INFECTION AND IMMUNITY, May 1995, p. 2021–2025 Vol. 63, No. 5
0019-9567/95/$04.00+0

Local and Systemic Antibody Responses to Dextran-Cholera Toxin B Subunit Conjugates

CHARLOTTA BERGQUIST, TERESA LAGERGÅRD, MARIANNE LINDBLAD, AND JAN HOLMGREN*

Department of Medical Microbiology and Immunology, Göteborg University, Göteborg, Sweden

Received 29 December 1994/Returned for modification 16 February 1995/Accepted 2 March 1995

This study was designed to test local and systemic immunity following mucosal immunization with a polysaccharide-protein conjugate. After preparing and characterizing dextran-cholera toxin B subunit (CTB) conjugates, we studied their immunogenicity in mice following systemic or mucosal immunizations. Dextran was chosen as a model polysaccharide antigen and conjugated via adipic acid dihydrazide and *N*-succinimidyl-3-(2-pyridyldithio)propionate to CTB. Mice were immunized either subcutaneously, intranasally, or perorally three times, and cholera toxin was used as an adjuvant for the mucosal immunizations. Three conjugates with different molecular weights for dextran (40,000 and 76,000) or varying dextran/CTB molar ratios were tested. Peroral immunizations with all conjugates evoked local immunoglobulin A (IgA) antibody responses against dextran in the small intestine, and intranasal immunizations did the same in the lung. Intranasal immunizations also elicited serum antibody titers that were significantly higher than or equal to those after subcutaneous immunizations. Intranasal immunizations evoked serum IgG antidextran titers which were dependent on the dextran/CTB molar ratio and inversely related to the local IgA response, which was not the case for subcutaneous immunizations. This is the first study of local and systemic immunity following mucosal immunization with a polysaccharide-protein conjugate. The results show that it is possible to evoke a local as well as a systemic antibody response against a polysaccharide by conjugating it to CTB and using an appropriate route of immunization.

Polysaccharides (PS) are T-cell-independent antigens of type 2; as such, they are poor immunogens, they induce mainly an immunoglobulin M (IgM) antibody response, and no booster effect is obtained after repeated immunizations. Infants under 15 months of age do not respond to PS antigens, and thereafter, the systemic immune response is correlated with age (30, 33). Many PS antigens are important bacterial virulence factors, i.e., capsular polysaccharides (CPS) or lipopolysaccharides (LPS). These types of antigens are of interest as vaccine candidates against a number of different bacterial pathogens, e.g., *Haemophilus influenzae*, group B streptococci, *Streptococcus pneumoniae*, *Staphylococcus aureus* types 5 and 8, *Salmonella typhi*, and *Vibrio cholerae*. By coupling the PS covalently to a protein carrier, such as tetanus or diphtheria toxoid, they can be converted to T-cell-dependent antigens, which results in an increase in the immunogenicity and a memory antibody response (3). This has been done successfully with several PS such as *H. influenzae* type b CPS (36), group B streptococcus CPS (26, 31), *Streptococcus pneumoniae* CPS (13), *Staphylococcus aureus* type 5 and 8 CPS (12), and *Salmonella typhi* Vi PS (46), although as yet *H. influenzae* type b CPS is the only PS used in a conjugate vaccine licensed for human use. All these vaccines and vaccine candidates are designed for systemic administration.

Since most bacteria enter the host through one of the mucosal surfaces and it has been shown for several bacterial pathogens that local antibodies secreted onto a mucosal surface can prevent colonization and subsequent disease (5, 6, 17, 48), it may be desirable to induce a local immune response against a bacterial PS antigen by mucosal vaccination. We have used cholera toxin B subunit (CTB) in this study as a carrier for a model PS antigen, dextran. CTB is the nontoxic binding moiety of cholera toxin (CT) and consists of a ring of five identical subunits with a total molecular weight of 58,000 (20, 38). CTB has recently attracted much attention as a promising mucosal carrier for different antigens such as proteins, peptides, or haptens (4, 9, 10, 21, 28), and it was therefore deemed important to test whether CTB would also provide a mucosal immune response to a conjugated PS antigen.

In this study, we have prepared different dextran-CTB conjugates and studied the antibody responses in serum as well as in different organs of mice following peroral (PO), intranasal (IN), or subcutaneous (SC) immunizations.

MATERIALS AND METHODS

Antigens. Dextrans with average molecular weights of 40,000 and 76,000 (designated dextran40 and dextran76) consisting mainly of linear α(1-6)-linked glycopyranosyl residues were used (Sigma Chemical Co., St. Louis, Mo.). Recombinant CTB was purified from *V. cholerae* 358 by using the plasmid pML LCTB tac1 and hexametaphosphate precipitation as described previously (27), followed by gel filtration on a Sephacryl S100 column (Pharmacia, Uppsala, Sweden).

Chemicals. The following reagents were used: *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) (Pharmacia Fine Chemicals, Uppsala, Sweden), adipic acid dihydrazide (ADH) (Fluka Chimie AG, Buchs, Switzerland), trinitrobenzenesulfonic acid (Eastman Chemical Products Inc.), CT (List, Campbell, Calif.), heparin (Lövens Kemiske Fabrik, Ballerup, Denmark), dithiothreitol (Calbiochem Corp., La Jolla, Calif.), orthophenylenediamine, *N*-hydroxysuccinimidobiotin, avidin, anthrone, and CNBr (Sigma Chemical Co.).

Antibodies. Anti-CTB mouse monoclonal IgG1 LT39, reacting specifically with the pentamer form of CTB, was prepared in-house (44). Antidextran mouse monoclonal IgG2b (F16B3), IgM (F4F7), and IgA (17-9) were gifts from C. Fernandez, Karolinska Institute, Stockholm, Sweden (14).

Conjugation procedure. The method of conjugation is a combination of two previously described methods (7, 8). The PS dissolved in water was activated with 0.32 mg of CNBr per mg of PS at pH 10.5, and this pH was maintained with 0.1 M NaOH for 5 to 7 minutes, after which ADH was added to a final concentration of 0.25 M in 0.25 M $NaHCO_3$. The pH was adjusted to 8.5, and the mixture was gently stirred overnight at 4°C. The derivatized PS was extensively dialyzed against H_2O and freeze-dried. We measured the amino groups by the trinitrobenzenesulfonic acid assay with ADH as a standard (23). In the second step, SPDP, a heterobifunctional coupling reagent, was used according to the manufacturer's description for protein conjugation with the following modifications.

* Corresponding author. Mailing address: Department of Medical Microbiology and Immunology, Guldhedsgatan 10A, S-413 46 Göteborg, Sweden. Phone: 4631-604911. Fax: 4631-820160.

TABLE 1. Overview of the immunization scheme

Group no.	Antigen	No. of mice	Immunization route	μg of dextran/dose (vol., μl)
1a	Dextran76 + -40 and CTB	8	SC	10 (200)
1b	Dextran76 + -40 and CTB	5	IN	10 (2 × 25)
1c	Dextran76 + -40 and CTB	4	PO	30 (500)
2a	Dextran40-CTB	8	SC	10 (200)
2b	Dextran40-CTB	8	IN	10 (2 × 25)
2c	Dextran40-CTB	8	PO	30 (500)
3a	Dextran76I-CTB	8	SC	10 (200)
3b	Dextran76I-CTB	8	IN	10 (2 × 25)
3c	Dextran76I-CTB	6	PO	30 (500)
4a	Dextran76II-CTB[a]	7	SC	10 (200)
4b	Dextran76II-CTB	6	IN	10 (2 × 25)

[a] The dextran76II-CTB conjugate was not given PO.

SPDP was added to the derivatized PS (10× molar excess over amino group content) in 0.1 M sodium phosphate buffer (pH 8.5)–0.1 M NaCl. SPDP was also added to CTB (3× molar excess) in sodium phosphate buffer (pH 7.5)–0.1 M NaCl. The 2-pyridyl disulfide containing PS was then reduced with 50 mM dithiothreitol, and the excess dithiothreitol, released *N*-hydroxysuccinimide, and pyridin-2-thione were removed from the PS with a Sephadex G-25 prepacked PD10 column (Pharmacia). The amount of available SH groups was determined with the Ellman reaction (11). The degree of 2-pyridyl disulfide derivatization of CTB was measured spectrophotometrically at 343 nm, and the derivatized CTB and PS were mixed with a 1:1 molar ratio of SH groups on PS and 2-pyridyl disulfide on CTB. The mixture was reacted overnight at room temperature, and the released pyridin-2-thione from CTB was quantitated spectrophotometrically as an indication of the degree of conjugation. The molar ratio of dextran to CTB in the conjugates was varied by adding different amounts of SPDP to the dextran. The conjugate was concentrated on an Amicon stirred concentration cell (Amicon) and purified by gel filtration on a Sephacryl S300 column (2 cm^2 by 60 cm) (Pharmacia). The hexose content was determined by the anthrone reaction (39) with uncoupled dextran as standard. Protein content of the fractions was monitored by measuring the A_{280}. Protein determination of the concentrated conjugate was performed with the Lowry protein determination kit from Sigma Chemical Co.

Estimation of unbound dextran in conjugate preparations. The conjugates were adsorbed onto GM1-coated polyethylene glycol-based beads in an Eppendorf test tube and incubated for 2 h at room temperature. The beads were centrifuged for 2 to 3 min, and supernatants were analyzed for dextran by the anthrone reaction. CTB was eluted from the particles with 50 mM sodium citrate buffer, pH 2.8. The GM1-coated beads were a gift from Jan-Erik Månsson, Department of Neurochemistry, Göteborg University, Göteborg, Sweden.

Immunizations. Male and female C57BL mice aged 10 weeks were used. C57BL mice were chosen for this study because they respond well to dextrans (16). The groups of mice and doses used in this study are shown in Table 1. The conjugates were diluted in phosphate-buffered saline (PBS) for SC and IN immunization and in PBS with 3% Na_2CO_3 for PO immunization. The doses were based on the dextran content, 10 μg for SC and IN immunizations and 30 μg for PO immunizations. CT was used as a mucosal adjuvant, 1 μg for IN and 5 μg for PO immunizations. Mice were sacrificed 1 week after the last immunization, and blood was drawn from the subclavian vein. The mice were then perfused with 0.1% heparin-PBS through the heart until all blood was removed, after which the lungs and small intestines were collected. The organs were washed in 0.1% heparin-PBS, frozen, and kept at −20°C. For analyses of antibody contents, they were then extracted with 2% (wt/vol) saponin-PBS, 1 μl/mg of organ, and allowed to thaw in the saponin in order to permeabilize the cell membranes (8a, 35). The organs were centrifuged, and the supernatants were analyzed for specific antibodies against dextran and CTB. In order to be able to estimate the amount of contaminating serum in the organ extracts, the following control experiment was performed. Mice were injected intravenously with the monoclonal antibody LT39. After 20 h, they were sacrificed and treated as described above. No organ extract contained more than 2% of the serum titer of LT39, which shows that these extracts were not significantly contaminated with serum and thus that the antibody levels in the organs in excess of 2% of the serum titer probably represent local synthesis.

Enzyme-linked immunosorbent assay (ELISA) methods. (i) Detection of antidextran antibodies. Plates coated with 3 μg of avidin per ml overnight and incubated with 3 μg of biotinylated dextran per ml were used (40). Test sera or organ extracts were added in threefold dilutions. Dextran-specific mouse monoclonal antibodies were used as positive controls. The titers of each plate were adjusted so that these controls were comparable.

(ii) Detection of anti-CTB antibodies (42). Plates coated with 0.3 μM GM1 overnight and incubated with 0.5 μg of CTB per ml were used. Test sera or organ extracts were added in threefold dilutions. LT39 was used as the positive control for detection of IgG. IgA and IgM controls were mouse serum and an extract of small intestine from a preliminary experiment used in the same way as for the dextran ELISA, respectively.

In both ELISA methods, anti-mouse IgM, IgG, and IgA horseradish peroxidase-conjugated antibodies (Jackson) were added and developed with orthophenylenediamine and H_2O_2. The antibody contents are expressed as the serum dilution giving an A_{450} of 0.4 above the background level, 0.05. The initial serum dilution was 1:100 for IgG and 1:30 for IgA and IgM. The initial organ dilution was 1:3. All antibody titers are given as geometric mean (GM) ± 1 standard deviation (SD).

Characterization of conjugates. The GM1-ELISA was used for detection of CTB and dextran bound to CTB following gel filtration. The fractions and reference CTB were added to GM1-coated plates blocked with 0.1% bovine serum albumin-PBS in threefold dilutions, and LT39 or antidextran monoclonal antibodies were used for detection.

Statistics. Analysis of antibody titers was performed on logarithmically transformed data. GM and SD were calculated. Student's *t* test was used to compare mean values of different groups. Statistical significance was designated as $P < 0.05$.

RESULTS

Characterization of conjugates. The degree of ADH derivatization of the dextrans was 4.4% (wt/wt) for dextran40 and 5.7% for dextran76 used for CTB conjugates, which corresponds to an ADH/dextran molar ratio of 10:1 and 25:1, respectively. The SPDP derivatization of CTB gave 1.2 to 2.9 for SPDP/CTB molar ratio. The GM1 binding capacity of CTB in the GM1 ELISA was not diminished by the SPDP derivatization as determined by GM1-ELISA (data not shown).

Typically, 50% or more of the SPDP-derivatized protein had reacted with the reduced dextran in the conjugation mixture after 24 h. Gel filtration elution profiles of one conjugate (dextran76I-CTB) and unconjugated CTB are shown in Fig. 1, and the other conjugates showed similar elution profiles (data not shown). The fractions corresponding to the highest molecular weight (eluting with the void volume) were collected and concentrated. The compositions and yields of the three conjugates are shown in Table 2. These conjugates reacted with both anti-CTB and antidextran monoclonal antibodies in a GM1 ELISA. The amount of free dextran in the conjugates was 13 to 22% and was assayed before each immunization.

Immune responses. (i) Serum antibody responses. Serum antibody titers are shown in Table 3. Preimmune sera contained no anti-CTB antibodies and only low levels of antidextran IgM antibodies, but neither IgG nor IgA antibodies were detected. Dextran given together with CTB but not conjugated (and CT for mucosal immunizations) elicited only IgM, not IgG or IgA, titers. The IgM titer increase in these groups was two- to fourfold. All dextran-CTB conjugates, however, elicited serum IgG titers against dextran. This strongly indicates that the dextran was functionally converted into a T-cell-dependent-type antigen. Titers were significantly higher ($P < 0.001$) after IN immunizations compared with after SC or PO immunizations. The molecular weight of the dextran or the PS/protein ratio did not significantly influence the magnitude of the serum antibody response to dextran following SC immunizations. After IN immunizations, on the other hand, groups 2b and 3b had significantly ($P < 0.001$) higher IgG titers compared with group 4b, which indicates that the PS/protein ratio is of importance. All mice immunized with conjugates or unconjugated material also responded against CTB.

(ii) Local antibody responses. The local IgA responses as measured in extracts of lung and small intestine are shown in Fig. 2. Neither conjugated nor unconjugated dextran elicited local IgA following SC immunization. In contrast, IN and PO immunization with conjugated dextran gave rise to local IgA responses against dextran. As opposed to the serum responses, both the size of the dextran and the dextran/CTB ratio influ-

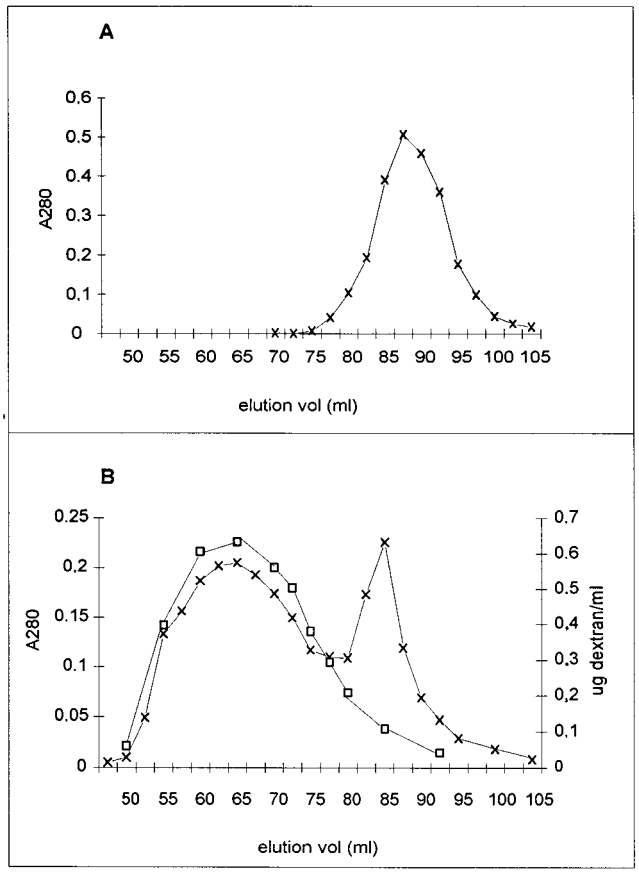

FIG. 1. Gel filtration of CTB alone (A) and CTB-dextran conjugate (B). X's represent A_{280}, and squares represent micrograms of dextran per milliliter.

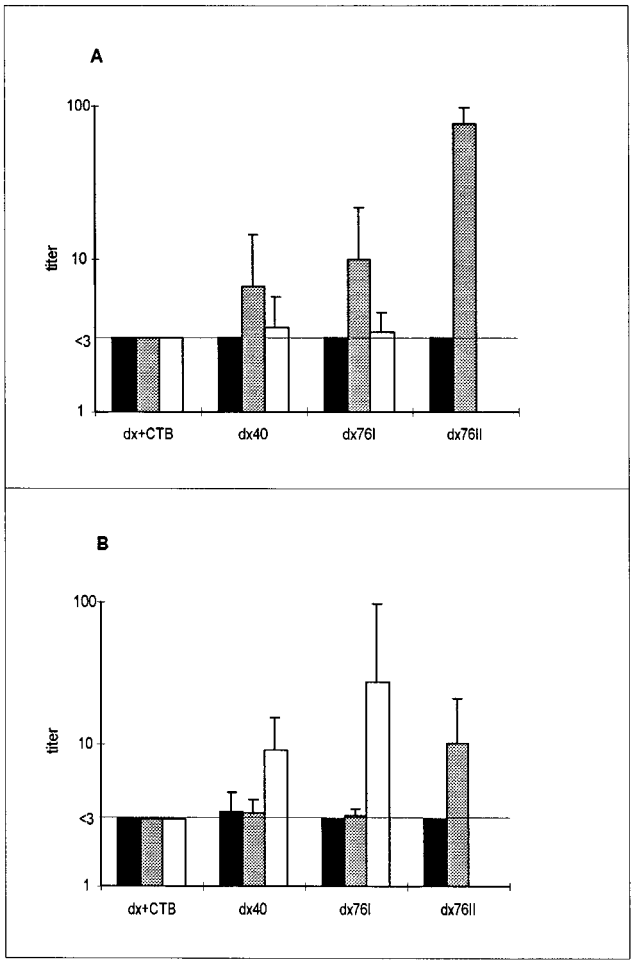

FIG. 2. Local antidextran IgA titers in extracts of lung (A) and small intestine (B). Black bars represent SC immunizations, gray bars represent IN immunizations, and white bars represent PO immunizations. Titers of <3 were not detected.

enced the magnitude of the immune response. The dextran76II-CTB conjugate elicited the highest IgA titers in both lung and small intestine following IN immunization ($P < 0.001$ and $P < 0.01$, respectively). After IN or PO immunization with the dextran40-CTB conjugate, the local IgA titers were slightly lower than with the dextran76I-CTB conjugate, but this difference was not statistically significant. The CTB responses were very similar whether any of the conjugates or unconjugated material was used; there was no significant difference between groups receiving different conjugates. The anti-CTB IgA titers in extracts of lung from representative groups were 3.5 (2.3 to 5.2) after SC immunization, 12,500 (5,300 to 29,400) after IN immunizations, and 86 (21 to 356) after PO immunizations. The corresponding data for extracts of the small intestine are 4.3 (2.9 to 6.2), 561 (312 to 1,010), and 2,760 (1,800 to 4,220). In conclusion, the IgA responses against both dextran and CTB were greater in the lung than in the small intestine following IN immunization and vice versa following PO immunization.

TABLE 2. Characteristics of conjugates used for immunizations

Conjugate	Molar ratio of PS to protein (wt ratio)	% (wt/wt) conjugated dextran	Yield after gel filtration (PS) (%)
Dextran40-CTB	1:2 (1:2.8)	78	38
Dextran76I-CTB	1:4.6 (1:3.3)	87	40
Dextran76II-CTB	1:1.4 (1:1)	85	28

DISCUSSION

In this study, we have shown that it is possible to elicit a local as well as a systemic antibody response against a model PS antigen by coupling it to CTB and administering it to a mucosal surface. We used dextran as the model antigen since it is a well-characterized PS that has been used as a model type 2 T-cell-independent-type antigen in many different studies (14–16, 30). By conjugation to a protein carrier, it will be transformed into a T-cell-dependent-type antigen. The mechanism for this is not known in detail, but it is believed that T-cell epitopes on the protein carrier will provide the T-cell help necessary for induction of B cells. CTB was chosen as carrier since it has proved to be both a good mucosal immunogen and an effective carrier for other types of antigens (9, 10, 21). One reason for this is probably that CTB binds to GM1 ganglioside receptors including those on M cells and thereby facilitates uptake of the antigen (21, 22). CTB and CT have been used successfully as carriers for PS antigens, Vi from *Salmonella typhi* (45), and *V. cholerae* LPS (18), but these conjugates were not intended for mucosal administration. We used CT as a mucosal adjuvant since it is one of the best-characterized mucosal adjuvants so far, with a proven effect (21, 22). The method of coupling was chosen in accordance with the follow-

TABLE 3. Serum antibody responses

Group	Antidextran serum titer (GM ± SD)			Anti-CTB serum titer (GM ± SD)		
	IgM	IgG	IgA	IgM	IgG	IgA
1a	116 (88–218)	102 (95–110)	<30	669 (472–1,100)	52,500 (37,100–74,400)	36.8 (24–56)
1b	141 (84–236)	<100	<30	2,813 (1,655–4,781)	643,000 (429,000–964,000)	16,500 (10,500–25,900)
1c	221 (124–394)	<100	<30	257 (104–634)	25,800 (16,200–41,100)	2,210 (1,250–3,900)
2a	1,890 (831–4,290)	2,760 (1,060–7,190)	<30	1,270 (769–2,080)	92,600 (63,300–135,000)	<30
2b	5,910 (2,310–15,100)	130,000 (57,200–293,000)	34 (14–81)	2,780 (1,720–4,510)	508,000 (347,000–741,000)	55,100 (26,200–116,000)
2c	234 (111–495)	1,290 (182–9,130)	<30	636 (323–1,250)	34,200 (16,700–69,900)	2,750 (2,150–3,520)
3a	1,820 (1,420–2,320)	2,880 (1,470–5,650)	<30	888 (538–1,470)	79,100 (43,000–145,000)	<30
3b	2,740 (1,370–5,470)	97,500 (38,100–249,000)	40 (14.3–113)	2,330 (1,070–5,060)	601,000 (247,000–1,460,000)	45,600 (23,200–89,500)
3c	253 (115–558)	1,350 (371–4,910)	33.4 (8.8–127)	763 (404–1,440)	87,800 (39,500–195,000)	1,300 (1,150–1,470)
4a	1,730 (578–5,160)	2,860 (1,270–6,440)	<30	187 (94–370)	14,600 (6,610–32,100)	<30
4b	1,600 (1,070–2,410)	2,640 (1,020–6,850)	46 (13–164)	355 (123–1,020)	202,000 (139,000–295,000)	2,570 (1,200–5,530)
0[a]	56 (26–119)	<100	<30	<30	<100	<30

[a] Preimmune sera (expressed as GM ± SD) of two randomly chosen mice from each group.

ing criteria. (i) CTB should retain GM1 binding capacity; (ii) the coupling reaction should be possible to control in order to allow defined variation in the degree of derivatization and the ratio of dextran to CTB; (iii) conjugates formed should be nontoxic; and (iv) no homoconjugates should be formed. The conjugates used in this study fulfilled all of these criteria. It might be preferable to use a coupling reagent that will not yield a disulfide bond that may be reduced in vivo; however, we have not yet found a coupling reagent that will be able to meet all set criteria.

Other studies show a compartmentalization within the common mucosal immune system (19, 34), and this seems to be true also for conjugated PS: the immune response in the small intestine is strongest following PO immunization and the immune response in the lung is strongest following IN immunization.

We have also found that the PS/protein ratio is of importance after IN administration. The conjugate with less protein (dextran76II-CTB) elicited more local antibodies than did the other conjugates. This can possibly be explained as an effect of the mucosal immunodominance of CTB. However, the comparison of serum antibody titers following IN immunization with these two conjugates showed that the dextran76II-CTB conjugate elicited lower antibody titers than dextran76I-CTB, which could be explained by a lower uptake of this conjugate.

Serum antibodies against bacterial CPS or LPS have been shown to be protective in animal models as well as in human studies (1, 2, 25, 37, 41). The most important mechanism for this is probably opsonization of the bacteria with IgG antibodies and complement activation. Most bacteria enter their host through one of the mucosal surfaces, and it has been shown for several bacterial pathogens in different animal models that IgA on a mucosal surface can prevent colonization and subsequent disease (5, 6, 17, 46). There are probably several mechanisms for this, e.g., steric hindrance of adhesion, aggregation of the microorganisms, blocking of adhesion molecules, or toxin neutralization. Since neither opsonization nor complement activity is likely to be an important mechanism on a mucosal surface, these mechanisms may apply to both locally produced secretory IgA and to systemic IgG transudating from blood. Systemic vaccination of children with *H. influenzae* type b conjugate vaccines not only prevents invasive disease but also reduces nasopharyngeal carriage, which might be explained both by IgG antibodies transudated from blood to the mucosal surface (24, 47) and by boosting of a naturally acquired yet insufficient mucosal immunity resulting from exposure to homologous or cross-reactive antigens (43). In accordance with the latter explanation, it is known that natural immunity against PS following colonization or infection also involves the mucosal immune system (29, 32).

We here show that it is indeed possible to evoke a mucosal immune response against a PS antigen by conjugating it to CTB. Our results give reason to believe that it might be possible to design conjugate vaccines for mucosal administration that could give both systemic and site-directed local mucosal protective immunity against several different bacterial pathogens.

ACKNOWLEDGMENTS

We thank Anna Rudin for critical reading of the manuscript.

This work was supported by Swedish Medical Research Council grant 16X-3383.

REFERENCES

1. **Acharya, I. L., C. V. Lowe, R. Thapa, V. L. Gurubacharya, M. B. Schrestha, M. Cadoz, D. Schultz, J. Armand, D. A. Bryla, B. Trollfors, T. Cramton, R. Schneerson, and J. B. Robbins.** 1987. Prevention of typhoid fever in Nepal with the Vi capsular polysaccharide of *Salmonella typhi*. N. Engl. J. Med. **317:**1101–1104.
2. **Anderson, P., R. B. Johnston, Jr., and D. H. Smith.** 1972. Human serum activities against *Haemophilus influenzae* type b. J. Clin. Invest. **51:**31–38.
3. **Avery, O. T., and W. F. Goebel.** 1931. Chemo-immunological studies on conjugated carbohydrate proteins. V. The immunological specificity of an antigen prepared by combining the capsular polysaccharide of type III *Pneumococcus* with foreign protein. J. Exp. Med. **54:**437–447.
4. **Bäckström, M., M. Lebens, F. Schödel, and J. Holmgren.** 1994. Insertion of a HIV-1-neutralizing epitope in a surface-exposed internal region of the cholera toxin B-subunit. Gene **149:**211–217.
5. **Bessen, D., and V. A. Fischetti.** 1988. Passive acquired mucosal immunity to group A streptococci by secretory immunoglobulin A. J. Exp. Med. **167:** 1945–1950.
6. **Cantey, J. R.** 1978. Prevention of bacterial infections of mucosal surfaces by immune secretory IgA. Adv. Exp. Med. Biol. **107:**461–470.
7. **Carlsson, J., H. Drevin, and R. Axén.** 1978. Protein thiolation and reversible protein-protein conjugation. *N*-Succinimidyl 3-(2-pyridyldithio) propionate, a new heterobifunctional reagent. Biochem. J. **173:**723–737.
8. **Chu, C., R. Schneerson, J. B. Robbins, and S. C. Rastogi.** 1983. Further studies on the immunogenicity of *Haemophilus influenzae* type b and pneumococcal type 6A polysaccharide-protein conjugates. Infect. Immun. **40:** 245–256.

8a. **Czerkinsky, C.** Personal communication.

9. **Czerkinsky, C., M. W. Russell, N. Lycke, M. Lindblad, and J. Holmgren.** 1989. Oral administration of a streptococcal antigen coupled to cholera toxin B subunit evokes strong antibody responses in salivary glands and extramucosal tissues. Infect. Immun. **57:**1072–1077.
10. **Dertzbaugh, M. T., and C. O. Elson.** 1993. Comparative effectiveness of the cholera toxin B subunit and alkaline phosphatase as carriers for oral vaccines. Infect. Immun. **61:**48–55.
11. **Ellman, G. L.** 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. **82:** 70–77.
12. **Fattom, A., R. Schneerson, S. C. Szu, W. F. Vann, J. Shiloach, W. W.**

Karakawa, and J. B. Robbins. 1990. Synthesis and immunological properties in mice of vaccines composed of *Staphylococcus aureus* type 5 and type 8 capsular polysaccharide conjugated to *Pseudomonas aeruginosa* exotoxin A. Infect. Immun. **58:**2367–2374.
13. **Fattom, A., W. F. Vann, S. C. Szu, A. Sutton, X. Li, D. Bryla, G. Schiffman, J. B. Robbins, and R. Schneerson.** 1988. Synthesis and physicochemical and immunological characterization of pneumococcus type 12F polysaccharide-diphtheria toxoid conjugates. Infect. Immun. **56:**2292–2298.
14. **Fernandez, C.** 1992. Genetic mechanisms for dominant VH gene expression. The VHB512 gene. J. Immunol. **149:**2328–2336.
15. **Fernandez, C., and G. Möller.** 1977. Immune response against two epitopes on the same thymus independent polysaccharide carrier. 1. Role of epitope density in carrier-dependent immunity and tolerance. Immunology **33:**59–68.
16. **Fernandez, C., and E. Sverremark.** 1994. Immune responses to bacterial polysaccharides: terminal epitopes are more immunogenic than internal structures. Cell. Immunol. **153:**67–78.
17. **Fubara, E. S., and R. Freter.** 1973. Protection against enteric bacterial infection by secretory IgA antibodies. J. Immunol. **111:**395–403.
18. **Gupta, R. K., S. C. Szu, R. A. Finkelstein, and J. B. Robbins.** 1992. Synthesis, characterization, and some immunological properties of conjugates composed of the detoxified lipopolysaccharide of *Vibrio cholerae* O1 serotype Inaba bound to cholera toxin. Infect. Immun. **60:**3201–3208.
19. **Haneberg, B., D. Kendall, H. M. Amerongen, F. M. Apter, J.-P. Kraehenbuhl, and M. R. Neutra.** 1994. Induction of specific immunoglobulin A in the small intestine, colon-rectum, and vagina measured by a new method for collection of secretions from local mucosal surfaces. Infect. Immun. **62:**15–23.
20. **Holmgren, J.** 1981. Actions of cholera toxin and the prevention and treatment of cholera. Nature (London) **292:**413–417.
21. **Holmgren, J., C. Czerkinsky, N. Lycke, and A.-M. Svennerholm.** 1994. Strategies for the induction of immune responses at mucosal surfaces making use of cholera toxin B subunit as immunogen, carrier and adjuvant. Am. J. Trop. Med. Hyg. **50S:**42–54.
22. **Holmgren, J., N. Lycke, and C. Czerkinsky.** 1993. Cholera toxin and cholera B subunit as oral-mucosal adjuvant and antigen vector systems. Vaccine **11:**1179–1184.
23. **Inman, J. K., and H. M. Dintzis.** 1969. The derivatization of crosslinked polyacrylamide beads. Controlled induction of functional groups for the purpose of special-purpose biochemical absorbents. Biochemistry **4:**4074–4080.
24. **Kauppi, M., L. Saarinen, and H. Käyty.** 1993. Anti-capsular polysaccharide antibodies reduce nasopharyngeal colonization by *Haemophilus influenzae* type b in infant rats. J. Infect. Dis. **167:**365–371.
25. **Klugman, K. P., H. J. Koornhof, R. Schneerson, M. Cadoz, I. T. Gilbertson, J. B. Robbins, D. Schultz, and J. Armand.** 1987. Protective activity of Vi capsular polysaccharide vaccine against typhoid fever. Lancet **ii:**1165–1169.
26. **Lagergård, T., J. Shiloach, J. B. Robbins, and R. Schneerson.** 1990. Synthesis and immunological properties of conjugates composed of group B streptococcus type III capsular polysaccharide covalently bound to tetanus toxoid. Infect. Immun. **58:**687–694.
27. **Lebens, M., S. Johansson, J. Osek, M. Lindblad, and J. Holmgren.** 1993. Large-scale production of *Vibrio cholerae* toxin B subunit for use in oral vaccines. Bio/Technology **11:**1574–1578.
28. **Liang, X., M. E. Lamm, and J. G. Nedrud.** 1988. Oral administration of cholera toxin-Sendai virus conjugate potentiates gut and respiratory immunity against Sendai virus. J. Immunol. **141:**1495–1501.
29. **Lue, C., A. Tarkowski, and J. Mestecky.** 1988. Systemic immunization with pneumococcal polysaccharide vaccine induces a predominant IgA2 response of peripheral blood lymphocytes and increases of both serum and secretory anti-pneumococcal antibodies. J. Immunol. **140:**3793–3800.
30. **Mosier, D. E., and B. Subbarao.** 1982. Thymus-independent antigens: complexity of B-lymphocyte activation revealed. Immunol. Today **3:**217–222.
31. **Paoletti, L. C., M. R. Wessels, A. K. Rodewald, A. A. Schroff, H. J. Jennings, and D. L. Kasper.** 1994. Neonatal mouse protection against infection with multiple group B streptococcal (GBS) serotypes by maternal immunization with a tetravalent GBS polysaccharide-tetanus toxoid conjugate vaccine. Infect. Immun. **62:**3236–3243.
32. **Pichichero, M. E., C. B. Hall, and R. A. Insel.** 1981. A mucosal antibody response following systemic *Haemophilus influenzae* type b infection in children. J. Clin. Invest. **67:**1482–1489.
33. **Pincus, D. J., D. Morrison, C. Andrews, E. Lawrence, S. H. Sell, and P. F. Wright.** 1982. Age-related response to two *Haemophilus influenzae* type b vaccines. J. Pediatr. **100:**197–201.
34. **Quiding-Järbrink, M., G. Granström, I. Nordström, J. Holmgren, and C. Czerkinsky.** 1995. Induction of compartmentalized B-cell responses in human tonsils. Infect. Immun. **63:**853–857.
35. **Sander, B., J. Andersson, and U. Andersson.** 1991. Assessment of cytokines by immunofluorescence and the paraformaldehyde-saponin procedure. Immunol. Rev. **119:**65–93.
36. **Schneerson, R., O. Barrera, A. Sutton, and J. B. Robbins.** 1980. Preparation, characterization and immunogenicity of *Haemophilus influenzae* type b polysaccharide protein conjugates. J. Exp. Med. **152:**361–376.
37. **Simoons-Smit, A. M., A. M. J. J. Verweij-Van Vught, and D. M. Maclaren.** 1986. The role of K antigens as virulence factors in *Klebsiella*. J. Med. Microbiol. **21:**133–137.
38. **Spangler, B. D.** 1992. Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. Microbiol. Rev. **56:**622–647.
39. **Spiro, R. G.** 1966. Analysis of sugar found on glycoproteins. Determination of neutral sugars. Methods Enzymol. **8:**4.
40. **Sutton, A., W. Vann, A. Karpas, K. E. Stein, and R. Schneerson.** 1985. An avidin-biotin based ELISA for quantitation of antibody to bacterial polysaccharides. J. Immunol. Methods **82:**215–224.
41. **Svennerholm, A.-M., and J. Holmgren.** 1976. Synergistic protective effect in rabbits of immunization with *Vibrio cholerae* lipopolysaccharide and toxin/toxoid. Infect. Immun. **13:**735–740.
42. **Svennerholm, A.-M., and J. Holmgren.** 1978. Identification of *Escherichia coli* heat labile enterotoxin by means of a ganglioside immunosorbent assay (GM1 ELISA) procedure. Curr. Microbiol. **1:**19–23.
43. **Svennerholm, A.-M., J. Holmgren, L. Å. Hanson, B. S. Lindblad, F. Quereshi, and R. J. Rahimtoola.** 1977. Boosting of secretory IgA antibody responses in man by parenteral cholera vaccination. Scand. J. Immunol. **6:**1345–1349.
44. **Svennerholm, A.-M., M. Wikström, M. Lindblad, and J. Holmgren.** 1986. Monoclonal antibodies to *Escherichia coli* heat-labile enterotoxins: neutralizing activity and differentiation of human and porcine LTs and cholera toxin. Med. Biol. **64:**23–30.
45. **Szu, S. C., X. Li, R. Schneerson, J. H. Vickers, D. Bryla, and J. B. Robbins.** 1989. Comparative immunogenicities of Vi polysaccharide-protein conjugates composed of cholera toxin or its B subunit as a carrier bound to high- or lower-molecular-weight Vi. Infect. Immun. **57:**3823–3827.
46. **Szu, S. C., D. N. Taylor, A. C. Trofa, J. D. Clements, J. Shiloach, J. C. Sadoff, D. A. Bryla, and J. B. Robbins.** 1994. Laboratory and preliminary clinical characterization of Vi capsular polysaccharide-protein conjugate vaccines. Infect. Immun. **62:**4440–4444.
47. **Takala, A. K., J. Eskola, M. Leinonen et al.** 1991. Reduction of oropharyngeal carriage of *Haemophilus influenzae* type b (Hib) in children immunized with an Hib conjugate vaccine. J. Infect. Dis. **164:**982–986.
48. **Williams, R. C., and R. J. Gibbons.** 1972. Inhibition of bacterial adherence by secretory immunoglobulin A: a mechanism of antigen disposal. Science **177:**697–699.