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CTA1-DD-Immune Stimulating Complexes: a Novel, Rationally Designed Combined Mucosal Vaccine Adjuvant Effective with Nanogram Doses of Antigen¹

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Mucosally active vaccine adjuvants that will prime a full range of local and systemic immune responses against defined antigenic epitopes are much needed. Cholera toxin and lipophilic immune stimulating complexes (ISCOMS) containing Quil A can both act as adjuvants for orally administered Ags, possibly by targeting different APCs. Recently, we have been successful in separating the adjuvant and toxic effects of cholera toxin by constructing a gene fusion protein, CTA1-DD, that combines the enzymatically active CTA1-subunit with a B cell-targeting moiety, D, derived from *Staphylococcus aureus* protein A. Here we have extended this work by combining CTA1-DD with ISCOMS, which normally target dendritic cells and/or macrophages. ISCOMS containing a fusion protein comprising the OVA_{323–339} peptide epitope linked to CTA1-DD were highly immunogenic when given in nanogram doses by the s.c., oral, or nasal routes, inducing a wide range of T cell-dependent immune responses. In contrast, ISCOMS containing the enzymatically inactive CTA1-R7K-DD mutant protein were much less effective, indicating that at least part of the activity of the combined vector requires the ADP-ribosylating property of CTA1. No toxicity was observed by any route. To our knowledge, this is the first report on the successful combination of two mechanistically different principles of adjuvant action. We conclude that rationally designed vectors consisting of CTA1-DD and ISCOMS may provide a novel strategy for the generation of potent and safe mucosal vaccines. *The Journal of Immunology*, 2001, 167: 3398–3405.

Mucosally active vaccines containing recombinant protein Ags would have many immunological and economic advantages for inducing protective immunity against mucosal and systemic pathogens. However, achieving this goal has been restricted by the fact that purified proteins usually induce tolerance rather than active immunity by the oral or intranasal routes (1, 2). Of the mucosally active adjuvants that have been investigated, we have concentrated on derivatives of cholera toxin (CT)³ and lipophilic immune stimulating complexes (ISCOMS) containing the adjuvant Quil A. Both of these approaches have their advantages and disadvantages. Although we and others have shown that CT is a potent inducer of most T cell-dependent responses when given orally, including Th1- and Th2-dependent immunity, as well as CD8⁺ cytotoxic T cells (3), other groups have reported that it may be less efficient at stimulating CD4⁺ Th1 cells than Th2 cells (4–6). In addition, the toxicity of CT and the related *E. coli* enterotoxin (lymphotoxin) pre-

cludes their general use in humans. Therefore, several groups have developed nontoxic mutants of CT or lymphotoxin, most of which have mutations in the CTA1-subunit that result in complete or partial disruption of the enzymatic activity. However, all of these mutant holotoxins retain their promiscuous binding to the GM1-ganglioside receptor, leaving a potential ability to bind to all nucleated cells and so gain access to unwanted tissues such as the CNS (7).

Our strategy to overcome the toxicity of CT has been to construct a gene fusion protein between the enzymatically active CTA1 and a dimer of the Ig-binding D moiety derived from *Staphylococcus aureus* protein A. In this way, the CTA1 enzyme is targeted selectively to B cells, and the resulting CTA1-DD fusion protein has no systemic toxicity. Importantly however, it has similar adjuvant properties to CT holotoxin when given by parenteral and intranasal routes (8, 9). However, our preliminary findings indicated that CTA1-DD may have only limited adjuvant effects when given orally (N. Lycke, unpublished observations). Because Ags incorporated into ISCOMS are highly immunogenic by all mucosal routes including orally, we postulated that the incorporation of CTA1-DD into ISCOMS may stabilize the molecule in the gastrointestinal tract and allow it to act as an oral adjuvant, providing a vector with adjuvant activity above that of either vector alone. ISCOMS induce strong T cell-mediated immune responses that include Th1-dependent delayed type hypersensitivity (DTH) and cytokine production, as well as very strong MHC class I-restricted CD8⁺ T cell responses (10), but CT is a stronger promoter of mucosal IgA and serum Ab responses (10). The complimentary effects of CT and ISCOMS on the immune system are consistent with our own and other findings that whereas B lymphocytes are important for the adjuvant effects of CT and CTA1-DD (8, 9, 11), ISCOMS are targeted to and activate macrophages and dendritic cells (DC) (12–15). In parallel, the mucosal adjuvant effects of CT

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³ Abbreviations used in this paper: CT, cholera toxin; ISCOMS, immune stimulating complexes; DC, dendritic cell; DTH, delayed type hypersensitivity.

are dependent on IL-4, but not on IL-12. ISCOMS show the opposite pattern of requirements (11, 15, 16). To examine the possibility that combining the distinctive properties of the two vectors might enhance their adjuvant effects and overcome some of their limitations, we have constructed a vector in which a CTA1-DD fusion protein containing the immunodominant OVA₃₂₃₋₃₃₉ peptide was incorporated into ISCOMS. Our results show that this novel formulation is nontoxic and is highly immunogenic by a variety of mucosal and systemic routes, using exceedingly low doses of Ag. This approach may form the basis of rationally designed vaccines for mucosal immunization with recombinant or peptide epitopes.

Materials and Methods

Animals

BALB/c mice (H-2^d) were purchased from Harlan Olac (Bicester, U.K.) and maintained under specific pathogen-free conditions in the Central Research Facility, University of Glasgow, or were obtained from B & K Universal (Sollentuna, Sweden) and bred in the Department of Microbiology and Immunology, University of Göteborg. All animals were first used at 6–8 wk of age.

Ags and adjuvants

OVA (fraction V) was obtained from Sigma (Poole, U.K.), whereas OVA₃₂₃₋₃₃₉ peptide was obtained from Sigma Genosys. Vectors encoding CTA1-DD and CTA1-R7K-DD fusion proteins were prepared as described previously (8, 9). For the generation of the fusion proteins CTA1-OVAp-DD and CTA1-R7K-OVAp-DD, harboring one copy of OVA₃₂₃₋₃₃₉ between the DD and the CTA1 moieties, a synthetic oligonucleotide encoding OVA₃₂₃₋₃₃₉, flanked by nonpalindromic *Aval* sites (17), was inserted head-to-tail into the *Bbs*I site in vectors pCTA1-DD and pCTA1-R7K-DD. For the production of fusion proteins, *Escherichia coli* TG-1 cells transformed with the different expression vectors were grown in 250-ml flasks overnight in 2× YT or Luria-Bertani, with 50 µg/ml kanamycin, at 37°C. After culture, the cells were collected by centrifugation, and the fusion proteins, produced as inclusion bodies, were solubilized by treatment with 6 M guanidine-HCl. After addition of distilled water to allow refolding, the fusion proteins were purified by affinity chromatography on IgG-Sepharose (Pharmacia, Peapack, NJ) and stored in 0.2 M HAc at 4°C.

Assessment of ADP-ribosyltransferase activity

Enzymatic activity was determined using the NAD-aggmatine assay as described earlier (18, 19). Briefly, the formation of ADP-ribosylaggmatine catalyzed by 10 µg of the different CTA1-DD mutants was assayed via the incorporation of [U-¹⁴C]adenine. Each sample contained 50 mM sodium phosphate (pH 7.5), 100 µM GTP, 5 mM MgCl₂, 100 mM [U-¹⁴C]adenine-labeled NAD, 10 mM agmatine, 0.1 mg/ml OVA, and the respective fusion proteins. After 3 h at 30°C, three 110-µl samples were transferred to AG1-X4 columns (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, U.K.). Samples plus eluates containing [U-¹⁴C]adenine-labeled ADP-ribosylaggmatine were collected for determination of radioactivity.

Preparation of ISCOMS

ISCOMS containing the CTA1-OVAp-DD fusion proteins were prepared by adding 1-mg aliquots of a purified, freeze-dried fraction of Quil A saponin (Quadri A) (20) to 1 ml of a 1 mg/ml solution of CTA1-OVAp-DD or CTA1R7K-OVAp-DD protein at room temperature in 0.2 M PBS, pH 6. After being allowed to dissolve using a magnetic stirrer, 40 µl of a lipid mixture containing 1% cholesterol and 1% phosphatidylcholine (Northern Lipids, Vancouver, Canada) dissolved in 20% Mega 10 (Bachem, Bubendorf, Switzerland) was then added, and the mixture was stirred for 3 h at room temperature, followed by dialysis against 0.2 M PBS, pH 6, at room temperature for another 2–3 h and then overnight at 4°C. The dialyzed material was then centrifuged for 5 min at 10,000 × g, and the supernatant was transferred in 300-µl aliquots to 4-ml plastic ultracentrifuge tubes containing 25% sucrose (w/w) in 0.2 M PBS, pH 6. After centrifuging the gradients for 5 h at 257,000 × g at 20°C, fractions were collected from the bottom of the tubes by puncturing with a needle. The fractions were analyzed for total protein content by the Bradford reaction (Bio-Rad) and for the incorporation of the CTA1-OVAp-DD constructs by rocket immunoelectrophoresis using a polyclonal rabbit antiserum raised against

CTA1-DD. The protein-rich ISCOMS fractions were pooled and dialyzed against 0.2 M PBS for 2 days at 4°C. Finally the ISCOMS preparations were concentrated using a centrifugal filter device to obtain a total protein concentration of 0.5 mg/ml, and the formation of intact ISCOMS was confirmed by electron microscopy (Fig. 1).

Immunization of animals

Mice were immunized s.c. (into one footpad) on one occasion, or intranasally on three occasions 10 days apart, with ISCOMS or purified fusion proteins containing 4 µg of CTA1-OVAp-DD or CTA1R7K-OVAp-DD (equivalent to 150 ng of OVA₃₂₃₋₃₃₉) in a total volume of 20 µl (intranasal) or 50 µl (s.c.). Control groups of mice received 150 ng of OVA₃₂₃₋₃₃₉ alone, admixed with alum s.c., or with 2 µg of CT intranasally. For oral immunization, mice were fed on days 1, 2, 3, 8, 9, and 10 with ISCOMS or purified fusion proteins containing 20 µg of CTA1-OVAp-DD or CTA1R7K-OVAp-DD (equivalent to 750 ng of OVA₃₂₃₋₃₃₉ per dose). Control mice received 750 ng of OVA₃₂₃₋₃₃₉ on each occasion.

Measurement of OVA-specific immune responses in vivo

Seven days after the last immunization, DTH was assessed by determining the increment in footpad thickness found 24 h after s.c. injection of 100 µg of heat-aggregated OVA in 50 µl of sterile saline. Mice were bled for primary serum Ab responses at this time and also 7 days after DTH challenge to assess secondary responses. Anti-OVA total IgG, IgG1, and IgG2a isotype responses were measured by ELISA, as described previously (21).

Measurement of OVA-specific immune responses in vitro

Seven days after the last immunization, draining popliteal lymph nodes or spleens were removed and single cell suspensions prepared in RPMI 1640 (Life Technologies, Paisley, Scotland) by rubbing through a stainless steel mesh and passing the resulting suspension through Nitex mesh (Cadisch & Sons, London, U.K.). After three washes in medium, the cells were resuspended at a final concentration of 10⁶ cells/ml and cultured in 200-µl aliquots in flat-bottom 96-well tissue culture plates (Nucleopore; Costar, High Wycombe, U.K.) in RPMI 1640 containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml fungizone, 2 mM L-glutamine, 25 mM HEPES, 50 mM 2-ME (all obtained from Life Technologies), either alone or with 1 mg/ml OVA. Proliferation was assessed by the addition of 1 µCi/well [³H]thymidine for the last 18 h of culture. Cell-bound DNA was harvested on filter mats, and [³H]TdR incorporation was measured on a Betaplate counter. To measure cytokine production, 4 × 10⁶ lymph node cells in 1-ml aliquots were cultured in 24-well tissue culture plates (Costar) either in medium alone or with 1 mg/ml OVA. Supernatants were harvested after 2–4 days and stored at –20°C until assayed. Cytokine production was quantified using sandwich ELISA techniques described in detail elsewhere (10, 22), using appropriate pairs of capture and biotinylated detecting Abs (all obtained from BD PharMingen, San Diego, CA). Ab binding was detected using extravidin-peroxidase (Sigma) and tetramethylbenzidine substrate as described above. Cytokine concentrations in test supernatants were determined with reference to a standard curve constructed using serial dilutions of recombinant cytokines (BD PharMingen).

Statistical analysis

Results expressed as means ± SD were compared using unpaired two-tailed Student's *t* test, whereas Wilcoxon's Signed Rank test was used to compare Ab levels. A *p* value of <0.05 was considered to be significant.

Results

Incorporation into ISCOMS enhances the systemic immunogenicity of the CTA1-DD adjuvant vector

Having successfully incorporated the CTA1-OVAp-DD fusion proteins into ISCOMS with the appropriate structural characteristics (Fig. 1), we went on to determine how the immunogenicity of the combined vector compared with the intact fusion protein. Mice were immunized s.c. on one occasion, and the subsequent systemic immune responses were assessed by measuring primary OVA-specific DTH responses, primary and secondary serum Ab responses, and in vitro T cell responses in the draining lymph node.

As anticipated, mice immunized with 150 ng of purified OVA₃₂₃₋₃₃₉ in saline showed little DTH response above background. In contrast, mice immunized with CTA1-OVAp-DD-ISCOMS fusion protein containing the same amount of OVA₃₂₃₋₃₃₉

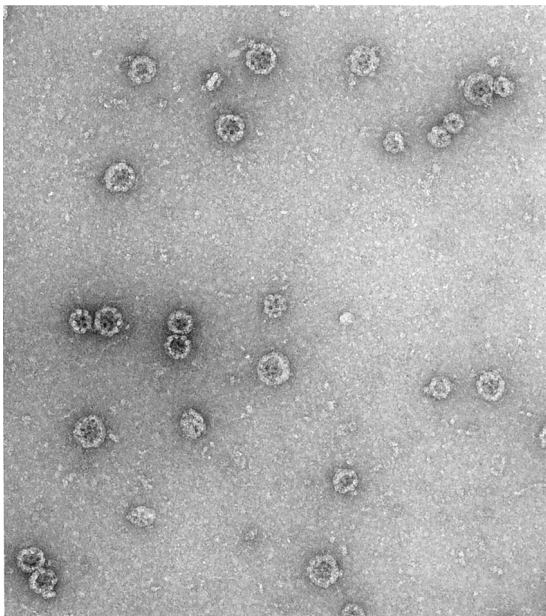


FIGURE 1. Successful formation of intact ISCOMS particles incorporating CTA1-OVAp-DD. Electron microscopic examination reveals characteristic cage-like particles of 40- to 70-nm diameter ($\times 100,000$).

had very strong DTH responses, which were significantly above background (Fig. 2A). These responses were dependent on the presence of enzymatically active CTA1, as mice receiving CTA1R7K-OVAp-DD in ISCOMS had no significant DTH responses above those in mice receiving peptide alone. Mice immunized with the CTA1-OVAp-DD fusion protein itself also had small, but significant DTH responses (Fig. 2A). The enzymatically inactive CTA1R7K-OVAp-DD fusion protein and OVAp in alum did not induce significant DTH responses (data not shown).

Primary serum Ab responses were not found after single immunization of any group (data not shown). However, secondary total IgG Ab responses did occur after s.c. challenge with heat-aggregated OVA. These followed a similar pattern to the DTH responses, with the highest levels of IgG anti-OVA being found in mice primed with CTA1-OVAp-DD-ISCOMS (Fig. 2B). Significant, but lower, IgG responses also occurred in mice given intact CTA1-OVAp-DD fusion protein itself. Again, animals receiving enzymatically inactive CTA1R7K-OVAp-DD-ISCOMS had low levels of total IgG Ab, which were identical with those in mice primed with OVAp alone.

Importantly, immunization with CTA1-OVAp-DD-ISCOMS primed for both IgG1 and IgG2a Ab responses, suggesting no bias toward priming of Th1 or Th2 cells by the vectors (Fig. 2, C and D). Immunization with CTA1-OVAp-DD alone was relatively ineffective at priming these IgG isotypes, although there was some increase in IgG2a Ab levels compared with those found in mice primed with OVAp alone (Fig. 2D). Priming of IgG2a, but not IgG1 responses, was also found in some mice given CTA1R7K-OVAp-DD-ISCOMS. Mice immunized with the enzymatically inactive CTA1R7K-OVAp-DD itself or with OVAp in alum had no total or isotype-specific IgG responses above those in mice receiving OVAp alone (data not shown).

The combined adjuvant vector augments T cell priming

Mice immunized s.c with CTA1-OVAp-DD-ISCOMS were also primed for very strong T cell-dependent immune responses in vitro, as assessed by OVA-specific proliferation (Fig. 3A) and production of IFN- γ (Fig. 3B) in draining popliteal LN cells. Immu-

nization with CTA1-OVAp-DD alone induced relatively good IFN- γ production and some proliferative responses. In both cases, these were substantially less than those in mice immunized with CTA1-OVAp-DD-ISCOMS. Immunization with OVAp alone primed for little or no proliferation or IFN- γ production, whereas ISCOMS containing the enzymatically inactive CTA1R7K-OVAp-DD fusion protein primed poor proliferative or IFN- γ responses (Fig. 3). Little or no IL-5 production was observed in any group, and no significant priming of in vitro T cell responses was found in mice immunized with CTA1R7K-OVAp-DD fusion protein or with OVAp in alum (data not shown).

We also used these parameters to assess the immunogenicity of the vectors by the nasal route. After intranasal immunization with CTA1-OVAp-DD-ISCOMS, splenic T cells responded strongly to the recall Ag in vitro with proliferation and IFN- γ production (Fig. 4). Again, ISCOMS containing the enzymatically inactive CTA1R7K-OVAp-DD mutant were inefficient at priming T cell responses in vivo, with low levels of IFN- γ being the only response observed (Fig. 4). The CTA1-OVAp-DD fusion protein itself was immunogenic by the nasal route, inducing relatively good IFN- γ responses and some proliferation, although both were significantly less than those found using CTA1-OVAp-DD-ISCOMS. This dose of OVAp given on one occasion with CT holotoxin intranasally failed to induce significant immune responses (data not shown). Together, these results confirm and extend our previous findings that enzymatically active CTA1-DD is an effective adjuvant for a broad range of systemic immune responses when given by parenteral and intranasal routes, by showing that incorporation into ISCOMS markedly enhances this activity.

Enhanced oral immunogenicity of the CTA1-DD-ISCOMS combined vector

In earlier work, we had found that CTA1-DD had little or no adjuvant activity by the oral route when coadministered with protein Ag (N. Lycke, unpublished observations). However, ISCOMS are extremely effective when given orally; therefore, we examined whether incorporation into ISCOMS could improve the mucosal adjuvant properties of CTA1-DD. Mice were fed on six occasions with the different vectors, a protocol we have found to be optimal in previous work with ISCOMS, and systemic immune responses were assessed as described above.

Oral immunization with CTA1-OVAp-DD-ISCOMS induced significant DTH responses, equivalent to those obtained after s.c. priming (Fig. 5A). CTA1-OVAp-DD itself also primed systemic DTH by the oral route, but again, this was significantly less than when the adjuvant construct was incorporated into ISCOMS. Some DTH responses appeared to occur in mice receiving the enzymatically inactive CTA1R7K-OVAp-DD vector, either alone or when incorporated into ISCOMS, but these were not significantly higher than the very small responses found in mice fed OVA₃₂₃₋₃₃₉ in saline (Fig. 5A).

The Ab responses in orally immunized mice after s.c. challenge with heat-aggregated OVA showed a similar pattern to those found after s.c. immunization, with the highest levels of IgG anti-OVA being found in mice immunized with CTA1-OVAp-DD-ISCOMS. Interestingly, significant IgG responses also occurred in mice given intact CTA1-OVAp-DD fusion protein itself, but animals receiving CTA1R7K-OVAp-DD-ISCOMS or CTA1R7K-OVAp-DD alone had very low amounts of total IgG Ab, which were similar to those in mice fed OVAp alone (Fig. 5B). Mice fed CTA1-OVAp-DD-ISCOMS were primed for both IgG1 and IgG2a Ab responses (Fig. 5, C and D). In confirmation of the pattern seen after s.c. immunization, mice primed with CTA1-OVAp-DD orally had low, but detectable

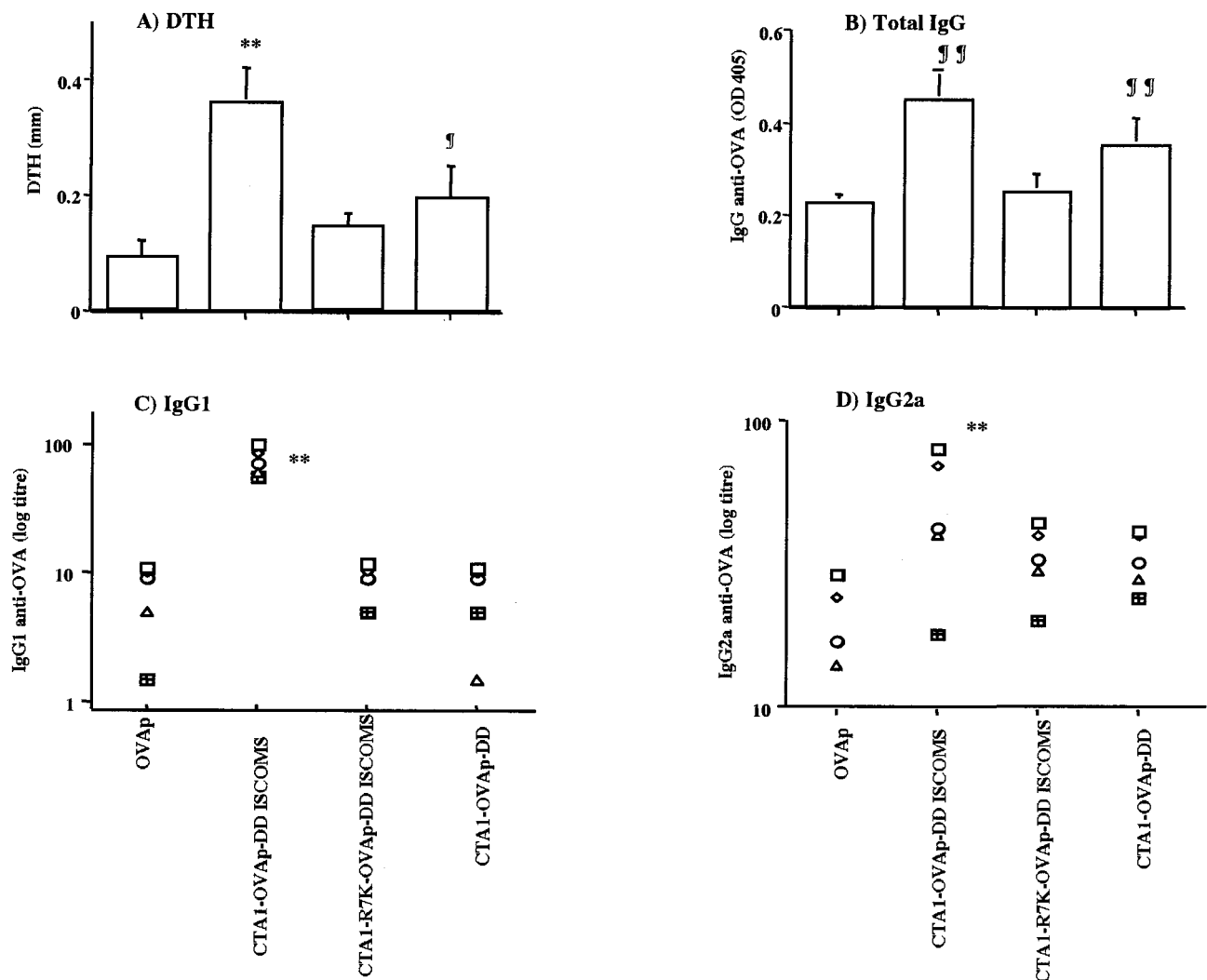


FIGURE 2. Induction of systemic immune responses by s.c. immunization with ISCOMS containing CTA1-OVAp-DD or enzymatically inactive CTA1-R7K-OVAp-DD. Control mice received CTA1-OVAp-DD alone or OVA₃₂₃₋₃₃₉ peptide alone. All mice received the equivalent of 150 ng of OVA peptide, and results shown are primary DTH responses measured 7 days after immunization (A), serum total IgG (B), IgG1 (C), and IgG2a (D) Ab levels measured 7 days after a s.c. challenge with soluble OVA given 7 days after primary immunization. The data are means \pm 1 SD for five mice per group and are representative of three similar experiments (**, $p < 0.05$ vs all other groups; ¶¶, $p < 0.05$ vs OVAp alone).

IgG2a responses after secondary challenge, but in this case, also had evidence of significant priming of IgG1 production. Oral immunization with CTA1R7K-OVAp-DD, alone, in ISCOMS, or with OVAp alone, primed no serum IgG1 or IgG2a responses (Fig. 5, C and D).

In agreement with these *in vivo* responses, spleen cells from mice immunized orally with CTA1-OVAp-DD-ISCOMS had excellent proliferative and IFN- γ responses *in vitro* (Fig. 6, A and B). CTA1-OVAp-DD itself also primed for these T cell responses *in vitro*, but again, these were markedly less than those found in CTA1-OVAp-DD-ISCOMS-primed animals. Little or no proliferation or cytokine production was observed in mice receiving OVAp or CTA1R7K-OVAp-DD alone, although small, but significant priming of proliferative responses was found in mice immunized orally with CTA1R7K-OVAp-DD-ISCOMS (Fig. 6). No IL-5 production was found in any group (data not shown).

Discussion

The results presented here show that it is feasible to incorporate a targeted, nontoxic derivative of CT into ISCOMS. The resulting combined vector is a very potent adjuvant for inducing a wide range of immune responses to small amounts of peptide immunogen after mucosal and parenteral administration.

These findings extend our previous work on the CTA1-DD vector (8, 9) by showing that defined peptide epitopes can be included in the construct. The combined vector also retains its adjuvant effects when incorporated into ISCOMS and when given by the oral, nasal, and parenteral routes. The responses induced included DTH and serum IgG Abs *in vivo*, Ag-specific T cell proliferation, and IFN- γ production *in vitro*. Although we were unable to detect IL-5 production in CTA1-OVAp-DD-ISCOMS-primed mice, these animals produced Abs of both the IgG2a and IgG1 isotypes, indicating that Th1 and Th2 cells were primed *in vivo*. The reasons for this discrepancy are not known, but a failure to find IL-5 (or IL-4) after restimulation with OVA was consistent throughout our experiments, despite the fact that Con A-stimulated lymphocytes produced significant amounts of IL-5 (data not shown). It may be that we examined for IL-5 priming too soon after immunization, or that secondary challenge *in vivo* may have been necessary to reveal detectable levels of Th2 cytokines *in vivo*.

As with the CTA1-OVAp-DD vector itself (8), the immune responses induced by ISCOMS containing the adjuvant construct required ADP-ribosylating activity, as ISCOMS containing the enzymatically inactive CTA1-R7K-DD construct were poorly immunogenic. Nevertheless, ISCOMS containing the enzymatically

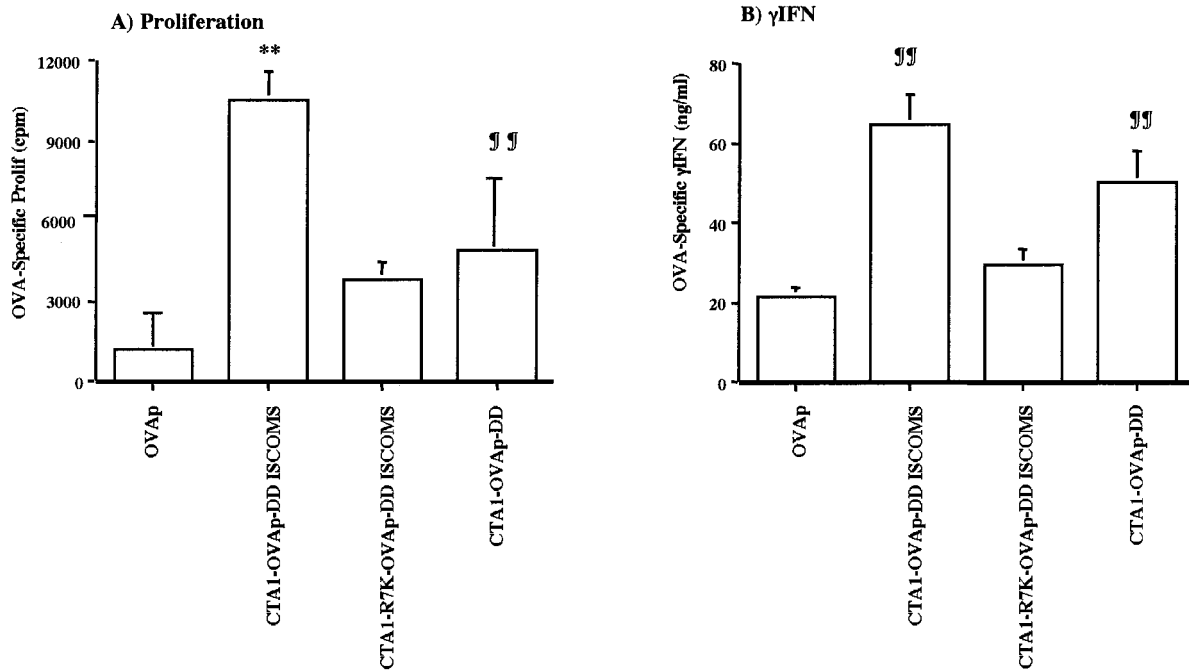


FIGURE 3. Priming of systemic T cells by s.c. immunization with ISCOMS containing CTA1-OVAp-DD or enzymatically inactive CTA1-R7K-OVAp-DD. Control mice received CTA1-OVAp-DD alone, or OVA₃₂₃₋₃₃₉ peptide alone. All mice received the equivalent of 150 ng of OVA peptide, and results shown are proliferation (A) and IFN- γ (B) levels measured in draining lymph nodes measured 7 days after immunization. The data are means \pm 1 SD for five mice per group and are representative of three similar experiments (**, $p < 0.05$ vs all other groups; ¶¶, $p < 0.05$ vs OVAp alone).

inactive CTA1R7K-OVAp-DD molecule induced some responses, supporting previous suggestions that ISCOMS containing peptides can induce humoral immunity in vivo (23). However, this work used much higher doses and larger peptides than in our experiments, indicating that the immunogenicity of ISCOMS may be enhanced by the DD fragment, perhaps because the combined vector can interact with B cells as well as the DC and/or macrophages

targeted by conventional ISCOMS (12–14, 24). In addition to targeting different APC, the CTA1-DD-ISCOMS vector contains two active adjuvants, Quil A and the ADP-ribosylating enzyme CTA1, which may have additive effects. The stable ISCOMS particle undoubtedly adds to the activity of the CTA1-OVAp-DD-ISCOMS by ensuring that Ag and the adjuvants are delivered directly to the same APC. In addition, targeting of the immunomodulating effect

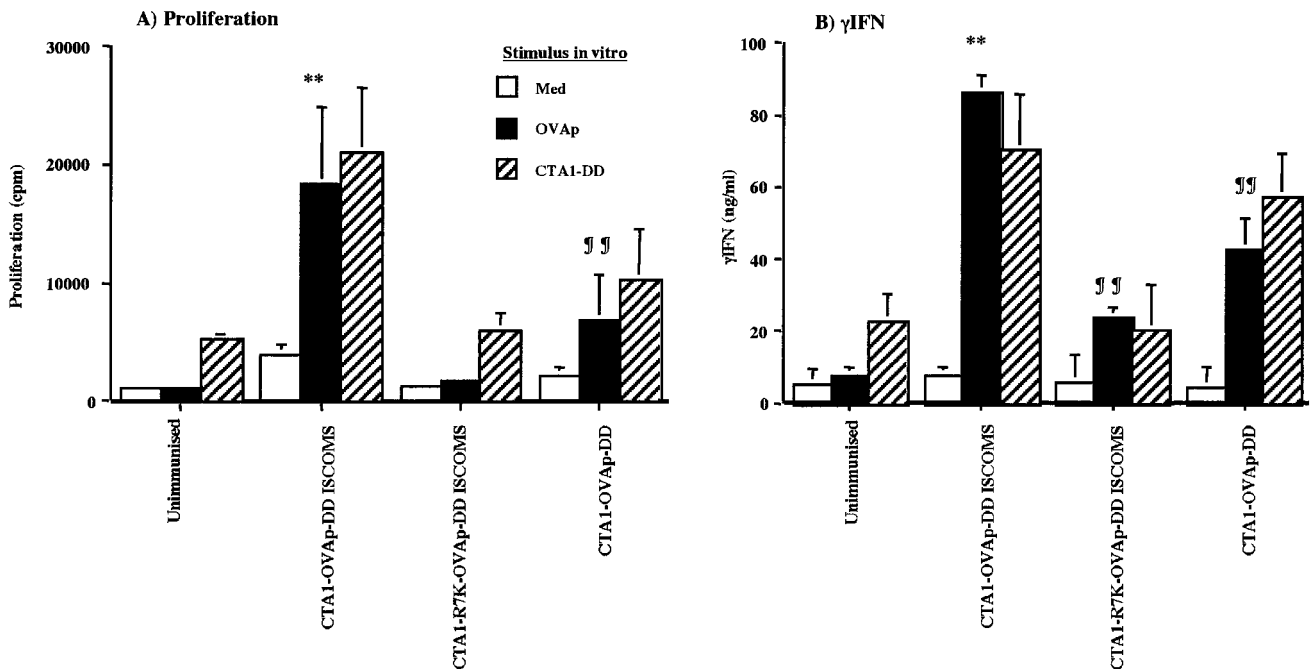


FIGURE 4. Priming of systemic T cells by intranasal immunization with ISCOMS containing CTA1-OVAp-DD or enzymatically inactive CTA1-R7K-OVAp-DD. Control mice received CTA1-OVAp-DD alone or OVA₃₂₃₋₃₃₉ peptide alone. All mice received the equivalent of 150 ng of OVA peptide, and results shown are proliferation (A) and IFN- γ (B) levels measured in the spleen 7 days after immunization. The data are means \pm 1 SD for five mice per group and are representative of three similar experiments (**, $p < 0.05$ vs all other groups; ¶¶, $p < 0.05$ vs OVAp alone).

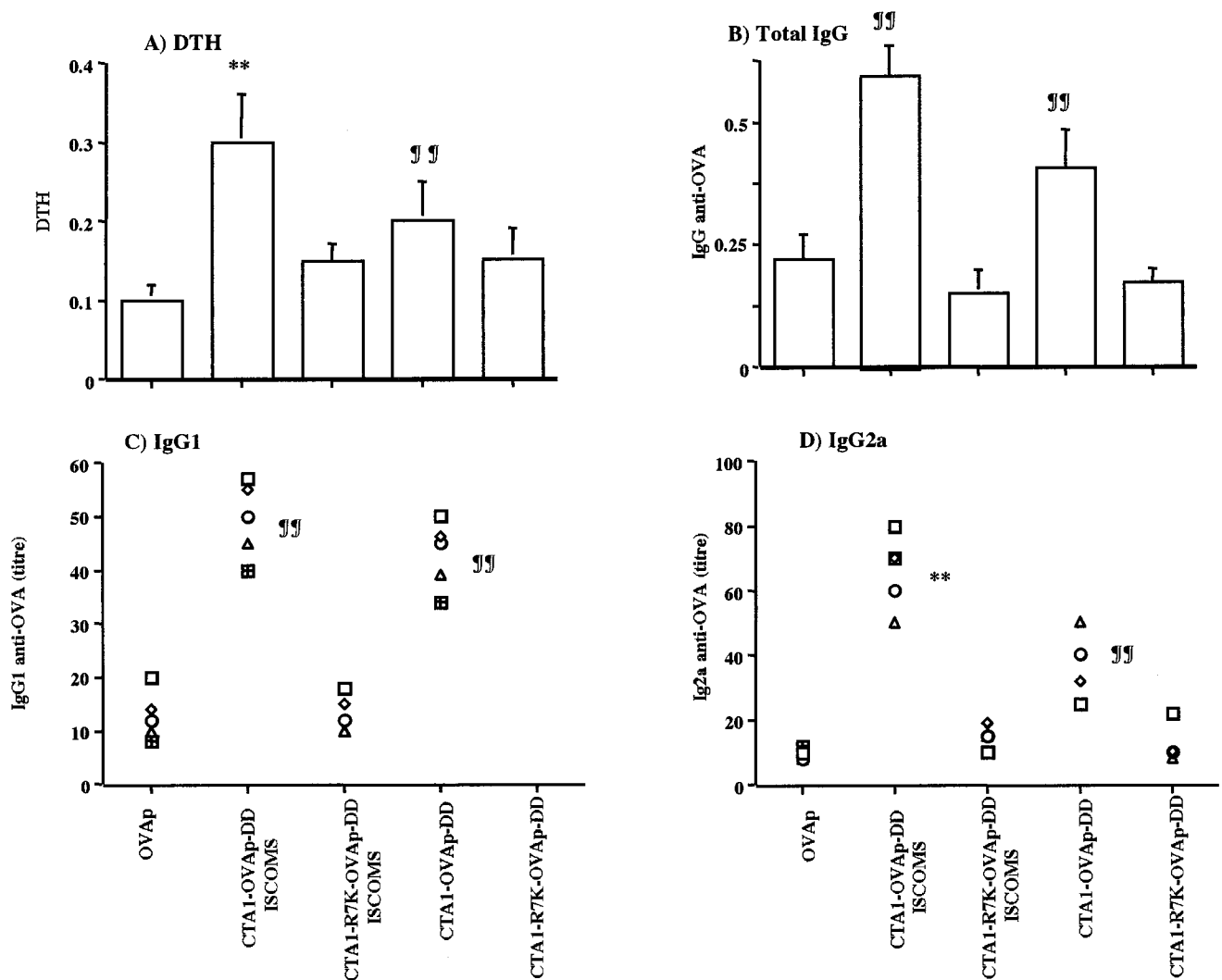


FIGURE 5. Induction of systemic immune responses by oral immunization with ISCOMS containing CTA1-OVAp-DD or enzymatically inactive CTA1-R7K-OVAp-DD. Control mice received CTA1-OVAp-DD alone, CTA1-R7K-OVAp-DD, or OVA₃₂₃₋₃₃₉ peptide alone. All mice received the equivalent of 750 ng of OVA peptide on six occasions, and the results shown are primary DTH responses measured 7 days after the last immunization (A), serum total IgG (B), IgG1 (C), and IgG2a (D) Ab levels measured 7 days after a s.c. challenge with soluble OVA given 7 days after the last immunization. The data are means \pm 1 SD for four to five mice per group and are representative of three similar experiments (**, $p < 0.05$ vs all other groups; ¶¶, $p < 0.05$ vs OVAp alone).

of CTA1-DD to DC by incorporation into ISCOMS may add further to the *in vivo* efficacy of the CTA1-DD vectors.

There are a number of other aspects of the behavior of the combined vector that deserve attention. First, extremely low doses of OVA peptide were able to prime systemic immunity by both mucosal and parenteral routes using the CTA1-DD-ISCOMS vector, with the equivalent of as little as 150 or 750 ng of peptide being effective by the s.c., intranasal, and oral routes, respectively. Secondly, CTA1-OVAp-DD-ISCOMS containing a minimal antigenic epitope induced strong immune responses that could be recalled with intact OVA protein, indicating that the CTA1-DD fusion protein does not interfere with the generation of MHC class II-restricted epitopes. Taken together, our results suggest that the combined vector gains access to physiologically relevant Ag-processing pathways in an extremely efficient manner. Finally, no toxicity was observed in mice given the combined adjuvant vectors by any route. This contrasts with the toxicity occasionally seen using vectors containing intact Quil A (25, 26), but extends our previous findings that the Quadri A fraction of Quil A and the

CTA1-DD fusion protein are themselves lacking significant toxicity, despite their potent adjuvant activities (Refs. 8, 9; and our unpublished observations). One surprising finding was that the free CTA1-DD fusion protein also had some adjuvant activity when given by the oral route. Despite its clear immunogenicity by parenteral and intranasal routes (8, 9), our earlier, preliminary findings had suggested that CTA1-DD was unstable in the intestine. However, in these previous studies, we used Ag admixed with free CTA1-DD in a single oral dosing protocol. In contrast, the current work shows that repeated oral immunization with a fusion protein that comprises both CTA1-DD and OVAp can induce some immune responses *in vivo*, emphasizing the importance of physical linkage between Ag and adjuvant. The adjuvant properties of intact CTA1-DD were entirely dependent on its ADP-ribosylating activity, but were less than those obtained when the fusion protein was inserted in ISCOMS. This again underlines the added potency of physically linking Ag and enzymatically active adjuvant in the combined ISCOMS vector. Incorporation into ISCOMS may also stabilize and protect the CTA1-DD enzyme from degradation in

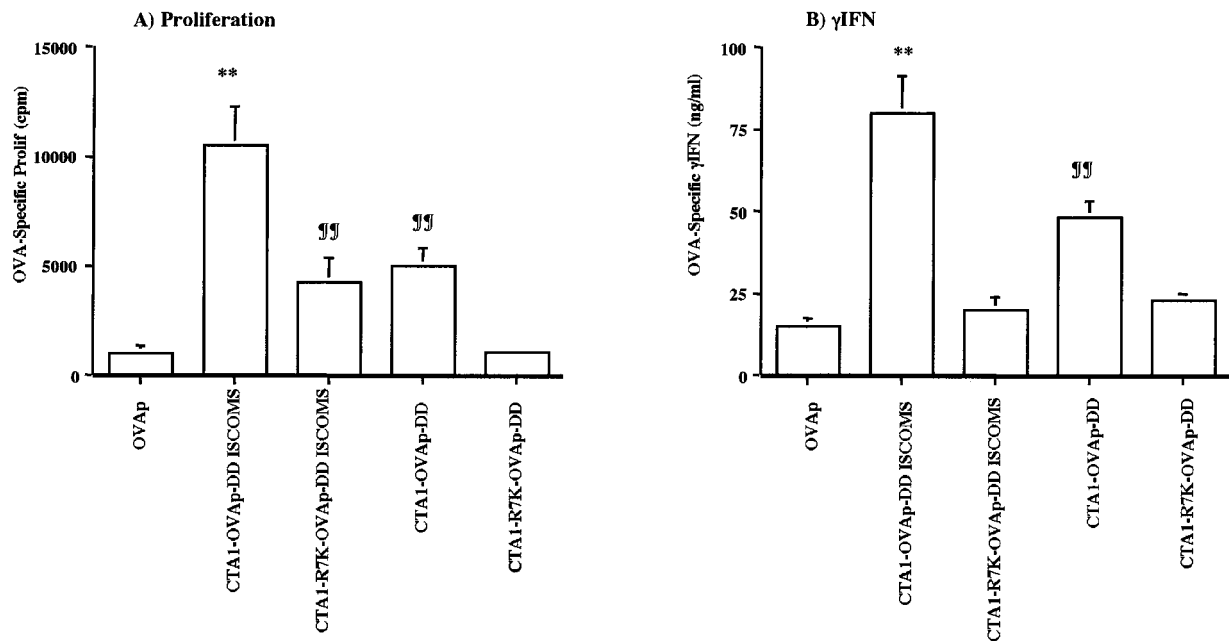


FIGURE 6. Priming of T cells by oral immunization with ISCOMS containing CTA1-OVAp-DD or enzymatically inactive CTA1-R7K-OVAp-DD. Control mice received CTA1-OVAp-DD alone, CTA1-R7K-OVAp-DD, or OVA₃₂₃₋₃₃₉ peptide alone. All mice received the equivalent of 750 ng of OVA peptide on six occasions, and the results shown are proliferation (A) and IFN- γ (B) levels measured in draining lymph nodes, measured 7 days after immunization. The data are means \pm 1 SD for four to five mice per group and are representative of three similar experiments (**, $p < 0.05$ vs all other groups; ¶, $p < 0.05$ vs OVAp alone).

the gastrointestinal tract. As when it was incorporated into ISCOMS, the CTA1-OVAp-DD itself induced the production of IFN- γ and IgG2a Abs, despite claims that CT-based adjuvants stimulate predominantly Th2-dependent responses (4, 5, 27).

The cellular and anatomical basis of the oral adjuvant properties of the CTA1-DD-containing vectors remain to be determined. B cells are required for the adjuvant properties when the fusion protein itself is used by parenteral routes (8, 9), and orally administered CTA1-DD may act via B cells in the follicles of Peyer's patches after uptake by M cells. Alternatively, the vectors may enter across the villus epithelium before being transported to B cells in the mesenteric lymph node via the draining lymph. Although both routes have been implicated in the intestinal immunogenicity of intact CT (11), CTA1-DD lacks the GM1-ganglioside binding activity that may assist the uptake of CT holotoxin by enterocytes (28). Thus CTA1-DD in ISCOMS may enter the immune system of the gut-associated lymphoid tissues by a distinctive mechanism. In conclusion, our results are encouraging evidence that by combining the distinctive adjuvant properties of ISCOMS and the nontoxic CTA1-DD derivative, it may prove possible to construct effective, safe, and stable subunit vaccines that are active by mucosal routes.

References

- Mowat, A. M. 1999. Oral tolerance: basic mechanisms and clinical applications. *Curr. Opin. Gastroenterol.* 15:546.
- Strobel, S., and A. M. Mowat. 1998. Immune responses to dietary antigens: oral tolerance. *Immunol. Today* 19:173.
- Wilson, A. D., M. Bailey, N. A. Williams, and C. R. Stokes. 1991. The in vitro production of cytokines by mucosal lymphocytes immunized by oral administration of keyhole limpet hemocyanin using cholera toxin as an adjuvant. *Eur. J. Immunol.* 21:2333.
- McGhee, J. R., J. Mestecky, M. T. Dertzbaugh, J. H. Eldridge, M. Hirasawa, and H. Kiyono. 1992. The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine* 10:75.
- McGhee, J. R., C. Czerkinsky, and J. Mestecky. 1999. Mucosal vaccines: an overview. In *Mucosal Immunology*, 2nd Ed. P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee, and J. Bienenstock, eds. Academic Press, San Diego, p. 741.
- Elson, C. O., and M. T. Dertzbaugh. 1999. Mucosal adjuvants. In *Mucosal Immunology*, 2nd Ed. P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee, and J. Bienenstock, eds. Academic Press, San Diego, p. 818.
- van Ginkel, F. W., R. J. Jackson, Y. Yuki, and J. R. McGhee. 2000. The mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. *J. Immunol.* 165:4778.
- Agren, L. C., L. Ekman, B. Lowenadler, and N. Y. Lycke. 1997. Genetically engineered nontoxic vaccine adjuvant that combines B cell targeting with immunomodulation by cholera toxin A1 subunit. *J. Immunol.* 158:3936.
- Agren, L. C., L. Ekman, B. Lowenadler, J. G. Nedrud, and N. Lycke. 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.* 162:2432.
- Grdic, D., R. E. Smith, A. M. Donachie, M. Kjerrulf, E. Hörnquist, A. Mowat, and N. Lycke. 1999. The mucosal adjuvant effect of cholera toxin and ISCOMS differ in their requirement for IL-12, indicating different pathways of action. *Eur. J. Immunol.* 29:1774.
- Vajdy, M., M. H. Kosco-Vilbois, M. Kopf, G. Kohler, and N. Lycke. 1995. Impaired mucosal immune responses in interleukin 4-targeted mice. *J. Exp. Med.* 181:41.
- Watson, D. L., N. A. Watson, C. Fossum, K. Lövgren, and B. Morein. 1992. Interactions between immune-stimulating complexes (ISCOMs) and peritoneal mononuclear leucocytes. *Microbiol. Immunol.* 36:199.
- Claassen, I. J., A. D. Osterhaus, and E. Claassen. 1995. Antigen detection in vivo after immunization with different presentation forms of rabies virus antigen: involvement of marginal metallophilic macrophages in the uptake of immune-stimulating complexes. *Eur. J. Immunol.* 25:1446.
- Claassen, I. J., A. D. Osterhaus, M. Poelen, N. Van Rooijen, and E. Claassen. 1998. Antigen detection in vivo after immunization with different presentation forms of rabies virus antigen. II. Cellular, but not humoral systemic immune responses against rabies virus immune stimulating complexes are macrophage dependent. *Immunology* 94:455.
- Smith, R. E., A. M. Donachie, and A. M. Mowat. 1998. Immune stimulating complexes as mucosal adjuvants. *Immunol. Cell Biol.* 76:263.
- Smith, R. E., A. M. Donachie, F. H. McLaren, and A. M. Mowat. 1998. Preservation of mucosal and systemic adjuvant properties of ISCOMS in the absence of functional interleukin 4 or γ interferon. *Immunology* 93:556.
- Lowenadler, B., A. M. Svennerholm, M. Gidlund, E. Holmgren, K. Krook, C. Svanholm, S. Ulf, and S. Josephson. 1990. Enhanced immunogenicity of recombinant peptide fusions containing multiple copies of a heterologous T helper epitope. *Eur. J. Immunol.* 20:1541.
- Spangler, B. D. 1992. Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiol. Rev.* 56:622.

19. Tsuji, T., T. Inoue, A. Miyama, K. Okamoto, T. Honda, and T. Miwatani. 1990. A single amino acid substitution in the A subunit of *Escherichia coli* enterotoxin results in a loss of its toxic activity. *J. Biol. Chem.* 265:22520.
20. Kamstrup, S., R. San Martín, A. Doberti, H. Grande, and K. Dalsgaard. 2000. Preparation and characterisation of Quillaja saponin with less heterogeneity than Quil-A. *Vaccine* 18:2244.
21. Maloy, K. J., A. M. Donachie, and A. M. Mowat. 1995. Induction of Th1 and Th2 CD4⁺ T cell responses by oral or parenteral immunization with ISCOMS. *Eur. J. Immunol.* 25:2835.
22. Garside, P., M. Steel, E. A. Worthey, A. Satoskar, J. Alexander, H. Bluethmann, F. Y. Liew, and A. M. Mowat. 1995. Th2 cells are subject to high dose oral tolerance and are not essential for its induction. *J. Immunol.* 154:5649.
23. Wejer, K., A. Pfauth, R. Van Herwijnen, O. Jarrett, R. H. Melen, C. Tomee, and A. D. Osterhaus. 1993. Induction of feline leukemia virus-neutralizing antibodies by immunization with synthetic peptides derived from the FeLV env gene. *Vaccine* 11:946.
24. Smith, R. E., A. M. Donachie, D. Grdic, N. Lycke, and A. I. Mowat. 1999. Immune stimulating complexes induce an IL-12 dependent cascade of innate immune responses. *J. Immunol.* 162:5536.
25. Johansson, M., K. Morein, and K. Lövgren-Bengtsson. 1999. ISCOMS with different Quillaja saponin components differ in their immunomodulating activities. *Vaccine* 17:2894.
26. Morein, B., K. Lövgren, B. Rönnerberg, A. Sjölander, and M. Villacres-Eriksson. 1995. Immunostimulating complexes: clinical potential in vaccine development. *Clin. Immunother.* 3:461.
27. Elson, C. O., and M. T. Derzbaugh. 1994. Mucosal adjuvants. In *Handbook of Mucosal Immunology*. P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee, and J. Bienenstock, eds. Academic Press, San Diego, p. 391.
28. Neutra, M. R., and J. P. Kraehenbuhl. 1996. Antigen sampling across epithelial barriers and induction of mucosal immune responses. *Ann. Rev. Immunol.* 14:275.