that the coupling of CpG ODN to CTB dramatically enhanced the immunostimulatory activity for human cells. The mechanism of action CTB-CpG conjugate may include both an increased uptake of CpG into endosomes and allowing for CpG to travel with CTB to the endoplasmic reticulum for interaction there with TLR-9.

Oral tolerance induction using cholera toxin B subunit

Mucosally induced immunological tolerance has become an attractive strategy for preventing and possibly treating illnesses resulting from the development of pathological immune reactions against allergens as well as self-antigens. However, while mucosal tolerance is usually effective in animal models for preventing inducible autoimmune diseases, its efficacy has been more variable and limited when attempted...
as an intervention strategy in animals in which the disease had already been induced or had spontaneously developed. This may explain in part the disappointing results of recent clinical trials of oral tolerance in patients with type I diabetes, multiple sclerosis, and rheumatoid arthritis, diseases in which there may be multiple target autoantigens that remain largely unknown.

A significant improvement has been achieved by co-administering CTB to enhance the tolerogenic activity of autoantigens or allergens given orally or nasally (9). When conjugated or co-administered with several autoantigens and allergens CTB has efficiently induced tolerance in already sensitized animals and suppressed progression of various autoimmune diseases. Recently, in patients with Behcet’s disease, an autoimmune eye disease often associated with extra-ocular manifestations and exhibiting an abnormal T cell reactivity to a specific peptide sequence of the human 60 kDa heat shock protein (hsp60), a small phase I/II trial has demonstrated the safety and clinical efficacy of treatment with a conjugate of the specific hsp60 peptide linked to CTB (10). The results, if confirmed in expanded placebo-controlled studies, give promise that the concept of oral tolerance as an immunotherapeutic modality may now be taken from vision to reality.

References

Corresponding author: Jan Holmgren
Email: jan.holmgren@microbio.gu.se

Horses for courses: Probiotic organisms as mucosal adjuvants

Paul Forsythe and John Bienenstock

The Brain Body Institute, St. Joseph’s Healthcare, McMaster University, Hamilton, Ontario, Canada.

Introduction

Probiotics are non-pathogenic live organisms that promote beneficial health effects when ingested [1]. Candidate probiotics are most frequently commensal organisms of the Lactobacilli or Bifidobacterium genera. There is increasing evidence to support a therapeutic role for probiotics in the treatment of GI infection, irritable bowel syndrome, inflammatory bowel diseases, allergy and autoimmune disorders [1,2]. While the mechanisms of action are unclear many of the beneficial effects of probiotics are mediated via immune regulation and in particular control of the balance of proinflammatory and anti-inflammatory cytokines. While the majority of studies have been carried out with fed probiotics it is clear their immunomodulatory and therapeutic effects are not restricted to the oral route of administration [3]. It is possible to discriminate two distinct effects of oral probiotics on the immune response. One is the suppression of an undesired immune response, for example allergic and autoimmune reactions; the other a generalized immunomostimulatory effect associated with adjuvanticity and increased intestinal non-specific IgA secretion [4].

Oral tolerance represents a significant obstacle to the development of effective mucosal vaccines for use in the gastrointestinal tract. Consequently, much research has been directed towards the development of mucosal adjuvants and antigen delivery systems to overcome the hyporesponsiveness to ingested proteins [5].

Adjuvants can act as vaccine delivery systems that target antigens into antigen presenting cells, immunostimulators that activate cells of the innate immune system or, particularly effectively, a combination of both. One approach for inducing efficient local immune responses relies on the development of live bacterial carriers. Attenuated pathogens are effective as vaccines against the pathogen itself but also show great potential as carriers for antigens of other pathogens for which an immune response is desired. However, these organisms are strongly immunogenic themselves making them unsuitable for use in immunocompromised individuals in whom they have the potential to be pathogenic. Also there is some evidence that prior exposure to the bacterial vector might compromise the efficacy of the vaccine construct [6]. For this reason attention has turned to the potential for non-pathogenic commensal or probiotic organisms to act as effective mucosal adjuvants [7].

Probiotics as adjuvants

In addition to increasing IgA expression in the mucosa, several strains of live lactic acid bacteria have been shown to induce the release of the proinflammatory cytokines TNF...
and IL-6, reflecting stimulation of non-specific immunity [8]. Oral administration of *L. casei* and *L. bulgaricus* activates the production of macrophages [9] and administration of *L. casei* or *L. acidophilus* activates phagocytosis in mice and humans [10,11]. Phagocytic activity results in the further recruitment of immunocompetent cells and the generation of an inflammatory response. In addition to these general immune responses there are also clear examples of candidate probiotic organisms demonstrating adjuvant activity in association with specific antigens.

Repetitive feeding of certain *Lactobacillus* strains can increase the virus neutralizing antibodies elicited after parenteral immunization with influenza haemagglutinin subunit vaccine. This has led to the suggestion that daily intake of lactobacilli before an annual flu vaccination should result in better immune memory and protection against the virus [12]. The influence of a range of *Lactobacillus* strains on IgG1 and IgG2a levels was measured following intraperitoneal immunization with trinitrophenylated chicken gamma-globulin (CGG-TNP) a thymus dependent antigen. Oral administration of *L. reuteri* caused an increase in anti-CGG activity and a decreased IgG1/IgG2a ratio of antibodies directed against CCG [13]. These results indicated that *L. reuteri* induces the cellular as well as humoral responses, characteristics common to vaccine adjuvants. However, the majority of *Lactobacillus* strains tested did not affect the systemic immune response. Nasal or oral immunization of mice with a range of *Lactobacillus* strains expressing fragment C of tetanus toxin (TTFC) has been used successfully to induce secretory and protective systemic responses against tetanus toxin including specific IgG, IgA and T-cell responses in the local lymph nodes [7]. While these results clearly show that it is possible to use probiotic organisms for mucosal immunization there has been limited success with other antigens. Furthermore, in contrast to these immunostimulating effects, many of the beneficial effects of probiotics appear to be related to their ability to induce anti-inflammatory and tolerogenic effects. Indeed, lactobacilli have been used to induce tolerance in mice against the Der p 1 epitope of the house dust mite [14] and when intranasally co-applied with recombinant Bet v 1, the major birch pollen allergen to increase levels of IgG2a antibodies, in vitro IFNγ production and suppression of allergen induced basophil degranulation [15].

### Strain dependent immunomodulation

Overall, evidence suggests that probiotics play a paradoxical role in immune regulation: they induce a tolerogenic response that may be utilized in therapy for allergic disorders and auto-immune disease and also mediate general immunostimulatory (adjuvant) responses that may be exploited for development of effective vaccine delivery systems.

Much of this disparity in immune response appears to be due to differing inherent characteristics of specific probiotic organisms that include colonization, adhesion and intrinsic immunogenicity. In this regard the cytokine profile elicited by a probiotic organism clearly plays an important role in determining the immunological outcome.

*L. rhamnosus* HNO01 enhances production of both T helper (Th1) and Th2 cytokines in mice primed with ovalbumin in alum adjuvant [16], while attenuation of a murine model of colitis by *L. salivarius* and *Bifidobacterium infantis* is associated with a reduced ability to produce Th1-type cytokines both systemically and mucusally [17]. In an extensive study of this issue Maassen et al analyzed eight different common *Lactobacillus* strains with respect to gut mucosal induction of pro-and anti-inflammatory cytokines in response to a parenterally administered antigen [13]. *L. casei* tended to induce the production of IL-10 and TGFβ. IL-10 and TGFβ have immunosuppressive effects on Th1 cells and are thought to be involved in oral tolerance. In contrast, the strains *L.reuteri* and *L.brevis* induced several pro-inflammatory and/or Th1 cytokines IL-1β, IL-2 and TNF but not anti-inflammatory or Th2 cytokines such as IL-10 and IL-4. These same strains were able to significantly enhance the systemic antibody response to the antigen. It is clear that realizing the true potential of probiotics as adjuvants will require a better understanding of the mechanisms behind the quantitative and qualitative differences in immune regulation that exist among candidate organisms.

### Determinants of the immune response

Historically, some of the most effective adjuvants are derived from bacterial components such as lipopolysaccharide, lipopeptides and the CpG motif of bacterial DNA. These components are now recognized as pathogen associated molecular patterns (PAMPs) that act as ligands for Toll-like receptors (TLR). Binding of PAMPs to TLRs causes dimerization of the receptor that enables attachment of an adaptor protein leading to activation of nuclear factor κB (NFκB) and consequent transcription of several genes including IL-1, 12 and TNF. The term PAMPs is a misnomer as these components are present in abundance on all microbes. However, like other commensals, probiotics do not naturally evoke a specific immune response through the innate immune system as to do so would result in a constant state of gut inflammation. As is the case for pathogens certain probiotic organisms may abrogate TLR activation by modifying the structure or expression of PAMPs, directly inhibiting TLR signaling or activating parallel receptors that interfere with TLR signaling [18]. Furthermore the specific
array of PAMPs expressed by an organism may trigger a particular immunological response. The first adaptor protein described was myeloid differentiation factor 88 (MyD88) [19]. Additional adaptor proteins have been described in recent years, including those identified by the acronyms TRAM, MAL/TIRAP and TRIF/TICAM [20]. Different adaptor proteins and combinations thereof may preferentially interact with particular homodimeric or heterodimeric TLRs to direct specific cellular responses. This system could thus act to orchestrate a range of potential immunological outcomes when exposed to the specific array of PAMPs expressed by individual micro-organisms.

TLRs are strategically expressed on cells that are the first to encounter pathogens or commensal organisms. The epithelial cell is the immediate interface between microbial organisms and the immune system and so the binding of PAMP to these cells likely has an important role in the immunomodulatory response to probiotics at the mucosal surface. A concept supported by the potent direct anti-inflammatory activity of L. reuteri on human epithelial cells [21]. Indeed one possible explanation for increased IgA secretion might be upregulation of the poly Ig receptor on the epithelium [22]. Once an organism, or antigen, has penetrated the epithelial barriers of the GI tract usually via M cells that are located in the Peyers patches it is met by macrophages and dendritic cells (DC) that act as antigen presenting cells (APC). Alternatively intestinal content can be sampled directly by DC that can protrude into the lumen through the tight junction of epithelial cells [23]. The microbial ligation of PAMPs on APC enhances the ability of the cell to present antigen and to stimulate T-cell activation resulting in increased adaptive immune responses. Here again there are differences seen in the effect of candidate probiotic organisms on DC function [24,25]. The capacity of lactobacilli to variably induce maturation and the cytokine profile expressed by DC indicates that strains of probiotics may differentially determine whether a DC drives Th1 Th2 or a Treg response. MacPherson and Uhr [23] demonstrated that intestinal DC retain small numbers of live commensals for several days, which allows the DC to selectively induce IgA. However, little is known about the fate of probiotic organisms in the gastrointestinal tract with only a few investigators addressing this issue [23,26]. In the future determining the extent of colonization, adhesion to epithelium and how and where probiotic organisms are exposed to APC may help determine their suitability for use as mucosal adjuvants or as an aid to inducing tolerance in allergic or autoimmune diseases.

When considering utilizing probiotic or commensal organisms as mucosal adjuvants it is important not to regard them as merely immunogenic particles. These organisms constantly monitor their environment and may alter their behavior and characteristics accordingly [27]. Indeed, it is interesting to note that probiotic bacteria were shown to modulate phagocytosis differently in healthy and allergic subjects. In healthy people there was an immunostimulatory effect whereas in allergic subjects there was down-regulation of the immune response [28]. Therefore the modulating effects of probiotic bacteria may depend on the immunologic state of the host.

Microbial organisms communicate and co-ordinate their actions through a system known as quorum sensing [29]. Recent reports suggest that signaling molecules used in quorum sensing can also have immunomodulatory actions on the host [30]. While most quorum sensing signaling molecules (QSSM) studied in this regard have been from gram-negative pathogens a number of metabolic products with anti-inflammatory properties have been described in probiotics [31,32]. Investigation of the QSSM from mostly gram positive probiotic species may yield important information regarding their immunomodulatory capacity.

Conclusions
It is clear that probiotic organisms have the potential to act as adjuvants. However, if this potential is to be exploited for use in mucosal vaccines further studies are required to gain an understanding of the complex interactions between bacteria and host that determine the immunomodulatory response. These interactions will depend on inherent characteristics of the probiotic as well as the initial immunological state of the host. Such understanding may also allow us to enhance the innate abilities of an organism a goal that could be achieved by co-expression of signaling molecules. In this regard, Lactococci expressing IL-10 and trefoil factors have proved therapeutically effective in treatment of experimental colitis models [33, 34] while intranasal immunization of mice with TTFC-expressing Lactococci that co-express IL-2 or IL-6 results in a more rapid response and higher levels of TTFC-specific antibodies [35]. Thus in the future, as knowledge of this area deepens, it should be possible to select appropriate organisms with high or low intrinsic adjuvanticity and appropriately modify them to obtain the desired immunomodulatory outcome for specific therapeutic applications.

References
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The use of CT as a nasal vaccine adjuvant in experimental animals is associated with accumulation of CT in the olfactory bulbs of the CNS and redirection of the co-administered vaccine antigen in the olfactory epithelium (5). Thus, not only is there a dire need to understand the mode of action of adjuvants, but there is also a critical need for new safe and effective mucosal adjuvants for use in humans. Recently, a collection of seemingly unrelated observations has provided likely clues to how adjuvants work and by extension implicated a key inflammatory cell, until now unrecognized, in the initiation of antigen-specific immunity. These findings include the observation that cytokines such as IL-1, IL-12, IL-18, and GM-CSF (6-11) and chemokines as such as lymphotactin, RANTES, MIP-1α, and MIP-1β (12-14) can be used quite effectively as mucosal adjuvants when co-administered with protein antigens without many of the harmful side effects of CT. Presumably, these cytokines/chemokines, along with the vaccine antigen, travel to the mucosal inductive tissues where they kick start the immune response in the host by initiating B and T cell responses (15, 16). Since mast cells are a major source of endogenous cytokines (17) and are usually abundant at mucosal sites typically used for vaccine administration (18, 19), these cells could potentially be tapped as an important source of endogenous cytokines that exhibit adjuvant properties. Indeed, several recent studies have suggested that cytokines derived from local mast cells at sites of infection play a key role in initiating the innate (20, 21), as well as certain key aspects of the adaptive immune response (22, 23). Although there is currently no data that the commonly used adjuvants such as CT and TLR ligands, including CpG, function by activating local mast cells, it is perhaps significant that mast cells possess receptors for many of these adjuvants (24-27).

Exogenous Cytokines are Highly Effective Mucosal Adjuvants

The adjuvant activity of CT has been associated with its ability to induce the production of proinflammatory

Mast cells and Mucosal Vaccines

Herman F. Staats, James B. McLachlan, Christopher P. Shelburne, Justin P. Hart, Salvatore V. Pizzo, Soman N. Abraham

Department of Pathology, Duke University Medical Center, Durham, NC 27710

Introduction

Many vaccine immunogens do not induce significant immune responses when administered alone, even when given in large doses and therefore need to be combined with adjuvants. Chemically, adjuvants are a highly heterogeneous group of compounds with only one thing in common: their ability to enhance the immune responses to coadministered antigens. How they achieve their effect is often unclear. In the absence of this information, there is considerable and sometimes unwarranted concern about the unintended consequences of adjuvants in immunized individuals (1). Because most infections originate at mucosal surfaces, there is growing interest in applying vaccines to mucosal sites so as to provoke vigorous mucosal immune responses. Cholera toxin (CT) is the most widely studied experimental mucosal adjuvant for the induction of antigen-specific systemic and mucosal immune responses (2, 3). Although this mucosal adjuvant initiates potent immune responses and is widely used in experimental animals, as little as 5 µg of CT administered gastrically to humans causes severe diarrhea (4). The use of CT as a nasal vaccine adjuvant in experimental animals is associated with accumulation of CT in the olfactory bulbs of the CNS and redirection of the co-administered vaccine antigen in the olfactory epithelium (5). Thus, not only is there a dire need to understand the mode of action of adjuvants, but there is also a critical need for new safe and effective mucosal adjuvants for use in humans.

Recently, a collection of seemingly unrelated observations has provided likely clues to how adjuvants work and by extension implicated a key inflammatory cell, until now unrecognized, in the initiation of antigen-specific immunity. These findings include the observation that cytokines such as IL-1, IL-12, IL-18, and GM-CSF (6-11) and chemokines as such as lymphotactin, RANTES, MIP-1α, and MIP-1β (12-14) can be used quite effectively as mucosal adjuvants when co-administered with protein antigens without many of the harmful side effects of CT. Presumably, these cytokines/chemokines, along with the vaccine antigen, travel to the mucosal inductive tissues where they kick start the immune response in the host by initiating B and T cell responses (15, 16). Since mast cells are a major source of endogenous cytokines (17) and are usually abundant at mucosal sites typically used for vaccine administration (18, 19), these cells could potentially be tapped as an important source of endogenous cytokines that exhibit adjuvant properties. Indeed, several recent studies have suggested that cytokines derived from local mast cells at sites of infection play a key role in initiating the innate (20, 21), as well as certain key aspects of the adaptive immune response (22, 23). Although there is currently no data that the commonly used adjuvants such as CT and TLR ligands, including CpG, function by activating local mast cells, it is perhaps significant that mast cells possess receptors for many of these adjuvants (24-27).

Exogenous Cytokines are Highly Effective Mucosal Adjuvants

The adjuvant activity of CT has been associated with its ability to induce the production of proinflammatory

continued on page 10
cytokines such as IL-1 (28). Accordingly, we and others evaluated recombinant cytokines such as IL-1α, IL-1β, IL-12, IL-18, and GM-CSF for their ability to provide adjuvant activity when coadministered by the nasal route with protein or peptide immunogens (6-11). Our studies determined that exogenously applied recombinant cytokines were effective adjuvants for nasally-delivered vaccines and augmented antigen-specific systemic and mucosal immune responses as effectively as CT. Our non-human primate studies determined that recombinant human IL-1α and GM-CSF exhibited significant adjuvant activity in the absence of any systemic adverse effects (fever, weight loss), and the host did not mount anti-cytokine antibody responses to the nasally-administered cytokines (9). In contrast, GM-CSF used as an adjuvant in combination with a TLR4 ligand and injected intramuscularly induced potent serum anti-GM-CSF neutralizing antibody responses (9). Despite the potential for the use of cytokines as adjuvants for mucosally-administered vaccines, their high cost will likely prevent their use in many vaccines.

A Physiological Role for Mast Cells
Mast cells were discovered in the frog mesentery over 150 years ago and until recently have remained an enigma. Mast cells have the capacity to release many presynthesized mediators, e.g., TNF, histamine, and tryptase, which are stored within specialized intracellular granules, as well as a myriad of de novo synthesized mediators, including most cytokines produced by the host (29-33). Because of their intrinsic capacity to undergo repeated cycles of degranulation and regranulation and to proliferate at sites of inflammation, mast cells are major mediators of inflammation in the body. Indeed, mast cells have been implicated in several pathophysiological conditions including, asthma, allergy, interstitial cystitis, scleroderma, inflammatory bowel disease and arthritis (34-46). Estimated concentrations of mast cell range from 500 to 4,000 per mm³ in the lungs, 7,000 to 12,000 per mm³ in skin, and 20,000 per mm³ in the bladder (47). Because many of these sites also happen to be portals of infection, mast cells may represent one of the first inflammatory or immune cells encountered by an invading pathogen. There is growing evidence that mast cells recognize and react, often in a beneficial manner, to a wide range of microorganisms and their products. Such interactions have lent credence to the notion that mast cells have the potential to markedly influence the immune responses to various microbial infections.

Mast cells as Modulators of Innate Immunity
Although it had been known that mast cells contribute to clearance of certain parasites, mast cell involvement in bacterial clearance was not seriously considered until recently. We and others noticed that mast cells had the capacity to specifically recognize and bind various bacteria, even in the absence of antibodies (48, 49). This observation, together with the fact that mast cells are located strategically at the portals of microbial entry and have the intrinsic capacity to release a myriad of proinflammatory cytokines in response to bacteria, led us to consider the possibility that these cells serve as key sentinel cells of the immune system. Our initial experiments revealed that upon exposure to bacteria, mast cells spontaneously degranulate and release their mediators. Because the overall inflammatory response of the host to a pathogen clearly involves the coordinated and often redundant actions of mediators from several cell types, it is difficult to ascertain the specific contribution of mast cells and their products. However, certain mutant mice virtually lacking mast cells are available, making it possible to evaluate the specific contribution of mast cells. One such mast cell deficient mutant is the WBB6F1/W/Wv mouse, which has defective c-kit proteins, the receptor for stem cell factor (previously called mast cell growth factor) (50-53). Many studies have demonstrated the value of this mouse model system for analyzing mast cell function. By quantifying differences in biological responses between W/Wv mice and their congenic mast cell sufficient (+/+ ) controls and then by analyzing the responses in W/Wv mice that have been selectively reconstituted with cultured mast cells, it is possible to precisely define the in vivo contributions of mast cells. To test the sentinel functions of mast cells in vivo, we compared the mortality of wild type (mast cell +/+ ) and mast cell deficient W/Wv mice following intraperitoneal challenge with E.coli. For ease of presentation, we will henceforth refer to the pair of WT and W/Wv mice as mast cell +/+ and mast cell +/- mice. We found that whereas all the mast cell +/+ and mast cell-reconstituted mast cell +/- mice survived after intraperitoneal instillation of E.coli, up to 80% of the mast cell +/- mice succumbed to infection, providing definitive proof of the importance of mast cells to the immune defense (54). The underlying mechanism of protection was shown to involve the release of TNF by local mast cells, which mediated the rapid recruitment of neutrophils to the site of infection. There was rapid clearance of bacteria in the mast cell +/- mice and in the repleted mast cell +/- mice, but not in the mast cell +/- mice, and consequently the latter group of mice succumbed to sepsis (54).

Mast cells as Modulators of Adaptive Immunity to Bacterial Infection
There are indications that mast cells also contribute to the adaptive immune responses of the host. These contributions range from secretion of immunoregulatory cytokines that influence specific lymphocyte responses to direct processing and presentation of bacterial antigens to immune cells of the host (54-58). The capacity of mast cells to release immunoregulatory cytokines, such as IL-1, IL-3 to IL-6, IL-
continued from page 10

8. IL-10 to IL-13, GM-CSF and TNF, and many chemokines (MCP-1, MIP-1α, and RANTES) indicates that mast cells have the potential of influencing the development of specific T-cell and B-cell responses (59-62). In spite of the potential for mast cells to modulate the adaptive immune responses to bacterial infection, direct evidence of this role and of the underlying mechanisms involved has been largely unavailable. In a study aimed at identifying the factors responsible for nodal enlargement during infection, we found a remarkable correlation between mast cell degranulation at sites of infection and corresponding enlargement of draining lymph nodes (21). To investigate the specific role of mast cells in this phenomenon, we injected E.coli J96 into the footpads of mast cell +/+ , repleted mast cell -/- and mast cell -/- mice. We observed a remarkable difference in growth of draining lymph nodes draining the site of infection (21). Whereas there was a two fold increase in the weight of nodes in the mast cell sufficient mice, a corresponding increase in nodal size in the mast cell -/- mice was not observed. Since enlargement of nodes was an early manifestation of the adaptive response in this organ, these observations indicated that mast cells at sites of infection were involved in initiating elements of the adaptive immune response. The underlying mechanism was shown to involve the lymphatic system, which is critical in draining the sites of infection and channeling mast cell products such as TNF directly into the nodes (21). Thus, mast cell-mediated modulation of lymph node activation occurs via remote control. The fact that the migration of dendritic cells from peripheral sites to the nodes was under regulation of mast cells following IgE mediated activation was recently shown by Marshall and coworkers (63). Thus, during infection, mobilization of lymphocytes, as well as dendritic cells, could occur. Cumulatively, these studies point to mast cells as critical modulators of the innate, as well as adaptive immune response to infectious bacteria.

Possible Use of Mast Cell Activators to Potentiate Adaptive Immune Responses

Upon activation by pathogens, mast cells release their mediators, including key cytokines, thereby orchestrating the intricate initiation of the host’s immune system. Since orchestrating mobilization and boosting functions of the adaptive immune response are precisely how adjuvants are believed to function, it was of interest to see if initiation of the certain key steps of the adaptive immune response could be triggered, even in the absence of the infecting pathogen, merely through the use of mast cell secretagogues. We found that intradermal instillation of a well known mast cell activator, compound 48/80 (64), resulted in significant hypertrophy of the draining lymph node and the extent of lymphocyte sequestration in the nodes was comparable to that seen during infection (21). The specific contribution of mast cells to lymph node hypertrophy was indicated by the observation that compound 48/80 induced lymph node hypertrophy in mast cell-sufficient mice, but not in mast cell-deficient mice (21). Although it remains to be seen whether this orchestration of immune cells triggered by mast cells can result in a productive immune response, this observation, nevertheless, shows that mast cell activators can mobilize essential elements of the immune response. If mast cell activators are co-administered with a vaccine antigen, it is likely that a vigorous immune response directed at the antigen will result and that these compounds could serve as a new class of effective adjuvants.

Concluding Remarks

Because of toxicity issues, many currently studied vaccine adjuvants have limited use in humans. Recent research has suggested that certain cytokines can be used quite effectively as adjuvants without harmful side effects. However, a decided disadvantage is the costs of these exogenous cytokine therapies are prohibitively expensive. In view of the fact that a prominent source of endogenous cytokines in the body are located within mast cells and that recent studies have implicated mast cell-derived cytokines in initiating the immune responses, including elements of the adaptive immune response, to infection, we hypothesize that local mast cells could be targeted to boost immune responses to vaccine antigens. There are several mast cell activators which potentially can serve as adjuvant candidates. Many of them are small molecule compounds and unlikely to evoke immune responses against themselves. While there are several advantages in using mast cell activators as adjuvants, such as low cost and relative stability of the compound and the fact that some have been used previously in humans with no long term side effects (65-67), it should be cautioned that rigorous efforts be taken to ensure some of the known harmful effects of mast cell activation, such as atopy and anaphylaxis, not be elicited in the mast cell activator-treated host.

References:
continued from page 11

47. Corresponding author: Soman N. Abraham
   Email: abrah006@m.c.duke.edu

Society for Mucosal Immunology
5272 River Road
Suite 630
Bethesda, MD 20816

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