Intestinal immunisation with *Escherichia coli* protects rats against *Escherichia coli* induced cholangitis

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Abstract

Background—Cholangitis, an infection of the biliary tract, is most commonly caused by Gram negative bacteria, particularly *Escherichia coli*. Factors governing the severity of cholangitis, including the role of biliary IgA, are poorly understood.

Aims—The aim of this work was to find out if biliary IgA directed against *E coli* protects rats against hepatobiliary infection with *E coli*.

Subjects—Male Sprague-Dawley rats weighing 270-350 grams were used in all of the experiments.

Methods—At laparotomy, rats were immunised by injecting killed *E coli* or normal saline (controls) into Peyer's patches. With or without subsequent antigenic boosting (by oral administration of killed *E coli*), bile was collected at a second laparotomy, and rats were infected by introducing viable *E coli* into the bile duct. Production of IgA anti-*E coli* antibody was measured by enzyme linked immunosorbent assay of bile, and the presence of hepatobiliary infection was determined by quantitative culture of liver homogenates.

Results—Systemic infection was present in six of 12 control rats and in one of 24 immunised rats ($p=0.005$) after death. There was an inverse correlation between immunisation and *E coli* colony counts in cultured liver homogenates ($p=0.024$).

Conclusion—The findings suggest that biliary IgA directed against *E coli* protected rats against hepatobiliary *E coli* infection and systemic sepsis.

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Keywords: cholangitis, biliary IgA, common bile duct, bile.

antibody response, but the functional role of biliary IgA has not been extensively studied. In particular, little is known about the potential of biliary IgA antibody to protect mammals against *E coli* induced cholangitis. The aim of this study was to discover if biliary IgA antibody, induced by injecting killed *E coli* into rat Peyer's patches, protects against biliary infection with *E coli*.

Methods

Animals

Male Sprague-Dawley rats weighing 270-350 grams were used in all of the experiments. The animals were housed under standard conditions and had free access to standard laboratory chow (Purina Rodent Laboratory Chow 5001) and water.

Bacteria

E coli (ATCC 25922; American Type Culture Collection, Rockville, Maryland) were grown on Mueller-Hinton agar (DIFCO Laboratories, Detroit, Michigan) and frozen for storage in vials of milk at -80°C . A single lot of *E coli* was used throughout the study.

Bacteria were killed by suspending thawed and recultured *E coli* in 15 ml of 10% formalin at 25°C for 15 minutes. The bacterial suspension was centrifuged, and the pellet was resuspended in 10 ml of sterile normal saline and re-centrifuged. This washing procedure was repeated four times, and the killed *E coli* were then suspended in sterile normal saline (2.5×10^8 organisms/ml) for injection into Peyer's patches. A 100 μl aliquot of each sample of killed *E coli* was plated on a Mueller-Hinton agar plate, which was then incubated overnight at 37°C to verify complete killing of the bacteria.

Experimental protocol

The study comprised two phases: phase 1 consisted of establishing the method for immunising rats (intra-Peyer's patch injection of killed *E coli*), and phase 2 determined whether immunisation could protect rats against bacterial cholangitis. Phase 2 involved two groups of rats (Table). Surgery was performed under aseptic conditions using intraperitoneal pentobarbital sodium (60 mg/kg body weight) for general anaesthesia. All surgical procedures were performed via a midline laparotomy after shaving the abdomen and

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Cholangitis, an infection of the biliary tract, is usually associated with obstruction of the common bile duct. Gram negative enteric bacteria, in particular *Escherichia coli*, cause most of these infections. The disease ranges from a mild transient illness to lethal sepsis, but the factors that govern the severity are poorly understood. One possibility is that the course is influenced by biliary IgA directed against the bacteria responsible for the infection.

Previous studies have shown that oral, intraduodenal, or intra-Peyer's patch administration of antigen to rats can lead to a biliary IgA

Experimental protocol for phase 2

Time (days)	Group 1		Group 2		
	Immunised (n=13)	Control (n=11)	Immunised		Control C (n=12)
0	killed <i>E coli</i> (IPP)	saline (IPP)	killed <i>E coli</i> (IPP)	killed <i>E coli</i> (IPP)	saline (IPP)
7	collect bile	collect bile	killed <i>E coli</i> (O)	killed <i>E coli</i> + CT (O)	saline (O)
10	live <i>E coli</i> (IBD)	live <i>E coli</i> (IBD)			
12	death	death	collect bile live <i>E coli</i> (IBD)	collect bile live <i>E coli</i> (IBD)	collect bile live <i>E coli</i> (IBD)
15			ligate CBD death	ligate CBD death	ligate CBD death

IPP: intra-Peyer's patch injection; IBD: intra-bile duct injection; CBD: common bile duct; O: oral administration; CT: cholera toxin.

preparing the skin with povidone-iodine solution (Betadine). At the conclusion of the procedure, the abdomen was closed in two layers using 5-0 Dexon and 4-0 silk.

Phase 1

Using a 30 gauge needle, the Peyer's patches of 25 rats were injected with 2.5×10^8 formalin killed *E coli* suspended in 1 ml of sterile normal saline. The number of Peyer's patches injected ranged from 7 to 11 per animal. Control rats (n=7) underwent intra-Peyer's patch injection with 1 ml of sterile normal saline and were otherwise treated the same as the immunised rats. At a second laparotomy 3, 5, 7, 9, or 14 days after injection, the common bile duct was cannulated with sterile PE-10 plastic tubing, and bile was collected. The rats were then killed by intra-cardiac injection of a lethal dose of pentobarbital sodium, followed by bilateral thoracotomy. Anti-*E coli* IgA was quantified in the bile samples by enzyme linked immunosorbent assay (ELISA).

Phase 2

Group 1 experimental rats (n=13) were immunised by injecting 2.5×10^8 formalin killed *E coli* into their Peyer's patches, and control rats (n=11) were injected with sterile normal saline by the same route. Seven days after injection, bile was collected by cannulating the common bile duct as described above. Live *E coli* (10^4 - 10^6 organisms in 0.1 ml of sterile normal saline) were then introduced into the bile duct, the cannula was removed, and the bile duct was ligated. Seventy two hours later, all surviving animals were anaesthetised by intra-peritoneal injection of pentobarbital sodium, and 1 ml of blood was obtained by sterile cardiac puncture for blood culture. The animals were then immediately killed as previously described. Bile and the right lobe of the liver were removed for post-mortem analysis.

Group 2 experimental rats (n=24) were immunised by injecting 2.5×10^8 formalin killed *E coli* into Peyer's patches as previously described. Seven days later, 12 of these rats were orally boosted via a blunt ended feeding needle with 6.9×10^8 killed *E coli*, and the remaining 12 rats were orally boosted with

6.9×10^8 killed *E coli* plus 0.04 mg whole cholera toxin (List Biological Laboratories, Campbell, California). Control rats (n=12) had sterile normal saline injected into their Peyer's patches, followed by sham oral boosting with saline seven days later. Five days after boosting, the common bile duct was cannulated, bile was collected, and 10^7 live *E coli* were introduced into the common bile duct, which was then ligated. Seventy two hours later, any surviving rats were killed in the same fashion as for group 1 rats. Intra-cardiac blood, bile, and the entire liver were collected for analysis.

ELISA for anti-*E coli* IgA in bile

The wells of flexible assay plates (Falcon 3912; Becton Dickinson, Oxnard, California) were coated with 100 µl of formalin killed *E coli* (10^8 organisms/ml suspended in 1M sodium carbonate, pH 9.5) for four hours at 25°C.¹ After washing with phosphate buffered saline (Dulbecco's PBS, pH 7.5; Sigma Chemical Co, St Louis, Missouri), the wells were blocked with 1% bovine serum albumin in deionised H₂O (essential fatty acid free bovine albumin; Sigma Chemical Co) for two hours at 25°C. After washing with PBS, 100 µl of bile (diluted in PBS from 1:50 to 1:3200) were added to duplicate wells, and the plates were incubated overnight at 4°C. The plates were again washed with PBS, and 100 µl of 1:1000 peroxidase conjugated antirat IgA (Zymed Laboratories Inc, South San Francisco, California) were added to the wells, and the plates were incubated for four hours at 25°C. After washing the plates with PBS, 100 µl of substrate solution consisting of 25 ml citrated phosphate buffer (pH 5.0), 10 µl 30% hydrogen peroxide, and 10 mg 0-phenylenediamine (Sigma Chemical Co) were added to the wells, and the reaction was allowed to proceed for 15 minutes in a light free container. The reaction was stopped by the addition of 50 µl of 2N sulphuric acid to each well. Plates were read at 490 nm in an automated ELISA reader (EL 310; BIO-TEK Instruments Inc, Winooski, Vermont). Each assay included bile from immunised and control rats, plus a rabbit antiserum control (rabbit anti-*E coli* antibody, followed by peroxidase conjugated swine antirabbit immunoglobulin, both obtained from Dako Corporation, Carpinteria, California). Mean optical density (OD₄₉₀) values were calculated for duplicate wells.

Polyacrylamide gel electrophoresis and western blotting

A lysate of *E coli* was prepared as outlined by Hitchcock and Brown.² The lysate was loaded on a 12% acrylamide gel and electrophoresed at 200V/100mA for 60 minutes.³ Proteins were transferred from gels to nitrocellulose sheets by electrophoresis at 100V/500mA for 60 minutes.⁴ After blocking with 1% casein for 30 minutes, each nitrocellulose sheet was cut into individual strips, which were incubated

Figure 1: Results of ELISA for anti-*E coli* IgA in bile from immunised rats ($n=13$) and from control rats ($n=11$) in phase 2, group 1. Anti-*E coli* IgA levels are expressed as optical density (OD_{490}). Mean values and standard deviations are shown.

overnight with bile samples diluted 1:100 in 0.1M TRIS/1% casein (pH 8.3) at 4°C. Next, the transblots were washed with PBS and incubated for 3.5 hours with a 1:500 dilution of alkaline phosphatase conjugated sheep anti-rat IgA (The Binding Site, University of Birmingham Research Institute, Birmingham, UK) in TRIS/1% casein (pH 8.3). The strips were then washed with PBS followed by further washing with TRIS buffer. A substrate solution, consisting of naphthol (1 mg/ml) and fast red (2 mg/ml; Sigma Chemical Co) in 50 mM TRIS buffer (pH 8.0), was added. The enzymatic reaction was allowed to proceed for 60 minutes. The development of pink bands, indicating binding of IgA to *E coli* antigens, was assessed visually.

Culture methods

Blood and bile were plated undiluted on Mueller-Hinton agar plates, which were incubated at 37°C for 24 hours. If no growth was seen at 24 hours, the plates were incubated for an additional 24 hours before being recorded as showing no growth.

In phase 2 group 1 rats, a 2.0 g piece of the right hepatic lobe was mechanically ground into a homogenate by trituration with sterile glass beads, and the homogenate was suspended in 2 ml of sterile normal saline. A 50 µl unfiltered aliquot of each homogenate was plated and cultured overnight at 37°C, and bacterial colonies were counted the next morning. The entire liver was removed from rats in phase 2 group 2. It was weighed and homogenised, and the homogenate was suspended in 20 ml of sterile normal saline. Each sample was filtered through fine mesh sterile gauze. A 100 µl filtered aliquot of each suspension was serially diluted in sterile PBS to a dilution of 1:30 000; four 20 µl drops of each diluted sample were plated to make four rows on a Mueller-Hinton agar plate.

Plates were incubated overnight at 37°C, and bacterial colonies were counted the next morning.

Results

Phase 1

Bile collected at 3, 5, 7, 9, and 14 days after injection from immunised and control rats was analysed by ELISA for anti-*E coli* IgA ('specific IgA'). Specific IgA was present in bile samples collected from immunised rats 7, 9, and 14 days after immunisation (data not shown).

Phase 2, group 1

Bile collected seven days after Peyer's patch injection with killed *E coli* (immunised rats) or with sterile saline (control rats) was analysed by ELISA. Specific IgA levels, expressed as OD_{490} values, were higher in bile from immunised rats than from controls, confirming immunisation (Fig 1). Western blotting showed that rats injected with killed *E coli* generated a biliary IgA response to multiple antigens of *E coli* (Fig 2).

One of the 11 control rats died of sepsis 48 hours after bacterial inoculation of the common bile duct. A bile leak was found in one immunised rat at postmortem examination. One control and one immunised rat died of anaesthetic complications before the bacterial inoculation step. All other immunised

Figure 2: Western blot of *E coli* antigens with biliary IgA from rats in phase 2, group 1. See text for method. (A) Lanes 1-5 show results for individual bile samples from control rats; (B) lanes 6-10 show results for individual bile samples from immunised rats. IgA from immunised rats binds to numerous antigens of *E coli*. Positions of molecular weight (MW) markers are shown (kDa).

Figure 3: Results of ELISA for anti-*E coli* IgA in bile from immunised rats (immunised A, $n=12$); immunised B, $n=12$) and from control rats (control C, $n=12$) in phase 2, group 2. Anti-*E coli* IgA levels are expressed as optical density (OD₄₉₀). Mean values and standard deviations are shown. See the Table for information about experimental conditions applicable to subgroups A and B.

rats ($n=11$) and control rats ($n=9$) survived until death 72 hours after bacterial challenge.

Blood, bile, and liver tissue collected at death were cultured. *E coli* was present in bile cultures from all rats in this group, but blood cultures were negative for bacterial growth. The results of the cultures of 50 μ l of undiluted liver homogenates suggested there was an inverse correlation between immunisation and bacterial colony counts from the liver. For example, the mean bacterial colony count for liver homogenates from nine control rats was 523 per culture plate. The corresponding mean value for liver homogenates from 11 immunised rats was 71 colonies per culture plate. While these results were statistically significant ($p=0.043$, Student's t test), only a 2.0 g piece of the right hepatic lobe from all rats in phase 2, group 1 was processed and cultured. Concern arose over whether this specimen was representative of bacterial distribution throughout the liver. Therefore, to check on the correlation between immunisation and bacterial colony counts, the entire liver was processed and cultured in group 2 rats.

Phase 2, group 2

The results of ELISA of bile samples from this group of rats were similar to the corresponding results for group 1 rats, in that specific IgA levels (OD₄₉₀ values) were higher in bile from immunised rats than from controls (Fig 3). No difference was seen in specific IgA levels between rats orally boosted with *E coli* plus cholera toxin versus *E coli* alone (Fig 3). The incidence of systemic sepsis after *E coli* inoculation of the bile duct was 50% in control rats (six of 12 controls) compared with 4% (one of 24) in immunised rats ($p=0.005$; χ^2 test). Of the 12 control rats, four died of sepsis and two others were clinically septic when

killed. All 12 rats injected with killed *E coli* and boosted with killed *E coli* plus cholera toxin, and 11 of 12 rats injected and boosted with killed *E coli* alone, survived until killed and were not bacteraemic or clinically septic. One rat that was immunised and boosted with killed *E coli* alone died of sepsis 48 hours after administration of viable *E coli*.

Blood, bile, and liver tissue were collected at death and cultured. Cultures of blood from the two control rats that were septic when killed grew *E coli*. There was no growth of *E coli* in blood cultures from the six control rats that were not septic when killed or from the 23 immunised rats that survived until killed. All bile cultures from immunised and control rats grew *E coli*. Serial dilution and culture of liver homogenates showed an inverse correlation ($p=0.024$; Student's t test) between immunisation and *E coli* colony counts (Fig 4).

Discussion

Immunoglobulin A is the predominant immunoglobulin of the mucosal immune system and the major antibody in bile.⁵⁻⁸ In rats, about 85% of biliary IgA is believed to be derived from hepatocyte transport and secretion of circulating polymeric IgA into bile.^{5-7 9-11} The remainder of biliary IgA is synthesised by antibody secreting cells located beneath the bile duct epithelium.^{5-7 12}

Previous work has shown that secretion of antigen specific IgA into bile can be induced by antigen administered by the following routes: oral^{6 13-15}; injection into the intestinal lumen^{10 16}; intra-peritoneal injection^{8 17}; and injection into Peyer's patches.^{6 12 13 17-19} The available evidence suggests that specific IgA contributes to protection of the biliary system and upper gastrointestinal tract by preventing attachment of bacteria, protozoan parasites, and viruses to epithelial cells.^{6 20} Furthermore, IgA directed against cholera toxin prevents intestinal secretion induced by this toxin.⁶ While IgA is not very effective for opsonisation or for activating the complement cascade, it may be involved in killing bacteria via antibody dependent cytotoxicity reactions.⁶

The objective of this study was to discover if the presence of antigen specific biliary IgA con-

Figure 4: Quantitative culture of *E coli* from liver homogenates from rats in phase 2, group 2, expressed as mean numbers of organisms per ml of liver homogenate. $p=0.024$, comparing subgroup C ($n=8$) with subgroups A ($n=11$) and B ($n=12$), by Student's t test.

ferred protection against a direct antigenic challenge in the hepatobiliary system. We found that high values of antigen specific biliary IgA were induced by immunisation, and that they did provide some protection from experimentally induced cholangitis. While the mechanism responsible for these effects has not been proved, the data suggest they are attributable to the induced specific IgA antibody.

Immunisation did not prevent infection altogether but mitigated its severity. The phase 2 rats, whether immunised or not, had cholangitis (culture results), but there was an inverse correlation between immunisation and the extent of infection. A correlation was suggested in the results from the group 1 rats (mean colony counts in controls versus immunised rats were 523 and 71, respectively). In group 2 rats this correlation was significant (Fig 4, $p=0.024$, Student's t test), which suggested that the immunisation inhibited intrahepatic proliferation of *E coli*.

Immunisation with killed *E coli* also provided (phase 2, group 2) considerable protection against systemic sepsis. In group 1, despite higher levels of specific biliary IgA in immunised rats than in controls, the clinical course after bacterial challenge was similar.

We concluded from the group 1 data that, while an effect of immunisation was present (based on liver homogenate colony counts), the severity of the bacterial challenge was insufficient to change the overall clinical course. In group 2, the bacterial challenge was increased, and the clinical effects were more striking. As noted above, systemic sepsis was present in six of 12 control rats and in one of 24 immunised rats in group 2. Therefore, the data suggest that, in addition to inhibiting bacterial proliferation within the hepatobiliary system, immunisation inhibited bacterial translocation into the systemic circulation. This may result from antibody antigen complex formation, which is known to prevent epithelial attachment^{6 20-22} and translocation.

In conclusion, our data show that intestinal immunisation with *E coli* in rats protected against *E coli* induced cholangitis and systemic sepsis. Additional studies are needed to further define the function of biliary IgA as well as other hepatobiliary factors that influence the severity of cholangitis, such as endotoxin (and anti-endotoxin IgA), bile composition, Kupffer cell function, and systemic factors, such as the effect of induced circulating immunoglobulins in preventing sepsis.

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The research protocol was approved by the Animal Studies Subcommittee, VA Medical Center, San Francisco.

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