
The immune responses to bacterial antigens encountered *in vivo* at mucosal surfaces

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Mammals have evolved a sophisticated immune system for handling antigens encountered at their mucosal surfaces. The way in which mucosally delivered antigens are handled influences our ability to design effective mucosal vaccines. Live attenuated derivatives of pathogens are one route towards the development of mucosal vaccines. However, some molecules, described as mucosal immunogens, are inherently immunogenic at mucosal surfaces. Studies on mucosal immunogens may facilitate the identification of common characteristics that contribute to mucosal immunogenicity and aid the development of novel, non-living mucosal vaccines and immunostimulators.

Keywords: mucosal vaccines; IgA; *Citrobacter*; enterotoxins

1. INTRODUCTION

During a lifetime individuals continuously encounter antigens at the mucosal surfaces (respiratory, gut, urogenital, corneal) of the body. Most of these are harmless environmental antigens, whereas others can be components of potentially life-threatening pathogens. Mounting an immune response to environmental antigens can be hazardous in terms of energy expenditure and the danger of autoimmunity and allergy. Since mammals have been continuously exposed to both environmentally derived and pathogen-associated antigens they have evolved mechanisms that enable them to tightly regulate immune responses to mucosally encountered antigens. In this there is a contradiction. There is a need to respond weakly to non-hazardous antigens but vigorously to pathogens. Mammals are further compromised by their dependence on the exchange of nutrients and gases with the environment. Rather than cover themselves in an impregnable shell they have evolved to exchange essential molecules with the environment via mucosal surfaces. These sites of nutrient exchange are the most vulnerable points for infection. Perhaps in response to these evolutionary pressures mammals have evolved a sophisticated mucosal-associated immune system that is integrated closely with the systemic immune system.

2. THE MUCOSAL IMMUNE SYSTEM: A BARRIER TO DELIVERING THERAPEUTIC AGENTS AND VACCINE ANTIGENS

The mucosal immune system presents a number of practical problems to the immunologist and vaccinologist. Most antigens are apparently poorly immunogenic when processed through mucosal surfaces (Levine & Dougan

1998). In this definition they are poor inducers of serum IgG or secretory IgA. However, early investigations demonstrated that animals can display systemic tolerance following a mucosal encounter with an antigen even in the absence of antigen-specific antibody production (Garside *et al.* 1999). Thus, an immune phenotype was present if the correct readout was measured. The physical location of the mucosal immune cells within the body also limited experimental access to immune inductive sites forcing investigators to use invasive or systemic techniques to measure immunological changes. In spite of these hurdles the interest in mucosal immunology has increased in recent years. This is partly because of the potential practical benefits of mucosally targeted therapies. These include mucosally deliverable vaccines against infectious agents or mucosal-tolerizing agents to treat autoimmune disease. The fear of needle contamination and the spread of infection (HIV or hepatitis) have also favoured oral or nasal delivery of antigens and drugs.

3. THE CONCEPT OF MUCOSAL IMMUNOGENS

Some antigens are clearly more immunogenic than others when delivered to mucosal surfaces. Antigens that have inherent immunogenicity when delivered to the mucosa can be described as mucosal immunogens. Mucosal immunogens fall into two classes, those that are alive and those that are non-living.

4. THE IMMUNOGENICITY OF LIVE MUCOSAL VACCINES

It makes sense that the immune surveillance system should be able to identify the presence of live pathogens. Exactly how this recognition works may be very complex but attenuated live micro-organisms are clearly a potential route towards vaccine development. Many effective

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vaccines have been based on this approach, the caveat being that attenuated strains must not be able to revert to virulence (Levine *et al.* 1997). Live vaccines have proven particularly effective when delivered through the mucosal route. Examples of mucosally deliverable vaccines include those based on attenuated mycobacteria, *Vibrio cholerae*, *Salmonella typhi* and polio virus. Attenuated live micro-organisms are likely to follow a similar preliminary route of colonization of the host compared to the fully virulent parent. This will include potential encounters with M and immune cells. In addition, direct contacts with eukaryotic cells and ligands (possibly leading to an intracellular phase) may be closely paralleled. Thus, live vaccines may be treated in a similar manner to virulent pathogens. Factors that may enhance the immunogenicity of live vaccines may include (i) the ability to produce antigens expressed only in the host and not on laboratory media, (ii) the ability to adhere to or colonize immune cells and deposit antigen directly into particular intracellular processing pathways, (iii) the ability to activate innate surveillance mechanisms through generic molecules such as lipopolysaccharides (LPS), (iv) the ability to produce metabolites that can activate surveillance cells, and (v) the ability to reach immune-inductive sites.

A number of pathogens (see above) have been attenuated to create live vaccines that can be delivered via mucosal routes. This approach was first explored soon after Pasteur had generated vaccines based on passaged micro-organisms. Indeed, BCG was extensively exploited as a mucosal (oral) vaccine. Mucosal vaccines based on live attenuated micro-organisms rely on the ability of the pathogen to target the mucosa and penetrate mucosal associated lymphoid tissues. Such vaccines, for example the Sabin polio vaccine, can be extremely effective. The underlying basis of attenuation does not have to be known in order for a live vaccine to be successful. However, with the improving knowledge of the molecular basis of infection and an emphasis on safety above all else, it would currently be difficult to register a live human vaccine without knowing the basis of attenuation. This knowledge can greatly simplify the process of vaccine quality control and can allow licensing agencies to evaluate the likelihood of reversion to virulence. Perhaps the biggest challenge facing new live vaccines is obtaining the balance between optimal immunogenicity with an absence of reactogenicity in the vaccine. This is a window of acceptability that has proved very difficult to hit using genetically characterized live vaccines. Much recent work on live mucosal vaccine development has focused on enteric bacteria. Work on shigella has been extensive, but so far no vaccine has been brought forward for registration. Although several candidate shigella vaccines have been developed, so far, all promising candidates have had problems with reactogenicity in the clinic (Formal *et al.* 1989; Klee *et al.* 1997; Levine *et al.* 1997; Coster *et al.* 1999). Work in *V. cholerae* has focused on cholera enterotoxin (CT) defective strains. One CT-negative candidate, CVD103, has been extensively evaluated in the clinic and in the field (Tacket *et al.* 1992; Kaper *et al.* 1995). This vaccine performed very impressively in the clinic during phase I and II immunogenicity and challenge studies. The vaccine was immunogenic in a single dose, eliciting serum and local anti-vibrio antibodies in most volunteers and was impressively protective. After

initial successes in the field this vaccine encountered some problems. A recent efficacy study in Indonesia generated disappointing protection against cholera. This may, in part, be due to the different intestinal environment in many individuals in developing compared to developed countries (Lagos *et al.* 1999). The normal flora and even gut architecture can differ enormously between the two groups, and higher doses of CVD103 vaccine were required in locals from the tropics (Thailand) to elicit similar levels of immunity to Westerner (in this case from the USA) (Tacket *et al.* 1992, 1999; Su-Arehawaratana *et al.* 1992). This could be due to competitive exclusion by the normal flora or activated or primed immunity in individuals from developing countries. Whatever the causes, this is an area of major interest to vaccine developers and must be better understood if we are to improve mucosal vaccine delivery.

Arguably, the most intensively studied area of live enteric vaccine development has involved *Salmonella*. This area has been driven by work using the murine model to identify virulence-associated genes in pathogenic *Salmonella* such as *Salmonella typhimurium*. In the last several years over 100 *Salmonella* genes have been implicated in virulence using the murine model (e.g. see Hensal *et al.* 1995). Many of these attenuating mutations have been proposed as potential components of live salmonella vaccines either in veterinary species or in man (usually as typhoid vaccines on a *S. typhi* background; Dougan *et al.* 1994). Out of these attenuating lesions, relatively few have been evaluated systematically in the murine model as components of live vaccines. In order to be useful in a live vaccine, an attenuating mutation must not over-attenuate (leading to poor protection) or under-attenuate (leading to reactogenicity). Many candidate attenuating mutations cannot be used in salmonella vaccines because they either over- or under-attenuate the vaccine strain (O'Callaghan *et al.* 1988). Of course this is a simplification because the mouse is not the ideal model for selecting candidate mutations for salmonella derivatives to be used as vaccines in other mammalian species. This we know, as some mutations that attenuate salmonella in the mouse do not attenuate in other mammals such as the cow (Tsolis *et al.* 1999). Nevertheless, the murine model has been used to underpin a number of candidate attenuating lesions for use in veterinary salmonellae and *S. typhi*. For example, an *S. typhi* Ty2 derivative based on *aroC*, *aroD* and *htrA* attenuation is currently in phase II clinical trial as a candidate human typhoid vaccine (Tacket *et al.* 1997). This strain, known as CVD908 *htrA* is highly attenuated in humans, not reaching the bloodstream in detectable numbers after oral immunization with as many as 10^9 viable bacteria. CVD908 *htrA* is also immunogenic, generating circulating B cells producing anti-*Salmonella* LPS antibodies. It is too early to say if this vaccine will perform well in the field.

Why are live salmonella and other pathogen-based vaccines effective mucosal immunogens? Like their wild-type parents, these candidate vaccine strains are likely to target the mucosal surface via M cells or epithelia and interact with antigen-presenting cells, such as dendritic cells and monocytes. We know *Salmonella* can invade mammalian cells and localize within a vacuole in both phagocytic and non-phagocytic cells (Finlay & Falkow 1997). From these sites there is plenty of opportunity to

interact with the immune system and at the same time avoid the attention of antibodies and other immune effectors. The expression of generic activating molecules such as peptidoglycan, lipoproteins and LPS, recognized by innate immune effectors, may help increase the immune potential of these live vaccines. Unfortunately, these are the same molecules that can activate the fever response potentially leading to reactogenicity.

5. THE MUCOSAL IMMUNOGENICITY OF NON-LIVING ANTIGENS

It is relatively easy to picture how a live pathogen may be immunogenic when administered via a mucosal surface. What about non-living antigenic preparations? Why are some more immunogenic than others? What molecular features of antigens might contribute to their mucosal immunogenicity? There are relatively few antigens (mucosal immunogens) that in a purified form are able to elicit significant levels of secretory IgA and serum IgG when administered to mucosal surfaces. Furthermore, some mucosal immunogens can only stimulate local IgA responses whereas others can stimulate both local and systemic immunity. In addition, these properties may, in part, be host dependent.

We know that the immunogenicity of antigens can be improved by using generic methods that protect the antigen from denaturation or degradation (e.g. encapsulation) (O'Hagen 1990). Antigens differ in their ability to resist the harsh conditions they are likely to encounter close to host body surfaces. Thus, antigens from pathogens that have evolved to retain biological function *in vivo* in body tissues might be more adapted to persist in the host and resist degradation and consequently be more immunogenic (Dougan 1994). Antigen persistence in the host may also be enhanced by an ability to bind mammalian cells and target mucosal surfaces. Aizapurua & Russell-Jones (1988) attempted to define classes of molecules that could act as mucosal immunogens by screening different antigens in a model oral immunization model. They were able to show that some, but not all, antigens that targeted mucosal surfaces had enhanced mucosal immunogenicity. Polymerized molecules such as flagella or fimbriae fell into the immunogenic class. This requirement for binding is not surprising as an antigen that can target the mucosa is likely to reach immune inductive sites and cells at a higher concentration than non-binding antigens. However, it is important to recognize that not all antigens that bind mucosal surfaces are necessarily mucosal immunogens as the nature of the binding site, coupled with antigen persistence, may be critical. For example, binding to enterocytes or epithelial mucus may not enhance antigen translocation to immune inductive sites in some tissues. Unfortunately, most mucosal immunogens are only moderately immunogenic in uncapsulated form and this factor has limited our ability to design and perform serious comparative experiments. Thus, data in this area are limited.

6. ENTEROTOXINS AS MUCOSAL IMMUNOGENS AND ADJUVANTS

Perhaps the best known example of a class of mucosal immunogens are the bacterial enterotoxins. Indeed, CT

and *Escherichia coli* (LT) enterotoxins are recognized as the most potent of all known mucosal immunogens. They are so immunogenic at mucosal surfaces that they can activate immune responses to co-administered, non-coupled, bystander molecules that are normally poorly immunogenic at mucosal surfaces (Elson & Ealding 1984; Lycke & Holmgren 1986). For example, mice will not normally mount significant secretory or systemic antibody responses to tetanus toxoid administered orally or intranasally. However, if tetanus toxoid is mixed with microgram quantities of CT or LT, mice will readily seroconvert and produce anti-tetanus toxoid IgA at the local mucosal surfaces as well as serum IgG (Jackson *et al.* 1994; Douce *et al.* 1995). As a consequence both LT and CT are referred to as mucosal adjuvants. This attractive property of these molecules is compromised by the fact that both LT and CT, although relatively weak toxins for mice, are highly toxic for humans and some other animals. This factor alone limits their value as practical mucosal adjuvants but does not preclude their use as experimental adjuvants in mice. The structures of both LT and CT have been defined using crystallography and the elegant structure of these enterotoxins has provided some clues as to why they are effective mucosal immunogens (Rappuoli *et al.* 1999). They have a very compact structure and the holotoxins are quite resistant to denaturation and degradation by proteases. Furthermore, they have the ability to target receptors (gangliosides and glycosylated proteins) at the surface of both epithelial and immune cells. Thus, their ability to target different types of cells may be an important characteristic. The influence of the cell-binding activities of LT (Nashar *et al.* 1998) and other ADP-ribosylating toxins such as pertussis toxin (Cropley *et al.* 1995; Roberts *et al.* 1995) have been studied using site-directed mutants or chemical inactivation. These studies have confirmed cell binding as a critical property contributing mucosal adjuvanticity and immunogenicity. However, it is important to note that there may be some situations in which non-binding enterotoxin derivatives may retain mucosal adjuvant activity.

How is adjuvanticity linked to other biological activities of the enterotoxins? Both CT and LT have a sophisticated tertiary structure. They belong to the AB class of bacterial enterotoxins and are composed of a pentameric B oligomer that binds receptors on the surface of eukaryotic cells and an enzymatically active A-subunit that is an ADP-ribosyltransferase responsible for toxicity. The A-subunit is associated with the B-subunit and together they form a tight complex which is highly protease resistant. The structure of both CT and LT has been probed by introducing site-directed amino-acid substitutions into both the A- and B-subunits. Mutations in the A-subunit have been identified that fully or partially inactivate the ADP-ribosyltransferase activity and hence reduce the toxicity of the molecule (Pizza *et al.* 1994). Careful studies using different A-subunit mutant derivatives of both LT and CT have been used to elucidate the contribution of holotoxin formation and enzymatic activity and/or toxicity to mucosal immunogenicity and adjuvanticity. This work has recently been reviewed in detail (Rappuoli *et al.* 1999) and will be described briefly here.

LT and CT mutants that have a destabilized AB structure in terms of subunit association or ability to resist

degradation or denaturation are less immunogenic. Thus, the compact structure of the molecule may be required to enhance resistance and persistence in tissues. Some non-toxic derivatives of LT retain significant mucosal adjuvant activity, e.g. LTK63 (Douce *et al.* 1995). Thus, toxicity and ADP-ribosyltransferase activity are not essential for adjuvanticity. Although the ADP-ribosyltransferase activity is not essential for mucosal adjuvant activity it can enhance this activity. For example, mutants that retain partial ADP-ribosyltransferase activity are better mucosal adjuvants than LTK63, e.g. LTR72 (Douce *et al.* 1997; Giuliani *et al.* 1998). Hence, by studying these mutant derivatives it has proved possible to identify combinations of important features that contribute to both immunogenicity and adjuvanticity at mucosal surfaces. What is the importance of this information for vaccine design? By breaking down the individual features of LT and CT that contribute to mucosal adjuvant activity it has been possible to use this information to design completely novel mucosal adjuvants. For example, by combining the antibody-binding domain of *Staphylococcus aureus* protein A with the A-subunit of CT, a novel mucosal adjuvant CTA1-DD has been designed as a prototype of a new class of artificial mucosal adjuvants (Agren *et al.* 1999).

7. UNDERSTANDING THE REGULATION OF MUCOSAL IMMUNE RESPONSES

If we are to improve methods for mucosal therapies and mucosal vaccination, we need to understand the fundamentals of how the immune system regulates the immune response to mucosally delivered antigens (Bienenstock *et al.* 1978; McGhee *et al.* 1992). What are the molecular mechanisms that initiate an active (IgA, serum IgG) compared to a tolerant immune response? Indeed, what is the basis of responsiveness compared to non-responsiveness and how is this balance maintained or changed? It could be argued that there are two fundamental elements interacting to maintain this balance. These elements are the formulation of the antigens derived exogenously from the environment (either environmental or pathogen derived) together with the mucosal immune regulator systems. Evidence has accumulated that T-cell responses to antigens presented via mucosal cells are tightly regulated. Both CD4⁺ and CD8⁺ T cells have been implicated in this form of regulation in experimental approaches involving vaccination and tolerance induction. A new class of regulatory T cell, producing high levels of interleukin 10 that proliferate poorly in response to antigen have been identified in mucosal tissues (Groux *et al.* 1997). The establishment of particular cytokine expression patterns by these and other regulatory cells may be critical in this phenomenon. It is also likely that the regulation of antigen presentation by antigen-presenting cells at mucosal surfaces is a critical regulatory step. Indeed, dendritic cells associated with mucosal surfaces may possess significantly different properties from other dendritic cell populations. If tight regulatory networks are operating *in vivo*, this will complicate any attempts to use *in vitro* methods of assessment of the function of mucosal immune cells since key signals may be missing. Thus, *in vivo* studies are essential if we

are to understand how the regulation of mucosal immune responses is achieved.

8. IN VIVO MODELS OF MUCOSAL IMMUNE DYSFUNCTION

The genetic manipulation of pathogens has provided a rich source of information on the role of bacterial gene products in infection and pathogenesis. Murine models have proved to be particularly fruitful and they now have the added value that both the pathogen and the host can be genetically manipulated. A murine model for analysing mucosal immunity and immune dysfunction could be particularly attractive. An *in vivo* approach to understanding mucosal T-cell regulation is evolving through the study of the pathogenic mechanisms of a family of bacterial pathogens that cause attaching and effacing lesions on gut enterocytes. The interaction of these pathogens with cells of the gastrointestinal tract is complex, but new studies (summarized below) have suggested that these bacteria can profoundly modulate mucosal T-cell responses. These bacteria may therefore represent useful tools with which to dissect elements of the intricate immune regulatory network present in the mucosa.

9. CITROBACTER RODENTIUM, AN EXPERT MUCOSAL IMMUNOMODULATOR

Citrobacter rodentium colonizes the distal colon of susceptible inbred mouse strains via the formation of attaching and effacing (AE) lesions on colonic enterocytes (Schauer *et al.* 1993a). Ultrastructurally, these AE lesions are indistinguishable from those caused by enteropathogenic *E. coli* (EPEC) infection in humans. AE lesions are characterized by the intimate attachment of bacteria to cup-like pedestals on the luminal side of the enterocyte cell membrane and the subsequent destruction of host cell microvilli. The bacterial virulence determinants required for AE lesion formation have been most extensively described in EPEC. Formation of AE lesions is dependent on expression of several bacterial proteins, which are encoded by genes located on a chromosomal pathogenicity island called the locus for enterocyte effacement (LEE). The *eae* gene, which encodes intimin, an outer membrane protein adhesin, was the first gene in the LEE to be associated with AE lesion formation (Jerse *et al.* 1990). In addition to intimin, the LEE encodes a type III secretion system, a translocated intimin receptor (Tir) and three EPEC secreted proteins required for protein translocation (reviewed in Frankel *et al.* 1998). Characterization of these LEE-encoded virulence determinants has been performed primarily by examining the interaction of EPEC bacteria with continuous human epithelial cell lines or human tissue explants. While these studies have helped facilitate a dissection of the events leading to AE lesion formation, they have not fully revealed the extent to which individual LEE-encoded proteins contribute to bacterial pathogenesis *in vivo*. Furthermore, these *in vitro* studies have not provided information on the extent, type or specificity of the infected host's response to EPEC antigens; information which is likely to be important in the rational design of vaccines or therapeutics to prevent

EPEC infections in humans. While *in vivo* studies of EPEC pathogenesis and immunity are clearly desirable, the inability of human isolates of EPEC to colonize small rodents has meant alternative animal models have been investigated. One of these models, *C. rodentium* infection of mice, has several features which make it attractive for furthering research into EPEC and also mucosal T-cell regulation.

10. IMMUNOBIOLOGY OF *CITROBACTER RODENTIUM* INFECTION

The *C. rodentium* chromosome has been shown to contain genes with homology to those located in the LEE pathogenicity island found in human EPEC strains (Schauer *et al.* 1993). The identification of LEE homologues in *C. rodentium* has facilitated the construction of defined mutants and their subsequent characterization in mice. An *eae* mutant of *C. rodentium* (strain DBS255) was shown to be avirulent. However, virulence could be restored by complementation with either the *eae* gene from *C. rodentium* (Schauer *et al.* 1993b), or the *eae* gene from the prototype EPEC strain E2348/69 (strain DBS255 (pCVD438) (Frankel *et al.* 1996). The fact that an *eae* mutant of *C. rodentium* is attenuated in mice is consistent with human studies demonstrating that an *eae* mutant of EPEC strain E2348/69 is attenuated in humans (Donnenberg *et al.* 1993). In addition to its role as an adhesin, intimin also contributes to the induction of colonic epithelial cell hyperplasia, the second characteristic feature of *C. rodentium* infection in mice. The colonic hyperplasia that occurs during *C. rodentium* colonization is associated with the expression of inflammatory cytokines (IL-1, tumour necrosis factor- α (TNF α)) and a strong T-cell infiltrate consisting predominantly of CD4⁺ T cells with a Th1 phenotype (Higgins *et al.* 1999). Interestingly, the characteristics of the T-cell infiltrate which occurs in the mouse colon during *C. rodentium* infection share striking similarity to the cellular events occurring in murine models of inflammatory bowel disease (IBD). Epithelial cell hyperplasia in mice can also be induced via intrarectal administration of formalin-killed *C. rodentium* or EPEC, but not by *eae* mutants of *C. rodentium* or EPEC, indicating a critical role for intimin in this effect (Higgins *et al.* 1999). Indeed, intimin-bearing bacterial cells appear to be sufficient for this effect, since intrarectal administration of formalin-killed *E. coli* K12 expressing intimin, but not *E. coli* K12 alone, also evokes colonic hyperplasia (Higgins *et al.* 1999). The mechanism through which intimin-associated bacteria promotes hyperplasia is not clear, but clues are provided by the ability of the C-terminal 280 amino acids of intimin to co-stimulate T cells *in vitro*, which potentially occurs as a result of intimin binding to cell-surface receptors (Higgins *et al.* 1999). If intimin does indeed bind resident or infiltrating T cells in the gut, then this may promote unregulated proliferation of these mucosal T cells, which under normal situations are hypo-responsive and require strong co-stimulatory signals before cytokine production occurs. Following mucosal T-cell activation, cytokine production has been shown to trigger a cascade of events, including production of the epithelial cell mitogen keratinocyte growth factor (KGF) by mesench-

ymal cells (Bajaj-Elliott *et al.* 1998). The uncontrolled epithelial cell proliferation characteristically seen during *C. rodentium* infection and in other models of IBD may result from inflammatory cytokine-driven overexpression of KGF. Although the colonic hyperplasia observed in *C. rodentium* infected mice has many similarities with other murine models of IBD, *C. rodentium* elicited hyperplasia represents a unique model of mucosal T-cell unregulation. This syndrome is caused by a specific microbial agent and is critically dependent in the presence of one well-defined molecule, intimin. Thus, intimin can be regarded as having a dual role as both a cell adhesin and as a molecule which the pathogen uses to modulate the function of immune cells *in vivo*. The epithelial cell hyperplasia observed during *C. rodentium* infection may be of benefit to the bacterium by providing the pathogen with a greater surface area to colonize and thereby increase shedding. Conversely, the peak of the hyperplastic response in mice occurs when *C. rodentium* numbers in the colon begin to subside. This may suggest that hyperplasia represents a component of a protective immune response. Although hyperplasia has not been regarded as a feature of EPEC infection there are reports describing hyperplasia and villus atrophy in small intestinal biopsies from EPEC-infected children. This suggests that hyperplasia resulting from the unregulation of mucosal T cells may occur in some individuals (Fagundes Neto *et al.* 1989; Hill *et al.* 1991).

The results summarized here highlight the importance of intimin in *C. rodentium* colonization and infection and form the rationale behind current studies that are designed to determine whether pre-existing immune responses to intimin can prevent *C. rodentium* infection of mice.

11. *CITROBACTER RODENTIUM* INFECTION OF MICE AS A MODEL FOR EVALUATING CANDIDATE EPEC VACCINE ANTIGENS

C. rodentium infection in mice can be exploited to address questions relating to enteric infections and the potential of LT based mucosal adjuvants for eliciting protective immunity. For example: Can combinations of intimin and LT-based adjuvants elicit protection in mice against *C. rodentium* colonization and/or disease? The oral infectious dose of a *C. rodentium* strain expressing intimin from EPEC (strain DBS255(pCVD438)) in CH3/Hej is approximately 10⁵ bacteria. At this dose, infected mice become detectably colonized for approximately 24 days and develop visible hyperplastic colons by days 10–14. The development of hyperplasia is also associated with weight loss, although this is reversed when the numbers of *C. rodentium* in the colon begin to subside at around day 16. At higher infectious doses, the peak bacterial load in the colon is reached earlier (around day 7), as is the development of hyperplasia and weight loss. Whilst a higher infectious doses leads to earlier onset of disease, it also results in more rapid clearance of the infecting bacterium, which is usually absent from the colons of infected mice 16–18 days post-infection. C3H/Hej mice were intranasally immunized three times with a highly purified preparation of the C-terminal 280 amino acids of intimin (Int280) from EPEC together with LT. Control mice were intranasally immunized with intimin alone, LT

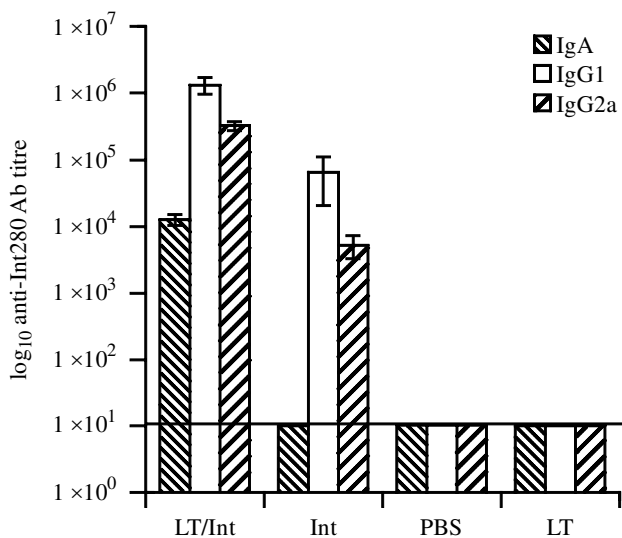


Figure 1. Co-delivery of Int-280 with the mucosal adjuvant LT elicits a strong serum IgG and IgA response to Int-280. Groups of 5 C3H/HeJ mice were intranasally immunized three times with 10 mg of Int-280 plus 1 mg of LT, 10 mg of Int-280 alone, 1 mg of LT alone or PBS alone. The data depicts the mean titre (plus standard error) of the antibody response to Int-280.

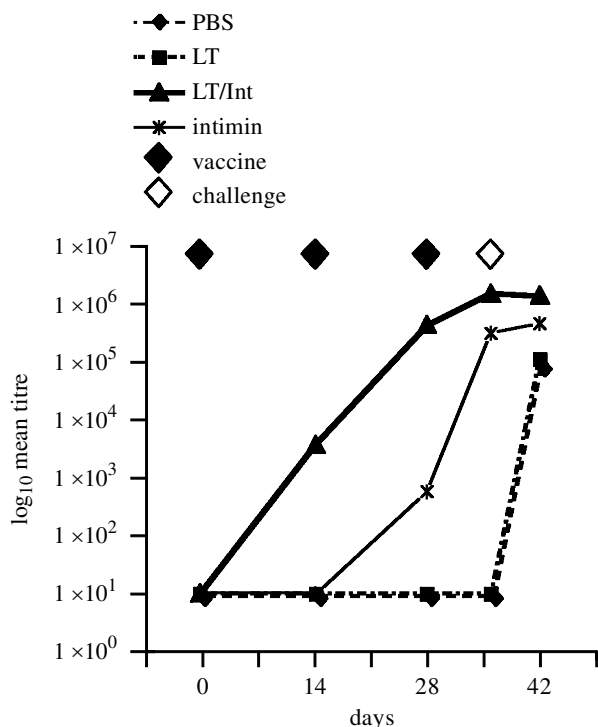


Figure 2. Kinetic of the anti-Int280 total Ig antibody response in immunized mice pre- and post-challenge with DBS255(pCVD438). The data depict the mean serum Ig titre against Int-280.

alone or phosphate buffered saline (PBS). After three immunizations, mice that received Int280 admixed with LT had developed the strongest serum anti-Int280 IgG and IgA antibody responses (figure 1). Mice immunized with Int280 alone mounted anti-Int280 IgG responses, but not serum IgA responses (figure 1). Immunized mice were orally challenged with DBS255(pCVD438) ten days

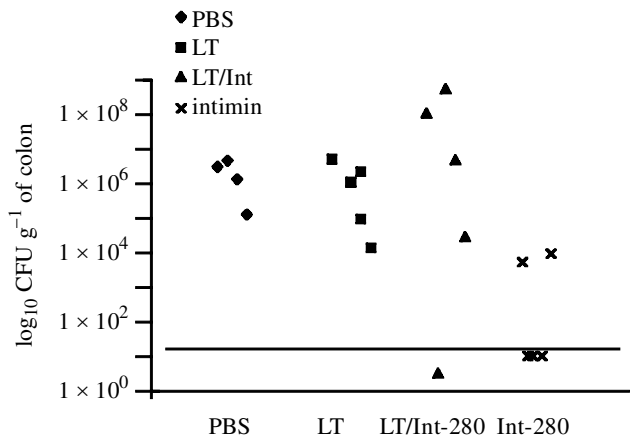


Figure 3. Pre-existing immune responses to Int-280 do not prevent bacterial colonization by *C. rodentium* DBS255(pCVD438). The data depict the number of viable DBS255(pCVD438) bacteria recovered from the colons of individual mice 14 days post-challenge.

after the last immunization. On day 14 post-challenge, mice not previously immunized with Int280 rapidly developed serum anti-Int280 antibodies (figure 2). The extent of bacterial colonization and hyperplasia was measured 14 days after being challenged with DBS255(pCVD438). Microbiological analysis of the colons of challenged mice suggested that the Int280-specific immune responses elicited by vaccination were not sufficient to protect mice from bacterial colonization nor the concomitant induction of colonic hyperplasia (figure 3). Other EPEC-associated determinants are currently under investigation as vaccine antigens in this model of *C. rodentium* infection. In addition, *C. rodentium* infection in other strains of mice has been evaluated with a view to investigating the immunological mechanisms through which *C. rodentium* promotes mucosal T-cell unregulation and hyperplasia. In summary, *C. rodentium* infection of mice represents a useful model in which to further our understanding of EPEC immunobiology. Furthermore, by using an 'expert' mucosal immunomodulator like *C. rodentium*, it is feasible to investigate mucosal T-cell regulation using a completely novel approach.

12. CONCLUDING REMARKS

We are in the middle of an intensive period of scientific investigations into the molecular basis of infectious diseases. As we begin to understand more about microbial pathogenicity, we appreciate the need to study pathogens *in vivo*, as they colonize, replicate and survive in host tissues. The design of novel and effective vaccines against mucosal pathogens will clearly benefit from detailed studies on the mechanisms of pathogenicity employed by the microbial pathogen and by monitoring the combined mucosal and systemic immune response associated with infection and recovery. In addition, many of the products of pathogenic bacteria might find specific use as mucosal vaccine components and/or more generic immunomodulators. The importance of studies in the natural host or whole animal systems cannot be over-emphasized. In terms of immunity, there are usually extremely complex

intercellular interactions occurring *in vivo* between different differentiated cell types and it is impossible to study many of these interactions *in vitro*. Indeed, the study of *in vitro* grown cells may provide misleading information on how regulatory networks are operating in real tissues. We are only just beginning to understand how the expression of immunity is regulated at mucosal surfaces and the use of bacteria and their products provides us with valuable tools for these ongoing investigations. As this work continues we can expect new and more effective vaccines to be designed and developed.

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