The LTR72 Mutant of Heat-Labile Enterotoxin of Escherichia coli Enhances the Ability of Peptide Antigens To Elicit CD4⁺ T Cells and Secrete Gamma Interferon after Coapplication onto Bare Skin

A.-S. Beignon, J.-P. Briand, R. Rappuoli, S. Muller, and C. D. Partidos

UPR 9021, Institut de Biologie Moléculaire et Cellulaire, CNRS, F-67084 Strasbourg, France, and IRIS, Chiron, SpA, 53100 Siena, Italy

Received 9 November 2001/Returned for modification 23 January 2002/Accepted 11 March 2002

Application of antigens with an adjuvant onto bare skin is a needle-free and pain-free immunization procedure that delivers antigens to the immunocompetent cells of the epidermis. We tested here the immunogenicity and adjuvanticity of two mutants of heat-labile enterotoxin (LT) of Escherichia coli, LTK63 and LTR72. Both mutants were shown to be immunogenic, inducing serum and mucosal antibody responses. The application of LTK63 and LTR72 to bare skin induced significant protection against intraperitoneal challenge with a lethal dose of LT. In addition, both LT mutants enhanced the capacity of peptides TT:830-843 and HA:307-319 (representing T-helper epitopes from tetanus toxin and influenza virus hemagglutinin, respectively) to elicit antigen-specific CD4⁺ T cells after coapplication onto bare skin. However, only mutant LTR72 was capable of stimulating the secretion of high levels of gamma interferon. These findings demonstrate that successful skin immunization protocols require the selection of the right adjuvant in order to induce the appropriate type of antigen-specific immune responses in a selective and reliable way. Moreover, the use of adjuvants such the LTK63 and LTR72 mutants, with no or low residual toxicity, holds a lot of promise for the future application of vaccines to the bare skin of humans.

Recently, bare skin has emerged as a potential alternative route for vaccine delivery (11, 27). This is because the skin is rich in immunocompetent cells (4, 36), and when antigens are applied with a suitable adjuvant either in solution (1, 11, 13, 34) or with a patch (14), they induce potent immune responses. The development of noninvasive immunization procedures, which can be needle free and pain free, is a top priority for public health agencies. This is because many current immunization practices are unsafe, particularly in developing countries due to the widespread reuse of nonsterile syringes (27). Therefore, the topical application of vaccines is attractive since it has the potential to make vaccine delivery more equitable, safer, and efficient. Furthermore, it would greatly facilitate the successful implementation of worldwide mass vaccination campaigns against infectious diseases.

For the induction of an effective immune response, the antigen is normally coapplied onto hydrated bare skin with an ADP-ribosylating exotoxin as an adjuvant (i.e., Vibrio cholerae cholera toxin [CT] or Escherichia coli heat-labile enterotoxin [LT]) (1, 11, 13, 34). Both CT and LT are composed of five nontoxic B subunits held together in a pentamer (responsible for binding to the cell membrane), surrounding a single A subunit, which is responsible for toxicity. The A subunit consists of two distinct structural domains: the A1 domain, which displays the ADP-ribosyltransferase activity in the cytosol of the target cells and the A2 domain that interacts with the B-subunit (35). These toxins are responsible for the cause of a debilitating watery diarrhea (35). Moreover, they are potent immunogens and exert an adjuvant effect on antigens presented simultaneously at the mucosal surfaces (26). When CT and LT are applied to bare skin it appears that they are well tolerated even at a high dose without any apparent sign of local or systemic toxicity (14). However, it is obvious that there would be some serious concerns for their use in humans. This has prompted researchers to genetically detoxify these toxins while retaining their adjuvanticity. By site-directed mutagenesis, several mutants have been generated with significantly reduced ADP-ribosylating activity and toxicity compared to the holotoxin (5, 6, 20, 30, 37). Two of these mutants, LTK63, which is devoid of enzymatic activity and toxicity (containing a serine-to-lysine substitution in position 63 of the A subunit), and LTR72, which retains ca. 1% of the wild-type ADP-ribosylating activity and reduced toxicity (containing an alanine-to-arginine substitution in position 72 of the A subunit), have been extensively tested and shown to be good adjuvants after mucosal coadministration with protein and peptide antigens (24, 29). Furthermore, these mutants have been shown to be very useful tools for examining the role of ADP-ribosylation in immunomodulation (32).

Since the LTK63 and LTR72 mutants are promising candidates for human use (28), we hypothesized that their use might allow us to circumvent the potential hazards of LT and CT after topical application. Therefore, we sought to investigate their immunogenicity and to test their adjuvanticity to peptide antigens after their coapplication to bare skin. Both mutants were shown to be effective immunogens, conferring protection against challenge with LT and enhancing the capacity of co-administered peptides to induce antigen-specific CD4⁺ T cells. In addition, the LTR72 mutant was shown to stimulate the secretion of high levels of gamma interferon (IFN-γ).
MATERIALS AND METHODS

Synthetic peptides. The synthetic peptides TT:380-843 [OYIKANSKIGITE (C)] and HA:307-319 [PYKVYKNTLKLAT (C)] representing promiscuous (non-major histocompatibility complex-restricted) T-helper epitopes from tetanus toxin (T) and influenza virus hemagglutinin (25), respectively, were synthesized by using Fmoc (9-fluorenylmethoxy carbonyl) chemistry. The influenza virus NSP:55-69 (RLIQNSLTERMLVS) peptide representing a T-helper epitope from nucleoprotein was synthesized by using the same chemistry and was tested as a control peptide. After cleavage, the peptides were purified by preparative high-performance liquid chromatography and then characterized by analytical high-performance liquid chromatography and mass spectrometry.

Mice. Female BALB/c mice, 6 to 8 weeks old at the start of the experiments, were purchased from Harlan (Gannat, France) and were maintained in the animal facility of the Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France.

Immunizations. Prior to immunization, mice were shaved on a restricted area of the abdomen (over an ca. 1- to 2-cm² surface area). During the immunization procedure the mice were under deep anesthesia after subcutaneous injection of 100 μl of solution of ketamine (Imalgene 1000 [15%]; Merial, Lyon, France) with xylazine (2% Rompun [9%]; Bayer AG, Leverkusen, Germany) for ca. 1 h to prevent grooming. Groups of BALB/c mice were immunized onto bare skin with a 30-μl volume of antigen solution (i) as a solution of 100 μg of TT:380-843 peptide with 50 μg of LT (Sigma) (eight mice), (ii) as a solution of 100 μg of TT:380-843 peptide with 30 μg of LTK63 (six mice), (iii) as a solution of 100 μg of TT:380-843 peptide with 50 μg of LTR72 (six mice), or (iv) as a solution of 100 μg of TT:380-843 peptide in saline (two mice). At 2 weeks after priming, the mice were boosted by the same route with the same dose and formulation of antigen. In a separate experiment, the avidity of 50 μg of each of LT mutants was tested after coapplication with 100 μg of HA:307-319 peptide onto bare skin of BALB/c mice (two mice/group). Control mice were immunized with a mixture of 50 μg of CT and 100 μg of synthetic oligodeoxynucleotide (ODN) containing the CpG motif 1668 (5'-TCC ATG ACG TTC CTG ATG CT-3') (18), purchased from MWG Biotech, Ebersberg, Germany. A booster application was given 14 days postpriming. No erythema was observed after the shaving procedure the mice were under deep anesthesia after subcutaneous injection of 100 μl of antigen solution (i) as a solution of 100 μg of TT:380-843 peptide with 50 μg of LT (Sigma) and then characterized by comparative high-performance liquid chromatography and then characterized by analytical high-performance liquid chromatography and mass spectrometry.

Vagina washes were collected by gentle pipetting on October 14, 2017 by guest http://iai.asm.org/ Downloaded from

RESULTS

Immunogenicity of LT mutants after application onto bare skin. Figure 1a shows that both LT mutants elicited primary and secondary serum antibody responses. However, the mutant LTR72 was significantly more immunogenic than LTK63 after the boost (P = 0.0002). When antibody responses were compared to those induced by LT, the LTR72 mutant elicited significantly higher antibody titers (P = 0.024), whereas the LTK63 elicited significantly lower titers (P = 0.005) (Fig. 1a). The predominant IgG subclass after the boost was IgG1, with the ratios of IgG1 to IgG2a ranging from 2.02 for LT (IgG1 titer, 4.92 ± 0.12; IgG2a titer, 2.43 ± 0.46), 1.89 for LTK63 (IgG1 titer, 4.44 ± 0.07; IgG2a titer, 2.35 ± 0.61), and 2.5 for LTR72 (IgG1 titer, 4.54 ± 0.15; IgG2a titer, 1.81 ± 0.36). Both mutants induced detectable levels of IgG (average titers of 4.21 ± 0.17 and 2.61 ± 0.17 for LTR72 and LTK63, respectively) and IgA (average titers of 3.6 ± 0.16 and 2.4 ± 0.18 for LTR72 and LTK63, respectively) antibodies in vaginal washes, with
Antibody titers and cytokine responses were measured upon in vitro restimulation with control peptide and 0.005 μg of LT. Responses were dose dependent and significantly higher in mice immunized with the homologous peptide compared to those of mice immunized with peptide alone (P < 0.0001). The levels of IL-2 secretion in culture supernatants of mice immunized with peptide TT:830-843 and LT were 22.5 ± 1.5, 17.85 ± 1.6, and 2.9 ± 1.75 U/ml in the presence of 5, 0.5, and 0.005 μg of homologous peptide/culture, respectively. Recall responses were also measured upon in vitro restimulation with various concentrations of TTx (Fig. 3d). After skin immunization, low levels of IL-2 secretion were also detected in the culture supernatants of inguinal lymph nodes restimulated with peptide but not with TTx (data not shown). Although peptide TT:830-843 induced proliferative T-cell responses, it did not elicit any detectable antibody responses (data not shown). This suggests that the peptide does not contain any B-cell epitope(s).

The secretion of IFN-γ is the hallmark feature of the Th1-type response that contributes to the clearance of viral infections and of other intracellular pathogens. Therefore, the IFN-γ response of immunized mice was measured in the supernatants of splenocyte cultures by ELISA. Mouse immunized with TT:830-843 peptide plus LTR72 gave a strong IFN-γ response of immunized mice compared to those of mice immunized with peptide alone (P < 0.0001). The levels of IL-2 secretion in culture supernatants of mice immunized with peptide TT:830-843 and LTK63 or LTR72 onto bare skin. Control mice were immunized by topically applying peptide TT:830-843 in saline or peptide TT:830-843 on the LT mutants.

The total IgE levels were measured in serum from groups of mice bled on days 14, 28, and 42 after priming. Panel b presents the average concentrations of total IgE levels ± the SD of groups of mice bled on day 28 after priming.

**FIG. 2.** Percent survival after intraperitoneal challenge with a lethal dose of recombinant LT (50 μg) of groups of BALB/c mice bare-skin immunized with LTR72 (n = 10, □) or LTK63 (n = 5, ▼). Also shown are data for control nonimmune mice (n = 11, ○).
response in the presence of homologous peptide or TTx (Fig. 4). In contrast, IFN-γ was not detectable in the supernatants of splenocyte cultures of mice immunized with peptide in saline or with LTK63 mutant (Fig. 4). Splenocyte cultures of mice immunized with peptide TT:830-841 plus LT secreted IFN-γ in the presence of homologous peptide but not with TTx (Fig. 4). Restimulation of splenocyte cultures with control peptide NP:55-69 did not produce any IFN-γ (data not shown).

**LTK63 and LTR72 mutants enhance the capacity of HA:307-319 peptide to induce proliferative T-cell responses.** Since both LT mutants were shown to exert an adjuvant effect to the coadministered TT:830-843 peptide and mutant LTR72 in particular stimulated the secretion of high levels of IFN-γ, their adjuvanticity was retested with peptide HA:307-319 as an antigen. In addition, a group of mice were bare-skin immunized with peptide HA:307-319 plus a mixture of CT and an ODN containing the CpG motif 1668 as a positive control. This was based on the observation that the CT-ODN CpG mixture stimulates the production of high levels of IFN-γ (2). As shown in Fig. 5a, splenocyte cultures of mice immunized with peptide and mutant LTR72 secreted high levels of IL-2 upon in vitro restimulation with the homologous peptide. This response was significantly higher than that measured in the splenocyte cultures of mice immunized with peptide and LTK63 mutant ($P = 0.0001$ for all peptide concentrations tested). Splenocyte cultures of mice immunized with peptide plus LTR72 secreted IL-2 after restimulation with heat-inactivated influenza virus but not splenocyte cultures of mice immunized with peptide and LTK63 (Fig. 5b).

Peptide HA:307-319, like peptide TT:830-843, did not in-
duce any detectable levels of serum anti-peptide antibodies (data not shown), suggesting that it does not contain any B-cell epitope(s).

When the production of IFN-γ was measured in the supernatants of splenocyte cultures, only groups of mice that were coimmunized with peptide and LTR72 mutant or with the CT-CpG ODN 1668 mixture gave an IFN-γ response (Fig. 6).

**DISCUSSION**

In this study the immunogenicity and adjuvanticity of mutants of *E. coli* was tested after application onto bare skin. Both LTK63 and LTR72 mutants were shown to be immunogenic, inducing serum and secretory antibody responses. However, mutant LTR72 was the more potent immunogen. This finding extends previous observations on the immunogenicity of LT after skin application (1, 34), demonstrating that two of its mutants—LTK63, which is devoid of ADP-ribosylating activity, and LTR72, which is partially active—can be immunogenic. Furthermore, their ability to generate secretory antibody responses suggests that after application onto bare skin and after their diffusion through the hydrated stratum corneum (that disrupts its barrier function) they might influence the microenvironment of the epidermis. This in turn could favor the migration of antigen-pulsed Langerhans cells to lymphoid organs committed to initiating mucosal immune responses (8).

Adverse reactions to immunization are common and are normally tolerated for the benefit of immunity. In several instances high levels of IgE responses have been noticed with diphtheria toxoid vaccines (19). In the present study, total IgE levels were elevated in the sera of mice receiving LT, LTK63, or LTR72 as adjuvants. Although the exact role of the IgE responses remains to be elucidated, there is always the possibility that the presence of high levels of IgE might be associated with high risk of anaphylaxis, particularly in individuals with atopic predisposition (33). On the other hand, antigen-specific IgE appears to correlate with protection in diseases such as schistosomiasis (17).

Since mutants LTR72 and LTK63 were found to be highly immunogenic, the LT challenge mouse model was selected to evaluate whether immunization onto bare skin can induce protective immune responses against lethal systemic challenge with LT. Despite the vigorous dose of LT, immune mice were significantly protected. This finding is in agreement with observations demonstrating that skin immunization can generate protective immune responses against challenge with a lethal dose of toxins such as CT (12), LT (1), or tetanus (13).

The LTK63 and LTR72 mutants were also effective adjuvants since they enhanced the capacity of topically coapplied peptide antigens to induce antigen-specific CD4+ T cells. Thus, LT mutants with low propensity for adverse side effects have adjuvant activity when they are topically applied, as has been demonstrated after their mucosal delivery (24, 29). Fur-

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FIG. 4. Secretion of IFN-γ by TT:830-843 peptide-specific immune splenocytes after in vitro restimulation with various concentrations of the homologous peptide (a) or TTx (b) (I, 0.5 μg/culture; □, 0.05 μg/culture; and ◯, 0.005 μg/culture). Mice were coimmunized onto bare skin with 100 μg of TT:830-843 peptide and 50 μg of LTK63 or with 50 μg of LTR72 or 50 μg of LT as adjuvants. Control mice were immunized with 100 μg of TT:830-843 peptide given alone. After 72 h of culture, supernatants were collected and assayed for IFN-γ by ELISA. The findings with medium alone are also indicated (□).
bation of CT with GM1 gangliosides prior to its application. This is also supported by demonstrating that binding was necessary for mucosal immu-
nogenicity and adjuvanticity (15, 23). This is also supported by the recent findings of Beignon et al. (1) showing that preincuba-
tion of CT with G₃₂₆ gangliosides prior to its application onto bare skin results in a significant reduction of systemic and mucosal anti-CT antibody responses. In general, these mutants exert their adjuvant activity mainly on APCs to upregulate major histocompatibility complex and costimulatory molecules and to secrete cytokines (16, 38). This enables Langerhans cells to take up antigens, mature, and migrate to regional lymph nodes, where they present the antigen for the initiation of an adaptive immune response (16).

The ability of small molecules such as synthetic peptides to induce antigen-specific CD4⁺ T cells after application onto bare skin is particularly important in the context of vaccine design and delivery, since CD4⁺ T cells help B cells to produce antibodies that neutralize viruses and bacterial toxins, enhance the magnitude of cytotoxic T-cell responses to clear virus-infected cells, and regulate the immune responses to foreign antigens on the basis of cytokine profile they secrete (22). Moreover, the finding that mutant LTR72 preferentially stimulates an IFN-γ response could be advantageous, particularly for the clearance of intracellular pathogens (3). Recent reports have indicated that mutants LTK63 and LTR72 preferentially stimulate Th1- and Th2-type immune responses, respectively, when they are administered in small quantities via the intranasal route (31, 32). However, the type of antigen and the mode of intranasal delivery might influence the induction of a particular Th phenotype, since it has been shown in other systems with peptides (24) or parasite protein antigens (3) that only the LTR72 mutant induces an IFN-γ response. This is also consistent with the results obtained in this study. The exact mechanism(s) of preferential stimulation of IFN-γ secretion by the LTR72 mutant is not clear. If we take into account that ADP-ribosylating exotoxins and their mutants bind to immunocompetent cells (i.e., T cells, B cells, and APCs), it is very difficult to speculate the exact series of in vivo events that favor the secretion of IFN-γ by the LTR72 mutant. However, it could be argued that certain levels of cyclic AMP are critical for the signaling events that will eventually lead to the secretion of IFN-γ (5). This is supported by the findings presented...
here demonstrating different IFN-γ secretion profiles elicited by LT and its mutants LTR72 and LTK63 (Fig. 4).

Taken together, these findings lead us to conclude that adjuvants such as the LTK63 and LTR72 mutants, which have no or low residual toxicity, hold much promise for the future application of vaccines onto bare skin of humans.

ACKNOWLEDGMENTS

This work was in part financed by the Centre National de la Recherche Scientifique, Virsol (Paris, France), and Biovector Therapeutics (Toulouse, France). One of the coauthors has competing financial interests.

We thank F. Mawas (National Institute for Biological Standards and Control, London, United Kingdom) for providing the TTx, G. Del Giudice (IRIS Research Center, Chiron, SpA, Siena, Italy) for reviewing the manuscript, and B. Jessel for animal husbandry.

REFERENCES


part of the document text is missing, but it seems to be discussing various aspects of immunology and adjuvant strategies in skin immunization.


