**Original Article**

Mucosal Vaccination Approach against Mosquito-Borne Japanese Encephalitis Virus

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**SUMMARY:** To investigate the potential applicability of mucosal vaccines against mucosa-unrelated pathogens, a non-parenteral vaccination approach was taken as a prophylactic strategy against mosquito-borne Japanese encephalitis virus (JEV). Intranasal (i.n.) immunization with a mouse brain-derived formalin-inactivated JE vaccine induced a robust virus-neutralizing antibody in mice, and this induction was augmented by co-administration with cholera toxin (CT) and pertussis toxin, but not with killed *Bordetella pertussis*. The antibody response induced by the i.n. administration of the JE vaccine with bacterial toxins was comparable in intensity to that induced by a parenteral immunization regime, and the former was considerably more effective in terms of delayed-type hypersensitivity and local antibody response. In addition, the adjuvant effects of bacterial toxins were much more prominent for the mucosal than the parenteral route. Two other non-invasive routes, oral and transcutaneous administration, were examined, but the i.n. route was by far the most effective. Finally, the vaccine efficacy of a chimeric fusion protein between the B subunit of CT and the JEV envelope protein showed some promise for the development of non-invasive JE vaccine. Our results suggest that the mucosal vaccination approach is feasible for a non-mucosal pathogen such as JEV, but that the adjuvant, carrier molecule, and administration route must be optimized for construction of an effective vaccine platform.

**INTRODUCTION**

Japanese encephalitis virus (JEV) is a mosquito-borne neurotropic virus and is the leading cause of viral encephalitis in Southeast Asia. In other parts of Asia, including Japan, the disease is no longer endemic, but still actively circulates between the vector mosquitoes and domestic animals such as pigs (1). JEV endemicity can be reduced by mosquito vector control and the use of prophylactic vaccines (2,3). Japan, South Korea, Taiwan, and some other Asian countries have licensed the use of a mouse brain-derived inactivated JE vaccine. New vaccines such as (i) a chimeric live attenuated vaccine (ChimeriVax-JE) that uses yellow fever 17D as a vector for expression of the JEV envelope (E) protein (4,5), (ii) a formalin-inactivated attenuated strain of JEV propagated in and purified from Vero cells (6), and (iii) peptide vaccines or DNA vaccines that are based on the E protein, are currently being investigated (7).

Unlike mosquito-borne JEV, the vast majority of pathogens initiate infection through mucosal portals of entry in the gastrointestinal, respiratory, and urogenital tracts. Accordingly, mucosal vaccinations with non-replicating particles or recombinant proteins in combination with an effective mucosal adjuvant have been proven effective at inducing local protective immunity against mucosal pathogens (8-10). In addition, due to their needle-free, non-invasive nature, topically administered vaccines may reduce the risk of infection from blood-borne pathogens, and may also be more cost-effective because their administration does not require trained medical or veterinary personnel. Despite these attractive features, their targets have been almost exclusively limited to mucosal infections, and their potential application to non-mucosal pathogens such as arthropod vector-borne viruses appears to be under-appreciated. In this study, we explored the use of mucosal vaccines against mosquito-borne JEV under the premise that a virus-neutralizing serum antibody would be elicited. Although the immunogenicity of soluble vaccines administered mucosally in the absence of adjuvants was considerably lower than that of parenteral immunization regimes, our results suggest the feasibility of developing non-invasive mucosal vaccines against mosquito-borne JEV, and possibly against other mucosa-unrelated pathogens.

**MATERIALS AND METHODS**

**Mouse brain-derived formalin-inactivated JE vaccines:** A JE vaccine (Beijing-1 strain) was purchased from the Chemo-Sero-Therapeutic Research Institute (Kaketsuken, Kumamoto, Japan) and was used for the preliminary studies.
shown in Fig. 1 and Table 2. The total protein concentration of this vaccine preparation was determined by BCA assay and was found to be approximately 300 ng/µl. The concentrated JE vaccine (Beijing-1 strain) used for the studies shown in Fig. 2 and Table 3 was a kind gift of the Research Foundation for Microbial Diseases of Osaka University (Biken, Osaka, Japan). A JE vaccine (Nakayama strain) used as a positive control to comparatively evaluate the efficacy of the recombinant subunit fusion proteins shown in Fig. 4 was also a gift of Osaka University (Biken).

Adjuvants: Cholera toxin (CT) was purchased from Sigma-Aldrich (C8052; St. Louis, Mo., USA). Pertussis toxin (PT) was purchased from List Biological Laboratories (Campbell, Canada). A killed Bordetella pertussis was purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Mice, immunization and sample collection: Five to six 7-week-old BALB/c or ddY mice (Japan SLC, Shizuoka, Japan) were immunized with the indicated amounts of the mouse brain-derived inactivated JE vaccine or cholera toxin B subunit (CTB)-JEV E protein fusion proteins (25 µg) through various routes for the indicated times without or with bacterial adjuvants. For intranasal (i.n.) immunization, 15 µl of vaccine materials was applied to the external nares by micropipette. For intragastric (i.g.) immunization, mice were starved overnight, and 100 µl of 3% sodium bicarbonate buffer was given by a ball-point needle prior to the vaccine administration (300 µl/dose of the Kaketsuken Beijing-1 vaccine). For intratail (i.t.) administration, the same vaccine (300 µl/dose of the Kaketsuken Beijing-1 vaccine) was directly injected into the intestinal ileal loop, after which the abdominal incision was sewn up and the animals were returned to the cage for recovery. For transcutaneous (t.c.) immunization the animals were returned to the cage for recovery. For transcutaneous (t.c.) immunization the animals were returned to the cage for recovery.

Expression of CTB-E protein chimeric fusion proteins in Escherichia coli: The CTB gene (GenBank accession no. U25679) was PCR-amplified from plasmid pM4 (a kind gift from Dr. Hiroshi Kyono at the University of Tokyo) with primers 5' -CGCATGTTAAATTTTGGTGTTTTGGTGGTGGTGGATAGGTTGTGCCTTCAGAGCCAG-3' (the hinge region is underlined) and 5' -CGGAATTCTCAAGATTTGCTGGGAATG-3' and 5' -CGGAATTCTTTGTGGATCCAAGACATTC-3' (the hexahistidine-tag is underlined) for domain I-II, digested with NcoI and EcoRI, and cloned at the unique EcoRI site of pETDuet-1 (Novagen, Madison, Wis., USA) to construct pBh. To construct plasmid pBΔLh containing the CTB gene devoid of the leader, another sense primer, 5' -CGCCATGTCACAAGAATTAGATTGAATG-3' was used in place of the sense primer above.

To construct the CTB-E protein domain III (163 aa: TYGMCTE...MSWITQG) or domain I-II (301 aa: FNCGLMG...ALKGTYY) chimeric gene, plasmid pJEVprM/E containing the entire prM/E region was used for PCR with the primers 5' -CGCAATTCACCTATGCGATCGACAG-3' and 5' -CGGAATTCTTCTAGGTGTTGTTGTTGTTGTCCTTTTGTTGATCACAAGACATC-3' (the hexahistidine-tag is underlined) for domain III or for the primers 5' -CGGAATTCCTTTTATTGTCTGGGAAATG-3' and 5' -CGGAATTCTCTAGTGTTGGTTGTTGTTGTCCTTTTGTTGATCACAAGACATC-3' (the hexahistidine-tag is underlined) for domain I-II, digested with EcoRI, and cloned at the unique EcoRI site of pBlunt at the cohesive end of pBl and pBl/III, respectively, or to construct pBl/I-II and pBl/III-I-I, respectively.

Transformed E. coli BL21(DE3) (Novagen) with the engineered plasmids were cultured in LB ampicillin, and expression was induced by 1 mM IPTG. Cells were lysed with Bugbuster reagent (Novagen) with lysozyme (Sigma-Aldrich) and benzamidine (Novagen) at 37°C for 1 h, and centrifuged (10,000 x g, 30 min) to collect the supernatant, which was then subjected to Ni²⁺-affinity chromatography (Amersham Biosciences, Piscataway, N.J., USA) for protein purification. Alternatively, for in vitro refolding of the chimeric protein (14), inclusion bodies isolated from cells transformed with the plasmid pBl/I-II or pBl/III were washed twice with 1 M urea in PBS, pH 7.2, and dissolved in 10 ml of 6.5 M urea, pH 2.0. A refolding buffer (20 ml, 2 M arginine, 50 mM Tris-HCl, pH 8.0) was added to the solubilized inclusion bodies,
and dialyzed against 500 ml of a refolding buffer (1 M arginine, 50 mM Tris-HCl, pH 8.0) at 4°C overnight; the buffer was exchanged every 4 h for a refolding buffer containing a twofold dilution of arginine. Finally, the samples were dialyzed against PBS.

GM1-ELISA and Western blot analysis: Microtiter plates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) were coated with 5 μg/ml of monosialoganglioside GM1 (Sigma-Aldrich) in bicarbonate buffer (15 mM Na2CO3, 35 mM NaHCO3, pH 9.6), blocked with 1% BSA in PBS, and incubated with samples. This was followed by incubation with rabbit anti-CT (Sigma-Aldrich) or anti-JEV antiserum, and AP-conjugated secondary antibodies and AP substrate. Absorbance was measured at 415 nm on a microplate reader (Bio-Rad Laboratories). For the Western blots, purified recombinant proteins were separated by a 10% SDS-PAGE and transferred to a PVDF membrane, and blots were probed with the same antibodies as used for the GM1-ELISA.

Statistics: Statistical significance of differences was determined by the Student’s t test. Differences were considered significant when P < 0.05.

RESULTS

Immunogenicity of bacterial compounds: It is often important to evaluate the immunogenicity of adjuvants used in vaccine formulations in addition to their immune-enhancing capacity. For this reason, we evaluated the immunogenicity of adjuvants used in this study. BALB/c mice were immunized with a i.n. or intraperitoneal (i.p.) route with CT (1 μg), PT (1 μg), or killed B. pertussis (Bp1, 2 × 108 CFU or Bp5, 1 × 108 CFU). Mice were immunized at weeks 0, 3, and 5, and were sacrificed a week after the last immunization for the measurement of specific antibodies in the serum. The adjuvants induced a serum antibody response, but the intensity and type of response was strongly influenced by the route of administration (Table 1). Bp given by the i.p. route induced the highest serum response, followed by PT and CT, while it was least immunogenic when given by the i.n. route. A mixture of CT/PT did not enhance, but rather, in many cases, suppressed the response against the other toxin.

Adjuvanticity of the bacterial compounds for the JE vaccine: The adjuvanticity of three bacterial compounds was examined (Table 2). BALB/c mice were immunized by the i.n. or i.p. route with 15 μl of the JE vaccine (4,500 ng of the total protein, Kaketsuken Beijing-1 strain) without or with the adjuvant (CT 1 μg, PT 1 μg, Bp1 or Bp5), at weeks 0, 3, and 5, and were either sacrificed a week after the last immunization for serum and mucosal sample collection or used for the delayed-type hypersensitivity (DTH) assay. CT and PT strongly enhanced serum IgG when administered by the i.n. route, but Bp had almost no effect. In contrast, all adjuvants were almost completely ineffective for serum IgG enhancement when given by the i.p. route. Although i.n. co-administration of individual bacterial toxins augmented the serum IgG response, mixtures of two adjuvants, i.e., CT/PT, did not further enhance the response, and in many cases it slightly suppressed the response as compared with that induced by the individual adjuvant. Specific S-IgA in nasal secretion was induced only by the i.n., and not by the i.p. route, while an adjuvant effect was more readily seen for nasal IgG by the i.p. than the i.n. route. In general, the enhancement of nasal IgG induced by the i.p. route was less pronounced than the enhancement of nasal S-IgA induced by the i.n. route. Administering the JE vaccine alone failed to induce any DTH response by either the i.n. or the i.p. route, but the response was markedly augmented when the vaccine was co-administered i.n. with CT or PT (but not with Bp). Interestingly, the DTH-enhancing effect was generally much more prominent by i.n. than by i.p. administration, and unlike in i.n. administration, no obvious difference in adjuvanticity was observed among the three bacterial compounds when the i.p. route was used. We concluded from these data that either CT or PT is significantly more effective as a mucosal than as a parenteral adjuvant. In addition, mucosal immunogenicity and adjuvanticity for antibody response were generally correlated, in that higher mucosal immunogenicity of a particular adjuvant tended to reflect higher mucosal adjuvanticity for heterologous antigens. We found that CT and PT were almost equally effective as mucosal adjuvants, but the use of CT seemed to be slightly more advantageous for induction of serum IgG against the JE vaccine.

Next, we investigated several non-parenteral immunization regimes for the JE vaccine (Fig. 1). To this end, BALB/c mice were i.g. immunized with 300 μl of the JE vaccine (Kaketsuken) without or with 5 μg of CT at weeks 0, 3, and 5. In contrast to the results by i.n. immunization (Table 2), we did not detect any specific serum IgG even in the presence of CT (Fig. 1A, upper panel). We speculated that the low oral immunogenicity was due to passage through a proteolytic environment in the stomach, and hence we administered the vaccine directly into the intestinal ileum to circumvent the stomach route. We observed improved immunogenicity with this method, and the adjuvant effect of CT (1 μg) appeared for both serum IgG and intestinal S-IgA responses.

Finally, we investigated a t.c. immunization regime (Fig. 1B). The JE vaccine alone (150 μl of 10 × concentrated Kaketsuken Beijing-1 vaccine, equivalent to 1,500 μl) or together with 100 μg of CT was topically applied to shaved skin at weeks 0, 3, and 5. t.c. immunization alone was not sufficient to elicit specific serum IgG or virus-neutralizing antibody even in the presence of CT. However, if one subcutaneous (s.c.) priming (100 μl of Kaketsuken Beijing-1 strain) was given, t.c. immunization became an effective booster.

Neutralizing virus-specific antibodies induced by i.n. JE vaccine: Based on the first set of preliminary experiments described above using the Kaketsuken Beijing-1 vaccine strain
From Fig. 1 and Table 2, we concluded that the i.n. route of immunization in combination with CT is the most effective, we sought to determine the efficacy of this particular vaccination regime in relation to the vaccine dose by using closed colony ddY mice (Fig. 2 and Table 3). Mice were immunized i.n. four times at weekly intervals with various amounts of the JE vaccine (Biken Beijing-1 vaccine strain) as indicated without or with 10μg of CT (Table 3). Approximately 1,200 ng of the vaccine determined as the amount of the total protein in the vaccine preparation was the minimum required for seroconversion and the induction of neutralizing virus-specific antibody in the presence of CT; however, at least 2,400 ng of the vaccine was required when CT was omitted.

The kinetics of virus-specific serum IgG response indicated that at least two doses were required, but an additional one or two boosters optimized the response (Fig. 2A). i.n. immunization with 2,400 ng of the vaccine induced the virus-neutralization titers above 600, and CT supplementation significantly augmented the response (>2,000) (Fig. 2B).

The mucosal adjuvant effect of CT is critically dependent on the GM1-ganglioside affinity of CT, and hence competitive binding with the GM1 completely abrogated the toxin’s mucosal immunogenicity and adjuvanticity, while the blocking effect was much less prominent when CT was administered by parenteral routes (data not shown). This observation is the...
rationale for the design of subunit vaccines in which vaccine antigens are linked to the GM1-binding B subunit of CT for efficient vaccine delivery. To this end, we designed CTB-JEV E protein chimeric fusion genes (Fig. 3A), and expressed them in *E. coli*. The full-length E protein was divided into two regions based on the three-dimensional structure of the E protein of the JEV and its related West Nile virus to construct fusion proteins with the CTB subunit. The C-terminal domain III, which comprises an independent physical entity separated from the other two domains, i.e., domains I and II, was fused with the CTB as an independent domain, while domains I and II, which cannot be detached from one another by genetic manipulation, were fused with the CTB as one connected entity, i.e., domain I-II (15-17). The chimeric rB:III protein was predominantly found in cell lysate as a soluble protein, retaining its specific binding affinity for GM1 (Fig. 3B). The presence of multimeric rB:III was confirmed by immunoblot detection of several high molecular mass protein bands, which upon heat treatment, integrated into a single band with an apparent molecular mass of approximately 27.5 kDa, a value close to the one expected for the fusion monomer (30.5 kDa) (Fig. 3C). The chimeric rB:I-II protein having a larger partner antigen (163 versus 301 aa for domains III and I-II, respectively) exhibited a marked reduction in pentamer level as compared with rB:III (Fig. 3B). In addition, the domain I-II was completely unreactive with JEV antiserum, probably due to the low expression level and/or improper folding. Thus, we attempted in vitro refolding by engineering plasmids pBΔL:III and pBΔL:I-II to express the CTB fusion protein devoid of its leader peptide (Fig. 3A). Unlike rB:III and rB:I-II, rBΔL:III and rBΔL:I-II were mainly found in inclusion bodies. Upon solubilization with urea and subsequent in vitro refolding, rBΔL:I-II became reactive with JEV antiserum (Fig. 3D). This acquisition of serum reactivity was most likely due to partial refolding of the domain I-II, because a control fusion protein between CTB and the in vitro-refolding incompetent malaria antigen, Pfs25H (18,19), failed to regain serum reactivity even after the same refolding process. A summary of the expression patterns for the chimeric proteins is provided in Table 4.

**Immunogenicity of the chimeric protein**: To test the mucosal immunogenicity of the chimeric proteins (rB:III or rBΔL:III), BALB/c mice were immunized i.n. or i.p. with 25 μg proteins without or with 1 μg of CT at weeks 0, 3, and 5, and immune sera collected a week after the last immunization were analyzed. Unexpectedly, unlike rB:I-II, rBΔL:II that acquired strong reactivity with JEV antiserum (Fig. 3D) failed to induce a JEV-specific IgG response (data not shown),

![Image](A) ![Image](B)

**Table 3. Dose-response and adjuvant effect of CT on JEV-specific immunity**

<table>
<thead>
<tr>
<th>JE vaccine dose (ng)</th>
<th>2,400</th>
<th>1,200</th>
<th>400</th>
<th>134</th>
<th>45</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Seroconversion?</td>
<td>CT (−) 60 (3/5) 0 (0/4) 0 (0/4) 0 (0/4) 0 (0/4) 0 (0/4)</td>
<td>(+) 100 (5/5) 100 (4/4) 75 (3/4) 25 (1/4) 25 (1/4) 0 (0/4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Neutralization?</td>
<td>CT (−) 100 (5/5) 0 (0/4) 0 (0/4) 0 (0/4) 0 (0/4) 0 (0/4)</td>
<td>(+) 100 (5/5) 100 (4/4) 100 (4/4) 0 (0/4) 0 (0/4) 0 (0/4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

?1: Determined based on JEV-specific serum IgG levels of mice i.n. immunized with the inactivated JE vaccine (Biken Beijing-1 strain) in the presence or absence of CT. Numbers within parenthesis indicate numbers of sero-positive mice out of the total numbers examined. Mice were considered seroconverted if the OD<sub>490</sub> for 100-fold diluted serum was at least twofold higher than that of the preimmune serum.

?2: Determined based on the 50% focus reduction neutralization test (FRNT<sub>50</sub>) as previously described (13). Immune sera were considered positive when titers became greater than 10 (49). Numbers within parenthesis indicate numbers of virus-neutralization titer positive mice out of the total numbers examined.

**Table 4. Summary of expression patterns of the recombinant CTB-JEV E protein fusion protein series**

<table>
<thead>
<tr>
<th>Recombinant protein</th>
<th>Presence of the leader peptide</th>
<th>Expressed cellular location</th>
<th>Soluble or inclusion body</th>
<th>Pentamer formation?</th>
<th>Antigenicity (CTB/JEV)</th>
<th>Immunogenicity (CTB/JEV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rB&lt;sup&gt;?&lt;/sup&gt;</td>
<td>Yes</td>
<td>Extracellular (secreted)</td>
<td>Soluble</td>
<td>Yes</td>
<td>Yes/No</td>
<td>Yes/No</td>
</tr>
<tr>
<td>rB:1-II</td>
<td>Yes</td>
<td>Intracellular</td>
<td>Soluble</td>
<td>Yes (but low)</td>
<td>Yes (weak)/No</td>
<td>Yes/No</td>
</tr>
<tr>
<td>rB:III</td>
<td>Yes</td>
<td>Intracellular</td>
<td>Soluble</td>
<td>Yes</td>
<td>Yes/Yes</td>
<td>Yes/Yes</td>
</tr>
<tr>
<td>rBΔL:1-II</td>
<td>No</td>
<td>Intracellular</td>
<td>Inclusion body</td>
<td>No, but yes after refolding</td>
<td>Yes/Yes (after refolding)</td>
<td>Yes/No</td>
</tr>
<tr>
<td>rBΔL:III</td>
<td>No</td>
<td>Intracellular</td>
<td>Inclusion body</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
</tr>
</tbody>
</table>

<sup>?</sup>: rB: Bacterially expressed CTB protein without any fused antigen. (Detailed results are not provided in this study.)

<sup>?</sup>: Determined by GM1-ELISA.

N/D, not determined.
and thus we decided to conduct the rest of the experiments with rB:III only. The rB:III elicited CTB- and JEV (Beijing-1 and Nakayama)-specific serum IgG by both the i.n. and i.p. routes, but the i.p. route was more efficacious than the i.n. route. However, i.n. co-administration with CT augmented the virus-specific response to levels comparable to that attained by the i.p. route (Fig. 4A), particularly for IgG2a and IgG2b (Fig. 4B). Weak IgM, but no IgA, was detected in serum (data not shown). i.n. immunization also induced virus-specific nasal S-IgA, while almost no IgG was detected in nasal secretion by the i.n. route (Fig. 4C). However, relatively high level of secretory IgG was induced by the i.p. route. Finally, the virus-neutralizing antibody-inducing capacity of the chimeric protein was determined (Fig. 4D). We found that chimeric rB:III induced a low level of virus-neutralizing antibody against the Beijing-1 strain of the virus when administered by the i.p., but not by the i.n. route. We concluded from these data that immunization with the chimeric protein could induce patterns of immune responses similar to those observed in the inactivated JE vaccine co-administered with CT.

DISCUSSION

Although protective mucosal immunity is difficult to induce by parenteral immunization, the majority of licensed vaccines are parenteral vaccines regardless of the mode of pathogen invasion (8). This dilemma has motivated many investigators to explore mucosal vaccines against mucosal pathogens, because these vaccines in combination with effective adjuvants could potentially provide a “double-layered” humoral (e.g., local S-IgA and systemic IgG) as well as cell-mediated protective immunity (20,21). However, target infections of mucosal vaccines have almost exclusively been limited to mucosal infections, and their potential application to non-mucosal pathogens has been largely neglected. Possible reasons for this omission are that the benefits of mucosal vaccines have been emphasized solely for the prevention of mucosal pathogens, and also that mucosal vaccines are generally perceived to induce immune responses with a strong bias towards Th2 type, which in some instances leads to undesired immunological consequences such as immune deviation, mucosal tolerance or allergic responses. Many investigators have demonstrated the potential efficacy of mucosal vaccines against mucosal pathogens. However, our interest in identifying the potential of mucosal vaccines has recently focused on their application to non-mucosal pathogens. Along this line, we have recently demonstrated a malaria mucosal transmission-blocking vaccine, by which complete prevention of parasite transmission from infected human blood to a vector mosquito was achieved (22). In this study we evaluated the mucosal immunogenicity of the mouse brain-derived inactivated JE vaccine for induction of antiviral immunity in a murine model. Although generally
less efficacious than a parenteral route. i.n. immunization was clearly shown to induce neutralizing JEV-specific antibody as assessed by a focus-reduction neutralization test, particularly when a mucosal adjuvant and/or carrier molecule was employed. We observed that both CT and PT exhibited more potent adjuvanticity for heterologous antigens when administered mucosally than parenterally. A strong DTH response induced by mucosal CT or PT is indicative of cell-mediated immunity, as it is known that CD4+ Th1 cells are responsible for generating the DTH response (23). Although virus-specific DTH was more potent when administered by the i.n. route than when given by the i.p. route, this was only true when the adjuvant was co-administered. Inactivated \( B. \) pertussis induced a strong humoral response against itself, especially when administered by the i.p. route; however, it was a very ineffective adjuvant, suggesting that bacteria-associated molecular patterns such as LPS and CpG-containing DNAs (CpG ODN), which are known to confer strong innate immunity, are not necessarily superior to enzymatically active bacterial toxins. These data may serve as a partial rationalization for an adjuvant and delivery system design strategy based on bacterial toxin-related molecules.

Rauthan et al. recently reported mucosal immunogenicity of the formalin-inactivated JE vaccine and an \( E. \) coli-expressed recombinant E protein administered via the i.g. route (24). Both vaccine preparations were found to be something effective, particularly when co-administered with CpG ODN, in inducing a virus-specific serum antibody response in mice, but the induced antibody was ineffective at neutralizing the virus in vitro and did not provide protection against the virus challenge (24). The results of this study were not inconsistent with our data, since the oral route is far less effective than the nasal route.

In order for CT to function as an effective mucosal adjuvant, it needs to breach mucosal barriers by binding to the cell surface receptor GM1-ganglioside (25). Thus, GM1-binding capacity may be critically important for mucosal immunogenicity and adjuvanticity, and it may become less important when parenteral administration routes are employed, which in part would explain the CT's less efficacious systemic adjuvant property. However, GM1 is essentially found in all nucleated mammalian cells, including professional antigen presenting cells (APCs) (26-28). This suggests that CT is not necessarily considered exclusively as a mucosal adjuvant (29), and indeed our results support this notion, because CT when co-administered i.p. with antigens significantly augmented humoral as well as DTH responses against heterologous...
antigens (Table 2).

Although bacterial toxins are strong mucosal adjuvants, their clinical use should be precluded due to their enterotoxicity and potential neurological effects on olfactory nerves (30). However, our preliminary mucosal JE vaccine studies, in which crossbred piglets were administered 200 μg of the JE vaccine together with 100 μg of CTB i.n., resulted in no pathologically noticeable adverse side effects, such as inflammation in the lung. In addition, we found that i.n. vaccination induced neutralizing antibody in 57% of the piglets (unpublished results).

Recently, the non-toxic CTB subunit or mutant CT has been shown to enhance immunity (31-33). A biologically active form of pentameric CTB has been successfully synthesized in several gene expression systems (34-36), which may pave the way for genetic conjugation between CTB and vaccine antigens and may further potentiate CTB-based recombinant mucosal vaccine strategies for infectious disease control (28,37). In addition to the use of CTB as a vaccine carrier, the specific targeting of professional APCs such as dendritic cells and B cells to deliver antigens or adjuvants may have a great potential for the application of mucosal vaccines in humans and animals (26,38-45). In addition to an expanding mucosal vaccination technology, the recent innovation of needle-free, non-invasive vaccines such as those using a dry powder or a skin patch is adding another attractive dimension to the concept of vaccine design (46-48). Thus, by rationally designing vaccine and adjuvant molecules in combination with novel targeting or delivery technologies, we may realize safer and easier-to-administer non-parenteral vaccines against infectious diseases.

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