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Induction of rat secretory IgA antibodies against cholera toxin by a synthetic peptide

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SUMMARY

There is accumulating evidence concerning the possible importance of secretory IgA antibodies in defence mechanisms against infections of the gastrointestinal tract, including cholera. Intestinal IgA antibodies are also thought to play a major role in protection against the diarrhoeogenic effects of cholera toxin. We therefore attempted to induce secretory IgA antibodies towards a reactive synthetic peptide from the cholera toxin B subunit sequence. We report that rat biliary secretory IgA antibodies against the CTP3 peptide (residues 50–64 of the B subunit) were obtained by three intra-Peyer's patch immunizations, at 2-week intervals, with CTP3 conjugated to tetanus toxoid in complete Freund's adjuvant. Purified secretory IgA fractions from bile of such immunized rats reacted with the carrier toxoid, but also with the CTP3 peptide, and with the native cholera toxin, they also partially neutralized its biological activity, as assayed by inhibition of *in vitro* cholera toxin-induced cAMP production in mouse thymocytes.

INTRODUCTION

Several reports have highlighted the importance of the local intestinal secretory IgA (sIgA) system in the antibody response against enterically administered cholera toxin (CT) in several species (Holmgren, Svennerholm & Ouchterlony, 1975; Svennerholm, Lange & Holmgren, 1978; Pierce, Cray & Sircar, 1978; Pierce, Cray & Sacci, 1982; Jertborn, Svennerholm & Holmgren, 1984; Svennerholm *et al.*, 1984). Moreover, Vaerman *et al.* (1985) demonstrated that rats immunized twice intraintestinally with CT responded by producing high levels of biliary sIgA antibodies. The sIgA fractions purified from cannulated bile precipitated with CT and neutralized CT-induced fluid accumulation in mouse and rat ligated jejunal loops. Simliar results were recently reported (Tamaru & Brown, 1985) further pointing to a possible role of sIgA antibodies in protection against CT.

The potential use of synthetic vaccines based on peptides corresponding to fragments of CT has been investigated (Jacob, Sela & Arnon, 1983; Jacob *et al.*, 1984b). Peptides called CTP1 (residues 8–20) and CTP3 (residues 50–64) of the B subunit of CT were found, after conjugation to tetanus toxoid as carrier, and parental immunizations of rats and rabbits, to induce serum IgG antibodies that effectively inhibited the biological activity of CT, as manifested both in secretion of fluid into intestinal loops of adult rats or rabbits, and in adenylate cyclase induction.

In view of the possible importance of local intestinal immunity in cholera, we attempted to induce rat sIgA antibodies against the synthetic CTP3 peptide. Here, we report that rat biliary sIgA antibodies against the CTP3 peptide were obtained that reacted with native CT and partially neutralized its biological activity.

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MATERIALS AND METHODS

Peptides, proteins and antisera

CT was purchased from Schwartz-Mann (Orangeburg, MO) and from Behring (Marburg, FRG). Tetanus toxoid (TT) was from Behring and from Rafa (Jerusalem, Israel). Peptide CTP3 (residues 50–64 of the B subunit of CT) was synthesized by the Merrifield (1963) solid-phase method and conjugated to TT as described elsewhere (Jacob *et al.*, 1983). Goat anti-rat IgA, rabbit anti-rat secretory component (SC), and rabbit anti-rat IgG were prepared as described previously (Vaerman *et al.*, 1975; Lemaître-Coelho, Jackson & Vaerman, 1977). Rabbit anti-whole-rat bile proteins were prepared by repeated subcutaneous injections of rat bile proteins in complete Freund's adjuvant (CFA). Purified goat anti-rat IgA antibodies were obtained by affinity chromatography from Sepharose-bound rat monoclonal IgA.

Immunization procedure

Four adult OFA rats (IFFA–CREDO, Belgium) were immunized three times at 2-week intervals with 100 μg of CTP3 conjugated to TT (CTP3-TT) in CFA into 8–12 Peyer's patches with a 30-gauge needle. Another four rats were immunized with native CT (100 μg) intraintestinally three times at 2-week intervals. Control rats were injected with saline.

Collection of bile

Four days after the last injection, the bile duct was cannulated as described elsewhere (Lemaître-Coelho *et al.*, 1977). The bile was collected for 5 days on ice, and in the presence of 1 mM iodoacetamide, 2 mM Na_2EDTA, 1 mM benzamidine and 15 mM NaN_3, to prevent proteolysis and bacterial growth. The bile samples were immediately filtered on AG1X8 resin columns (Biorad, Richmond, VA) to remove bile salts and pigments (Vaerman *et al.*, 1985). Then, the daily samples from individual rats were pooled and concentrated 10-fold (by vacuum dialysis) with respect to their starting volume.

Purification of bile sIgA fractions

The concentrated biles (5–7 ml) were gel-filtered on a 5 × 90 cm Ultrogel AcA22 column in 20 mM Tris-HCl buffer, pH 8·0, containing 0·34 M NaCl and 15 mM sodium azide. The sIgA fractions were quantified by OD at 280 nm using an extinction coefficient of 13·4.

Bile protein analysis

Agarose gel electrophoresis and immunoelectrophoresis were carried out according to standard procedures. The Ouchterlony test was used for the detection of antibodies against double dilutions of TT, starting at 0·5 mg/ml.

Solid-phase radioimmunoassay (RIA)

This assay was performed on antigen-coated (0·5 μg/well) V-bottomed flexible microtitre plates by the addition of three-fold serial dilutions of the test samples, followed by ^{125}I-labelled purified anti-rat IgA antibodies (10^5 c.p.m./50 μl/well). The washed and dried wells were cut and counted in a gamma counter.

Immunoblotting

CT was separated into its A and B subunits on a 5–15% SDS–polyacrylamide gel and transferred to a nitrocellulose sheet (Towbin, Stakelin & Gordon, 1979). The blot was preincubated for 1 hr with 9 mM Tris-HCl buffer, pH 7·4, containing 0·9 mM NaCl and 3% (w/v) bovine serum albumin before it was cut into strips. The strips were incubated for 2 hr with 0·3 mg/ml of the different bile sIgA fractions. After thorough washing, the strips were further incubated with ^{125}I-labelled anti-IgA antibodies, again thoroughly washed, dried and finally autoradiographed.

Cyclic AMP assay

Mouse thymocytes were isolated from 4–6-week- old SJL mice (Zick, Cesla & Shaltiel, 1979). Adenylate cyclase was activated by the incubation of $1{\cdot}25 \times 10^7$ cells with 100 ng of CT for 2 hr. At the end of the incubation period, the cells were ruptured by the addition of 0·1 ml of 0·1 M HCl to the centrifuged cell pellet and heated for 3 min at 95°. The samples were then transferred to 4° and neutralized with 30 μl of 0·25 M Tris, 20 mM EDTA, pH 12·5. The cAMP content in each sample was determined using the assay kit provided by Amersham International (Amersham, Bucks, U.K.) (TRK 432). For the inhibition of CT-induced cAMP by sIgA antibodies, CT (100 ng in 25 μl) was incubated with 25 μl of sIgA at various concentrations for 1 hr at room temperature, followed by the addition of the mixture to the $1{\cdot}25 \times 10^7$ cells for activation of adenylate cyclase. Results were expressed as percentaged of CT-induced cAMP reduction, with

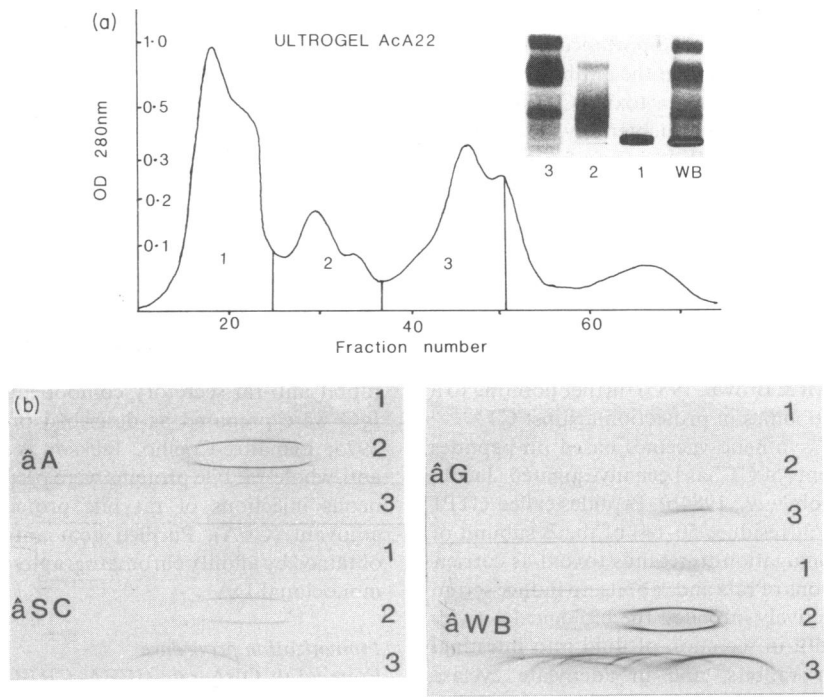

Figure 1. Representative gel-filtration of 10-fold concentrated bile from a rat immunized with CTP3-TT into Peyer's patches. (a) Elution pattern with agarose gel electrophoresis (inset) of the three pooled fractions of the eluate and of whole bile (WB). (b) Immunoelectrophoresis of the three gel-filtration fractions with anti-rat IgA (âA), anti-rat secretory component (âSC), anti-rat IgG (âG) and anti-whole-rat bile (âWB).

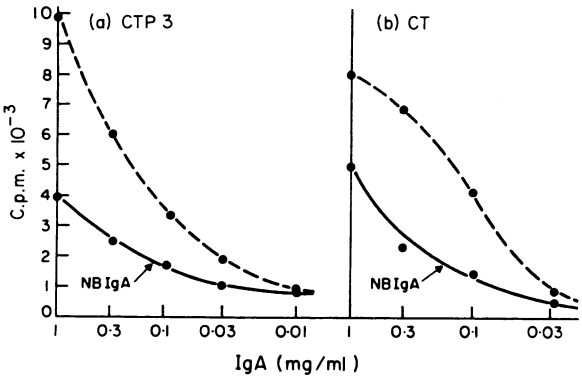

Figure 2. Binding of bile sIgA from rats immunized with CTP3-TT (a) with the homologous CTP3 peptide (broken line) and (b) with native cholera toxin (broken line) in comparison with bile sIgA from control rats (solid lines), revealed by ^{125}I-labelled anti-rat IgA antibodies. Points are means of the three responder rats. Variation between rats was always less than 10%.

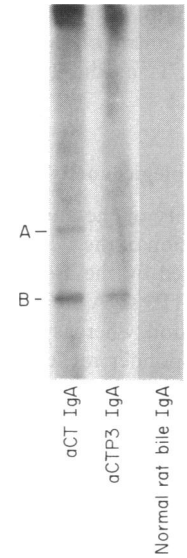

Figure 3. Immunoblot analysis of cholera toxin after SDS–PAGE electrophoresis, after reaction with bile sIgA from rats immunized with cholera toxin intraintestinally (aCTIgA), bile sIgA from rats immunized with CTP3-TT into Peyer's patches (aCTP3IgA), and bile sIgA from control rats. This was revealed with ^{125}I-labelled anti-rat IgA antibodies and autoradiography.

100% being the reduction given by a diluted (1/10) pool of serum from rats parenterally hyperimmunized with CT (Jacob *et al.*, 1984b).

RESULTS

Purification of bile sIgA

Figure 1a illustrates a representative gel-filtration elution pattern of 10-fold concentrated bile from a rat immunized with CTP3-TT into Peyer's patches. The eluates were divided into three fractions, which were analysed by agarose gel electrophoresis (Fig. 1a, inset). Fraction 1 contained essentially lipid and lipoprotein material, as well as traces of sIgA and IgM (not shown). Fraction 2 contained virtually all the sIgA, as well as some alpha-macroglobulin contaminants (Lemaître-Coelho *et al.*, 1977). Fraction 3 consisted of large amounts of albumin, free SC, transferrin and IgG, in order of decreasing electrophoretic mobility. These results were confirmed by the immunoelectrophoreses (Fig. 1b). As demonstrated, Fraction 1 only contained traces of material reacting with any of the antisera used. Fraction 2 reacted strongly with anti-IgA, at the same mobility, with anti-SC, but did not react with anti-IgG. Fraction 3 reacted strongly with anti-SC at a different mobility to Fraction 2. It also reacted with anti-IgG, but not with anti-IgA. Fraction 3 also contained several proteins, which were only revealed with the antiserum against whole rat bile proteins.

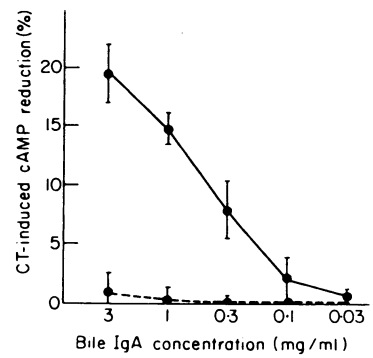

Figure 4. Inhibition of cholera toxin-induced cAMP production by bile sIgA from rats immunized with CTP3-TT (solid line) in comparison with sIgA from bile of control rats (broken line). Points are mean (±SEM) of the three responder rats. One hundred percent reduction was that given by a serum pool (1/10) of rats parenterally hyperimmunized with CT.

Immunological reactivity

The sIgA fractions of the CTP3-TT-immunized rats were further analysed for their antibody reactivity. Anti-TT reactivity was demonstrated by Ouchterlony tests (not shown). The sIgA fraction of all four rats, at 4–8 mg/ml, precipitated with TT at concentrations from 15 to over 1000 μg/ml. The same sIgA fractions of rats immunized with CT or from control rats did not precipitate with TT at any concentration.

Anti-CTP3 reactivity was shown in the bile sIgA fractions of three out of four CTP3-TT-immunized rats by RIA in comparison with the bile sIgA of control rats (Fig. 2a). Moreover, these sIgA antibodies cross-reacted with native CT to a level similar to that of their reactivity with CTP3 (Fig. 2b). Rats immunized three times intraintestinally with 100 μg of CTP3-TT did not respond to CTP3; their bile behaved like that of the control rats (unpublished results). This shared antigenicity with CT was confirmed by immunoblotting experiments. As shown in Fig. 3, the sIgA fraction of CTP3-TT-immunized rats reacted with the B subunit of CT, whereas the bile sIgA fraction of rats

immunized intraintestinally with native CT reacted with both A and B subunits of CT. Control bile sIgA fractions did not react with any subunit of CT, as did the bile sIgA of the non-responder rat.

Neutralization of the biological activity of CT

The neutralizing capacity of the anti-CTP3 sIgA fraction was evaluated by the inhibition of the CT-induced adenylate cyclase activation, as manifested by the assay of the cyclic AMP. As shown in Fig. 4, anti-CTP3 sIgA inhibited up to 20% of the CT-induced cAMP production, whereas control sIgA, as well as bile sIgA from the non-responder rat, did not inhibit at all at the same total sIgA concentrations.

DISCUSSION

There is much evidence for the role of intestinal sIgA antibodies in defence mechanisms against several bacterial and viral infections of the gastrointestinal tract in different species (Ogra *et al.*, 1968; Kapikian *et al.*, 1980; McNabb & Tomasi, 1981; LaBrooy, Shearman & Rowley, 1982; Freter & Jones, 1983), including experimental cholera (see the introduction). It was therefore of interest to induce IgA antibodies towards a synthetic reactive peptide from the CT–B subunit sequence, and to test their effect on the activity of CT. In general, it is difficult to induce strong sIgA responses against soluble proteins. For native CT or its B subunit, however, sIgA antibodies are consistently obtained by various immunization protocols (Elson & Ealding, 1984), but this does not apply to other soluble proteins, even if they have intestinal wall-binding properties (Woogen, Ealding & Elson, 1985). The availability of a synthetic peptide that contains a neutralizing epitope of CT, and which was used as a basis for a synthetic cholera vaccine (Jacob *et al.*, 1983), prompted us to induce sIgA antibodies against it. Our data indicate that this is now possible, although responses were still rather weak. In contrast to systemic IgG responses, protocols aimed at inducing sIgA responses against soluble proteins are often ill defined and usually induce no detectable or only relatively weak sIgA responses. We used Peyer's patch immunizations, which reportedly induce good primary and secondary rat bile sIgA responses, at least with particulate antigens (Hall *et al.*, 1979; Andrew & Hall, 1982a, b). Using such a protocol, we were able to induce sIgA antibodies against the synthetic CTP3. These sIgA antibodies cross-reacted with the native CT and also partially neutralized one of its biological activities. The assay of cAMP reduction was chosen because it is at least five times more sensitive than the gut loop assay, and because relatively few antibodies were expected to cross-react with CT after immunization with CTP3-TT. Moreover, a high correlation between the two types of biological assays had been demonstrated earlier (Jacob *et al.*, 1984b). CTP3 was well suited for such a study because it was previously shown (Jacob, Pines & Arnon, 1984a) to induce neutralizing antibodoes against several heat-labile toxins of the coli/cholera family. Others (Klipstein *et al.*, 1983) also obtained local sIgA antibodies against a synthetic peptide, namely the heat-stable enterotoxin of *Escherichia coli*, conjugated covalently to the B subunit of its heat-labile toxin. They primed by the intraperitoneal route with CFA and gave two oral boosters. The immunized animals were protected against both heat-stable and heat-labile toxins. Recently results for a similar antigen were obtained using four oral immunizations without intraperitoneal priming (Klipstein, Engert & Houghten, 1984). Such a protocol seems indeed more physiological than intra-Peyer's patch injections, and more applicable to humans. We certainly realize the poor physiological relevance of our Peyer's patch immunizations, as well as their relatively weak efficiency in inducing the relevant cross-reactive anti-CT sIgA antibodies, despite their strong sIgA reactivity for the TT carrier, attested by its precipitation. Compared with systemic immunization with CTP3-TT (Jacob *et al.*, 1983), the present cross-reactive sIgA anti-CT titres were rather low, and this probably explains why a neutralization of only 20% of the adenylate cyclase activity was reached, compared to 60–70% for systemic IgG antibodies. In addition, protection against CT-induced fluid accumulation in intestinal loop assays, which was demonstrated with anti-CTP3-TT IgG, remains to be shown with sIgA anti-CTP3 antibodies. Various immunization protocols, with various forms of CTP3 and various adjuvants, are now in progress in order to obtain higher titres of CT-reactive sIgA antibodies, aiming at a better neutralization of both *in vivo* and *in vitro* biological activities of CT.

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