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Cholera toxin-induced fluid secretion in rat gut ligated loops: influence of bile from normal or cholera toxin-immunized rats

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SUMMARY

Fresh normal rat bile premixed with cholera toxin (CT) did not significantly affect the CT-induced fluid accumulation in rat jejunal ligated loops. Bile from rats intrajejunally (i.j.) immunized three times with CT definitely inhibited CT-induced fluid secretion. Bile duct ligature (BDL) for 1–4 days in unimmunized rats, in contrast with mice, did not significantly affect subsequent CT-elicited fluid secretion in their ligated loops. BDL for 4 days in rats i.j. immunized with CT, only slightly decreased the CT-neutralizing ability of their gut loops. Passive transfer during 24 hr of bile from i.j.-immunized rats, but not from normal rats, into gut of normal recipient rats with BDL, significantly protected loops made in such recipients. The affinity-purified antibodies of immune bile, mixed with CT, neutralized its effect. Our data show that, unlike mice, rat bile acids are not required for expression of the CT effect in gut loops. In addition, bile from i.j.-immunized rats contains enough anti-CT antibodies to be protective on its own, but is not necessary for substantial gut protection against CT in i.j.-immunized BDL rats. Our results confirm a major and complementary role of both biliary and intestinal secretory IgA antibodies in protection of the rat gut mucosa against CT-induced fluid secretion.

INTRODUCTION

It is generally admitted (Lange & Holmgren, 1978; Lycke, Eriksen & Holmgren, 1987; Pierce, Cray & Sircar, 1978; Svennerholm, Lange & Holmgren, 1978; Svennerholm *et al.*, 1984) that protection of the intestinal epithelial wall against cholera toxin (CT) and related enterotoxins is mainly due to secretory IgA (sIgA) antibodies (Ab), quantitatively the predominant immunoglobulins in the gut (Brandtzaeg, 1981; Jonard *et al.*, 1984). These sIgA Ab are mainly synthesized by the numerous IgA-containing plasmacytes found in the lamina propria of the intestinal mucosa (Crabbé, Carbonara & Heremans, 1965; Nash *et al.*, 1969; Pierce & Gowans, 1975). In rats, these polymeric IgA (pIgA) Ab may reach the gut by two pathways. In the first, a direct one, the pIgA Ab bind to secretory component (SC), their glycoprotein receptor expressed on the baso-lateral membrane of enterocytes (Brandtzaeg, 1978; Brown, Isobe & Nakane, 1975; Crago *et al.*, 1978; Lemaître-Coelho, Naccache-Corbic & Vaerman, 1977b). Thereafter, they are endocytosed in small vesicles, actively transferred through the cells, and finally secreted, by exocytosis and proteolytic cleavage, at their apical pole, into the gut lumen (Brandtzaeg, 1981; Brown *et al.*, 1975). Those pIgA Ab that were not transferred through the enterocytes by the first route, reach the blood, via the lymph (Vaerman *et al.*, 1973), and are, in rats, rabbits and mice, actively secreted into bile through hepatocytes by the SC-mediated vesicular transfer (Jackson *et al.*, 1978; Delacroix, Malburny & Vaerman, 1985; Vaerman & Lemaître-Coelho, 1979; Vaerman *et al.*, 1982). In the rat model, biliary pIgA Ab are bound to SC and occur at concentrations 7–15 times higher than in serum (Delacroix *et al.*, 1983; Lemaître-Coelho, Jackson & Vaerman, 1977a, 1978a,b; Vaerman, Lemaître-Coelho & Jackson, 1978). After bile duct ligature (BDL), serum IgA levels rapidly rise 10–20 times (Lemaître-Coelho *et al.*, 1978a). If bile is diverted from the gut by cannulation for 48 hr, the sIgA content of upper gut washings decreases by nearly 90% (Lemaître-Coelho *et al.*, 1978b). Rat bile thus represents an important source of gut sIgA Ab (Vaerman *et al.*, 1978).

Two groups found that repeated intraintestinal or oral immunizations with CT elicited high levels of sIgA anti-CT Ab in rat bile. They suggested that they were protective, as they inhibited CT-induced fluid secretion in ligated intestinal loops (Tamaru & Brown, 1985; Vaerman *et al.*, 1985). On a molar basis, affinity-purified bile sIgA anti-CT Ab were about sevenfold more efficient than similarly purified serum IgG anti-CT Ab obtained after parenteral immunization (Pierre, Langendries & Vaerman, 1988).

Others demonstrated that, in mice, bile acids were necessary for activation of enterocyte adenylate cyclase by CT (Lange, Hansson & Lönnroth, 1983; Lange & Lönnroth, 1982). Among the various bile acids, cholic acid, deoxycholic acid and

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chenodeoxycholic acid were most potent (Lange *et al.*, 1983). The same group also outlined the protective role, against CT, of a pituitary protein called anti-secretory factor (ASF) (Lange & Lönnroth, 1986, Lange, Lönnroth & Nygren, 1984, Lönnroth & Lange, 1984, 1986). ASF was induced after repeated oral administrations of CT, and inhibited gut adenylate cyclase activation by CT. The excretion of ASF in small amounts in bile and milk of mice orally given CT was further reported by them (Lange & Lönnroth, 1984; Lönnroth & Lange, 1986).

In this report, we have investigated the contribution of whole native bile and of its Ab content for gut protection against CT in the rat model.

MATERIALS AND METHODS

Animals

OFA male rats, weighing 250–300 g, were obtained from IFFA-Credo (St. Germain-sur-l'Arbresle, France).

Cholera toxin (CT)

CT, affinity-purified on insoluble Gm1-ganglioside (Tayot *et al.*, 1981) and kindly given by Institut Mérieux (Lyon, France), was used for immunizations. For the intestinal ligated loop assay, we used CT from Sigma (St Louis, MO).

Immunization procedures

Rats were anaesthetized with ether, and intrajejunally (i.j.) immunized three times, at 2-week interval, just beyond the Treitz angle, with 25 μg of CT in 0·5 ml of phosphate-buffered saline (PBS), pH 7·2, with 0·02% gelatin (PBSG).

Collection of bile

Bile was obtained by cannulation of the bile duct as described previously (Lemaître-Coelho *et al.*, 1977a). The immune animals were cannulated 4 days after the last i.j. injection of CT. Bile was immediately processed.

CT neutralization test

The classical ligated intestinal loop assay, described for mice (Lange & Holmgren, 1978), was adapted to 24-hr fasted rats. Briefly, two ligated loops of 5–7 cm length were prepared in the upper part of the jejunum of ether-anaesthetized rats, with care to avoid ligature of large vessels. The upper loop was injected with 1·0 μg of CT in PBSG, alone or premixed with bile or anti-CT Ab, up to a total volume of 500 μl. The distal loop received 500 μl of PBSG as control. The abdominal wall was closed and animals were then kept without food and water until killed. Four hours after CT challenge, rats were killed by ether overdose, and loops were dissected, measured (cm) and weighed. Results are expressed as loop weights, i.e. differences between weight/length ratios (mg/cm) of CT- and PBSG-challenged loops. CT-challenged loops were considered as protected if their weight was smaller than 50 mg/cm. This value corresponds to the fluid accumulation induced by 50 ng of CT (unpublished results) and can thus be considered as 95% neutralization of the injected CT dose.

Bile duct ligature (BDL)

Bile flow was stopped by two ligatures on the bile duct (Lemaître-Coelho *et al.*, 1978a). Loops were CT challenged before and on Day 1, 2, 3 and 4 after BDL in unimmunized rats, but only before and on Day 4 after BDL in immunized rats.

In vivo *bile transfer from immune to normal rats*

Six i.j.-immunized rats were cannulated on Day 3 after the last i.j. injection. A day later, the free end of the cannula was passed through the abdominal wall of an unimmunized rat, and surgically inserted into his duodenum. Bile ducts of the six recipient rats were ligated during the same operation. After 24 hr of recovery, the action of CT was tested in the six recipients by the ligated loop assay. The same procedure was applied to six donor–recipient pairs of unimmunized rats used as controls.

Isolation of anti-CT Ab

Six 1-ml samples of fresh bile, collected from six rats, obtained at 4 days after the third i.j. immunization, were pooled. This immune bile pool (6 ml) was passed through a small column of CT (5 mg) coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). After recovery of the unretained proteins with PBS, and further washings with 1 M NaCl and PBS again, Ab were eluted with 0·1 M Na citrate buffer, pH 2·4, and immediately neutralized with solid Tris base. The unretained fraction was passed again on the immunoadsorbant. Both the second unretained and the two pooled acid-eluted fractions were dialysed against PBS and concentrated to their initial volume. For neutralization assays, 0·5 ml of each fraction, premixed *in vitro* for about 30 min with 1 μg of CT, were injected into ligated loops made in normal rats.

Antibody determination

Specific anti-CT IgG or IgA was measured by an enzyme-linked immunosorbent (ELISA). Briefly, 96-well microtitre plates (Greiner, Nurtingen, FRG) were coated overnight at room temperature with CT (2 μg/ml, 100 μl/well), and saturated with 2% fetal calf serum (FCS 100 μl/well), diluted in glycine-buffered saline, pH 9·2, (GBS: 18·5 mM glycine, 31·5 mM NaCl). Bile (100 μl) was applied for 2 hr at 37° in 5X-GBS containing 50 mM EDTA, 0·1% Tween 80 and 0·1% bovine albumin. Specific rabbit anti-rat IgG and goat anti-rat IgA (Vaerman *et al.*, 1973) coupled with horseradish peroxydase (Nakane & Kawaoi, 1974) were used as conjugates and o-phenylene-diamine as substrate. Reaction was halted with 25 μl/well of 2 M H_2SO_4. Plates were read at 492 and 620 nm with a Titertek Twinreader (Flow Labs, Rockville, MD). The titre was the reciprocal of the highest dilution giving a 0·1 OD signal above the same dilution of normal fresh bile.

Statistical analysis

Significance of results was analysed by the unpaired Student's *t*-test.

RESULTS

Influence of normal rat bile on CT-induced fluid secretion

Various amounts of normal fresh bile, when premixed *in vitro* with CT, had little effect on the CT challenge, as measured by loop weights in jejunal ligated loops (Table 1). With larger amounts of normal bile (200 and 400 μl), fluid accumulation

Table 1. Fluid accumulation after injection, into ligated gut loops constructed in normal rats, of 1 μg of CT alone or premixed with various amounts of normal or immune rat bile

Volume of bile mixed with CT	Normal bile ($n=5$)	No. protected loops*	Immune bile ($n=6$)	No. protected loops
No bile	305±38†‡§	0/5		
50 μl	274±35	0/5	166±213	3/6
200 μl	227±84¶	0/5	129±110¶	2/6
400 μl	242±81§**	0/5	47±137‡**	5/6

* Protected = loop weight at 4 hr after CT challenge was < 50 mg/cm.

† All figures are mean loop weights (mg/cm) ± SD; $n=5$ without bile and with normal bile; $n=6$ with immune bile.

‡ $P=0.003$.

§ $P=0.151$.

¶ $P=0.137$.

** $P=0.022$.

Table 2. Influence of bile duct ligature on CT-induced fluid secretion in ligated loops constructed in normal and CT-immunized rats

Duration of ligature	Normal rats	No. protected loops*	Immune rats	No. protected loops
No ligature	305±38†‡§¶**	0/5	11±26¶††	5/5
1 day	294±65	0/5	ND	ND
2 days	272±31‡	0/5	ND	ND
3 days	284±72	0/5	ND	ND
4 days	254±47§‡‡	0/5	101±159**††‡‡	4/5

* Protected = loop weight at 4 hr after CT challenge was < 50 mg/cm.

† All figures are mean loop weights (mg/cm) ± SD, $n=5$.

‡ $P=0.172$.

§ $P=0.097$.

¶ $P<0.001$.

** $P=0.023$.

†† $P=0.251$.

‡‡ $P=0.071$.

ND, Not done.

induced by CT decreased slightly, but none of the five rat loops tested was protected. Even with 400 μl of normal bile, the mean loop weight was not statistically different from that obtained using CT without bile ($P=0.151$), and not a single loop was protected either.

Influence of immune rat bile on CT-induced fluid secretion

In contrast, fresh bile collected from animals i.j. immunized three times was protective (Table 1). As little as 50 μl of immune bile already protected 3/6 rat loops, with the three other loops unprotected. This resulted in a mean loop weight not statistically different from that obtained with CT only ($P=0.189$), nor from that given by CT premixed with various amounts of normal bile (all P values >0.3). However, 200 or 400 μl of immune bile were protective in 2/6 or 5/6 loops, respectively. With 400 μl, the mean loop weight was significantly lower than that due to CT alone ($P=0.003$), or to CT premixed with the same amount of normal bile ($P=0.022$).

Table 3. Effect of passive 24 hr transfer of bile from normal or CT-immunized rats on CT-induced fluid secretion in gut loops constructed in normal recipient rats with bile duct ligature

Transfer of normal bile		Transfer of immune bile	
Loop weight	No. protected loops*	Loop weight	No. protected loops
287±65·7†	0/6	33·9±21·3†	9/10

* Protected = loop weight at 4 hr after CT-challenge was < 50 mg/cm.

† Figures are mean loop weights (mg/cm) ± SD: $P<0.001$.

Influence of BDL in normal rats

Withdrawal of bile supply to the gut by BDL during 1–4 days only gave rise to a slight decrease of CT potency in the challenged loops (Table 2). The mean decrease in loop weight was never significant, even when it was most pronounced, i.e. at 4 days after BDL ($P=0.097$). In this series of CT challenges performed in ligated jejunal loops of rats with BDL, not a single loop out of 20 was protected.

Influence of BDL in CT-immunized rats

In rats i.j. immunized with CT, the effect of BDL on loop challenge with CT was only examined after 4 days of BDL. At this time, 4/5 loops were still protected, despite prolonged absence of bile supply (Table 2). The mean loop weight was not significantly different ($P=0.251$) from that of non-BDL immunized rats. If compared, however, to the mean loop weight of normal rats at Day 4 of BDL, the difference was just below significance ($P=0.071$), despite the fact that none of the five loops were protected in the normal group, in contrast with 4/5 in the immunized group.

Passive transfer of bile from normal or CT-immunized rats into gut of normal recipients

The physiological bile flow of normal rats was surgically diverted during 24 hr into the duodenum of unimmunized rats with BDL (Table 3). When challenged with 1 μg of CT, none of the six recipient loops were protected. Their mean loop weight (287 ± 66 mg/cm) was not statistically different ($P=0.603$) from that given by 1 μg of CT in normal untreated rats (305 ± 38 mg/cm), nor from that in unimmunized rats with 24 hr of BDL (294 ± 65 mg/cm; $P=0.860$).

Bile from immunized rats was similarly diverted into the gut of unimmunized recipients with BDL. When jejunal loops in the 10 recipient rats were similarly challenged with CT, 9/10 were

Table 4. Influence of CT immunosorbent-retained Ab-like fraction, and of unretained fraction of immune bile, premixed with CT, on its fluid secretory effect in ligated gut loops prepared in normal rats

Unretained fraction (0·5 ml)	No. protected loops*	Retained Ab-like fraction (0·5 ml)	No. protected loops
226±94‡	0/6	7±10†	6/6

* Protected = loop weight at 4 hr after CT-challenge was < 50 mg/cm.

† Mean loop weight ± SD with $n=6$; $P<0·001$.

protected, and their mean loop weight (33·9±21·3 mg/cm) was significantly smaller than that obtained in recipients of normal bile (287±66 mg/cm, $P<0·001$).

Antibody-like behaviour of CT-neutralizing factor in immune bile

After passage of 6 ml of immune bile through a small CT immunosorbent, none of the six loops challenged with CT premixed *in vitro* with 500 μl of the unretained fraction was protected; their mean loop weight (226±94 mg/cm) was not significantly smaller ($P=0·774$) than that obtained with CT premixed with 400 μl of normal bile (242±81 mg/cm) (Table 4). In contrast, all six loops challenged with CT premixed with 500 μl of the eluted Ab-like fraction were protected; their mean loop weight (11±10 mg/cm) was very significantly smaller ($P<0·001$) than that obtained when CT was premixed with the unretained bile proteins.

The ELISA IgA anti-CT titres, in native immune bile, its immunosorbent unretained and retained fractions were, respectively, 800, 0 and 600. All sIgA anti-CT Ab were retained after two passes through the immunosorbent. Similar data were obtained for the small amount of IgG anti-CT found in immune bile (respective titres: 100, 0 and 60). Protection is thus associated with the bile fraction containing specific anti-CT Ab, most of which are of the IgA class.

DISCUSSION

The first question addressed in this paper was whether normal rat bile contained substances capable of influencing the fluid secretion induced by CT, when premixed with CT *in vitro* and tested in ligated jejunal loops constructed in unimmunized rats. Various amounts of normal fresh bile premixed with a given amount of CT did not significantly affect its fluid secretory effect in normal rat loops (Table 1). Moreover, loops tested with CT in unimmunized rats with 1–4 days of BDL did not reveal a major effect of deprivation of bile supply on the secretory effect of CT (Table 2). Thus, in rats, in contrast with mice (Lange & Lönnroth, 1982; Lange *et al.*, 1983), bile acids are not required for manifestation of CT effects in ligated gut loops. In addition, although we had observed earlier (Vaerman *et al.*, 1985) that normal rat bile inhibited the secretory effect of CT when tested in mouse loops, this was not found when rat bile was mixed with CT and tested in rat loops, obviously a more physiological situation. Such discrepancies could reflect important interspecies differences.

A second question was whether fresh rat bile from i.j.-immunized rats could neutralize CT, not only when premixed *in vitro* with CT and tested in loops constructed in unimmunized rats, but also when immune bile had, thanks to appropriate surgery, flowed almost physiologically during 24 hr into gut from unimmunized recipients with BDL. Various amounts of fresh immune bile, premixed *in vitro* with CT, clearly protected ligated loops constructed in normal rats (Table 1), confirming earlier reports (Tamaru & Brown, 1985; Vaerman *et al.*, 1985). More significantly, we also showed that the gut from an unimmunized rat could be passively protected against CT merely by receiving, during 24 hr, the normal bile supply of an i.j.-immunized rat. Nine out of 10 ligated loops constructed in such rats were definitely protected. These results demonstrate that bile from i.j.-immunized rats, on its own, can protect normal rat gut against CT-challenge in a ligated loop. These data further outline the importance of rat bile in enhancing the local immunity of rat gut, as suggested when the high sIgA Ab content of rat bile was described (Vaerman *et al.*, 1978).

A further question, answered in this article, concerns the nature of the factor(s) protective against CT present in bile from intestinally immunized rats. Previous reports (Jacob & Vaerman, 1986; Tamaru & Brown, 1985; Vaerman *et al.*, 1985) suggested that the sIgA anti-CT Ab present in immune bile were by far the predominant carriers of its CT-protective ability. Recently, we purified such sIgA anti-CT Ab from sIgA isolated from rat immune bile, by absorption on and acid-elution from a CT-immunosorbent column (Pierre *et al.*, 1988). The sIgA Ab precipitated CT, and their CT-neutralizing ability was stronger than that of IgG Ab similary purified from serum of rats i.v. immunized with CT. Both purified IgG and sIgA anti-CT Ab, premixed *in vitro* with CT, protected rat gut against CT. In this report, we show that the protective fraction of immune bile is also the fraction containing specific sIgA anti-CT. This confirms the importance of this Ab class for rat gut protection.

However, it was recently reported that repeated oral doses of CT given to rats induced the production of a protective protein, called ASF for anti-secretory factor, in their pituitary extracts and gut mucosa, with small amounts also excreted in their bile and milk (Lange *et al.*, 1984; Lange & Lönnroth, 1986; Lönnroth & Lange, 1984, 1986). We do not think that ASF contributed much to our results for several reasons. (i) Bile only contains minute amounts of ASF (Lange & Lönnroth, 1986; Lönnroth & Lange, 1986), probably insufficient to elicit a significant protection. (ii) The molecular weight (MW) of the isolated protective sIgA antibody fraction (>400,000) from immune bile (Jacob & Vaerman, 1986; Vaerman *et al.*, 1985), is much larger than the 43,000–60,000 proposed for ASF (Lönnroth & Lange, 1986). (iii) Similar amounts of monoclonal mouse IgA anti-CT Ab and of rat biliary sIgA anti-CT Ab were protective (P. G. Pierre *et al.*, unpublished results). (iv) In mice, the immune response to CT is highly thymus-dependent (Lycke *et al.*, 1987).

Bile from i.j.-immunized rats, although shown here to be sufficient by itself for gut protection in the passive transfers, was not absolutely required, as demonstrated by our challenges of loops constructed in i.j.-immunized rats with BDL for 4 days (Table 2). This is easily explained by a direct secretion, through enterocyte SC-mediated transport, of IgA anti-CT Ab synthetized by the numerous anti-CT IgA-secreting plasmacytes found in gut mucosa after i.j. immunizations with CT (Lycke *et al.*, 1987; Pierce & Gowans, 1975). The high serum titres of anti-CT sIgA found in i.j.-immunized rats after BDL (Vaerman, Pierre & De Rijk-Langendries, 1987) do not contribute to the gut loop

protections observed here, as sIgA Ab are not actively secreted by hepatocytes into bile (Fisher *et al.*, 1979; Vaerman *et al.*, 1982), and thus probably also not by enterocytes into gut lumen. However, since oral CT immunization also elicits high serum levels of IgG anti-CT Ab (Elson & Ealding, 1984; Vaerman *et al.*, 1987), a minor contribution of these to gut protection is possible, by passive transsudation into the gut lumen (Jonard *et al.*, 1984; Pierce & Reynolds, 1974).

Intestinal as well as biliary sIgA Ab alone were experimentally shown here to protect the gut against CT; however, in the normal situation, they must reinforce each other and thus confer a very strong protection to the rat gut against CT.

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