Mucosal immunogenicity and adjuvant activity of the Escherichia coli heat-labile enterotoxin

Haan, Lolke de

Summarizing discussion and concluding remarks

Many pathogens invade their host via mucosal surfaces. The mucosal immune system forms a first line of defense against such pathogens, through secretion of pathogen-specific antibodies of the IgA isotype, preventing infection of the host. Current vaccination strategies, which generally involve intramuscular injection of live or inactivated pathogens, or antigens thereof, mainly induce systemic IgG antibodies, but rarely result in a mucosal immune response. New-generation vaccines should preferably be aimed at the induction of a more broad-spectrum immunity, including mucosal immunity. However, for induction of mucosal immune responses the vaccine needs to be administered locally, which is usually fairly ineffective due to the low immunogenicity of protein antigens upon local immunization. Therefore, an efficient mucosal immunoadjuvant is required to induce such responses. Escherichia coli heat-labile toxin (LT) and Vibrio cholerae cholera toxin (CT) represent important candidates in this respect. However, the intrinsic toxicity of these molecules has thusfar precluded their use in human vaccination strategies. Aim of the study presented in this thesis was to investigate if the toxicity of LT can be dissociated from its immunogenic and adjuvant properties. Final goal of this project is the development of recombinant vaccines, using nontoxic variants of LT as mucosal immunoadjuvants.

1. LT and LT subunits as mucosal immunogens

LT and CT are the most potent mucosal antigens known to date. Both are not only of interest because of their ability to stimulate antibody responses towards coadministered antigens, but are also potentially important antigens in vaccines directed against diarrheal disease mediated by enterotoxigenic *E. coli* (ETEC) and *V. cholerae* (108). Large-scale field trials with experimental whole-cell cholera vaccines have shown that inclusion of the CTB subunit greatly improves the efficacy of the vaccine (108), possibly due to the immune-stimulatory properties of CTB (see below). The basis of induction of antitoxin antibodies is not well understood. The results from early studies suggested that the antitoxin antibodies are almost exclusively directed against the B subunit (103,119,136,187,198). In our studies, we used recombinant LT variants to study the role of the individual LT subunits in the induction of LTB- and LTA-specific responses upon intranasal administration to mice. It is demonstrated that not only LT, but also the recombinant LTB pentamer alone is an effective immunogen when administered intranasally to mice (**Chapter 2,3**). LT is even more immunogenic, especially with respect to its capacity to induce LTB-specific S-IgA responses at distant mucosal effector sites. Thus, it is clear that the A subunit stimulates the induction of LTB-specific antibody responses. The results in **Chapter 3** show that a nontoxic LT mutant, LT-E112K, completely retains the immunogenic properties of wild-type LT. Therefore, the ADP-ribosyltransferase activity of LTA does not play a major role in the stimulation of LTB-specific responses, indicating that other features of LTA are responsible for the stimulation of such responses. This is in agreement with recent observations of others with respect to the role of ADP-ribosylation activity in the immunogenicity of LT (60,61,63,271, 272). In agreement with Nashar *et al.* (175), we show that G_{M1}-binding activity is essential for the immunogenicity of LTB; the immunogenicity of an LTB mutant

lacking affinity for G_{M1} was markedly reduced when administered nasally to mice (**Chapter 6**). In addition, we demonstrated that also for the potent immunogenicity of LT, G_{M1}-binding affinity is essential (**Chapter 6**). We were the first to show that wild-type LT and a nontoxic LT mutant induce comparable LTA-specific serum and mucosal antibody responses (**Chapter 7**). On the other hand, when mice were immunized with LTA alone, no antigen-specific antibodies were induced, indicating that linkage to the B-pentamer is essential for the immunogenicity of LTA.

Taken together, the above results indicate that the potent immunogenicity of LT stems primarily from its capacity to bind with high affinity to the G_{M1} ganglioside, present on mucosal epithelial cells. This ensures efficient cellular uptake of the toxin. After transcytosis, the B-pentamer probably interacts directly with antigen-presenting cells, and B and T cells, resulting in activation of G_{M1}-coupled signal transduction pathways within these cells. This eventually results in modulation of the mucosal immune response (see also section 2.2). In case of the LT/CT holotoxin, the A subunit probably influences the immune-stimulatory process upon G_{M1}-binding-mediated internalization of the toxin. It is likely that this immune-stimulatory mechanism is similar to that involved in adjuvanticity of LTA (as described below). Also, T-helper epitopes of the A subunit may influence the induction of antitoxin antibody responses. However, further characterization of the structure-function relationships of the A subunit is required to delineate which properties, epitopes, or amino acid residues are essential for immune stimulation by LTA. Such studies would involve the evaluation of the immunogenicity of LT(A) variants in which mutations have been placed at random, or LT(A) deletion mutants.

2. LT and LT subunits as mucosal immunoadjuvants

2.1 Role of the individual subunits in adjuvanticity of LT

Most adjuvant studies with bacterial enterotoxins (LT, CT, PT, etc) have been performed with CT. However, more recent studies have also focussed on LT, mainly because LT is less toxic than CT; in addition, the structure-function relationships of LT have been studied with aid of the known 3D-structure of the molecule (210-212), while the 3D-structure of CT has only recently become available (163,273,274). At the beginning of this study, the role of the individual subunits in the immunoadjuvant properties of LT/CT was not clear. Especially results obtained with commercially available and recombinant CTB/LTB were highly inconsistent (41,98,140,146,228-236, 258). In addition, more recent results obtained with LT/CT mutants, lacking ADP-ribosylation activity, have been contradictory as well. While Lycke *et al.* (146) showed that the nontoxic LT-E112K mutant lacks adjuvant activity, several other investigators have demonstrated that different nontoxic LT/CT mutants retain adjuvanticity (60,61,63,271,272). With respect to the role of ADP-ribosylation activity, we unambiguously demonstrate that the enzymatically inactive LT-E112K mutant completely retains the adjuvant properties of wild-type LT upon intranasal administration to mice (**Chapter 4, 5, 8**). This adjuvant activity not only pertains to the induction of strong serum IgG responses to coadministered antigens, but also to the induction of brisk S-IgA responses in mucosal secretions. Thus, clearly, ADP-

ribosyltransferase activity of LTA is not essential for the adjuvant properties of LT towards admixed antigens. This is in agreement with recent publications on the adjuvant activity of nontoxic LT/CT mutants (60,61,63,271,272). On the other hand, Lycke *et al.* (146) observed that LT-E112K lacks adjuvant activity towards orally administered KLH. We suspect that the route of immunization, intranasal vs. oral, is responsible for this discrepancy. In fact, none of the nontoxic LT mutants described thusfar, has been administered via the oral route to result in generalized mucosal immune responses. There is a growing awareness that indeed for induction of generalized mucosal immune responses intranasal immunization is more efficient than oral immunization (266). Possible explanations for this phenomenon are: (i) more efficient uptake of antigens in the nasal cavity, due to a lower total antigen load; (ii) a milder physiological environment in the nasal cavity compared to the gut, where antigens may be degraded by acid pH or proteolytic activity; and, (iii) differences in the nasal (NALT) and gut-associated lymphoid tissue (GALT). With respect to the latter, it is clear that there are differences in the T/B cell, naive/memory T cell, and $CD8^+/CD4^+$ T cell ratios in NALT and GALT (125,265,266). This suggests that compared to GALT, mucosal immune responses in NALT are induced and regulated differently. The fact that recently CT-E112K was found to be an effective adjuvant upon subcutaneous administration (271), supports our view that indeed the route of immunization is crucial for the outcome of the experiment.

The results described in **Chapter 4**, **5**, and **8** clearly show that recombinant LTB has *bona fide* adjuvant activity. Recombinant LTB induced both strong serum and mucosal antibody responses towards a variety of coadministered antigens. Furthermore, the observed antibody responses were comparable to those obtained with either LT-E112K or wild-type LT. Therefore, it is clear that neither the ADP-ribosylation activity of LTA, nor the presence of LTA is essential for effective immune stimulation. While most investigators have found that recombinant LTB/CTB lack adjuvanticity (41,140,146,271,272), several others observed the opposite (64,98). This may be explained by the fact that the majority of these studies were performed using the oral route of administration, while only in a few cases adjuvanticity of LTB/CTB upon intranasal administration was evaluated. Thus, again the route of immunization may be crucial. Also, most studies were performed with CTB, and not with LTB. While CTB binds exclusively to G_{M1}, LTB also binds to a variety of other receptors (66,83,104,105). This different receptor binding capacity is likely to play a role in the observed adjuvant effect, especially since it was recently observed that LTB and nontoxic LT mutants are more potent stimulators of mucosal immune responses than CTB or nontoxic CT mutants (64).

To our knowledge, we were the first to study the role of G_{M1}-binding in the adjuvant properties of LT and LTB. In general, G_{M1}-binding is considered to be essential for adjuvanticity. Remarkably, we found that an LT mutant, which lacks G_{M1}-binding affinity, acts as a potent adjuvant towards coadministered antigens (**Chapter 6**, **8**). Furthermore, an LT double mutant, LT-E112K/G33D, lacking both ADP-ribosylation activity and G_{M1}-binding activity, also retained adjuvant activity comparable to that of wild-type LT. On the other hand, G_{M1}-binding is essential for the adjuvanticity of LTB (**Chapter 6**, **8**). These findings are suggestive of a G_{M1}-binding-independent adjuvant effect of LTA. Furthermore, immune stimulation by

LTA does not require ADP-ribosylation activity. Interestingly, the immunogenicity of the non-G_{M1}-binding LT mutants is markedly reduced. This suggests that the mode of induction of antitoxin antibodies vs. induction of antibodies to coadministered antigens may be different. Recent studies, involving the use of IL-4 knock-out mice, appear to support this idea (182,246). While in these mice responses to orally administered CT were normal, antibody responses to coadministered antigens were absent. This is probably because the antibody response towards coadministered antigen is solely dependent on a Th2-type helper response, which is inhibited in the absence of IL-4, while both Th1- and Th2-type responses support the production of antitoxin antibodies (182,246).

In agreement with the results in **Chapter 6**, **Chapter 7** and **8** show that LTA by itself can act as a powerful intranasal mucosal adjuvant towards a coadministered antigen. Therefore, we are the first to present direct evidence that both the recombinant A and B subunit of LT have adjuvant activity. Furthermore, by using an LTA mutant, devoid of ADP-ribosylation activity, we showed that this activity is not involved in the immune stimulation by LTA (**Chapter 8**). These findings are in agreement with and extend the findings of Ågren *et al.* (2), who showed that a targeted CTA1 fusion protein has potent adjuvant activity towards intranasally or intravenously administered antigens. These investigators showed that a CTA1 fusion protein, composed of CTA1 fused to 2 copies of the D domain of staphylococcal protein A, has potent adjuvant activity. Furthermore, it was shown that the DD-moiety targets CTA1 to B cells, which is considered to be the basis of the observed adjuvant effect. We now demonstrate, that also in the absence of a specific targeting mechanism, LTA and a nontoxic LTA mutant strongly stimulate antibody responses towards coadministered antigens. Clearly, this has important implications for the design of LT/CT variants as vaccine adjuvants. Such strategies have, until now, focussed mainly on the use of the nontoxic B subunit or nontoxic LT/CT mutants. However, we have shown that also nontoxic CTA/LTA variants may be used as vaccine adjuvants.

Although it is clear that LT is a potent mucosal immunoadjuvant, there appear to be limitations to its adjuvant properties. We observed that LT failed to induce mucosal secretory responses against pneumolysin (**Chapter 5**), a malaria antigen (TBV25H), and recombinant HIV gp120 (De Haan *et al.*, unpublished observations), upon intranasal administration to mice. At this moment, we do not have an explanation for the observed antigen-specificity of the adjuvant effect. Possibly, the physical-chemical properties of the antigen, such as solubility, molecular size or weight, and hydrophobicity, play a role in the adjuvanticity of LT. However, there are indications that adjuvanticity of LT towards such antigens can be restored by coupling antigen and adjuvant, resulting in a targeted antigen/adjuvant complex (41).

2.2 Mechanism of adjuvanticity of LT and LT subunits

LT and CT are well-recognized mucosal immunoadjuvants. Although we have established the requirements for adjuvanticity of LT and LTB, the immunological basis of their adjuvant properties is still poorly understood. Clearly, adjuvant activity of LTB is directly related to G_{M1}-binding affinity (**Chapter 6**, **8**). Therefore, G_{M1}-

binding not only mediates efficient uptake of the toxoid, but is also involved in the stimulation of antibody responses directed against coadministered antigens. Although the exact mechanism of adjuvanticity of LTB is likely to be extremely complex, two separate stages can be distinguished: (i) binding of LTB to the mucosal surface and enhancement of the uptake of luminal antigens, and (ii) specific modulation of mucosal cellular responses towards coadministered antigen. It is known that upon binding of CTB to the mucosal cell surface, the permeability of the mucosal membrane barrier is enhanced (87,88,145). This facilitates more efficient absorption of antigens coadministered with CTB or LTB. After transcytosis through mucosal epithelial cells or M-cells, the B pentamer interacts directly with immunocompetent cells via its G_{M1}-binding capacity. Subsequently, G_{M1}-coupled cell-signaling pathways within these cells are activated, eventually resulting in cytokine expression, selective stimulation of proliferation of $CD4^+$ Th2-type cells, inhibition of proliferation and/or apoptosis of $CD4^+$ Th1-type and $CD8^+$ T cells, and stimulation of B cell proliferation. For instance, LTB and LT/CT strongly stimulate production of Th2-type cytokines by $CD4^+$ T cells and macrophages (147,173,226,267,268,271,272). By binding to the cell surface of B cells and macrophages, LTB enhances their antigen-presenting capacity (79,143,174). Also, expression of co-stimulatory molecules ICAM-1, B7, CD25, and CD40 on B cells is enhanced by binding of LTB (175). For most of these effects the requirement for G_{M1}-binding has been established in studies involving the use of LTB and CTB mutants, which lack G_{M1}-binding affinity (73,79, 174).

As indicated above, our present results show that also the A subunit alone has immunoadjuvant properties, which are independent of ADP-ribosylation activity. The immunological basis of immunomodulation by LTA alone is not clear. However, recently, Ågren *et al.* (2) have shown that CT and CTA1, but not CTB, enhances the expression of CD80 and CD86 co-stimulatory molecules on B cells. Also, in a recent study, we demonstrated that LT, LT-E112K, LTB and LTA induce antigen-specific IgG1, IgG2a and IgG2b (DeHaan *et al.* manuscript in preparation). Thus, these molecules induce both Th1 and Th2-type responses, which support the production of these antibody subtypes (166,238). However, the kinetics of induction of especially IgG2a by LTA and LTA-E112K is different, indicating that the mode of induction of mucosal immune responses by LTA alone is different. It is possible that these molecules also induce altered cytokine profiles. No doubt, determination of these cytokine profiles, in combination with studies performed in cytokine gene knock-out mice, would give further insights in the mechanisms of adjuvanticity of LT and its subunits.

3. Perspectives for vaccine development based on the use of nontoxic LT variants as mucosal immunogens and adjuvants

LT and CT represent important adjuvant candidates for locally administered vaccines, which induce adequate serum IgG in addition to mucosal IgA responses against mucosal pathogens. There is a great need for these types of vaccines, not only in developing countries, but also in the western world, where infectious diseases threaten the health of young children in particular. Therefore, the observation made

in this thesis that nontoxic variants of LT retain the immunogenic and adjuvant properties of the wild-type toxin, has important implications for future vaccine design against cholera, enterotoxigenic *E. coli* (ETEC), and other infectious diseases.

Current experimental ETEC and cholera vaccines are composed of whole inactivated bacteria, supplemented with recombinant B subunit (35). Although the protective capacity of antitoxin antibodies alone is limited, coadministration of B subunit clearly improves the efficacy of these vaccines (35). Our studies demonstrate that LT mutants which lack ADP-ribosylation activity are more immunogenic than recombinant LTB upon intranasal administration to mice. Furthermore, it was recently shown that a nontoxic LT mutant induced toxin-neutralizing antibodies against both the A and B subunit (191). Therefore, a promising approach for improvement of current whole-cell cholera and ETEC vaccines would be replacement of the B subunit with a nontoxic mutant.

All of the adjuvant studies presented in this thesis have focussed on the direct measurement of antibody titers. For the use of LT and LT variants as vaccine adjuvants it is crucial to relate these titers to protection against infection with a mucosal pathogen. Recently, we have evaluated the protective capacity of an experimental influenza vaccine against a homologous virus challenge (Verweij *et al.*, manuscript in preparation). The vaccine consisted of influenza subunit antigen supplemented with either LT or LTB. The obtained results show that in contrast to mice immunized subcutaneously with subunit antigen (the currently used immunization procedure), mice that received either the LT or LTB-supplemented vaccine are completely protected against virus challenge, indicating that the experimental vaccine offers superior protection (Verweij *et al.*, manuscript in preparation). Clearly, the antigen-specific antibody responses induced by LT and LT variants, presented in this thesis, are relevant for providing protection against infection with a mucosal pathogen. Therefore, the main conclusion from the work in this thesis is that nontoxic LT variants, which retain the immunoadjuvant properties of the wild-type toxin have great potential, and may be used as adjuvants for new-generation vaccines, which will elicit a more broad-spectrum immunity, using the local route of administration. The use of LT variants as vaccine adjuvants is not restricted to induction of mucosal respiratory immunity, relevant for pathogens which enter their host via the respiratory tract, such as influenza virus and respiratory syncytial virus. Because of the existence of generalized mucosal immunity after intranasal immunization, LT variants may also be used for induction of immunity against pathogens which enter the host via different mucosal sites. In this respect, the observation that nontoxic LT variants induce brisk vaginal IgA responses, offers attractive perspectives for intranasal vaccination against sexually-transmitted pathogens, such as hepatitis, herpes and human papilloma virus.