Vaccination by cholera toxin conjugated to a herpes simplex virus type 2 glycoprotein D peptide

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Immunization of BALB/cJ mice with a peptide corresponding to residues 1 to 23 of glycoprotein D [gD(1-23)] from herpes simplex virus type 2 (HSV-2) elicits antibody responses which correlate with protection against lethal HSV-2 infection. In the present study, we examined the ability of cholera toxin (CTX) to act as an immunogenic carrier for gD(1-23). The number of gD(1-23) residues conjugated to CTX affected its binding to GM₁ ganglioside and physiological toxicity, both of which are factors affecting oral immunogenicity. The antibody response elicited after intraperitoneal (i.p.) immunization with the CTX-gD(1-23) conjugate was protective against a lethal i.p. challenge with HSV-2. In other experiments, mice were immunized i.p. on day 0 and subsequent immunizations conducted on days 14 and 28 were administered either intragastrically or intravaginally (i.vag.). Intrapерitoneal priming followed by either i.p. or intragastric boosting resulted in anti-HSV-2 antibodies in vaginal washings and in protection against a lethal i.vag. challenge with HSV-2. Intrapерitoneal priming followed by i.vag. boosting did not elicit anti-HSV-2 antibodies in vaginal washings and did not protect mice against a lethal i.vag. challenge with HSV-2. These results suggest that CTX can act as a systemic and an oral delivery molecule for the covalently linked gD(1-23) peptide and that such conjugates can elicit protective immune responses against systemic and genital HSV-2 infection.

Introduction

Herpes simplex virus type 2 (HSV-2) infects the genital and oral mucosae (Corey & Spear, 1986; Rawls, 1985). After primary mucosal infection, HSV-2 can spread to the central nervous system and other organs. Recurrent disease occurs by activation of latent virus persisting in ganglia (Corey & Spear, 1986; Rawls, 1985; Walz et al., 1974). Although in vivo and in vitro studies indicate that immunity to and recovery from primary HSV-2 infection involves humoral (Balachandran et al., 1982; Simmons & Nash, 1985; Shore et al., 1979; Engler et al., 1981) and cellular (Morahan et al., 1980; Plaeger-Marshall et al., 1983; Larsen et al., 1983; Rosenthal et al., 1987; McDermott et al., 1989) effector mechanisms, parenteral vaccination inducing systemic immunity presently lacks sufficient efficacy for use against genital HSV-2 infections. An explanation, in part, for these observations is that humoral immunity in the female genital tract is derived from both mucosal and systemic sources. Uterine secretions contain transudated serum IgA and IgG and few plasmacytes are present in the endometrium (Tourville et al., 1970; Kutteh et al., 1988; Parr & Farr, 1990). Secretory component (SC) in the uterine epithelium (Parr & Farr, 1989) suggests that serum IgA might be transported into the uterine lumen by a mechanism analogous to the SC-mediated transport of IgA into biliary, lacrimal, salivary and mammary secretions (Jackson et al., 1978; Sheldrake et al., 1984; Underdown & Schiff, 1986; Mestecky et al., 1978). In contrast, cervicovaginal tissue displays hallmarks of local immunity (Tourville et al., 1970; Waldman et al., 1971; Rebello et al., 1975; Kutteh et al., 1988). IgA plasmacytes predominate here and increase in number following exposure to antigen (Chipperfield & Evans, 1972). Locally synthesized IgA is transported by SC in the cervicovaginal epithelium (Waldman et al., 1971; Rebello et al., 1975; Kutteh et al., 1988; Thapar et al., 1990a, b). Furthermore, serum-derived uterine secretions flow into the cervicovaginal canal and contribute to local immunity.

Immunity can be induced in the genital tract. Parenteral immunization with attenuated HSV-2 (McDermott et al., 1984, 1990), its envelope glycoprotein D (gD) (Stanberry et al., 1987; Berman et al., 1988) or
synthetic peptides derived from gD (Watari et al., 1987; Geerligs et al., 1989; Brynestad et al., 1990) elicited serum antibodies and conferred complete or partial protection against intravaginal (i.vag.) challenge with HSV-2. Similarly, exposing the genital mucosa to soluble (Batty & Warrack, 1955; Yang & Schumacher, 1979; Thapar et al., 1990a, b), cellular (Kerr & Robertson, 1953; Beer & Heaves, 1978; Chipperfield & Evans, 1972; Clarke, 1984) or viral (Ogra & Ogra, 1973) antigens, including HSV-2 (Mendis et al., 1981; Merrinran et al., 1984; McDermott et al., 1984, 1987, 1990), resulted in specific IgA and IgG antibody responses in cervicovaginal secretions which were not mirrored in serum. Indeed, the observation that mucosal immunity in the genital tract resembles that found at other sites (McDermott & Bienenstock, 1979; McDermott et al., 1980) supports the notion that this site is part of the common mucosal immune system in which the various mucosas are linked together by the selective migration of IgA plasmacyte precursors originating in the intestinal and bronchus-associated lymphoid tissues (McDermott & Bienenstock, 1979; Weiss-Carrington et al., 1979; McDermott et al., 1980). Together, these results suggest that enteric immunization which stimulates IgA plasmacyte precursors destined to reside in the genital mucosa plus parenteral antigen exposure, might be an efficacious means of generating immunity to HSV-2 genital infections.

Enteric immunization requires large quantities of nonviable antigen (Mestecky et al., 1978; Czerkinsky et al., 1987; Mestecky, 1987) or replicating organisms (Melnick, 1978; Nichols et al., 1978). However, intragastric (i.g.) administration of cholera toxin (CTX), the most potent enteric immunogen known (Pierce & Gowans, 1978), induces immunity in serum and local secretions (Elson & Ealding, 1984; Liang et al., 1988). When covalently conjugated to poorly immunogenic antigens and given enterically, CTX facilitates local and systemic immunity to such antigens (Elson & Ealding, 1984; McKenzie & Halsey, 1984; Lycke & Holmgren, 1986; Liang et al., 1988; Czerkinsky et al., 1989; Tamura et al., 1989; Kikuta et al., 1990). These responses reflect the ability of CTX to bind GM1 ganglioside and activate adenyl cyclase (Holmgren, 1981; Lycke & Holmgren, 1986; Lebman et al., 1988). Thus, genital immunity to HSV-2 infection might be engendered by using CTX to deliver viral epitopes enterically.

We examined the ability of CTX to deliver a peptide derived from HSV-2 glycoprotein D (Eisenberg et al., 1984). Our results show that CTX--peptide conjugates elicited antiviral antibodies in sera and in intestinal and vaginal washings after a primary parenteral immunization alone or after a primary parenteral immunization plus secondary parenteral, enteric or i.vag. immunization. These results demonstrate that CTX can serve as a HSV-2 epitope delivery system which elicits both systemic and mucosal immunity to HSV-2.

### Methods

**Conjugation of gD(1–23) to CTX using succinimidyl-4(4-p-maleimidophenyl) butyrate (SMPB).** Synthetic gD(1–23), a peptide corresponding to residues 1 to 23 of HSV-2 gD, with cysteine added to the C terminus (Bachem) was conjugated to CTX (List Biological Laboratories) or horse spleen ferritin (Sigma) using SMPB (Pierce). All reactions occurred at room temperature. SMPB (10 mg/ml in dimethyl formamide) was added to 1 mg of protein diluted in 100 μl of activation buffer (0.01 M Na2HPO4, 0.15 M NaCl pH 8.5) at SMPB to CTX ratios ranging from 2.5 to 80:1 or at an SMPB to ferritin ratio of 20:1. After 30 min the unreacted SMPB was removed with a Sephadex G-25 column (Pharmacia) equilibrated with coupling buffer (0.01 M NaH2PO4, 0.15 M NaCl, 5 mM-EDTA pH 6.7). A 100-fold molar excess of gD(1–23) was added to SMPB-activated protein and the reactants were incubated for 30 min. Unconjugated gD(1–23) was separated with a Sephadex G-25 column equilibrated with PBS.

**Characterization of CTX-gD(1–23) conjugates.** The number of gD(1–23) residues conjugated to CTX was determined radiochemically. 125I-labelled gD(1–23) was prepared using Bolton-Hunter reagent (Amersham) and the [125I]gD(1–23) was conjugated to CTX using SMPB. Unconjugated [125I]gD(1–23) was removed by ultrafiltration (Centri-cent 30 microconcentrators; Amicon). Radioactivity associated with the CTX-[125I]gD(1–23) conjugate was determined (1282 Compgamma Universal Gamma Counter; LKB). The number of gD(1–23) residues per CTX was estimated from the specific activity of [125I]gD(1–23) (1.73 TBq per mmol). Conjugation of [125I]gD(1–23) to CTX was confirmed by HPLC (TSK 2000 column; Beckman).

**Sheep erythrocyte (SRBC) binding assay.** An SRBC binding assay was used to determine whether the conjugation of gD(1–23) to CTX altered its ability to bind GM1 ganglioside displayed on cell membranes. All reactions occurred at 37 °C. SRBC (Cedarlane) were washed with 1% foetal bovine serum in PBS (FBS-PBS) and resuspended in an equal volume of FBS-PBS. Aliquots (125 μl) of the cell suspension were added to a two-fold dilution series of CTX or CTX-gD(1–23) conjugates in FBS-PBS, beginning at 4 μg/ml. After 1 h, the cells were washed with FBS-PBS and resuspended for 1 h in 125 μl of FBS-PBS containing 100 μg/ml of IgG monoclonal antibodies specific for the CTX B subunit (CTB). Hybridoma cells secreting these antibodies were developed in our laboratory from BALB/cJ mice (Charles River) immunized with CTX and were proven specific for CTB by ELISA and Western blot analysis (data not shown). The antibodies were purified chromatographically from ascites fluid using Protein A-Sepharose (Pharmacia).

The SRBC were washed with FBS-PBS and resuspended for 1 h in 125 μl of goat anti-mouse IgG (Sigma) diluted 1000-fold in FBS-PBS. Cells were washed with FBS-PBS, resuspended in 250 μl of borate buffer (15 mM-boric acid, 0.15 M NaCl pH 9.5) containing 1 mg/ml of p-nitrophenyl phosphate (Sigma) and incubated for 15 min. The cells were centrifuged and the absorbance of the supernatant was measured spectrophotometrically at 405 nm (Titertek Multiskan Plus MKII; ICN).

**Rat skin bluing test.** A skin test in male Sprague-Dawley rats (Charles River) was used to assess the CTX-mediated physiological toxicity of the CTX-gD(1–23) conjugates (Craig, 1971). Shaved flanks were injected intracutaneously with PBS or 100 μl of PBS containing 50 ng of CTX or CTX-gD(1–23) conjugates containing 50 ng of CTX. Twenty-
four hours later, 200 μl of a saturated Coomassie blue dye solution diluted 1:20 in PBS was injected intravenously. After a further 24 h, the areas of blue induration wheals surrounding the injection sites were determined on the serosal skin surfaces.

**Immunization of mice and collection of samples.** Female BALB/cJ mice aged 6 to 8 weeks (Charles River) were used (McDermott et al., 1990). Mice were immunized intraperitoneally (i.p.) on days 0, 14, 28 and 42 with 10 μg of the immunogens in 200 μl of PBS or PBS alone.

Doses of CTX-gD(1-23) conjugates were standardized to contain 10 μg of CTX without allowance for the mass of conjugated gD(1-23). The amount of CTX in each conjugate was determined by ELISA using unconjugated CTX as a standard. All reactions occurred at 37°C. Microtitre plate wells (Immulon 2; Dynatech) were incubated with 100 μl of anti-CTX IgG monoclonal antibodies in PBS (1 μg/ml) for 2 h. The wells were washed with 0.05% Tween 20 in 0.02 M-Tris–HCl pH 7.4, containing 0.15 M-NaCl and 0.005 M-MgCl₂ (referred to as Tris-T) and incubated for 30 min with 150 μl of 0.1% BSA in 0.02 M-Tris-HCl buffered saline at pH 7.4 (referred to as diluent). After washing with Tris-T, the wells were incubated for 1 h with 100 μl of a dilution series of CTX standards of known protein concentration or with CTX–gD(1-23) conjugates in diluent. Wells were washed with Tris-T and incubated for 1 h with 100 μl of 10 μg/ml anti-CTX IgA monoclonal antibodies in diluent. After washing, the wells were incubated for 1 h with 100 μl of alkaline phosphatase-conjugated goat anti-mouse IgA (H chain-specific, Sigma). The wells were washed with Tris-T and 100 μl of 1:0 m-diethanolamine buffer pH 9.8 containing 0.05 M-MgCl₂ and 1:0 mg/ml p-nitrophenyl phosphate (Sigma) was added to each well. After 30 min incubation, the absorbance of the solution in each well was determined (Multiskan Titertek Plus MKII). Standard curves relating absorbance to the log₃ (CTX concentration) were developed using the CTX standards and used to estimate the CTX content of the CTX–gD(1-23) conjugates.

In other experiments, mice were immunized i.p. on day 0 and subsequent immunizations conducted on days 14 and 28 were administered either i.p., i.g. or i.vag. Intragastric immunizations consisted of 10 μg of various immunogens in 0.2 m-NaHCO₃ administered using polyethylene tubing (PE 50; Becton-Dickinson) to anaesthetized mice. Intravaginal immunization was accomplished by instilling 10 μg of immunogen in 20 μl of PBS into the vaginas (McDermott et al., 1990). Blood and vaginal washing were obtained on day 35 (McDermott et al., 1990). Intestinal washings were obtained on day 42 using the method of Elson et al. (1984). Sera, intestinal or vaginal washings from individual mice were pooled for analyses.

**Measurement of antibody titres by ELISA.** An ELISA was used to detect and quantify HSV-2-specific antibodies present in sera, intestinal and vaginal washings (McDermott et al., 1990). Threefold serial dilutions of fluid specimens beginning with dilutions of 1/27, 1/9 or 1/3, respectively, were analysed using microtitre plate wells coated with a virus-infected Vero cell lysate. Anti-HSV-2 antibodies were detected by adding alkaline phosphatase-conjugated goat anti-mouse IgG (H + L chain-specific, Sigma). In other experiments, anti-HSV-2 antibodies were detected by adding alkaline phosphatase-conjugated goat antisera specific for mouse IgG, IgA or IgM (Sigma). p-Nitrophenyl phosphate (50 μl) (1 mg/ml in 1:0 m-diethanolamine buffer pH 9.8) was added to each well and the absorbance was determined (Titertek Multiskan Plus MKII). Anti-CTX antibodies were evaluated similarly except that the wells were coated with CTX (5 μg/ml in PBS) overnight at 4°C. Antibody titres were calculated using the method of Steinke et al. (1991).

**Virus propagation and inoculation of mice.** Wild-type HSV-2 strain 333 and ΔTK-HSV-2 were propagated and stored as described previously (McDermott et al., 1984, 1987). Female BALB/cJ mice, aged 6 to 8 weeks (Charles River) were inoculated i.p. or i.vag. with 1 × 10⁶ p.f.u. of virus suspended in 10 or 200 μl of PBS, respectively. The dose of HSV-2 was 1000-fold greater than the LD₅₀, whereas the dose of non-pathogenic ΔTK-HSV-2 readily induced antiviral humoral and cellular immune responses in mice and conferred protection against lethal HSV-2 challenge (McDermott et al., 1984, 1987, 1989, 1990). HSV-2-inoculated mice were observed for signs of infection, neurological disease and death. For statistical analyses, surviving and deceased mice were assigned scores of 1 and 0, respectively (Drew & Owen, 1988). Analysis of variance was performed on these scores using the Statistical Analysis System (SAS Institute, 1985) and differences between groups were analysed using orthogonal contrasts (P ≤ 0.05).

**Results**

**Conjugation of CTX and gD(1-23)**

To determine the number of gD(1-23) moieties that could be coupled to CTX, the toxin was activated with various molar ratios of SMPB and then coupled to [¹²⁵I]gD(1-23). Fig. 1 shows that the [¹²⁵I]gD(1-23)-associated radioactivity bound to CTX was maximal at a SMPB to CTX ratio of 40:1. Using the estimated specific activity of [¹²⁵I]gD(1-23) (1.73 TBq/mmol), the number of gD(1-23) residues bound to CTX was estimated to range from two at an SMPB to CTX ratio of 5:1, to 15 at 40:1. HPLC of a 20:1 CTX–[¹²⁵I]gD(1-23) conjugate revealed that the ¹²⁵I was associated with the CTX fractions and these were eluted earlier than unconjugated CTX, thus demonstrating that the molecular size of the conjugate was greater than that of unconjugated toxin (data not shown).

To determine whether conjugation of gD(1-23) to CTX would impair its ability to bind GM₁, ganglioside and induce physiological toxicity, CTX–gD(1-23) conjugates were synthesized using SMPB to CTX ratios ranging from 5 to 40:1. Conjugate binding to GM₁, ganglioside expressed on SRBC and induction of physiological toxicity using a rat skin bluing test were evaluated. Fig. 2 demonstrates that over a 64-fold dilution range, the conjugation of the peptide to CTX using SMPB to CTX ratios of 5, 10 or 20:1 did not affect the ability of CTX to bind GM₁, ganglioside. Only at an SMPB to CTX ratio of 40:1 [the ratio that enables maximal conjugation of gD(1-23) moieties to CTX] was the GM₁ ganglioside binding ability of the toxin impaired. Fig. 3 shows that the skin test responses induced by CTX–gD(1-23) conjugates decreased appreciably at SMPB to CTX ratios of 10 and 20:1 and that a response was not measurable at 40:1. These results suggest that the conjugation of large numbers of gD(1-23) residues to CTX might alter its enteric immunogenicity by impairing both GM₁ binding and the ability to induce physiological responses.
Fig. 1. Conjugation of CTX to gD(1–23) peptide using a heterobifunctional cross-linking reagent. SMPB was reacted with CTX at various SMPB to CTX ratios. A molar excess of 125I-labelled gD(1–23) was coupled to the SMPB-activated CTX. The radioactivity associated with CTX-gD(1–23) conjugates was determined by scintillation counting (○). The molar ratio of gD(1–23) to CTX in the conjugates (■) was calculated using the specific activity of the [125I]gD(1–23).

Fig. 2. Effect of CTX-gD(1–23) peptide conjugation on binding to GM1 ganglioside. SRBC were added to a dilution series of CTX (○) or CTX-gD(1–23) prepared using SMPB to CTX ratios of 5 (●), 10 (□), 20 (■) or 40:1 (▲). Washed SRBC were then reacted with IgG monoclonal antibodies specific for CTB. Following treatment with alkaline phosphatase-conjugated goat anti-mouse IgG, washed cells were resuspended in buffer containing p-nitrophenyl phosphate. Subsequently, the SRBC were removed by centrifugation and the A405 of the supernatants was determined. The abscissa describes the concentration of CTX contained in the conjugates.

Systemic immunogenicity of various CTX–gD(1–23) conjugates

To examine the immunogenicity of various CTX–gD(1–23) conjugates, mice were immunized with conjugates prepared using SMPB to CTX ratios from 5 to 40:1. Following four i.p. immunizations with CTX–gD(1–23) conjugates or unconjugated CTX, the anti-CTX serum antibody response on day 49 was 12(log3) (Fig. 4). Thus, despite the effects of viral peptide conjugation on GM1 ganglioside binding and physiological toxicity, peptide conjugation to CTX had no effect on the systemic immunogenicity of the CTX. The antiviral antibody titres were also unaffected by the number of gD(1–23) residues conjugated to CTX; all CTX–gD(1–23) conjugates elicited anti-HSV-2 serum antibody titres of 9(log3), a level that was 2(log3) less than that noted following immunization with ΔTK–HSV-2. In contrast, gD(1–23) or a ferritin–gD(1–23) conjugate prepared using an SMPB to CTX ratio of 20:1 were poorly immunogenic, thus demonstrating the efficacy of CTX as a parenteral immunogenic carrier.
Immunity to cholera toxin–peptide conjugates

Table 1. Protection against systemic HSV-2 infection elicited by systemic immunization with CTX–gD(l–23) peptide conjugates

<table>
<thead>
<tr>
<th>Immunogen*</th>
<th>Survival (%)</th>
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<tr>
<td>PBS</td>
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<td>gD(l–23)</td>
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</tr>
<tr>
<td>CTX</td>
<td>0</td>
</tr>
<tr>
<td>Ferritin–gD(l–23)</td>
<td>0</td>
</tr>
<tr>
<td>5:1 CTX–gD(l–23)</td>
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</tr>
<tr>
<td>10:1 CTX–gD(l–23)</td>
<td>63†</td>
</tr>
<tr>
<td>20:1 CTX–gD(l–23)</td>
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<td>40:1 CTX–gD(l–23)</td>
<td>63†</td>
</tr>
<tr>
<td>ATK–HSV-2</td>
<td>80</td>
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</table>

* Groups of eight to 10 female BALB/cJ mice were inoculated i.p. on days 0, 14, 28 and 42, with PBS, gD(l–23) peptide, CTX, ATK–HSV-2 or conjugates of gD(l–23) and ferritin or CTX synthesized using SMPB. CTX–gD(l–23) conjugates were synthesized using the indicated molar ratios of SMPB to CTX. On day 56, mice were inoculated i.p. with 1 × 10^6 p.f.u. of wild-type HSV-2 and the proportion of animals surviving on day 66 was determined.
† The survival difference between mice receiving CTX–gD(l–23) conjugates compared to those receiving CTX, gD(l–23) ferritin–gD (l–23) or PBS was significant (P < 0.05).

Intraperitoneal immunization of mice with ATK–HSV-2 and all CTX–gD(l–23) conjugates correlated with protection from a lethal i.p. challenge with HSV-2 (Table 1). However, i.p. immunization with PBS, CTX, gD(l–23) or the ferritin–gD(l–23) conjugate failed to protect against a lethal i.p. challenge with the virus. These results indicated that CTX–gD(l–23) conjugates given systemically elicited antiviral serum antibody responses which correlated with protection against a lethal systemic challenge with HSV-2. Moreover, the magnitude of such immunity was independent of whether the toxin–peptide conjugates could bind to GM1 ganglioside and trigger physiological sequelae (compare Fig. 2 and 3).

Immunogenicity of a CTX–gD(l–23) conjugate after immunization by various routes

Since no differences in immunity were detected for any of the CTX–gD(l–23) conjugates used (Fig. 4 and Table 1), subsequent studies were done with CTX–gD(l–23) conjugates prepared using an SMPB to CTX ratio of 5:1. This conjugate contains approximately 2 mol of gD(l–23) per mol of CTX (Fig. 1), has maximal GM1 binding ability (Fig. 2) and will induce potent physiological responses (Fig. 3). The number of immunizations was decreased to three as antiviral serum antibody titres did not improve after the third i.p. immunization (data not shown).

Fig. 5 shows that anti-CTX antibody responses in sera were similar in both titre and isotype distribution for CTX or CTX–gD(l–23) conjugates after systemic priming and boosting by various routes. This supports the...
notion that the conjugation of gD(1-23) to CTX does not affect serum responses when priming occurs systemically.

There was a pronounced effect of the immunization route on the antibody titres and isotype distribution of the anti-CTX responses in intestinal washings (Fig. 5). I.p/i.p. immunization with either CTX or the CTX–gD(1-23) conjugate elicited an IgG response and a smaller IgA response, whereas i.p./i.g. immunization resulted primarily in an IgA response with smaller IgG titres. Anti-CTX antibody titres in intestinal washings after i.p./i.vag. immunization with CTX or CTX–gD(1-23) conjugate were reduced compared to the other routes. Anti-CTX antibodies were detected in vaginal washings after immunization by all routes (Fig. 5). The anti-CTX IgG titres induced by CTX and CTX–gD(1-23) conjugate were similar for all immunization routes although IgA antibody titres were highest after i.p./i.p. immunization followed by i.p./i.g. and i.p./i.vag. immunizations. Anti-CTX antibodies were not detected in sera, intestinal or vaginal washings after inoculation with PBS, gD(1-23) or ΔTK–HSV-2.

Peptide gD(1-23) alone did not elicit an anti-HSV-2 response in serum or washings regardless of the immunization route (Fig. 6). Antiviral serum antibodies were detected after immunization with CTX–gD(1-23) administered by each route and IgM titres exceeded IgG titres. Immunization with ΔTK–HSV-2, however, elicited higher IgG than IgM anti-HSV-2 titres. Other studies using an enzyme-linked immunosorbent spot assay (Lycke, 1986) supported the observation that CTX–gD(1-23) elicits predominantly anti-HSV-2 IgM plasma-cytes (data not shown). The antiviral serum antibody titres elicited by ΔTK–HSV-2 were similar for all routes. The CTX–gD(1-23) conjugate, however, elicited the highest antiviral serum titres following i.p./i.p. immunization with lower responses after i.p./i.g. and i.p./i.vag. immunization.

Anti-HSV-2 responses in intestinal washings consisted of IgA only (Fig. 6). Immunization with CTX–gD(1-23) by the i.p./i.g. route resulted in an IgA titre of 4(log3) in intestinal washings compared to 2(log3) after i.p./i.p. or i.p./i.vag. immunizations., ΔTK–HSV-2 also elicited an anti-HSV-2 titre of 2(log3) in intestinal washings after i.p./i.p. immunization.

Anti-HSV-2 titres in vaginal washings following immunization with CTX–gD(1-23) were highest after i.p./i.p. immunizations, small after i.p./i.g. immunization and not detectable after i.p./i.vag. immunization. This response consisted of IgG and IgA after i.p./i.p. immunization whereas only IgA was detectable after i.p./i.g. immunization. ΔTK–HSV-2 elicited high anti-HSV-2 IgG and IgA titres in vaginal washings regardless of the immunization route.

Viral challenge

Mice were inoculated once via the i.p. route alone (i.p./-) or by the i.p./i.p., i.p./i.g. and i.p./i.vag. routes with the CTX–gD(1-23) conjugate, attenuated ΔTK–HSV-2 or CTX or gD(1-23) peptide and lethally challenged i.vag. with HSV-2 (Table 2). As expected, mice immunized with ΔTK–HSV-2 survived regardless of the immunization route (McDermott et al., 1984, 1987). Mice immunized with CTX–gD(1-23) by the i.p./i.p. or i.p./i.g. routes had survival rates that were
Table 2. Protection against i.vag. HSV-2 infection elicited by immunization with CTX–gD(1–23) peptide conjugates

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Routes of immunization*</th>
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<td>PBS</td>
<td>i.p./-</td>
<td>i.p./i.p</td>
<td>i.p./i.g</td>
<td>i.p./i.vag</td>
</tr>
<tr>
<td>gD(1–23)</td>
<td>20</td>
<td>10</td>
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<td>10</td>
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<td>20</td>
<td>40</td>
<td>40†</td>
<td>20†</td>
</tr>
<tr>
<td>ΔATK-HSV-2</td>
<td>100†</td>
<td>100†</td>
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* Groups of 10 BALB/cJ mice were immunized i.p. on day 0 and subsequently on days 14 and 28 by the i.p., i.g. or i.vag. route as described in Table 1. The CTX–gD(1–23) conjugate was prepared using a SMPB to CTX ratio of 5:1. On day 45, mice were inoculated i.vag. with 1 x 10⁶ p.f.u. of wild-type HSV-2 and the proportion of animals surviving on day 55 was determined.
† Mice in these groups had survival frequencies significantly greater than 0 (P < 0.001).

Discussion

In this study, we demonstrate that the ability of CTX to bind GM₁ ganglioside and induce physiological responses was impaired by conjugation to the gD(1–23) peptide. Despite this, the immunogenicity of CTX and its ability to act as a carrier for gD(1–23) after systemic immunization were unaffected by the conjugation of gD(1–23) peptide. Indeed, the antiviral antibody response elicited by repeated systemic immunization with CTX–gD(1–23) correlated with protection against a lethal i.vag. infection with HSV-2.

CTX, the most potent enteric immunogen known, can function as a systemic or oral delivery molecule for soluble (Elson & Ealding, 1984; McKenzie & Halsey, 1984; Czerkinsky et al., 1989) or particulate (Liang et al., 1988) antigens. The properties of CTX are due to its ability to bind GM₁ ganglioside and induce immunomodulation (Holmgren, 1981; Lycke & Holmgren, 1986; Lebman et al., 1988). Covalent linkage of antigen to CTX can enhance antibody responses compared to mixing of antigen and CTX (McKenzie & Halsey, 1984); however, this can negatively affect GM₁ ganglioside binding and the induction of physiological responses by CTX (Liang et al., 1989).

In the present study, the ability of CTX to bind GM₁ ganglioside and induce physiological responses decreased as more gD(1–23) peptide was conjugated to the toxin (Fig. 2 and 3). Despite this, antiviral antibody responses elicited by the various CTX–gD(1–23) conjugates were independent of the molar ratio of CTX to gD(1–23) peptide (Fig. 4). The binding of CTX to GM₁ ganglioside may be less important after i.p. than i.g. immunization; CTX that is not bound to GM₁ ganglioside expressed on gut epithelium is excreted. A reduction in CTX-induced physiological responses due to peptide conjugation may also be relatively unimportant since only minute amounts of active toxin are required systemically to potentiate the immune response to CTB (Lycke & Holmgren, 1986).

The systemic immunogenicity of N-terminal peptides from HSV requires large doses of 50 to 150 µg (Geerligs et al., 1989; Watari et al., 1987). In the present study, the peptide dose ranged from 0.7 µg for the 5:1 CTX–gD(1–23) conjugate to 5 µg for the 40:1 CTX–gD(1–23) conjugate, suggesting that conjugation of peptide to CTX is an extremely potent approach to the delivery of viral epitopes.

CTX can act as a systemic or mucosal adjuvant. This is particularly important for expression of immunity in the...
female genital tract since immunoglobulins secreted there are derived from both systemic and mucosal sources (Tourville et al., 1970; Waldman et al., 1971; Rebello et al., 1975; Kutteh et al., 1988; McDermott et al., 1990). The dissemination of gut immune response to distant mucosal sites via lymphocyte traffic (McDermott & Bienenstock, 1979; McDermott et al., 1980) and transudation of serum antibodies into genital secretions (Kutteh et al., 1988) support oral immunization as a route for the induction of genital immune responses (McDermott & Bienenstock, 1979; McDermott et al., 1980). Oral immunization with replicating antigens can elicit secretory IgA in vaginal washings (Nichols et al., 1978), whereas non-viable antigen given orally provokes poor antibody responses in genital secretions unless local antigen exposure also occurs (Parr et al., 1988; Thapar et al., 1990a, b). Intraperitoneal priming and i.g. boosting (i.p./i.g.) with CTX–gD(1–23) resulted in a greater antiviral IgA response in gut washings as compared to i.p./i.p. or i.p./i.vag. administration. Intraperitoneal/i.vag. immunization resulted in antiviral IgA in vaginal washings which was probably due to local synthesis (such antibodies were not detected in sera) and may be a result of IgA plasmocyte precursor migration to genital tissue (McDermott & Bienenstock, 1979; McDermott et al., 1980).

Intraperitoneal/i.vag. immunization with the CTX–gD(1–23) conjugate was ineffective in stimulating antiviral antibodies in vaginal washings. Antigen uptake across the vaginal epithelium is inefficient (Parr & Parr, 1990) and, thus, i.vag. boosting might not have occurred. Intraperitoneal/i.vag. administration of CTX–gD(1–23) elicited IgG and IgA anti-HSV-2 responses in sera and vaginal washings, respectively, which correlated with resistance to i.vag. challenge with the virus. However, such antibodies in vaginal washing were probably locally synthesized since serum IgG does not transudate into vaginal secretions in mice (McDermott et al., 1990) and anti-HSV-2 IgA was not detectable in sera.

The safety of non-viable subunit vaccines makes them an attractive alternative to attenuated vaccines. The gD(1–23) peptide is a possible component for a paternal subunit vaccine (Watari et al., 1987; Geerligs et al., 1989; Brynestad et al., 1990). This study has shown that i.p./i.p. or i.p./i.vag. immunization with gD(1–23) conjugated to CTX elicits significant protection against both systemic and mucosal infection by HSV-2 and, thus, induces broader resistance to infection. This is a useful model for assessing vaccine materials that target antigen to specific receptors on gut epithelium and modulate systemic and mucosal immune responses.

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References


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