Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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

Gordon Dougan, Marjan Ghaem Maghami, Derek Pickard, Gad Frankel, Gill Douce, Simon Clare, Sarah Dunstan and Cameron Simmons

Phil. Trans. R. Soc. Lond. B 2000 **355**, 705-712
doi: 10.1098/rstb.2000.0610

Email alerting service Receive free email alerts when new articles cite this article - sign up in the box at the top right-hand corner of the article or click **here**

To subscribe to *Phil. Trans. R. Soc. Lond. B* go to: **http://rstb.royalsocietypublishing.org/subscriptions**

This journal is © 2000 The Royal Society

Downloaded from rstb.royalsocietypublishing.org

THE ROYAL SOCIETY

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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

Gordon Dougan*, Marjan Ghaem-Maghami, Derek Pickard, Gad Frankel, Gill Douce, Simon Clare, Sarah Dunstan and Cameron Simmons

Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2Z, UK

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

)uring a lifetime individuals continuously encounter ntigens at the mucosal surfaces (respiratory, gut, urino-enital, corneal) of the body. Most of these are harmless nvironmental antigens, whereas others can be compo-ents of potentially life-threatening pathogens. Mounting n immune response to environmental antigens can be azardous in terms of energy expenditure and the danger f autoimmunity and allergy. Since mammals have been ontinuously exposed to both environmentally derived nd pathogen-associated antigens they have evolved echanisms that enable them to tightly regulate immune esponses to mucosally encountered antigens. In this there ; a contradiction. There is a need to respond weakly to on-hazardous antigens but vigorously to pathogens. Mammals are further compromised by their dependence n the exchange of nutrients and gases with the environ-ent. Rather than cover themselves in an impregnable nell they have evolved to exchange essential molecules vith the environment via mucosal surfaces. These sites of utrient exchange are the most vulnerable points for nfection. Perhaps in response to these evolutionary pres-ures mammals have evolved a sophisticated mucosal-ssociated immune system that is integrated closely with ne 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 ractical problems to the immunologist and vaccinologist. Most antigens are apparently poorly immunogenic when rocessed 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

*Author for correspondence (g.dougan@ic.ac.uk).

© 2000 The Royal Society

Downloaded from rstb.royalsocietypublishing.org

accines have been based on this approach, the caveat eing that attenuated strains must not be able to revert to irulence (Levine *et al.* 1997). Live vaccines have proven articularly effective when delivered through the mucosal oute. Examples of mucosally deliverable vaccines include hose based on attenuated mycobacteria, *Vibrio cholerae*, *almonella typhi* and polio virus. Attenuated live micro-rganisms are likely to follow a similar preliminary route f colonization of the host compared to the fully virulent arent. This will include potential encounters with M and nmune cells. In addition, direct contacts with eukaryotic ells and ligands (possibly leading to an intracellular hase) may be closely paralleled. Thus, live vaccines may e treated in a similar manner to virulent pathogens. actors that may enhance the immunogenicity of live accines may include (i) the ability to produce antigens xpressed only in the host and not on laboratory media, ii) the ability to adhere to or colonize immune cells and eposit antigen directly into particular intracellular rocessing pathways, (iii) the ability to activate innate urveillance mechanisms through generic molecules ıch as lipopolysaccharides (LPS), (iv) the ability to roduce metabolites that can activate surveillance cells, nd (v) the ability to reach immune-inductive sites.

A number of pathogens (see above) have been attenu-ted to create live vaccines that can be delivered via ıucosal routes. This approach was first explored soon fter Pasteur had generated vaccines based on passaged ıicro-organisms. Indeed, BCG was extensively exploited s a mucosal (oral) vaccine. Mucosal vaccines based on ve attenuated micro-organisms rely on the ability of the athogen to target the mucosa and penetrate mucosal ssociated lymphoid tissues. Such vaccines, for example he Sabin polio vaccine, can be extremely effective. The nderlying basis of attenuation does not have to be known ı order for a live vaccine to be successful. However, with he improving knowledge of the molecular basis of infec-ion and an emphasis on safety above all else, it would urrently be difficult to register a live human vaccine ithout knowing the basis of attenuation. This knowledge an greatly simplify the process of vaccine quality control nd can allow licensing agencies to evaluate the likelihood f reversion to virulence. Perhaps the biggest challenge acing new live vaccines is obtaining the balance between ptimal immunogenicity with an absence of reactogenicity ı the vaccine. This is a window of acceptability that has roved very difficult to hit using genetically characterized ve vaccines. Much recent work on live mucosal vaccine evelopment has focused on enteric bacteria. Work on higella has been extensive, but so far no vaccine has been rought forward for registration. Although several candi-ate shigella vaccines have been developed, so far, all romising candidates have had problems with reacto-enicity in the clinic (Formal *et al.* 1989; Klee *et al.* 1997; evine et al. 1997; Coster *et al.* 1999). Work in *V. cholerae* has ocused on cholera enterotoxin (CT) defective strains. One T-negative candidate, CVD103, has been extensively valuated in the clinic and in the field (Tacket *et al.* 1992; aper *et al.* 1995). This vaccine performed very impress-ely in the clinic during phase I and II immunogenicity nd challenge studies. The vaccine was immunogenic in a ingle dose, eliciting serum and local anti-vibrio antibodies ı 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 patho-genic *Salmonella* such as *Salmonella typhimurium*. In the last several years over 100 *Salmonella* genes have been impli-cated 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 muta-tions for salmonella derivatives to be used as vaccines in other mammalian species. This we know, as some muta-tions 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, gener-ating 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

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

nteract with the immune system and at the same time void the attention of antibodies and other immune effecors. The expression of generic activating molecules such s peptidoglycan, lipoproteins and LPS, recognized by nnate immune effectors, may help increase the immune otential of these live vaccines. Unfortunately, these are ne same molecules that can activate the fever response otentially leading to reactogenicity.

5. THE MUCOSAL IMMUNOGENICITY OF NON-LIVING ANTIGENS

It is relatively easy to picture how a live pathogen may e immunogenic when administered via a mucosal urface. What about non-living antigenic preparations? Vhy are some more immunogenic than others? What nolecular features of antigens might contribute to their nucosal immunogenicity? There are relatively few antiens (mucosal immunogens) that in a purified form are ble to elicit significant levels of secretory IgA and serum gG when administered to mucosal surfaces. Furthernore, some mucosal immunogens can only stimulate ocal IgA responses whereas others can stimulate both ocal and systemic immunity. In addition, these properties nay, in part, be host dependent.

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

6. ENTEROTOXINS AS MUCOSAL IMMUNMOGENS AND ADJUVANTS

Perhaps the best known example of a class of mucosal nmunogens 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

Downloaded from rstb.royalsocietypublishing.org

BIOLOGICAL SCIENCES THE ROYAL B SOCIETY PHILOSOPHICAL TRANSACTIONS OF

egradation or denaturation are less immunogenic. Thus, he compact structure of the molecule may be required to nhance resistance and persistence in tissues. Some non-oxic derivatives of LT retain significant mucosal adjuvant ctivity, e.g. LTK63 (Douce *et al*. 1995). Thus, toxicity nd ADP-ribosyltransferase activity are not essential for djuvanticity. Although the ADP-ribosyltransferase ctivity is not essential for mucosal adjuvant activity it an enhance this activity. For example, mutants that etain partial ADP-ribosyltransferase activity are better nucosal adjuvants than LTK63, e.g. LTR72 (Douce *et al*. 997; Giuliani *et al*. 1998). Hence, by studying these nutant derivatives it has proved possible to identify ombinations of important features that contribute to oth immunogenicity and adjuvanticity at mucosal urfaces. What is the importance of this information for accine design? By breaking down the individual features f LT and CT that contribute to mucosal adjuvant activity has been possible to use this information to design ompletely novel mucosal adjuvants. For example, by ombining the antibody-binding domain of *Staphylococcus ureus* protein A with the A-subunit of CT, a novel nucosal adjuvant CTA1-DD has been design as a prototype of a new class of artificial mucosal adjuvants (Agren *al*. 1999).

. UNDERSTANDING THE REGULATION OF MUCOSAL IMMUNE RESPONSES

If we are to improve methods for mucosal therapies nd mucosal vaccination, we need to understand the undamentals of how the immune system regulates the nmune response to mucosally delivered antigens Bienenstock *et al*. 1978; McGhee *et al*. 1992). What are the nolecular mechanisms that initiate an active (IgA, serum gG) compared to a tolerant immune response? Indeed, what is the basis of responsiveness compared to non-esponsiveness and how is this balance maintained or hanged? It could be argued that there are two fundamental elements interacting to maintain this balance. These elements are the formulation of the antigens erived exogenously from the environment (either envirnmental or pathogen derived) together with the mucosal nmune regulator systems. Evidence has accumulated hat T-cell responses to antigens presented via mucosal ells are tightly regulated. Both $CD4^+$ and $CD8^+$ T cells ave been implicated in this form of regulation in experimental approaches involving vaccination and tolerance nduction. A new class of regulatory T cell, producing igh levels of interleukin 10 that proliferate poorly in esponse to antigen have been identified in mucosal issues (Groux *et al*. 1997). The establishment of particular ytokine expression patterns by these and other regulaory cells may be critical in this phenomenon. It is also kely that the regulation of antigen presentation by ntigen-presenting cells at mucosal surfaces is a critical egulatory step. Indeed, dendritic cells associated with nucosal surfaces may possess significantly different roperties from other dendritic cell populations. If tight egulatory networks are operating *in vivo*, this will complicate any attempts to use *in vitro* methods of assessment of he function of mucosal immune cells since key signals nay 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*. 1993*a*). 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

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

:PEC infections in humans. While *in vivo* studies of EPEC athogenesis and immunity are clearly desirable, the nability of human isolates of EPEC to colonize small odents has meant alternative animal models have been ıvestigated. One of these models, *C. rodentium* infection of nice, has several features which make it attractive for ırthering research into EPEC and also mucosal T-cell egulation.

0. IMMUNOBIOLOGY OF *CITROBACTER RODENTIUM* INFECTION

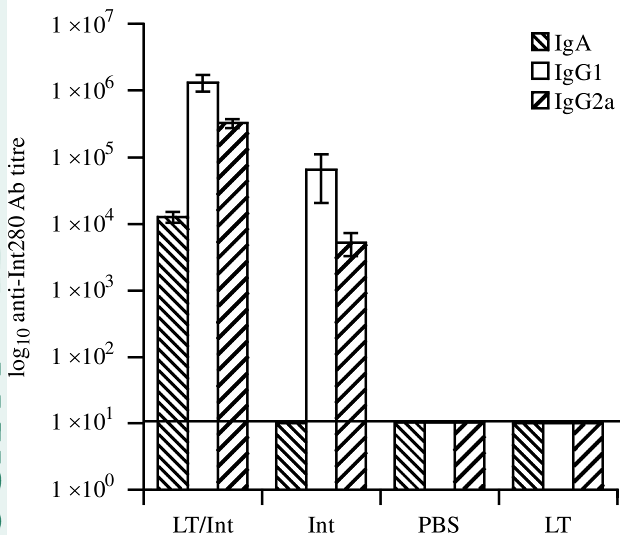
The *C. rodentium* chromosome has been shown to ontain genes with homology to those located in the LEE athogenicity island found in human EPEC strains Schauer *et al.* 1993). The identification of LEE homolo-ues in *C. rodentium* has facilitated the construction of efined mutants and their subsequent characterization in nice. An *eae* mutant of *C. rodentium* (strain DBS255) was ıown to be avirulent. However, virulence could be estored by complementation with either the *eae* gene rom *C. rodentium* (Schauer *et al.* 1993*b*), or the *eae* gene rom the prototype EPEC strain E2348/69 (strain)BS255 (pCVD438) (Frankel *et al.* 1996). The fact that n *eae* mutant of *C. rodentium* is attenuated in mice is onsistent with human studies demonstrating that an *eae* ıutant of EPEC strain E2348/69 is attenuated in humans Donnenberg *et al.* 1993). In addition to its role as an dhesin, intimin also contributes to the induction of olonic epithelial cell hyperplasia, the second character-stic feature of *C. rodentium* infection in mice. The colonic yperplasia that occurs during *C. rodentium* colonization is ssociated with the expression of inflammatory cytokines IL-1, tumour necrosis factor-alpha (TNFα)) and a rong T-cell infiltrate consisting predominantly of $CD4^+$ ' cells with a Th1 phenotype (Higgins *et al.* 1999). Inter-stingly, the characteristics of the T-cell infiltrate which ccurs in the mouse colon during *C. rodentium* infection hare striking similarity to the cellular events occurring ı murine models of inflammatory bowel disease (IBD). pithelial cell hyperplasia in mice can also be induced ia intrarectal administration of formalin-killed *'. rodentium* or EPEC, but not by *eae* mutants of *'. rodentium* or EPEC, indicating a critical role for intimin ı this effect (Higgins *et al.* 1999). Indeed, intimin-earing bacterial cells appear to be sufficient for this ffect, since intrarectal administration of formalin-killed *'. coli* K12 expressing intimin, but not *E. coli* K12 alone, lso evokes colonic hyperplasia (Higgins *et al.* 1999). The ıechanism through which intimin-associated bacteria romotes hyperplasia is not clear, but clues are provided y the ability of the C-terminal 280 amino acids of ıtimin to co-stimulate T cells *in vitro*, which potentially ccurs as a result of intimin binding to cell-surface eceptors (Higgins *et al.* 1999). If intimin does indeed ind resident or infiltrating T cells in the gut, then this ıay promote unregulated proliferation of these mucosal ' cells, which under normal situations are hyporesponsive nd require strong co-stimulatory signals before cytokine roduction occurs. Following mucosal T-cell activation, ytokine production has been shown to trigger a cascade f events, including production of the epithelial cell ıitogen keratinocyte growth factor (KGF) by mesenchymal 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.

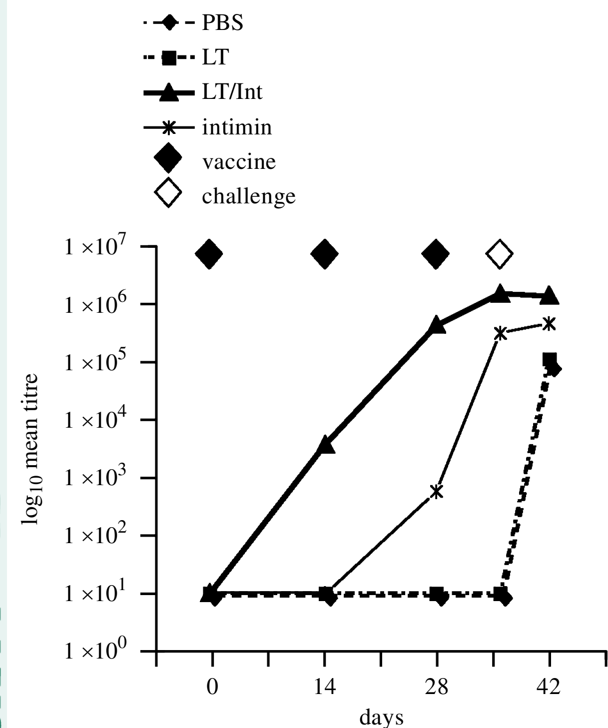
11. *CITROBACTER RODENTIUM* INFECTION OF MICE AS A MODEL FOR EVALUATING CANDIDATE EPEC VACCINE ANTIGENS

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 10^5 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

Downloaded from rstb.royalsocietypublishing.org



igure 1. Co-delivery of Int-280 with the mucosal adjuvant ,T elicits a strong serum IgG and IgA response to Int-280. ;roups of 5 C3H/Hej mice were intranasally immunized three mes with 10 mg of Int-280 plus 1 mg of LT, 10 mg of nt-280 alone, 1 mg of LT alone or PBS alone. The data epicts the mean titre (plus standard error) of the antibody esponse to Int-280.



igure 2. Kinetic of the anti-Int280 total Ig antibody esponse in immunized mice pre- and post-challenge with)BS255(pCVD438). The data depict the mean serum Ig titre gainst Int-280.

lone or phosphate buffered saline (PBS). After three nmunizations, mice that received Int280 admixed with ,T had developed the strongest serum anti-Int280 IgG nd IgA antibody responses (figure 1). Mice immunized /ith Int280 alone mounted anti-Int280 IgG responses, ut not serum IgA responses (figure 1). Immunized mice /ere orally challenged with DBS255(pCVD438) ten days

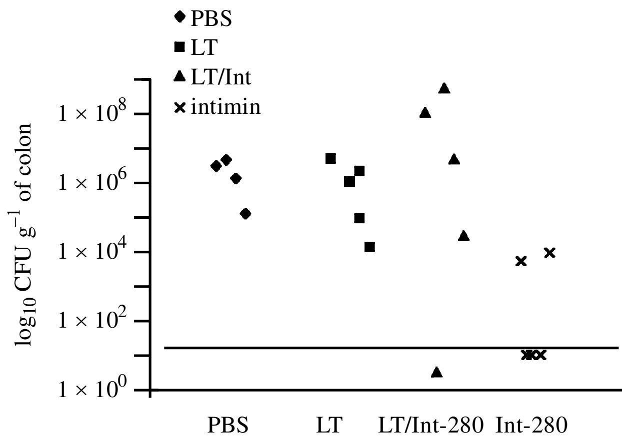


Figure 3. Pre-existing immune responses to Int-280 do not prevent bacterial colonization by *C. rodentium* DBS255(pCVD438). The data depict the number of viable DBS255(pCVD438) bacteria recovered from the colons of individual mice 14 days post-challenge.

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

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

ıtercellular interactions occurring *in vivo* between ifferent differentiated cell types and it is impossible to tudy many of these interactions *in vitro*. Indeed, the study f *in vitro* grown cells may provide misleading information n how regulatory networks are operating in real tissues. Ve are only just beginning to understand how the expres- .on of immunity is regulated at mucosal surfaces and the se of bacteria and their products provides us with valu- ble tools for these ongoing investigations. As this work ontinues we can expect new and more effective vaccines ɔ be designed and developed.

REFERENCES

.gren, L. C., Ekman, L., Lowenadler, B., Nedrud, J. G. & Lycke, N. Y. 1999 Adjuvanticity of the cholera toxin A1-based gene fusion protein, CTA1-DD, is critically dependent on the ADP-ribosyltransferase and Ig-binding activity. *J. Immunol.* **15**, 2432–2440.

.izapurua, D. H. & Russell-Jones, A. 1988 Oral vaccination: identification of classes of proteins that provoke an immune response upon oral feeding. *J. Exp. Med.* **167**, 440–451.

ajaj-Elliott, M., Poulsom, R., Pender, S. L., Wathen, N. C. & MacDonald, T. T. 1998 Interactions between stromal cell-derived keratinocyte growth factor and epithelial transforming growth factor in immune-mediated crypt cell hyperplasia. *J. Clin. Invest.* **102**, 1473–1480.

ienenstock, J., McDermott, M., Befus, D. & O'Neil, L. M. 1978 A common mucosal immunological system involving the bronchus, breast and bowel. *Adv. Exp. Med. Biol.* **107**, 53–69.

:oster, T. S. (and 10 others) 1999 Vaccination against shigellosis with attenuated *Shigella flexneri* 2a strain SC602. *Infect. Immun.* **67**, 3437–3443.

:ropley, I., Douce, G., Roberts, M., Chatfield, S., Pizza, M., Rappuoli, R. & Dougan, G. 1995 Mucosal and systemic immunogenicity of a recombinant, non-ADP-ribosylating pertussis toxin: comparison of native and formaldehyde-treated proteins. *Vaccine* **13**, 1643–1648.

)onnenberg, M. S., Tacket, C. O., James, S. P., Losonsky, G., Nataro, J. P., Wasserman, S. S., Kaper, J. B. & Levine, M. M. 1993 Role of the *eaeA* gene in experimental enteropathogenic *Escherichia coli* infection. *J. Clin. Invest.* **92,** 1412–1417.

)ouce, G., Turcotte, C., Cropley, I., Roberts, M., Pizza, M., Dominghini, M., Rappuoli, R. & Dougan, G. 1995 Mutants of *Escherichia coli* heat-labile toxin lacking ADP-ribosyltransferase activity act as non-toxic mucosal adjuvants. *Proc. Natl Acad. Sci. USA* **92**, 1644–1648.

)ouce, G., Fontana, M., Pizza, M., Rappuoli, R. & Dougan, G. 1997 Mucosal immunogenicity and adjuvanticity of site-directed mutant derivatives of cholera toxin. *Infect. Immun.* **65**, 2821–2828.

)ougan, G. 1994 The molecular basis for the virulence of bacterial pathogens: implications for oral vaccine development. Colworth Lecture. *Microbiology* **140**, 215–224.

.lson, C. J. & Ealding, W. 1984 Generalised systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. *J. Immunol.* **132**, 2736–2743.

agundes Neto, U., Ferreira, V. D. C., Patricio, F. R., Mostaco, V. L. & Trabulsi, L. R. 1989 Protracted diarrhea: the importance of the enteropathogenic *Escherichia coli* (EPEC) strains and *Salmonella* in its genesis. *J. Pediatr. Gastroenterol. Nutr.* **8,** 207–211.

inlay, B. B. & Falkow, S. 1997 Common themes in microbial pathogenicity revisited. *Microbiol. Mol. Biol. Rev.* **61**, 136–169.

ormal, S. B., Hale, T. L., Kapfer, C. 1989 Shigella vaccines. *Rev. Infect. Dis.* **11** (Suppl. 3), S547–S551.

Frankel, G., Phillips, A. D., Novakova, M., Field, H., Candy, D. C., Schauer, D. B., Douce, G. & Dougan, G. 1996 Intimin from enteropathogenic *Escherichia coli* restores murine virulence to a *Citrobacter rodentium eaeA* mutant: induction of an immunoglobulin A response to intimin and EspB. *Infect. Immun.* **64,** 5315–5325.

Frankel, G., Phillips, A. D., Rosenshine, I., Dougan, G., Kaper, J. B. & Knutton, S. 1998 Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Mol. Microbiol.* **30,** 911–921.

Garside, P., Mowat, A. M. & Khoruts, A. 1999 Oral tolerance in disease. *Gut* **44**, 137–142.

Giuliani, M. M., Del Giudice, G. D., Giannelli, V., Dougan, G., Douce, G., Rappuoli, R. & Pizza, M. 1998 Mucosal adjuvanticity of LT72, a novel mutant of *Escherichia coli* heat-labile enterotoxin with partial knock-out of ADP-ribosyltransferase activity. *J. Exp. Med.* **187**, 1123–1132.

Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J. E. & Roncarolo, M. G. 1997 A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* **16**, 737–742.

Hensel, M., Shea, J. E., Gleeson, C., Jones, M. D., Dalton, E. & Holden, D. W. 1995 Simultaneous identification of bacterial virulence genes by negative selection. *Science* **269**, 400–403.

Higgins, L. M., Frankel, G., Connerton, I., Goncalves, N. S., Dougan, G. & MacDonald, T. T. 1999*a* Role of bacterial intimin in colonic hyperplasia and inflammation. *Science* **285,** 588–591.

Hill, S. M., Phillips, A. D. & Walker-Smith, J. A. 1991 Enteropathogenic *Escherichia coli* and life threatening chronic diarrhoea. *Gut* **32**, 154–158.

Jackson, R. J., Staats, H. F., Xu-Amano, J., Takahashi, I., Kiyono, H., Hudson, M. E., Gilley, R. M., Chatfield, S. N. & McGhee, J. R. 1994 Oral vaccine models: multiple delivery systems employing tetanus toxoid. *Annls NY Acad. Sci.* **730**, 217–234.

Jerse, A. E., Yu, J., Tall, B. D. & Kaper, J. B. 1990 A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proc. Natl Acad. Sci. USA* **87**, 7839–7843.

Kaper, J. B., Morris Jr, J. G. & Levine, M. M. 1995 Cholera. *Clin. Microbiol. Rev.* **8**, 48–86.

Klee, S. R., Tzschaschel, B. D., Falt, I., Karnell, A., Lindberg, A. A., Timmis, K. N. & Guzman, C. A. 1997 Construction and characterization of a live attenuated vaccine candidate against *Shigella dysenteriae* type I. *Infect. Immun.* **65**, 2112–2118.

Lagos, R., Fasano, A., Wasserman, S. S., Prado, V., San Martin, O., Abrego, P., Losonsky, G. A., Alegria, S. & Levine, M. M. 1999 Effect of small bowel bacterial overgrowth on the immunogenicity of single-dose live cholera vaccine CVD 103-Hg. *J. Infect. Dis.* **180**, 1709–1712.

Levine, M. M. & Dougan, G. 1998 Optimism over vaccines administered through mucosal surfaces. *Lancet* **351**, 1375–1376.

Levine, M. M., Woodrow, G. C., Kaper, J. B. & Cobon, G. S. 1997 *New generation vaccines*, 2nd edn. New York: Marcel Dekker, Inc.

Lycke, N. & Holmgren, J. 1986 Strong adjuvant properties of cholera toxin on gut mucosal immune responses to orally presented antigens. *Immunol.* **59**, 301–308.

McGhee, J. R., Mesteky, J., Dertbaugh, M. T., Eldridge, J. H. M. & Kiyono, H. 1992 The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine* **10**, 75–81.

Nashar, T. O., Williams, N. A. & Hirst, T. R. 1998 Importance of receptor binding in the immunogenicity, adjuvanticity and therapeutic properties of cholera toxin and *Escherichia coli* heat-labile enterotoxin. *Med. Microbiol. Immun. (Berlin)* **187**, 3–10.

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

O'Callaghan, D., Maskell, D., Liew, F. Y., Easmon, C. S. F. & Dougan, G. 1988 Characterisation of aromatic-dependent and purine-dependent *Salmonella typhimurium*: studies on attenuation, persistence and ability to induce protective immunity in BALB/c mice. *Infect. Immun.* **56**, 419–423.

O'Hagan, D. T. 1990 Novel non-replicating antigen delivery systems. *Curr. Opin. Infect. Dis.* **3**, 393–401.

Pizza, M. G., Domenighini, M., Hol, W., Gianneli, V., Fontana, M. R., Giuliani, M., Magagnoli, C., Peppeloni, S., Manetti, R. & Rappuoli, R. 1994 Probing the structure–function relationship of *Escherichia coli* LT-A by site-directed mutagenesis. *Mol. Microbiol.* **14**, 51–61.

Rappuoli, R., Pizza, M., Douce, G. & Dougan, G. 1999 Structure and mucosal adjuvanticity of cholera and *Escherichia coli* heat-labile enterotoxins. *Immunol. Today* **20**, 493–500.

Roberts, M., Bacon, A., Rappuoli, R., Cropley, I., Douce, G., Dougan, G. & Chatfield, S. N. 1995 A mutant pertussis toxin molecule that lacks ADP-ribosyltransferease activity, PT-9K/129G, is an effective mucosal adjuvant for intranasally delivered proteins. *Infect. Immun.* **63**, 2100–2108.

Schauer, D. B. & Falkow, S. 1993*a* Attaching and effacing locus of a *Citrobacter freundii* biotype that causes transmissible murine colonic hyperplasia. *Infect. Immun.* **61**, 2486–2492.

Schauer, D. B. & Falkow, S. 1993*b* The *eae* gene of *Citrobacter freundii* biotype 4280 is necessary for colonization in transmissible murine colonic hyperplasia. *Infect. Immun.* **61**, 4654–4661.

Schauer, D. B., Zabel, B. A., Pedraza, I. F., O'Hara, C. M., Steigerwalt, A. G. & Brenner, D. J. 1995 Genetic and biochemical characterization of *Citrobacter rodentium* sp. *J. Clin. Microbiol.* **33**, 2064–2068.

Su-Arehawaratana, P. (and 14 others) 1992 Safety and immunogenicity of different immunization regimens of CVD 103-HgR live oral cholera vaccine in soldiers and civilians in Thailand. *J. Infect. Dis.* **165**, 1042–1048.

Tacket, C. O., Losonsky, G., Nataro, J. P., Cryz, S. J., Edelman, R., Kaper, J. B., Levine, M. M. 1992 Onset and duration of protective immunity in challenged volunteers after vaccination with live oral cholera vaccine CVD 103-HgR. *J. Infect. Dis.* **166**, 837–841.

Tacket, C. O., Sztein, M. B., Losonsky, G., Wasserman, S. S., Nataro, J. P., Edelman, R., Pickard, D., Dougan, G., Chatfield, S. & Levine, M. M. 1997 Safety and immune response in humans of a live oral *Salmonella typhi* vaccine strain deleted in *htrA* and *aroC*, *aroD* and demonstration in mice of their use as vaccine vectors. *Infect. Immun.* **65**, 452–456.

Tacket C. O. (and 11 others) 1999 Randomized, double-blind, placebo-controlled, multicentered trial of the efficacy of a single dose of live oral cholera vaccine CVD 103-HgR in preventing cholera following challenge with *Vibrio cholerae* O1 El for inaba three months after vaccination. *Infect. Immun.* **67**, 6341–6345.

Tsolis, R. M., Adams, L. G., Ficht, T. A. & Baumler, A. J. 1999 Contribution of *Salmonella typhimurium* virulence factors to diarrheal disease in calves. *Infect. Immun.* **67**, 4879–4885.