A mouse model for direct evaluation of cholera vaccines

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Sweden 2009

“When things go wrong, don’t go with them”

– Elvis Presley

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Abstract

Cholera continues to be an important cause of morbidity and mortality in large parts of the developing world and is a significant negative factor for economic development. *Vibrio cholerae* bacteria of the O1 or O139 serogroup can cause disease due to their ability to colonize the intestine and produce an enterotoxin, cholera toxin (CT). An effective oral vaccine against *V. cholerae* O1 is available, whereas vaccine against O139 is lacking. Development and pre-clinical evaluation of cholera vaccines have been hampered by the fact that man is the only natural host for *V. cholerae*. Although various animal models have been described, there exists no convenient and inexpensive model that allows evaluation of vaccine-induced protection against a challenge infection.

The main objective of this thesis was to develop a model that allows direct evaluation of the immunogenicity and protective efficacy of cholera vaccine candidates in conventional adult mice. Paper I demonstrates that strong serum and mucosal antibody responses to *V. cholerae* O1 or O139 lipopolysaccharide (LPS) can be induced in adult mice vaccinated intranasally or orally with either live or formalin-killed bacteria. Standardized intestinal IgA antibody responses estimated using extracts prepared from faecal pellets or from intestinal mucosa were found to correlate significantly, hence validating the use of the more convenient fecal pellets extracts for measuring gut mucosal antibody responses in vaccinated hosts. Paper II describes an adult mouse model for studying intestinal colonization by *V. cholerae* and associated immune responses. It was shown that oral pre-treatment of mice with streptomycin (Sm) allows intestinal colonization by Sm-resistant *V. cholerae* O1 or O139 bacteria, and that mice immunized with viable or inactivated *V. cholerae* as described in Paper I were comparatively refractory to colonization following infection/challenge with the immunizing strain, with protection resulting in accelerated clearance of the challenge organisms correlating inversely with the intestinal IgA anti-LPS response. In paper III this model was further used to evaluate immune responses and protection by orally administered live and killed O1 and O139 whole cell vaccines and the impact of co-administration of CT on the immunogenicity and protective effect. CT proved to be an effective adjuvant, markedly potentiating antibody responses and also increasing the protective effect against both serogroup homologous and heterologous challenge. The results presented in this thesis suggest that the new adult mouse model may be used to broaden our understanding of immune protection against *V. cholerae* infection, and thus be a useful tool in the pre-clinical evaluation of oral cholera vaccines.

Keywords: *Vibrio cholerae*, cholera vaccine, cholera toxin, LPS, anti-bacterial immunity, IgA, challenge, protection, colonization

ISBN 978-91-628-7777-4

Original papers

This thesis is based on the following papers, which are referred to in the text by the given Roman numerals (I-III):

I. Nygren E, Holmgren J, Attridge SR
Murine antibody responses following systemic or mucosal immunization with viable or inactivated *Vibrio cholerae*
Vaccine. 2008 Dec 9;26(52):6784-90.

II. Nygren E, Li Bl, Holmgren J, Attridge SR
Establishment of an adult mouse model for direct evaluation of the efficacy of vaccines against *Vibrio cholerae*
Submitted

III. Nygren E, Holmgren J, Attridge SR
Immunogenicity of live and killed *Vibrio cholerae* O1 and O139 oral vaccines in an adult mouse model: cholera toxin adjuvants intestinal antibody responses and serogroup homologous and cross-reactive protection
Submitted

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Abbreviations

Ace	Accessory cholera enterotoxin
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
BSA	Bovine serum albumin
C	Caecum
cAMP	Cyclic adenosine monophosphate
CFU	Colony forming units
CPS	Capsule
CT	Cholera toxin
CTA	Cholera toxin A-subunit
CTB	Cholera toxin B-subunit
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ETEC	Enterotoxigenic *Escherichia coli*
Fk	Formalin-killed
FP	Faecal pellet
FPE	Faecal pellet extracts
GM	Geometric mean
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IMCM	Infant mouse cholera model
IN	Intranasal
IP	Intra-peritoneal
KLH	Keyhole limpet haemocyanin
Km	Kanamycin sulphate
LB	Luria–Bertani
LI	Large intestine
LPS	Lipopolysaccharide
LT	Heat-labile enterotoxin of *E. coli*
MP	Membrane preparation
OD	Optical density
ORS	Oral rehydration solutions
PBS	Phosphate buffered saline
PERFEXT	Perfusion–extraction technique
PF	Protection factor
PMSF	Phenylmethylsulfonyl fluoride
PO	Peroral
RITARD	Reversible intestinal tie adult rabbit diarrhea model
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SI	Small intestine
Sm	Streptomycin sulphate
SNs	Supernatants
STI	Soybean trypsin inhibitor
TCP	Toxin-coregulated pilus
TcpA	Structural subunit of TCP
TLR	Toll-like receptor
VPI	*Vibrio* pathogenicity island
WT	Wild type
Zot	Zonula occludens toxin

Introduction

Vibrio cholerae

The diarrheal disease cholera is caused by the non-invasive Gram-negative curved rod bacterium now known as *Vibrio cholerae.* This organism was originally microscopically identified by Filippo Pacini in 1854. The role of *V. cholerae* in cholera disease was however not widely recognized until the rediscovery and isolation of the "comma-bacillus" by Robert Koch in 1884 (78, 85, 158). This polar monotrichous bacterium can, on the basis of the composition of the cell wall lipopolysaccharide O antigens, be classified into serogroups (see Table 1). Today more than 200 different serogroups have been described, yet for a long time only the O1 serogroup, and since 1992 also the O139 serogroup, are known to cause epidemic outbreaks (48). Importantly, strains of the O1 serogroup can also be biotyped as either classical or El Tor bacteria and further divided using serological methods into three subtypes, with names denoting their historical origin, Ogawa, Inaba and Hikojima (28). The later subtype is however rare and unstable (78).

Table 1. General description of epidemic *V. cholerae*

Serogroup	Biotypes	Serotypes	TCP biotype	Capsule
O1	El Tor	Inaba or Ogawa	El Tor	na
	classical	Inaba or Ogawa	classical	
O139	na	na	El Tor	Polysaccharides

na, not applicable

Cholera epidemiology

Cholera continues to be an important cause of morbidity and mortality in several developing regions of the world, officially accounting for between 100,000 – 300,000 cases annually during the last ten years (2). However, the WHO recognizes that the disease is grossly underreported and that the actual number of cases may be 3-5 million per year, causing at

least 120,000 deaths (182). The disease is often associated with poverty, low socioeconomic status, overcrowding and malnutrition (54, 182). Children under 5 years of age living in endemic areas and persons of blood group O are also at higher risk of developing severe cholera when infected (31, 42, 47). Studies by Clemens et. al. (31), conducted in Bangladesh at a time when the classical and El Tor biotype were cocirculating and causing similar numbers of disease, have revealed that the link between blood group O and severity of disease was only valid for *V. cholerae* O1 El Tor infection. Later, during the emergence of *V. cholerae* O139 it was observed that individuals of blood group O are also more susceptible to O139 infection than those with other blood groups (47).

V. cholerae appear to have relatively simple nutritional requirements for survival outside the human body, as indicated by the observation that the bacteria is normally found in surface water of various aquatic environments (50, 177), such as the Gulf of Mexico, the costal regions and fresh water reservoirs of many African countries and most importantly in the Ganges river; the later area being the endemic homeland for *V. cholerae* (78, 133). It is known that cholera has existed in the Indian subcontinent since the beginning of records and that the disease since at least 1817 has spread outside this area, causing seven distinct pandemics. The 6th pandemic and possibly also the 5th pandemic were caused by classical *V. cholerae* O1 strains (48, 111), whereas the current 7th pandemic is caused by strains of the El Tor biotype. In contrast to the previous pandemics the latest and still ongoing pandemic began in Indonesia on the island of Sulawesi (formerly known as Celebes) in 1961 (17). The El Tor biotype was however originally isolated by Felix Gotschlich already in 1905 from Indonesian pilgrims to Mecca, at the quarantine station in El Tor, Egypt (133). During the 1960's El Tor bacteria spread to most countries in South-East Asia where it, except for a brief period in time, displaced the classical strains totally (94, 138). During the early 1970's it continued to spread from Asia to the Middle East and also to sub-Saharan West Africa (54). In January 1991 cholera returned to South America for the first time in a century, causing epidemic outbreaks in Peru and soon thereafter affecting almost all countries in South America, where it is now endemic (182).

In 1992-1993 a second serogroup (O139) with epidemic potential emerged in India and southern Bangladesh (7, 112). Several investigations have suggested that *V. cholerae* O139 arose from an O1 El Tor strain by acquisition of DNA coding for the synthesis of the antigenically-determining (serogroup specific) O antigen (19, 159). Since this new serogroup

initially displaced the current *V. cholerae* O1 strains and also in the following years spread to several Asian countries (3, 7, 112), it was feared that this new organism may cause an 8th cholera pandemic in parallel to the ongoing 7th *V. cholerae* O1 El Tor pandemic. Although recent data have shown that *V. cholerae* O139 today exists side by side with O1 strains in India and Bangladesh, occasionally giving rise to epidemic outbreaks (49, 152), *V. cholerae* O1 El Tor is still the predominant cause of cholera world-wide accounting for more than 95% of all cases (182). Yet, based on the experiences with the El Tor biotype, which more than 50 years after its first isolation started a large pandemic, the risk that O139 may become pandemic remains.

Most recently it has become evident that different variants of *V. cholerae* O1 El Tor producing CT of the classical biotype are circulating in Bangladesh, India, Mozambique and Vietnam (9, 10, 58, 106, 111). It has further been shown that since 2001 only El Tor strains expressing CT of the classical biotype have been isolated in Bangladesh (111). Similar findings have also been made for the O139 serogroup (J. Sanchez and J. Holmgren, unpublished). Intriguingly the shift in CT expression profile appears to have caused the bacteria to become more virulent (111, 168), although solid evidence is still missing.

Clinical features

Cholera is a disease of the small intestine. Following ingestion of toxogenic *V. cholerae* and successful colonization of the intestinal mucosa the bacteria effectively release cholera toxin, which via its high affinity interaction with the monosialoganglioside GM1 on the intestinal epithelium is endocytosed. Subsequently CT activates adenylate cyclase inside the epithelial cells and induces a cascade reaction which ultimately gives rise to the characteristic profuse, and sometimes life-threatening, diarrhea and dehydration. In addition to CT, there are also several “minor toxins” which contributes to the pathogenicity of *V. cholerae*, as indicated by the observation that attenuated live cholera vaccines unable to produce CT still are able to cause diarrhea in healthy volunteers (91, 165). Although the colon is also affected by CT, it has been estimated that approximately 90% of the secreted fluids originate from the small intestine (67, 133). In contrast to what was originally described by Robert Koch (85), *V. cholerae* does not normally disrupt the intestinal epithelium, hence the resulting diarrheal fluid contains only minor amounts of protein and blood cells (67, 78). Instead the volumes stools contain large amounts of sodium, chloride, bicarbonate and potassium (133). In severe

cases (*cholera gravis*) continuous diarrhea, if untreated, often results in hypovolemic shock and collapse after only a few hours. Death can occur already 12 hours after onset of disease (133), making cholera a much feared infectious disease. A large proportion of *V. cholerae* infections do however remain inapparent and do not result in severe disease (78, 104, 126, 149).

The incubation period and the severity of disease appear to be effected by the size of the inoculum (78, 108). Whereas the infectious dose to cause severe cholera in healthy adult volunteers has been shown to be ca. 10^8 bacteria (78, 133), malnourished, hypochlorhydria and immunodeficient individuals has been shown to be more prone to develop disease (134, 180), with doses as low as 10^3 bacteria commonly being cited (78, 133) . If the bacteria is ingested together with food or other agents with the capacity to neutralize the gastric acidity e.g. bicarbonate, the minimal pathogenic dose is decreased significantly; with 10^6 vibrios being able to induce diarrhea in 90% of volunteers (67, 72).

Although *V. cholerae* clearly spreads via the faecal-oral route, direct person-to-person spread is probably less common than ingestion of contaminated water or food (128). *V. cholerae* passaged through the human intestine has recently been found to be more infectious in infant mice than *in vitro* cultured bacteria (105). This transient hyper infectious phenotype may be one mechanism that contributes to the explosive outbreaks of cholera often seen during epidemics. Another such mechanism by which *V. cholerae* increases its spread during epidemics may be the capability to cause inapparent infections, leading to dissemination of bacteria for an extended period of time following infection (83).

Patients with severe cholera may require intravenous fluid rehydration, especially when the rate of fluid loss by diarrhoea exceeds that of what can be compensated by oral rehydration. However, most cases of cholera can be successfully treated with simple oral rehydration solutions (ORS), containing both salts and glucose (133). When properly administered the ORS can decrease the cholera case fatality rate to below 1% even in developing countries (2). Although effective antibiotic treatment can be used to shorten the duration of cholera disease and decrease the risk for further spread of the infection, the WHO only recommends treatment with antibiotics in severe cases of cholera. Due to uncontrolled use of antibiotics in several cholera-struck regions, numerous pathogenic *V. cholerae* strains are today resistant against

some of the most common antibiotics on the market, including Streptomycin, Trimethoprim, Kanamycin, Ampicillin, Gentamycin and Tetracycline.

Despite the high rate of inapparent infections and the efficacy and simplicity of treatment, the annual morbidity/mortality toll in developing regions of the world means that vaccine research is warranted.

Pathogenicity and virulence factors

Studies of the processes occurring in the intestine after ingestion of pathogenic *V. cholerae* O1 or O139 have shown that those vibrios that do survive passage through the acidic stomach rely on temporal expression/action of several different secreted and cell-associated components for colonization of the intestinal mucosa and subsequent induction of cholera disease. Most important for the colonization, which is a prerequisite for production of CT, is 1) the sheathed polar flagellum, 2) the mucinase (soluble hemagglutinin) and 3) the toxin-coregulated pilus (TCP). The former two are thought to be involved in the early phase of colonization and TCP in the late phase. In addition it appears that LPS also plays an important role (28, 67, 78).

The key virulence factors of *V. cholerae* are encoded in two distinct areas on chromosome I of the bacteria, the CTX element and the vibrio pathogen island (VPI). Both of these elements are believed to originate from different bacteriophages (39, 139). The CTX element is the genome of an integrated filamentous phage, CTXΦ and contains the genes encoding the cholera toxin (173). Importantly the receptor for CTXΦ has been shown to be TCP, which in turn is encoded in the VPI island (79, 80, 86). Studies by Boyd and Waldor have however suggested that the bacteriophage CP-T1 provides an alternative TCP independent mechanism for horizontal transfer of CTXΦ between pathogenic and non-pathogenic *V. cholerae* strains (23). In addition, there are also virulence determinants that are located outside the CTX and VPI elements, LPS probably being the most important (7).

CT

Undoubtedly, cholera toxin is one of the more potent bacterial toxins known to man; capable of causing severe diarrhoea even with doses as low as 5-10 μg (89). The existence of this enterotoxin was first postulated by Rober Koch and later separately identified independently

by both De and Dutta in 1959 (40, 45). A few years later it was shown that CT is a complex protein consisting of one A subunit (28 kDa) and five B subunits (11.6 kDa each) in a ring (95). Following colonization the bacteria upregulates the genes encoding CT (*ctxAB*) and express CT which after assembly in the periplasm, via a so-called type II secretion system, is efficiently, transported across the outer membrane and released into the surroundings. The excreted toxin binds to GM1 ganglioside receptors on the intestinal epithelium via the non-toxic B-pentamer (69), which allows for the toxin to be internalised (139). Following proteolytic cleavage of the CTA into its CTA1 and CTA2 parts, the former becomes active. The CTA1 component then activates adenylate cyclase by catalysing the ADP-ribosylation of CTP-binding regulatory component of the cyclase system. Subsequently, abnormal levels of intracellular cyclic adenosine monophosphate (cAMP) accumulates and modulates electrolyte and fluid transport across the epithelium. It is generally accepted that the volumes diarrhea results from increased active anion secretion, mostly chloride and bicarbonate, in combination with simultaneous inhibition of sodium absorption. These processes result in increased sodium outflow, and drives water secretion which gives rise to the characteristic cholera diarrhea (67, 78, 139). The heat labile toxin (LT) of enterotoxigenic *E. coli* (ETEC) has been shown to function similarly to CT in many aspects (140). Importantly the toxic effects of both CT and LT can be antagonized by cross-reacting antibodies to the B-subunit (70).

TCP

The toxin-coregulated pilus (TCP) is a homopolymer of repeated subunits of the major pilin component TcpA, similar to other enterobacterial type 4 pili (170). *V. cholerae* O1 El Tor and O139 bacteria produce a closely related pilli, whereas bacteria of the classical biotype produce an antigenically dissimilar pili (77, 129, 181). Studies initially performed in infant mice showed that mutants unable to express TCP are incapable of colonizing the intestinal epithelium (13, 170). When wild-type *V. cholerae* were mixed with mutant bacteria unable to express TCP and fed to infant mice, the mutants were found to be outcompeted with regards to the number of colonizing bacteria, giving rise to a very high competition index (13). These findings have also been confirmed in human volunteer challenge studies (65, 167).

Although the exact role of TCP in colonization is not fully understood, it has been suggested that there might exist an intestinal receptor for TCP (84). However, reports have also shown that TCP promotes bacterial interaction and that the pilli is crucial for microcolony formation (84, 93, 170). These results do however not contradict each other and the function of TCP in colonization may be multifactorial.

The *V. cholerae* flagella

The polar flagellum of *V. cholerae* is partially covered by a protrusion of the LPS containing outer membrane (53). The function of the flagella is complex, contributing to the motility of *V. cholerae* by operating together with chemotactic receptors and intracellular sensor molecules (20). Most likely the flagella contributes to the patogenicity of the bacteria by mediating some of the initial steps of colonization (15). Directed motility towards the intestinal cell wall by *V. cholerae* has been shown to depend on the propelling movement of the polar flagella and deletion of genes encoding the sodium-driven flagella motor proteins results in flagellated but non-motile bacteria (20). Human volunteer trails conducted with attenuated live cholera vaccine candidates have suggested that the reactogenicity observed with most attenuated *V. cholerae* strains are at least partially caused by flagella driven motility (82). Importantly it was shown by Taylor *et al.* (169), that infection with high doses of a filamentous motility-deficient mutant *V. cholerae* O1 El Tor (Peru 14), originally identified to have reduced capacity to move in soft-agar, produced only marginal reactogenicity in a volunteer study. Studies performed using different animal infection models to compare *V. cholerae* strains with various combinations of defined motility and/or structural mutations have also demonstrated that motility is an important component of *V. cholerae* colonization and pathogenicity in experimental cholera (87, 130). Further, it has been shown that flagellin, the major component of the inner structure of the flagella, can induce inflammatory responses via TLR5 (63). The exact role for the flagella in colonization and virulence of *V. cholerae* O1 El Tor and O139 strains is however not very well understood (20, 153).

Mucinase (soluble hemagglutin)

The soluble hemagglutinin, which is encoded by the *hapA* gene, is one of several hemagglutinins produced by *V. cholerae* O1 of both biotypes and O139 (64). The soluble hemagglutinin (or HapA and HA/protease) is a secreted zinc-dependent protease (22, 153) that has been shown to cleave several mucus associated components including mucin, fibronectin and lactoferrin (51). Translocation of *V. cholerae* through mucin-containing gels has also been shown to required expression of *hapA* (155). In addition it has also been shown to be involved in the activation of the cholera toxin A-subunit (21). Further, the soluble hemagglutinin is also excreted via the same general secretion pathway as CT (141). These findings fit well with the idée that the initial phase of *V. cholerae* colonization most likely

depends on the capacity of the bacteria to penetrate the intestinal mucus layer (153). In contrast to findings in infant mice and rabbits, safety and immunogenicity studies with an attenuated cholera vaccine candidate *V. cholerae* 638, lacking the CTXΦ and also *hapA* have recently indicated that the soluble hemagglutinin contributes to the pathogenicity and adverse effects often observed with live attenuated cholera vaccines (55, 153)

Other virulence factors of *V. cholerae*

In addition to CT and mucinase, *V. cholerae* also produce several other soluble factors with pathogenic potentials. Among these are the Accessory cholera enterotoxin (Ace); Zonula occludens toxin (Zot) which might be both a morphogenetic phage protein and an enterotoxin; the pore-forming and vacuolating hemolysin A, the S-CEP (Chinese hamster cell elongating protein) cytotonic protein; and the actin-crosslinking RTX toxin (67, 78, 142). Although these factors have been suggested to singly or jointly contribute to some of the adverse reactions observed with live attenuated cholera vaccines, the importance of these factors in human disease remains some-what unclear (91, 166, 169).

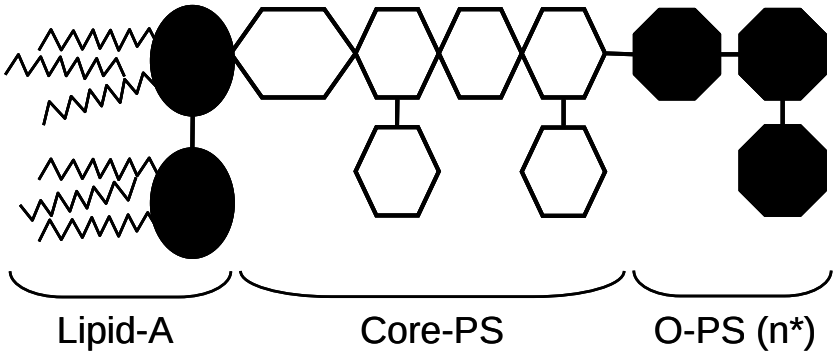

Fig. 1. Schematic structure of *V. cholerae* lipopolysaccharide.
* The number of monosaccharide repeating units of the O-PS is variable between *V. cholerae* serogroup O1 (n = 12-18) and O139 (n = 1).

The cell walls of *V. cholerae* O1 and O139, similarly to other Gram-negative bacteria, contains both protein structures and LPS which interact with the host and facilitates pathogenicity (28, 78). The LPS of *V. cholerae* is a highly immunogenic and retains this property after both heat and formalin treatment (28, 162). The genes encoding the biosynthesis of LPS are located on chromosome 1 of *V. cholerae* and give rise to the

characteristic polysaccharide structure consisting of the lipid-A, core-PS and O-PS (see Figure 1). The core-PS and O-PS parts project outward from the bacteria while the lipid-A region of LPS is attached to the outer membrane (27). In addition to its general function in preventing bile salts and other bactericidal molecules found in the intestine, LPS may also be directly involved in gut colonization, as indicated by the observations that the O-PS is important for intestinal colonization in infant mice (11, 172). Further, it has also been shown *in vitro* that attachment of *V. cholerae* to a monolayer of mucin-secreting cells can be partially inhibited by the addition of LPS (18).

The apparent lack of cross-protection between *V. cholerae* O1 and O139, observed during the emergence of the O139 serogroup in 1992 to 1993, also stresses the importance of the O-antigens for pathogenicity (7, 126, 133). In the years following after the emergence of *V. cholerae* O139, it was shown that the O139 O-PS lack perosamine, a characteristic sugar found in *V. cholerae* O1 of both El Tor and classical biotype; and that the O-PS of O139 consists of only one subunit instead of several, as in the O1 serogroup (27, 126). In addition to the LPS, *V. cholerae* O139 similarly to several other non-O1 vibrios also produce a capsule consisting of polymerized O-antigen subunits (CPS), that are antigenically very similar underlying O-PS (27, 126). Functional studies of the role of the capsule have suggested that presence of CPS contributes to virulence by decreasing the sensitivity to serum killing and by increasing binding to intestinal epithelial cells (76, 172). More recent studies have suggested however, that production of O-antigen by O139 strains is of greater importance than the presence of CPS material for virulence (11).

Outer membrane proteins (Omps) of *V. cholerae* have been implicated to participate in bacterial interactions in the host intestinal milieu. Although TCP and the flagellum are major components of these interactions, immune sera collected following vaccination or infection with *V. cholerae* also recognize other non-LPS antigens (38, 147, 156). Some of these immunogenic proteins have also been observed to be protective antigens in experimental cholera models, such as OmpU (38-42 kDa) and OmpW (19-22 kDa) (38, 148, 156). A so called ”cholera protective antigen” (18 kDa) has also been suggested to contribute to protection against experimental cholera (145).

Studies the mannose-sensitive hemagglutinin (MSHA, 17 kDa) which is expressed on *V. cholerae* O1 El Tor and O139 but not on classical bacteria, have shown that even though this

pilus is immunogenic (122, 124), monoclonal antibodies to MSHA do not protect infant mice against *V. cholerae* infection (13). Importantly, a human volunteer vaccine study with a MSHA deficient *V. cholerae* O139 strain CVD 112 have also failed to prove a role of this pili in colonization of the human intestine (167). The function of MSHA is probably instead related to its persistence in aquatic environments (74, 175). Further, it has also been suggested that repression of MSHA expression following infection is important for avoiding IgA mediated blocking of *V. cholerae* colonization, in a mannose-sensitive, non-antibody specific manner (73, 74).

Immunity to cholera

Protection against cholera is most likely a combination of adaptive immune responses elicited by previous infection or vaccination and innate immune mechanisms. Little is however known about the latter. Recently though, whole-genome microarray studies conducted on duodenal biopsies collected during the acute phase of the infection, have shown broad upregulation of genes known to be involved in innate immunity (60). This observation is in line with findings by Qadri *et al*. (52), showing increased numbers of neutrophil polymorphs in the lamina propria and upregulation of several innate mediators e.g. myeloperoxidase, lactoferrin and α-defensin in adults suffering from acute watery cholera diarrhea.

Infection with *V. cholerae* O1 or O139 can induce lasting protective immunity against re-infection, especially if severe enough to cause clinical disease (108, 121). The nature of this protective effect has been well studied and there is abundant evidence that along with CT, *V. cholerae* lipopolysaccharide is the predominant protective *V. cholerae* antigen (56, 67, 88). Serological studies utilizing the bactericidal assay, which measures the ability of antibodies to kill *V. cholerae* in the presence of complement, have shown that vibriocidal antibodies increase with age in areas where *V. cholerae* is endemic and that the attack rate is inversely related to the vibriocidal antibody titre (34, 88, 164). Although a minor proportion of the vibriocidal response may be directed against outer membrane proteins, the major component of the response is immunoglobulin M directed against serotype specific LPS (57, 97, 109, 110, 113). The large epidemic outbreaks of *V. cholerae* O139 during 1992 to 1993 in India and Bangladesh, where the El Tor biotype at that time was endemic (12, 97, 114), further illustrates that acquired immunity to LPS is of utmost importance for protection against disease. Despite the fact that *V. cholerae* O1 El Tor and O139 share several characteristics

and produce identical CT and TCP (3, 7, 112), the epidemic outbreaks of *V. cholerae* O139 in 1993 were distinguished by an unusually large proportion of infected adults (5, 129).

Even though serum vibriocidal antibodies induced by infection or oral immunization is the best known predictor for protection against disease caused by *V. cholerae* (3), these antibody responses are most likely surrogate markers for immunity to cholera. Importantly, there exists no threshold vibriocidal titre above which a person is protected against developing symptomatic cholera (35, 109, 164). Secondly it is also known that even though parenteral cholera vaccines elicit very high serum vibriocidal titres, such vaccines only confer short-lived (6 months) and limited protection (around 50%) (62, 137). More likely, protection against disease induced by infection or vaccination results from a broader gut derived IgA immune response to *V. cholerae* (16, 59, 62, 124).

Several studies in experimental cholera models have convincingly shown that intestinal synthesis of IgA antibodies to CT are protective (62, 67, 71, 150). Importantly the CTB component by itself also has the capability to induce strong protection against experimental cholera (119, 120, 161), and to provide about 60% cross-reactive protection for 6 months against diarrhea caused by heat labile toxin (LT) producing enterotoxigenic *E. coli* (ETEC) (33).

Serum IgA, but not IgG, antibodies to CTB have also recently been found to predict protection against *V. cholerae* O1 El Tor infection in a cohort household study (62). This study also shows that serum antibodies to TCP, which can been found in approximately half of all tested individuals previously infected with *V. cholerae* (16, 61), are also associated with protection against both *V. cholerae* O1 and O139 (62). Although antibodies to TCP are sufficient to protect infant mice from colonization by *V. cholerae* (151, 160), direct protection induced by TCP remains to be de demonstrated in humans.

Vaccines against cholera

Following the initial attempts by Jaime Ferrán in 1884 to vaccinate against cholera using ordinary broth cultured *V. cholerae*, several vaccines against cholera have been developed (59). A parenteral cholera vaccine containing a mixture of killed whole cell *V. cholerae* O1 of different serotype and biotype is still available in some countries. However, this type of

vaccine does not only provide limited and short-lived protection (around 50% for less than six months), but are also associated with frequent adverse reactions (50% local reaction and 30% general) (59). For these and other reasons WHO does not recommend the use of any injectable vaccine. Instead WHO recommends the use of oral cholera vaccines (OCV) in certain endemic and epidemic situations (67). Two principally different types of oral cholera vaccines have been licensed for wider use in humans: 1) a killed whole cell oral vaccine with or without the nontoxic B-subunit of cholera toxin and 2) an attenuated live oral vaccines (59, 67); however only the first type is currently available on the market.

Killed whole cell oral vaccines

As a result of several studies on the immunogenicity and protective effect of purified CTB and also killed *V. cholerae* administered orally, the Dukoral™ whole cell cholera vaccine was developed during the 1980s (66). The present vaccine consists of 1 mg of purified recombinant CTB in combination with 2.5 x 10^{10} killed *V. cholerae* O1 El Tor (Inaba) and Classical (Inaba and Ogawa) bacteria (96). The protective efficacy of the Dukoral™ vaccine has in two studies carried out in Bangladesh and Peru been shown to be ca. 85% for six months (67). Recently the vaccine was also administered to a large population in Mozambique with a high seroprevalence of HIV infection without markedly reducing its efficacy (98). In long term studies of Dukoral™ vaccinated individuals in Bangladesh, the protective efficacy against cholera after three years has also been shown to be about 50% (174). Importantly the added CTB component has also been shown to have a synergistic protective effect together with the whole cell component, increasing the protective efficacy of the vaccine significantly during the initial eight months of observation (30, 32). Dukoral™ has also been found to function well as a travelers' vaccine if two doses are administered prior to departure (143), and further to give rise to heard protection (20). Currently the Dukoral™ vaccine is produced by Crucell (formally SBL vaccines) and licensed in more than 50 countries (67).

Technology transfer from Sweden has also allowed for a simplified *V. cholerae* O1 vaccine lacking the CTB-component to be produced and licensed in Vietnam (67, 96). More recently, production of the Vietnamese cholera vaccine has been improved and the composition altered so that it now also contains killed *V. cholerae* O139 bacteria (8). Noteworthy, the reformulated vaccine contains almost 40% more LPS antigen as compared to the earlier Vietnamese vaccine (103). Following successful phase II studies with this bivalent

formulation, the production technique is currently being transferred from Vietnam to WHO-approved facilities in India and Indonesia. A large phase III placebo-controlled, randomized trail undertaken in Kolkata, India, with support from the International Vaccine Institute (IVI) is also currently being evaluated.

CVD 103-HgR and other attenuated live oral cholera vaccines

Several different live attenuated *V. cholerae* strains lacking at least the A-subunit of CT have been developed as vaccines against cholera. One of these strains CVD 103-HgR (Orachol^{TM}) has also been licensed in a few countries for use in travelers to endemic areas (67). Orochol is based on the *V. cholerae* O1 classical Inaba strain 569B and has been genetically detoxified to contain only the B-subunit of CT. A mercury resistance gene has also been introduced into the hemolysin locus, allowing for this strain to be easily identified in environmental samples (90). CVD 103-HgR has been shown to be safe and immunogenic in many studies, eliciting strong antibacterial responses with only a single dose (96). The protective data are however some what contradictory. Vaccination with this classical strain has been shown to provide protection in US volunteers against later challenge with *V. cholerae* O1 El Tor (163), but a large double-blind, placebo-controlled field trial carried out in Indonesia with CVD 103-HgR unfortunately failed to show significant protection during the four-year observation period (13.5% overall efficacy) in a cholera-endemic setting (132). Yet, a retrospective study of a vaccination campaign in Micronesia, carried out as part of a cholera outbreak response, estimated the vaccine efficacy of CVD 103-HgR to be 79% (25). Importantly this vaccine does not appear to be protective against *V. cholerae* O139 (6). Production of the CVD 103-HgR vaccine was halted in 2004 (1, 96).

In addition to the CVD 103-HgR, a handful of attenuated live El Tor vaccines (Peru 15, CVD 110, IEM101 and *V. cholerae* strain 638) or O139 vaccines (Bengal 15 and CVD112) are currently in various states of clinical evaluation (67). Although these live attenuated vaccines are highly immunogenic most have also been reported to elicit reactogenic responses or at least mild diarrhea (142).

Cholera toxin as immunomodulator

In addition to the enterotoxic effects of CT and LT, these toxins are highly immunogenic and also remarkable adjuvants. The immunomodulatory property of CT was initially observed using the systemic route of immunisation (68, 81). When CT a few years later was given orally to mice together with keyhole limpet haemocyanin (KLH) it was observed that the intestinal IgA antibody response to KLH was significantly potentiated, indicating that CT was also an adjuvant for unrelated antigens delivered mucosally (46). Later studies confirmed these findings and also revealed a critical temporal effect of CT-administration on intestinal antibody formation (100, 139), simultaneous administration of CT and KLH giving rise to the strongest responses.

The adjuvant effects of CT and LT observed following administration into the intestinal tract has been suggested to largely depend on the fact that these toxins are remarkably resistant to degradation by proteases, bile salts and other compounds in the intestine; bind with high affinity to GM1 ganglioside receptors, which are highly expressed on intestinal epithelial cells as well as on the M-cells of lining the Peyers patches; and stimulates uptake of co-administered antigens by increasing permeability of the intestinal epithelium leading to enhanced accessibility for antigen-presenting cells (139, 140). On the cellular level, CT and LT enhances antigen presentation by various antigen presenting cells, promotes isotype switching to IgA in B-cells, and modulates complex stimulatory as well as inhibitory pathways regulating T-cell proliferation and cytokine production by both innate and adaptive immune cells (99, 139, 140, 142). Although the adjuvanticity of CT and LT mainly resides in the enzymatic activity of the A1 component, the underlying mechanisms are not yet fully understood (127).

Animal models for intestinal *V. cholerae* infection and disease

V. cholerae is a strict human enteropathogen, the adults of no other species being naturally susceptible to infection (26, 41, 44, 157, 171). All animal models in adult hosts are therefore unnatural in one way or another, often requiring chemical, antibiotic and/or surgical manipulations. Yet, information obtained from various animal cholera models has been found to predict the outcome of several human infection and protection studies (131).

The pioneering work by De *et al.* (40) in the identification of the cholera toxin was performed using the rabbit ligated intestinal loop model (41). False-positive results are however not

uncommon with this model. Several other animals, for example rats, mice and chickens, have been similarly treated and used for studies of the secretory response of CT (131). Importantly studies using the mouse ligated loop assay have provided direct evidence for the importance of intestinal IgA in protection against CT (161). The Removable Intestinal Tie Adult Rabbit Diarrhea (RITARD) model developed by Spira *et al.* (157), has also been of great importance for studies of induction as well as protection against lethal watery diarrhea (101, 130). Similarly to the ligated loop assays this model does require surgical manipulation prior to challenge i.e. ligation of the small intestine with a slip knot tie directly proximal to the caecum. Two hours after inoculation the tie is removed and the rabbits are monitored for diarrhea and lethality, usually occurring within a few days in unimmunized animals infected with *V. cholerae* (131, 157). An alternative to these invasive models is the canine cholerae model of Sack and Carpenter (135) which do not require any surgical work. Despite the usefulness and reproducibility of this model it is not commonly used because of the high cost and more rigid animal care associated with it.

Much less expensive are infant (or suckling) mice, which following oral infection with *V. cholerae* bacteria do become intestinally colonized. This model, originally developed by Ujiiey *et al.* (171), is commonly used for competition experiments between wild type (WT) *V. cholerae* and mutant bacteria expressing some sort of marker that allows identification. Important information about the relative significance of TCP and MSHA for colonization was obtained using this model (13). Other very young animals such as infant rabbits can also readily be colonized with *V. cholerae* (44). However, these models are limited by their immunological and physiological immaturity. Yet studies of passive immunity have successfully identified antigenic targets which can block colonization (151, 160). As these animals become older and acquire an intestinal bacterial flora they do however rapidly become refractory to colonization.

In contrary to conventional mice, adult gnotobiotic mice can also become colonized with *V. cholerae* (24). Although such mice have been used occasionally to study acquired immunity to different antigens expressed by *V. cholerae* (37), the usefulness of this model has been questioned. Not only is the intestinal immune system in germfree mice relatively immature, their handling and housing is also costly (102, 107).

Aims of this study

The general aim of this thesis was to develop a model that will allow direct evaluation of the immunogenicity and protective efficacy of cholera vaccine candidates in conventional adult mice.

The specific aims were:

- To define immunization protocols for viable and killed *V. cholerae* which consistently elicit both systemic and mucosal antibacterial antibody responses.
- To evaluate the validity of using faecal pellet extracts for assessment of intestinal antibody responses.
- To define protocols for establishing intestinal *V. cholerae* O1 and O139 colonization in streptomycin treated adult mice
- To examine if vaccinated hosts are comparatively refractory to colonization following challenge with *V. cholerae*.
- To evaluate the impact of co-administration of cholera toxin on vaccine-induced antibody responses and protection.
- To examine correlates of immunity with protection against colonization.

Materials and methods

Bacterial strains and culture conditions

V. cholerae strains used in this thesis (See Table 2) were stored at -80°C in Luria-Bertani (LB) medium containing 20% glycerol, and grown in LB at 37 °C with agitation (150 rpm). When necessary for *in vivo* studies, Sm-resistant variants were selected by growth on LB agar plates containing Sm (200 μg/ml). For immunization or challenge of mice, bacteria were grown to OD_{600} 1.0-1.2, harvested by centrifugation and washed twice in PBS before use. Wild-type *V. cholerae* strains H1 and N1696 are of O1 serogroup (biotype El Tor, serotype Ogawa and Inaba respectively), while JBK70 is an atoxigenic mutant of the latter (91). MO10 and AI-1838 are encapsulated serogroup O139 isolates. AF3 is an unencapsulated variant derived from AI-1838 (11) which was used for isolation of O139 LPS. A *tcpA*::Km (kanamycin) derivative of AI-1838 (13) was used to investigate the role of TCP in colonization.

Table 2. Presentation of *V. cholerae* strains and their variants used in this thesis

Parent strain	Serogroup	Serotype	Biotype	Variants
H1	O1	El Tor	Ogawa	WT
				tcpA::Km^R mutant
N16961	O1	El Tor	Inaba	WT
				Δ*ctx* (JBK 70)
MO10	O139	na	na	WT*
AI-1838	O139	na	na	WT*
				tcpA::Km^R mutant
				Unencapsulated (AF3)

na, not applicable
*, encapsulated

Formalin-inactivation of *V. cholerae*

Vibrio cultures grown and harvested as above were washed twice with PBS and resuspended to a final concentration of 10^{10} cells/ml in 1% formaldehyde-PBS. Following incubation at 37°C for 2 hours the cells were washed twice and resuspended in PBS. Aliquots were plated to confirm sterility before addition of sodium azide and storage at 4°C. Formalin-killed (fk)

bacterial suspensions were enumerated using a Neubauer Improved cell counting chamber (0.02mm, Neubauer, Germany) and used within 14 days of preparation. Prior to immunization the suspension was again washed twice in PBS and resuspended to the desired concentration.

Isolation of *V. cholerae* antigens

LPS was extracted from *V. cholerae* O139 strain AF3 (11) using the phenol–water method of Westphal and Jann (176). After phenol extraction crude preparations were treated with Deoxyribonuclease I (Sigma-Aldrich D5025; 0,4 mg/ml) and Ribonuclease A (Sigma R5000; 0,4 mg/ml) in Tris/HCl-buffer (20 mM, pH 8.0) containing $MgCl_2$ (1 mM) and NaCl (10 mM) for 24 h at room temperature (RT); followed by treatment with Proteinase K (Sigma P8044; 2% w/w) in Tris/HCl-buffer (20 mM, pH 8.0) containing $CaCl_2$ (1 mM) for 24 h at RT. After a second phenol-water extraction, the preparation was dialyzed extensively against distilled water and then lyophilized. Protein contamination was < 1% as judged by Micro BCA Protein Assay Kit (Pierce Biotechnology) and by optical density at 280 nm relative to total weight. *V. cholerae* O1 LPS (Inaba 569B) was purchased from Sigma Aldrich (L-0385) and described as having <3 % protein contamination. Membrane proteins from *V. cholerae* O1 strain JBK70 was prepared as described previously (4). Briefly, the bacteria were sonicated and subjected to low-speed centrifugation to remove intact bacteria. Cell membranes were pelleted by centrifugation (30 min at 10k x g), resuspended in PBS and optically quantified as above.

Animals

Female BALB/c, C57BL/6, C3H/HeN, SJL and CD1 mice were purchased from Charles River Laboratories (Sulzfeld, Germany), provided food and water ad libitum, individually marked and generally used one week after delivery at the age of 8 weeks. In a few experiments mice of younger ages were used in competition studies (se below). All animals in this study were treated and housed under specific-pathogen free conditions at the Laboratory for Experimental Biomedicine at University of Gothenburg as stipulated by the Ethical Committee for Laboratory Animals in Gothenburg.

In vivo experiments with *V. cholerae*

a) Immunization with viable or inactivated *V. cholerae*

Mice were immunized without any prior antibiotic treatment. IN immunization was performed by administering 10 µl of bacterial suspension (prepared as described above in LB), dropwise to the external nares of each animal using an air displacement pipette. Mice to be immunized either intragastrically or intraperitoneally (IP) were lightly anaesthetized with isoflurane (Isoba vet, Schering-Plough Animal Health, Stockholm). For IP immunization, mice were injected with 200 µl bacterial suspension (prepared as described above in PBS). Immediately prior to intragastrical inoculation, refered to as peroral (PO) immunization throughout our studies, the bacterial suspension was mixed with an equal volume of 1 M $NaHCO_3$ in LB, then 200 µl was administered using a 1 ml syringe and a disposable feeding needle with silicon tip (Fuchigami Ltd., Kyoto, Japan). When immunizations were performed with viable *V. cholerae*, the number of bacteria (CFU) administered to the animals was determined retrospectively by plating suitable dilutions of the suspensions onto LB-plates. In all experiments additional age-matched mice were set aside as untreated controls.

b) Infection/challenge with viable *V. cholerae*

Naïve or immunized mice were treated with Sm prior to challenge with pathogenic *V. cholerae*. Animals were provided with Sm-containing water *ad libitum* according to schedules described in the figure legends. Dosing was performed as described for PO immunization above; the number of viable bacteria administered was estimated retrospectively by viable counting.

c) Estimation of *in vivo* colonization

The duration and intensity of *V. cholerae* colonization was generally estimated by monitoring excretion of bacteria in freshly-collected faecal pellets (FPs). Four FPs (ca. 0.07 g) were collected into 1 ml of ice-cold PBS, homogenized and plated in serial dilutions onto agar plates containing Sm. Initially collection tubes were weighed prior to and after collection of FPs, to allow calculation of CFUs per mg faecal material, but this did not significantly alter the data and was deemed unnecessary.

In some experiments additional mice were infected to allow enumeration of bacteria persisting within the small intestine (SI), caecum (C) and large intestine (LI). Tissues were

extensively rinsed in ice-cold PBS to remove debris and non-adherent vibrios, homogenized in 2 mls PBS and serial dilutions plated onto agar plates containing Sm. (Previous testing had shown that recoveries from Sm-plates were comparable to those obtained using thiosulfate/citrate/bile/sucrose plates). Representative colonies were examined for agglutination using monoclonal antibodies directed against the relevant LPS. The limit of detection was 20 CFU; negative samples were given a value of 10 CFU for statistical analysis.

d) Competition studies

The colonization potentials of wild-type and *tcpA*::Km-mutant bacteria were directly compared in competition experiments as described previously (13). BALB/c and CD1 mice of various ages were infected with a mixed suspension of the two strains, the input ratio being determined retrospectively by spreading the inoculum on plates containing Sm or both Sm and Km. After 24 hours the SIs were excised and homogenised and the resulting suspensions again plated on both media for determination of output ratios.

Sample collection

Blood samples were collected by tail bleeding and the resulting sera stored at -20°C. Two procedures were used to gather samples for estimation of intestinal IgA responses following immunization. Supernatants were prepared from homogenates of fresh FPs (24) and in some experiments additional animals were immunized and sacrificed to allow for preparation of tissue extracts using the perfusion-extraction technique (PERFEXT) (178).

For preparation of FP supernatants, seven fresh FPs were collected into Eppendorf tubes containing 600 µl of ice-cold PBS buffer with 0.1 mg of soybean trypsin inhibitor (STI; Sigma) per ml, 1% (wt/vol) bovine serum albumin (BSA), 25 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 50% (v/v) glycerol (24). The pellets were emulsified and left at 4°C for 4 h. Debris was removed by centrifugation (15.5k x g, 4°C) and the resulting supernatants stored at -20°C. FPs were collected and supernatants stored in tubes which had been pre-blocked with 1% BSA-PBS overnight at 4°C. When analyzing the antibody content in the FP supernatants the samples were always kept on ice.

Mucosal tissue extracts were prepared using a modified version of the PERFEXT method (178). Briefly, animals were anaesthetized and perfused with at least 20 ml 0.1% heparin-PBS

per mouse before removal of the lung, small intestine, caecum and large intestine. Tissue samples were then stored at –20°C in 450 µl PBS solution containing 2 mM PMSF, 0.1 mg/ml of STI, and 0.05 mM EDTA. The tissue samples were later thawed and permeabilized by addition of saponin (Sigma) to a final concentration of 10% (vol./vol). After incubation at 4°C overnight, SNs were collected by centrifugation at (15.5k x g, 10 min) and frozen at -20°C.

Analysis of antibody responses

a) Enzyme-linked immunosorbent assay (ELISA)

Antibody responses to LPS, CTB and membrane proteins (MP) were analyzed as described before using ELISA (75). Briefly, high-binding plates (Greiner, Germany) were sensitized with (O1 or O139) LPS or (O1) MP (all 5 µg/ml in PBS) overnight at 4°C. Low-binding plates (Nunc, Denmark) were similarly sensitized with GM1 ganglioside (0.3 nmol/ml in PBS) and then further incubated with CTB (0.5 µg /ml). Following blocking with 1% BSA-PBS test-samples and a positive control of known activity were titrated in three-fold falling dilution and incubated for 4 hours at 37°C. The following conjugates were used according to the manufacturers description: goat-anti-mouse IgA conjugated to horseradish peroxidase (HRP) and goat-anti-mouse IgG-HRP (Both from Southern Biotech) or goat-anti-mouse IgG-HRP (Jackson ImmunoResearch Europe Ltd).

Because of variations in the immunoglobulin content of the tissue and FPs, IgA ELISA titres were standardized per mg of total IgA as described previously (179), with the following modifications: High Binding ELISA trays were sensitized with goat anti-mouse IgA (1 µg/ml, Southern Biotech) in PBS; samples and standard (purified mouse IgA, Southern Biotech) were titrated in three-fold falling dilutions; and goat-anti-IgA-HRP conjugate was used as above.

b) Bactericidal assay

Serum samples of specific interest were also tested for the presence of (complement-dependent) bactericidal antibodies in a microtitre plate assay, described elsewhere (12). Briefly, *V. cholerae* O1 or O139 strain H1 and AI-1838 respectively was cultured to early-log-phase in LB medium and spread (ca. 10^4 CFU) onto LB-agar plates containing streptomycin (Sm; 200 µg/ml). After incubation for 18 h at 37°C, the resulting growth was

harvested with PBS. The OD_{600} was used to guide dilution of the suspension to a final concentration of ca. 2 x 10^5 vibrios per ml in PBS, containing Sm (200 µg/ml) and 20 % guinea pig serum as the source of complement. This suspension was then added to a microtitre tray (50 µl/well) containing equal volumes of samples (or a standard of known activity) previously serially titrated in PBS, resulting in a final bacterial concentration of 10^5 per ml and a final complement concentration of 10%. Negative control wells (negative for bacterial growth) received the same solution without bacteria. After incubation for 70 min at 37°C, 100 µl pre-warmed 4x LB medium (containing Sm) was added to each well. Incubation was continued for approximately 5 h at 37°C, until the positive control wells (bacterial suspension added to PBS with no antibody) reached OD_{600} ca. 0.35 (Labsystems Multiskan MS spectrophotometer). The mean ODs of negative and positive control wells (a and b, respectively) were used to calculate an OD that represented a 70% inhibition of bacterial growth (OD = a + 0.3[b – a]). This value was then used to assign a lytic endpoint to each test sample, this being the highest dilution causing ≥70% killing.

Statistical analyses

The Prism software system GraphPad 4.03 (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical analyses. Multigroup comparisons were performed using either one-way or two-way-(repeated measurement)-ANOVA with Bonferroni's post-test if not noted otherwise. The protection factor (PF) was calculated by dividing the geometric mean excretion of the control group with the excretion observed in the sample of interest at the same time-point. The relationship between corresponding antibody estimates or between different antibody estimates and colonization (or PF) was evaluated using a Pearson correlation test. Two-sided P-values < 0.05 were considered as significant and asterisks or crosses denote probability values (* $P < 0.05$, ** or $^{++}$ $P < 0.01$, *** or $^{++}$ $P < 0.001$).

Results and comments

Induction of strong immunity to viable and killed *V. cholerae* following systemic or mucosal vaccination of conventional adult mice (Paper I)

Viable *V. cholerae* O139 administered via the IN, PO or IP route is immunogenic

The inability of pathogenic *V. cholerae* to colonize the conventional adult mouse gut has made it difficult to elicit consistently strong antibacterial immunity (24). Recent reports have however shown that attenuated live *V. cholerae* O1 El Tor vaccines administered by the intranasal IN route as four doses over a period of 56 days induce strong serum antibacterial immunity (29, 154). Therefore initial experiments were performed in our laboratory with viable *V. cholerae* O1 or O139 administered IN on days 0 and 28. These experiments convincingly showed that our simplified dosing regime induced not only strong serum antibacterial immunity but also intestinal antibodies to LPS. In the context of enteric defence it was of interest to extend this observation to a comparison of intestinal responses following IN immunization or PO immunization. Adult BALB/c mice were therefore immunized IN or PO on days 0 and 28 with ca. 10^9 viable *V. cholerae* O139. In parallel, mice were also vaccinated IP with ca. 10^7 O139 bacteria. Analysis of the serum anti-O139 LPS response using ELISA revealed that IP and IN immunization effectively induced serum antibody responses with similar kinetics and high IgG1:IgG2a subclass ratios, that in strength were significantly greater than those induced by the PO route. Later studies revealed that higher immunizing doses are required to elicit comparable serum anti-LPS responses by the oral route.

In addition to analyzing serum antibody responses following vaccination, two sampling techniques were compared for deriving samples suitable for estimation of gut IgA responses using ELISA. Supernatants were prepared either from intestinal tissue extracts - prepared using a modified version of the perfusion-extraction technique (PERFEXT) (178) - or from homogenates of fresh FPs (24). The latter method is far more convenient and is widely used, but in the mouse the hepato-biliary transport of IgA from serum via bile to the gut means that FP IgA titres might not accurately reflect local antibody synthesis in the intestine (43). We

found that, for a given immunization route, the strength of the intestinal anti-LPS IgA antibody response monitored using tissue extracts was comparable to that detected in corresponding FP samples. Importantly, these indices of anti-LPS immunity were found to correlate significantly ($r \geq 0.85$ and $P < 0.0001$ for small intestinal, caecum and large intestinal vs. FP samples). The disproportionately low content of IgG, and high content of IgA, antibodies to LPS in the PERFEXT extracts, relative to the corresponding serum levels, precludes the possibility of significant transudation of serum antibody into the gut. Collectively our data provide an important validation of the use of FP supernatants as a convenient guide to local IgA antibody production. Consistently the PERFEXT and FP-samples also showed that the PO and IN routes generated similarly strong intestinal IgA responses. Intestinal responses generated by IN immunization however, tended to be more consistent than those following PO vaccination. In contrast to the mucosal routes, IP immunization did not generate any significant IgA responses.

Formalin-killed *V. cholerae* is immunogenic in adult mice

Ongoing research into inactivated cholera vaccines made it of interest to evaluate the mucosal immunogenicity of inactivated *V. cholerae* O139. Two experiments were therefore performed with fk whole cell vaccines administered either IN or PO using ca. 5×10^8 and 5×10^9 bacteria per dose respectively. In each experiment two groups were immunized on days 0 and 28 with viable or fk *V. cholerae* O139 strain AI-1838. A third group was also immunized more intensively, receiving six doses of fk AI-1838 on days 0, 1, 2, 28, 29 and 30. For both the IN and PO route, six doses of killed bacteria were similarly immunogenic to two doses of viable bacteria. Mice in these groups mounted significantly stronger serum IgG and intestinal IgA anti-LPS ELISA responses than animals given only two doses of inactivated bacteria. Importantly the mice immunized with six doses of killed bacteria mounted more consistent serum and intestinal antibody responses than those receiving only two doses.

V. cholerae O1 and O139 display similar mucosal immunogenicity

Because of our interest in bivalent O1 and O139 vaccines we also wished to investigate the relative immunogenicity of these two serogroups. Viable *V. cholerae* O1 or O139 were therefore administered IN or PO, using the same dosing protocols as in the previous experiment. Two weeks after the secondary immunization on day 28 these mice were sampled for serum and FPs. Mice were also sacrificed for collection of PERFEXT whole small

intestinal samples. The standardized IgA responses detected in these samples are expressed as fold-rises above the GM O1 or O139 titre of 8 control samples prepared from unimmunized animals.

V. cholerae O1 and O139 elicited very similar levels of intestinal IgA antibodies following IN or PO immunization, whether estimated using FPs (Fig. 2) or PERFEXT extracts. Irrespective of the serogroup we observed significant correlations between the standardized anti-LPS IgA titres detected using the two sampling techniques ($r = 0.89$ and $P < 0.0001$ for O1; $r = 0.84$ and $P = 0.0002$ for O139). The serum IgG responses were also very similar regardless of the immunization route or strain.

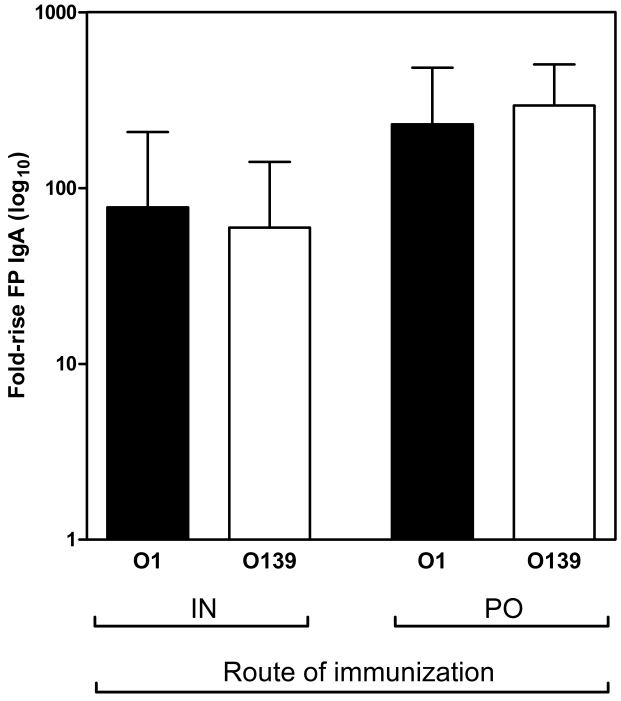

Fig. 2. Intestinal IgA antibody responses in BALB/c mice immunized with viable *V. cholerae* O1 or O139. Four groups of mice were inoculated with the O1 strain H1 (closed bars) or the O139 strain AI-1838 (open bars) on days 0 and 28, using doses of 5×10^8 CFUs IN (n = 8 / group) or 5×10^9 CFUs PO (n = 7 / group). Since background titres differ in the O1 and O139 ELISA systems, standardized FP IgA anti-LPS ELISA responses detected on day 42 are expressed as (GM + SD, $\log_{10}$) fold-rise in antibody titre, in relation to the GM pre-immunization control titres. Statistics: $P > 0.05$ for all comparisons between the O1 and O139 groups.

Wild type *V. cholerae* O139, as well as the attenuated live O139 vaccine CVD112, has been shown in volunteers to have strong protective efficacy (≥80%) against challenge (108, 165). Conflicting data have however been published regarding the capacity of *V. cholerae* bacteria of the O139 serogroup to induce significant serum bactericidal responses (36, 108, 125, 165). These responses, which measures the ability of serum antibodies to serogroup specifically kill *V. cholerae* in the presence of complement, and which successfully have been used to guide the development of the presently licensed oral O1 cholera vaccines (67, 92), was therefore recently further studied by Attridge *et al.* (12, 14). Although in these studies the conflicting

results observed for the O139 bactericidal responses were indicated to reflect variations in the assays used to determine the bactericidal titres, the relevance of these estimates in evaluation of the immunogenicity elicited by *V. cholerae* O139 has recently been questioned (137). This study shows that the immunogenicity of O1 and O139 vaccines can be compared in the new adult mouse model, and indicates that the two serogroups are similarly immunogenic. Further studies comparing both the immunogenicity and protective capacity of O1 and O139 vaccines administered via different routes has therefore been initiated.

Establishment of intestinal *V. cholerae* colonization in conventional adult mice (Paper II)

Streptomycin treatment makes adult mice receptive to colonization by both *V. cholerae* O1 and O139

It has been known for a long time that germfree adult mice can be consistently colonized with pathogenic *V. cholerae* following oral infection (136), and that the normal gut flora is a major impediment to successful gut colonization by *V. cholerae* in several animal species (131). These observations prompted us to examine the possibility of depleting the intestinal flora using oral Sm treatment to possibly make mice receptive to colonization by Sm-resistant *V. cholerae* bacteria. Initial studies revealed that mice administered Sm-containing drinking water (5 mg/ml) before inoculation with viable *V. cholerae* excreted viable bacteria for significantly longer than untreated age-matched controls. Mice maintained on Sm post-inoculation (0.2 mg/ml) were also found to excrete significantly higher numbers of viable *V. cholerae* for a longer period of time than those receiving Sm only prior to inoculation. Therefore a Sm-dosing protocol involving continuing exposure to antibiotic, beginning 24 hours prior to administration of *V. cholerae*, was adopted for further use.

In the following study we could also show that oral inoculation with 10^9 bacteria was uniformly well-tolerated and that there was no significant difference in the colonization profiles between the *V. cholerae* wild-type O1 (El Tor strain H1) or O139 (MO10) bacteria. These observations were confirmed in two later repeat experiments, and *V. cholerae* O139 strain AI-1838 also resulted in similar patterns of colonization. Our observations are in

agreement with human volunteer studies showing that O1 El Tor and O139 cholera has similar clinical characteristics (108).

V. cholerae colonize the intestine following infection of streptomycin treated mice

Cholera is mainly a disease of the small intestine. *V. cholerae* bacteria can however be recovered from all regions of the intestine of infected patients. Therefore we examined the localization of *V. cholerae* O139 in the gut of Sm-treated adult mice. At various time points following infection with 10^9 bacteria we sacrificed mice and enumerated bacterial persistence in the small intestine (SI), caecum (C) and large intestine (LI). Bacterial recoveries from carefully washed intestinal tissue samples were found to correlate significantly with the levels of bacteria excreted in FPs ($P<0.0001$ for all comparisons and $r = 0.79$ for SI, 0.90 for C and 0.91 for LI vs. FP). Although much greater numbers of epithelium-associated bacteria were consistently recovered from the large bowel compared with the SI, indicating a limitation of the model, the highly significant relationship between gut colonization and bacterial excretion shows that the latter provides a convenient way to monitor intestinal *V. cholerae* infection. Experiments with lower inocula importantly showed that the bacteria clearly replicates within the gastro-intestinal tract of Sm-treated mice. Recoveries of challenge organisms in different gut tissues or in fresh FPs were consistently greater than the initial challenge inoculum for periods of 4 or 8 days respectively.

Refined protocols for infection improve colonization

Initial challenge studies showed that it was possible to protect adult mice against colonization by vaccination with either live or killed *V. cholerae* vaccines (Se below). However in these initial experiments we also observed variable excretion patterns among challenged naïve control mice, precluding comparison of protection beyond day 6 at best. Several different variables possibly effecting colonization were therefore examined. Initial experiments revealed, similarly to other gastro-intestinal infection models, that the mouse strain greatly affected the patterns of colonization. Whereas BALB/c, C57BL/6, and SJL mice gradually cleared the infection over a period of about two weeks, C3H/HeN mice continued to excrete significant numbers of vibrios for at least three weeks post-infection. Colonization data from two pooled experiments with BALB/c and C3H/HeN mice are shown in Figure 3A; exemplifying the two types of colonization patterns observed. Interestingly the serum IgG responses detected three weeks after inoculation did not correlate with these two patterns of

colonization (Fig. 3B), indicating that prolonged colonization is not required for development of antibacterial immunity in Sm-treated adult mice. The prolonged colonization seen in the C3H/HeN mice did however suggest that such hosts might provide a longer interval for demonstration of the protective effects of prior immunization. As shown in a later challenge study (Fig. 5) this was however not the case and so no further studies were performed with this strain.

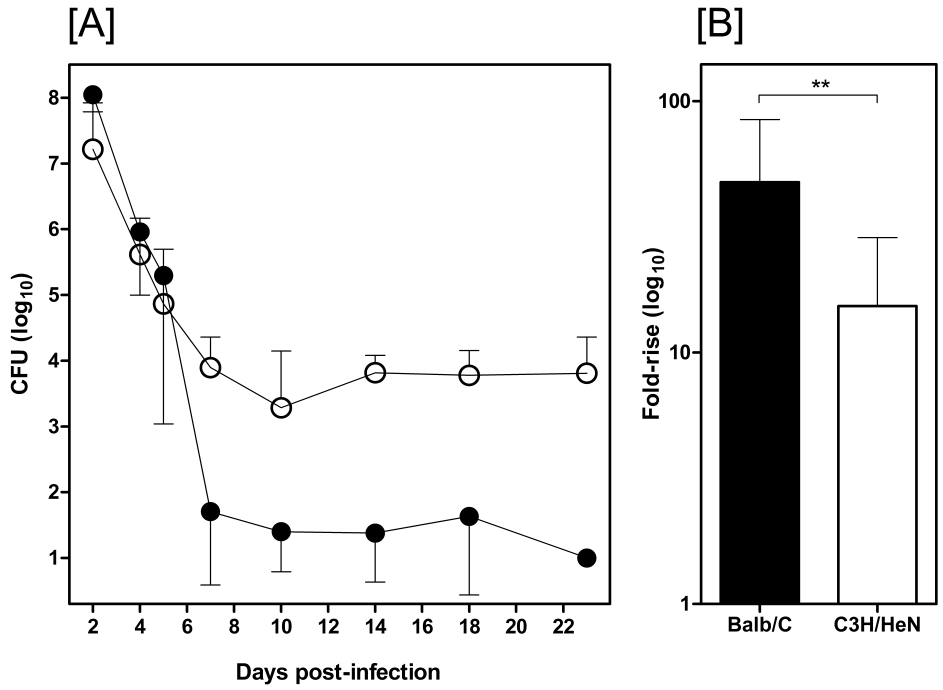

Fig. 3. Bacterial excretion and serum antibody responses in two mouse strains following oral infection with *V. cholerae* O139. Pooled results from two experiments with BALB/c (closed circles/bar, n = 13) and C3H/HeN (open circles/bar, n = 13) mice are shown. In panel A the mice received Sm-containing drinking water (5 mg/ml) for 24 hours before inoculation with ca. 10^9 viable Sm-resistant *V. cholerae* O139 strain MO10 on day 0. The mice also received Sm-water (0.2 mg/ml) after challenge. Faecal bacterial excretion was monitored and the CFUs are shown as (GM + SD) on a $\log_{10}$ scale. In panel B serum collected on day 21 post infection were analyzed for antibody responses to O139 LPS using ELISA. The IgG responses are expressed as fold-rise in antibody titre (GM + SD) on a $\log_{10}$ scale, in relation to the GM pre-immunization control titres.

A series of experiments was therefore set up to compare various modified Sm dosing regimes and also the impact of adding saccharin to the Sm-containing drinking water as a sweetener to increase intake of antibiotic. Although saccharin had only a very marginal effect, we did observe a slight improvement in colonization among mice treated with Sm more intensely i.e.

involving a longer period (48 hours) of dosing pre-challenge and maintenance on a higher concentration (1 mg/ml) of Sm post-challenge. Applying this dosing strategy we finally tested whether a lower infectious dose might be sufficient to provide consistent colonization of unimmunized hosts. Counter-intuitively, we found that FP excretion was significantly prolonged following infection of 14-week old mice with 10^7 *V. cholerae* O139 as compared with the previously used challenge dose of 10^9 bacteria. Importantly the variability at each time point was much reduced, and also the level of excretion was maintained above 10^7 CFU even 8 days after infection. Later experiments using the refined dosing protocol confirmed these findings (Fig. 7D) and also showed that there was again no significant difference in the small intestinal colonization profiles between the O1 and O139 serogroup (Fig. 4). Why a 100-fold lower challenge dose should result in more sustained and consistent colonization of naïve mice remains unexplained. However, it may be that a higher infectious dose induces stronger activation of the innate immune system of the gut, thereby which inhibiting *V. cholerae* persistence. Such responses have been described in patients with severe cholera (52, 123).

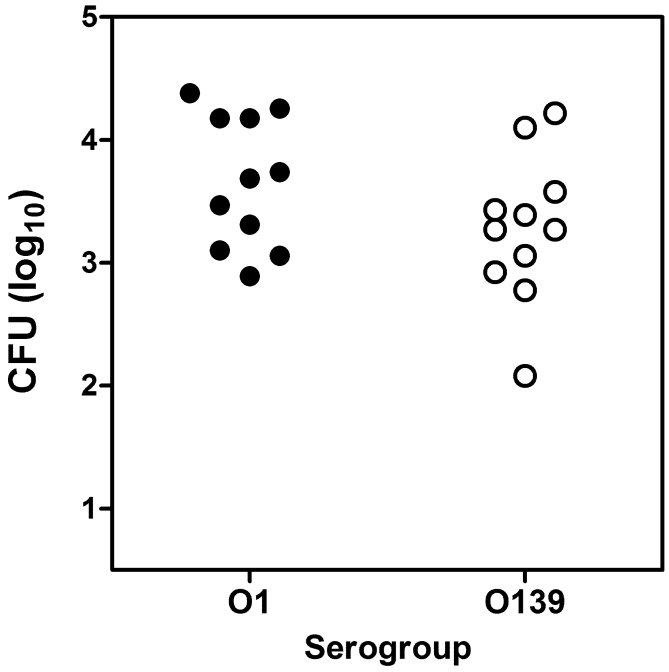

Fig. 4. Recoveries of *V. cholerae* O1 or O139 from the small intestine following oral infection of Sm-treated BALB/c mice. Two groups of BALB/c mice were treated with a more intensive Sm dosing regimen (5mg/ml Sm for 48 hours prior to inoculation and 1 mg/ml after challenge). The animals were inoculated with ca. 10^7 *V. cholerae* O1 or O139 strain H1 and MO10 respectively. Epithelium-associated vibrios recovered from small intestines three days after inoculation were enumerated as described in Methods. Each point represents bacterial recovery from an individual mouse ($\log_{10}$ CFU).

Expression of CT and TCP are not required for colonization

CT and TCP are critical virulence determinants which enable *V. cholerae* to establish infection and disease in the human intestine (65, 91, 167). To evaluate the significance of these factors in our modified model we assessed the colonization potential of mutant strains unable to express either factor. Similarly to volunteer studies with atoxigenic live cholera vaccines (65, 91), we found that CT was not required for persistent colonization. Our findings are also in agreement with colonization studies in infant mice (170), and with recent observations by Olivier *et al.* (116, 117) in Ketamine and Sm-treated, juvenile C57BL/6 mice.

In a preliminary experiment comparing FP bacterial excretion profiles of mice infected with wild-type *V. cholerae* or isogenic mutants lacking functional TCP expression, we observed no significant dependence of this virulence factor for colonization. This was unexpected given earlier findings in the IMCM, which have shown dramatic attenuation and colonization impairment associated with mutations affecting TCP expression (13, 170). We therefore conducted mixed infection competition experiments, to compare the potential of a *tcpA*::Km mutant strain to colonize the small intestine in comparison with wild-type bacteria, in mice of different ages. Experiments in both adult outbred CD1 and inbred BALB/c mice showed that our preliminary studies were correct, and that colonization in mice of intermediate age were, at best, only marginally dependent on TCP. Similarly Olivier *et al.* also recently reported that TCP expression was not required for colonization of the juvenile mice in their system (116, 117).

In conclusion these results preclude any examination in the adult mouse *V. cholerae* challenge model of the role of the TCP pili in enhancement of the immunogenicity of inactivated cholera vaccines. The apparent disparity in dependence of TCP for colonization in infant mice as compared with colonization in older mice is however fascinating. Although it may reflect differences in the models used for establishing colonization or age-dependent changes in the intestinal epithelium, it could also be that the host intestinal milieu changes following acquisition of a commensal flora. Experiments in germ free mice of different ages comparing colonization of WT bacteria and mutants lacking TCP are therefore warranted.

Protection against colonization by live and killed *V. cholerae* O1 and/or O139 vaccines (Papers II and III)

Immunization with live or killed *V. cholerae* O139 protects against colonization (Paper II)

Pre-clinical studies of the determinants of direct protection following infection or immunization with *V. cholerae* have previously been primarily performed in model systems that are fairly expensive and surgically demanding (131). In contrast, adult mice are very convenient, inexpensive experimental animals and the availability of inbred and congenic mutant strains facilitates evaluation of immunogenicity. Having established effective immunization protocols and also demonstrated that Sm-resistant *V. cholerae* can colonize the intestine of Sm-treated adult mice, it was of interest to investigate whether colonization would be curtailed in hosts which had been previously immunized using these protocols. Challenge experiments with 10^9 *V. cholerae* (conducted prior to our later refinement of the Sm-dosing regime) were therefore set up, generally using the IN or PO routes of immunization. Since the IP route is commonly used in immunogenicity studies mice immunized in this way were also tested in a few experiments. Immunization on days 0 and 28 with live *V. cholerae* O139 administered via either the IN, PO or IP route was found to elicit significant protective immunity, although no differences in the levels of excretion of challenge organisms were apparent until 4 days after challenge. At this time point the GM excretion was most reduced in the IN immunized mice - about 3900- fold as compared with the unimmunized controls. In contrast, excretion by mice immunized PO or IP was only reduced about 100- or 300-fold respectively. Subsequent PO immunization experiments were therefore performed with slightly higher immunizing doses.

To ascertain whether the present model would be applicable to evaluation of inactivated vaccine candidates we inoculated mice PO with 5 x 10^9 fk *V. cholerae* O139 on days 0, 1, and 2 for primary immunization and 28, 29 and 30 for secondary immunization. Mice in other groups were immunized PO as before, on days 0 and 28 with 5 x 10^9 live or fk *V. cholerae*. Two weeks after the last immunization these groups and age-matched naïve controls were challenged homologously with 10^9 vibrios. The results indicated that vaccine viability is important for induction of host protective immunity. Mice immunized with two doses of viable *V. cholerae* showed significantly faster clearance of the challenge organisms than

unvaccinated controls, or mice given two doses of fk bacteria. Importantly however, six doses of killed *V. cholerae* conferred immunity to challenge, indicating that the model could be applied to the testing of inactivated vaccine candidates. Similarly to the previous experiment there was a clear lag period of 4 days before expression of immunity in the group immunized with live bacteria.

When the protective effect induced by PO vaccination with live *V. cholerae* O139 was evaluated in C3H/HeN mice we again observed this delayed onset of immunity. Further, we also observed that although the immunized animals excreted significantly fewer challenge organisms than the age-matched controls on days 4, 5 and 6 post-challenge, vaccination was not sufficient to confer sterilizing immunity in this strain (Fig. 5).

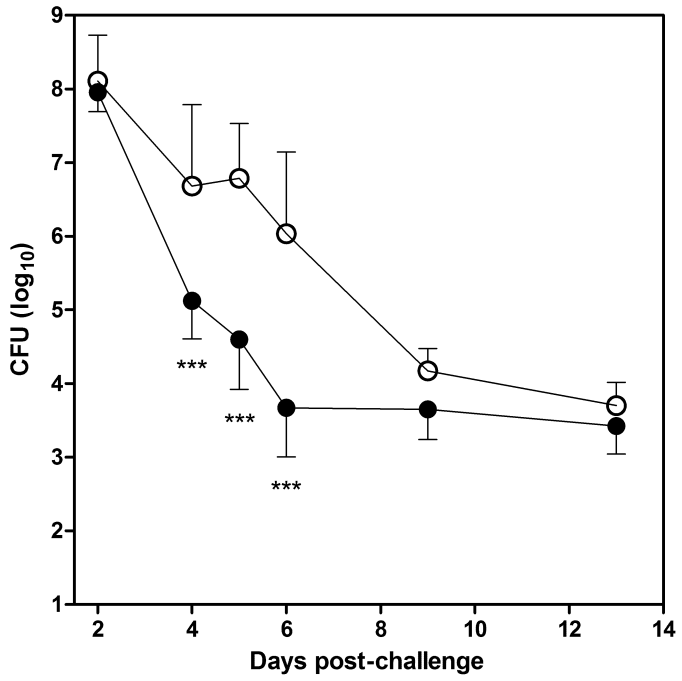

Fig. 5. PO immunization with viable *V. cholerae* O139 confers immunity to homologous challenge. One group of C3H/HeN mice were immunized PO with *V. cholerae* O139 strain MO10 (closed circles, n = 6), as described in the legend to Figure 2. Another group of mice were left unimmunized (open circles, n = 8). Two weeks after the last immunization these mice were Sm-treated and orally challenged with ca 10^9 MO10 bacteria as described in the legend to Figure 3. FP excretion of challenge organisms was monitored and is shown as (GM ± SD) log_{10} CFU.

Intranasal vaccination elicits serogroup-specific protection against colonization (Paper II)

Having improved the challenge protocol as described above, we examined whether protective immunity was serogroup-specific in the new adult mouse model, as is inferred to be the case in man. Two groups of mice were immunized IN with 5 x 10^8 viable *V. cholerae* O1 or O139 using a dosing schedule shown to generate comparable immune responses to the two

serogroup antigens (Fig. 2). Following homologous or heterologous challenge with ca. 10^7 *V. cholerae* O1 or O139 on day 42, bacterial excretion profiles were compared. The patterns of colonization within the two unimmunized control groups were found to be similar for both serogroups, confirming our previous observations (Fig. 4). Also we observed for both serogroups that vaccination did confer significant immunity to homologous challenge, whereas the patterns of colonization were not significantly altered by prior immunization with *V. cholerae* of heterologous serogroup. These differences were also apparent when comparing the GM day 17 protection factor (PF) for each group above the controls (Fig. 6). One animal out of six immunized with *V. cholerae* O139 and challenged heterologously with O1 bacteria was however partially protected, as indicated by the more variable PF in this group.

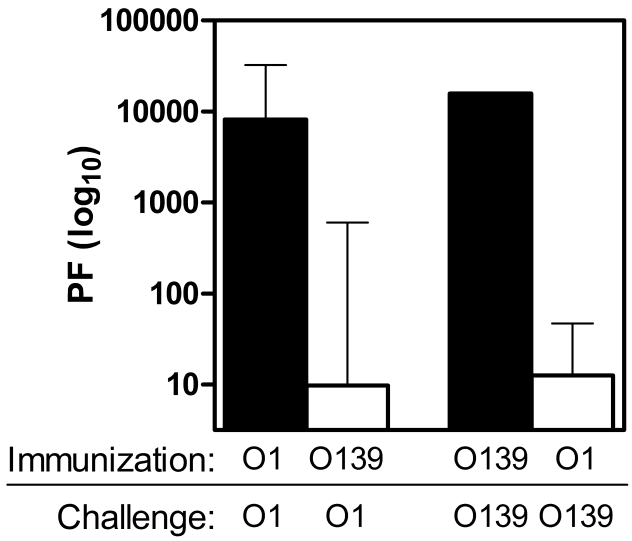

Fig. 6. IN immunization with viable *V. cholerae* confers immunity to homologous re-challenge. Two groups of twelve BALB/c mice were immunized IN with *V. cholerae* O1 strain H1 or O139 strain AI-1838, as described in the legend to Figure 2. One extra group of 12 age-matched controls was left unimmunized. Sm-treatment was commenced on day 40 and on day 42 each group was sub-divided for oral challenge with ca. 10^7 H1 or MO10. FP excretion of challenge organisms was monitored and used to calculate the day 17 PF (log_{10} GM + SD), as described in Methods.

It is fascinating that even after lowering the challenge inoculum 100-fold, host immunity was clearly still delayed, and surprisingly even more so than in our earlier experiments using the higher challenge dose (Fig. 5). Nevertheless this experiment, which was also later confirmed, showed that the fortuitous improvement in the duration and reproducibility of colonization observed using the lower challenge dose made the model more robust and provided a longer window for expression of protective immunity.

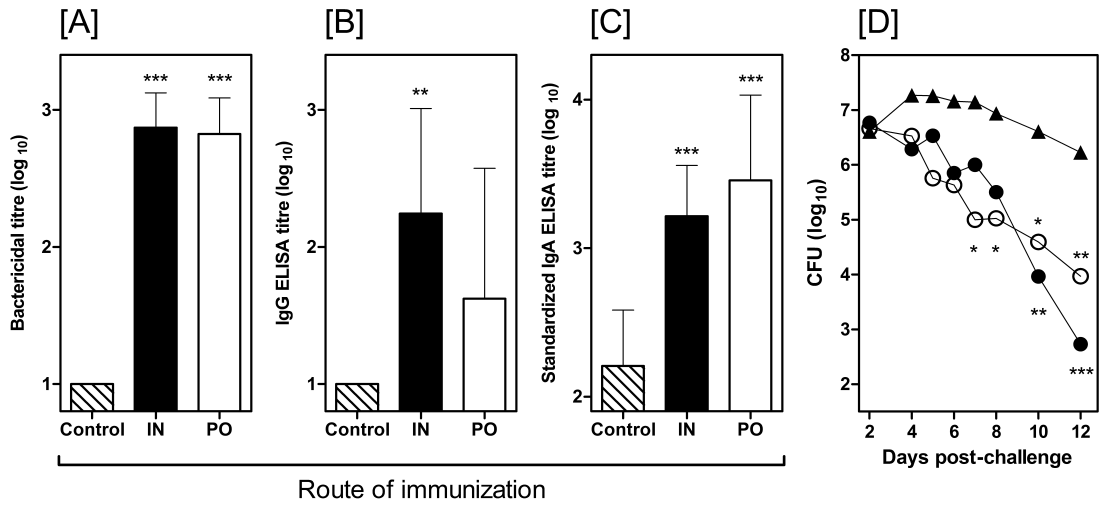

Fig. 7. Antibacterial immune responses and protection following mucosal immunization using a compressed schedule. Two groups of fourteen mice were inoculated with viable *V. cholerae* strain AI-1838 on day 0 and 14, using doses of 5 x 10^8 CFUs IN (closed bars or ●) or 5 x 10^9 CFUs PO (open bars or ○). Yet another group of twelve age matched controls were left untreated (striped bars or ▲). Specimens for estimation of antibody responses to *V. cholera* O139 responses were collected on day 24. Serum was analyzed for both bactericidal (panel A) and anti-LPS IgG ELISA (panel B) antibodies. The standardized anti-LPS IgA responses in extracts from fresh FP (panel C) were also measured using ELISA. On day 26 half of the animals in each group were also challenged with *V. cholerae* O139 strain MO10 as described in Methods (panel D), doses being ca. 10^7 bacteria per mouse. Results are expressed as $\log_{10}$ (GM ± SD).

Co-administration of CT enhances homologous and heterologous protection following oral immunization with live or formalin-killed *V. cholerae* O139 (Paper III)

In our earlier studies in this model we could show that mucosal vaccination conferred immunity to homologous challenge with *V. cholerae* (See Figure 5 and 6), although expression of significant immunity was delayed; especially so in the previous IN immunization experiment using a challenge dose of 10^7 bacteria. It was therefore of great interest to ascertain whether the strength of the host anti-bacterial immune response could be improved, either by inclusion of extra immunizing doses, and/or, in the present context, by the addition of exogenous CT as adjuvant at the time of vaccination. In addition, we wanted to compress the immunization/challenge protocol to make the model more convenient to use. Since the oral route of immunization is the route of choice for both live attenuated and killed cholera vaccines, we initially immunized mice either IN or PO on days 0 and 14 with live *V. cholerae* O139. Additional unimmunized age-matched mice were set aside as controls. When examining host antibacterial responses ten days after the last immunization (day 24) we found that both routes elicited strong serum bactericidal responses and intestinal IgA titres to

O139-LPS, although the serum IgG response was significant only in the IN immunized group (Fig. 7A-C). Further, we also challenged the immunized groups and the naive controls on day 26 with ca. 10^7 *V. cholerae* O139 strain MO10. When analyzing the patterns of colonization following challenge we found that both of the immunized groups on several occasions excrete significantly fewer challenge organisms than the unimmunized control group (Fig. 7D). These results were encouraging with regards to compressing the model, yet fully sterilizing immunity was again delayed; especially so in the PO group. Further, no reduction in the initial colonization was observed for any of the immunized mice, prompting experiments with more intensive dosing protocols.

When such protocols were applied in a preliminary experiment we observed that repeated PO inoculations with 5 x 10^8 bacteria were well tolerated, while higher doses were not. Hence in the following experiment we adopted a protocol involving orally immunization on days 0 and 2 for primary immunization and on days 14 and 16 for secondary immunization, followed by challenge on day 26.

Our interest in the development of bivalent O1/O139 *V. cholerae* vaccines (67) prompted a study to evaluate the immunogenicity and protective potential of bivalent O1/O139 immunization in conventional adult BALB/c mice. Animals were orally immunized as described above, with each dose comprising ca. 5 x 10^8 *V. cholerae* (H1 and AI-1838 bacteria in a 1:1 mixture). A second group of mice received the same bacterial suspensions supplemented with CT (7.5 μg/dose). Ten days after the last dose the immunized groups and a group of age-matched naive controls were orally challenged with ca. 10^7 *V. cholerae* O139 strain MO10.

A comparison of the bacterial excretion patterns following challenge showed that *V. cholerae* O139 colonization was reduced in vaccinated hosts. Interestingly the switch to four immunizing doses did not appear to enhance the anti-bacterial impact of immunization with viable bacteria, since these hosts excreted only marginally less challenge organisms that the controls during the early post-challenge period and expression of immunity was still considerably delayed. Strikingly however, the extent of protection in the CT-adjuvanted group was significantly better than in the non-adjuvanted group at most time-points. Not only was the duration of colonization greatly reduced in this group, but FP excretion of challenge bacteria on day 2 post-challenge was also ca. 130-fold lower than in the control group. These

observations were not paralleled by differential anti-LPS responses in the two vaccinated groups however. Whether assessed using tissue extracts or FP supernatants, the (standardized) anti-O139 LPS IgA titres revealed highly significant intestinal antibody responses which were not affected by co-administration of CT. Yet these IgA responses were found to correlate inversely with colonization as early as 2 days after challenge ($r = -0.50$ and $P = 0.0406$), and with even stronger significance from day 8 ($r = -0.85$, $P < 0.0001$) and onwards.

Co-administration of CT enhances anti-bacterial immunity following oral immunization with fk *V. cholerae* O139 (Paper III)

Encouraged by the observations in the previous study we wished to examine if oral immunization with inactivated whole cell cholera vaccine could similarly induce protective immunity, and if so to determine the potential adjuvant effect of co-administered CT. We therefore inoculated mice PO with fk *V. cholerae* O139 with and without CT (7.5 μg/dose) on days 0, 1, and 2 for primary immunization and 14, 15 and 16 for secondary immunization. Extra mice were similarly treated with CT alone or left unimmunized. Six weeks after the last immunization these groups were Sm-treated and two days later challenged with ca. 10^7 *V. cholerae* O139; additional animals were also challenged 10 days after immunization as in the previous experiment. When analyzing bacterial excretion following challenge we observed that immunization with CT alone did not induce protection against *V. cholerae* colonization (shown for the O139 challenge in Figure 8). These observations indicate that neither adaptive nor innate immune mechanisms induced by CT alone are protective in the adult mouse model.

In contrast, and regardless of the interval between vaccination and challenge, mice immunized with fk *V. cholerae* O139 displayed immunity to homologous challenge. The protective potential was most pronounced with the longer interval (challenge on day 60, Fig. 9), suggesting a maturation of the protective potential of host antibacterial immune responses. In neither case did CT significantly enhance the anti-bacterial immunity elicited by fk bacteria. Nevertheless the greatest impact on colonization was observed in the CT-adjuvanted group, with a 270-fold lower excretion of the challenge organism two days after challenge, compared with only 15 -fold reduction in the group not receiving CT (Fig. 9).

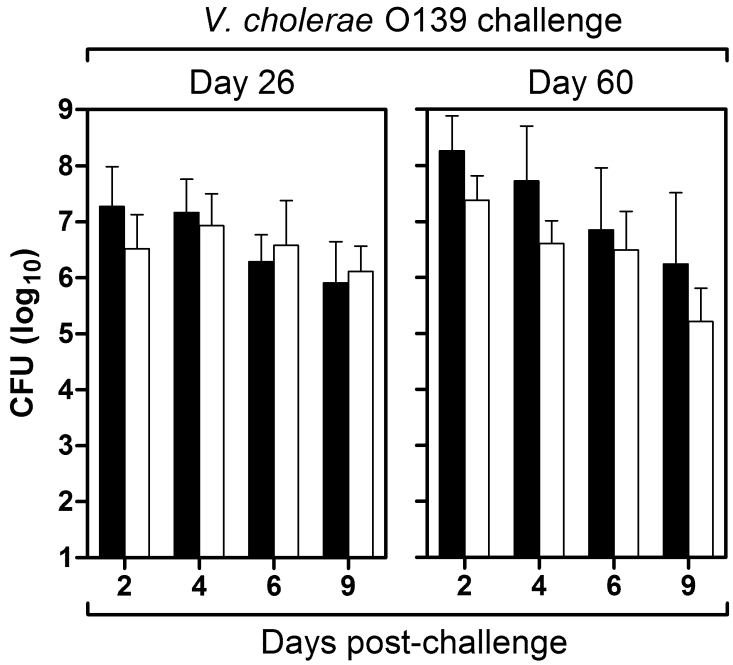

Fig. 8. Immunization with CT does not induce protection. BALB/c mice were inoculated PO on days 0, 1, 2, 14, 15 and 16 with CT (7.5 μg/dose) or left unimmunized. On days 60 these groups were challenged with *V. cholerae* O139 strain MO10 as described in Methods, doses were ca. 10^7 bacteria per mouse ($n \geq 6$ per group). Fecal excretion of challenge organisms was monitored and is shown as (GM + SD) $\log_{10}$ CFU.

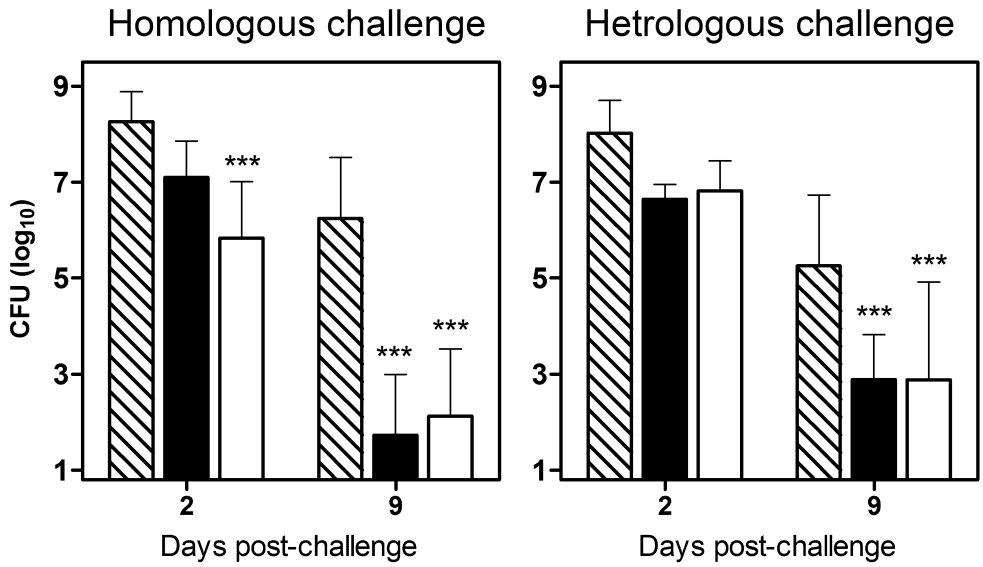

Fig. 9. Co-administration of CT improves protection of fk *V. cholerae* O139. BALB/c mice were immunized PO on days 0, 1, 2, 14, 15 and 16. One group of animals were immunized with 5 x 10^8 fk *V. cholerae* O139 strain AI-1838 (closed bars, n = 34). A second group were also administered CT (7.5 μg/dosing) together with the fk bacteria (open bars, n = 32). A third group of age-matched controls were left untreated (striped bars, n = 26). On day 60 these groups were challenged either homologously with *V. cholerae* O139 strain MO10 or heterologously with *V. cholerae* O1 strain H1 as described in the legend to Figure 8 ($n \geq 6$ per group and time point). Colonization on days 2 and 9 post-challenge is shown as (GM + SD) $\log_{10}$ CFU.

Because *V. cholerae* O1 El Tor and O139 bacteria share several surface antigens (38, 181) we also examined whether PO immunization with fk *V. cholerae* O139 could induce serogroup cross-protection. Subsets of the animals immunized as described above were therefore challenged heterologously on days 26 and 60 with *V. cholerae* O1 El Tor strain H1. The O1 colonization patterns showed that mice immunized with fk *V. cholerae* O139 were significantly protected against colonization only when challenged on day 60 (Fig. 9). This result contradicts our earlier conclusion that protection in this model is serogroup-restricted. However these earlier results (see Figure 6) emanated from studies using a 14-day interval between IN vaccination and challenge, which now appears sub-optimal. Possibly the buildup of strong immunity prior to challenge at day 60 allowed detection of weaker but significant cross-reactive immunity not detected in our earlier experiments. Interestingly the importance of the immunization/challenge protocol appears to be minimized by adjuvanting with CT, since mice immunized with both bacteria and CT excreted significantly fewer challenge organisms than controls whether challenge occurred early or late after vaccination. The GM day 9 PFs of animals challenged heterologously on day 60 was however only about 2500, compared with PFs ca.100,000 following homologous challenge.

CT enhances mucosal antibody responses to LPS and also to non-LPS antigens shared between the O1 and O139 serogroups (Paper III)

In a search for possible correlates of the homologous and heterologous protection data described in Figure 9, serum and FP samples collected just prior to challenge (days 24 or 58) were analyzed for antibody responses to various *V. cholerae* antigens. Both groups immunized with fk bacteria (± CT) displayed high levels of both serum IgG and intestinal IgA antibodies to LPS. Only the latter response was significantly increased by co-administration of CT with the fk bacteria. Consistent with our previous findings using the intranasal route of immunization (Fig. 6), anti-O139 LPS titres correlated significantly with protection ($P < 0.0001$ and $r \geq 0.81$ for both IgG and IgA vs. PF).

The divergent effects of CT on intestinal IgA responses to live and killed *V. cholerae* may be explained by the fact that viable bacteria represent a more powerful tool for eliciting an immune response than killed whole cell formulations (115). Hence the use of the former may have maximised the immune response, obscuring any effect of CT in these experiments.

Alternatively, since it has been known that CT has to be co-administered or at least given within a few hours before or after antigen administration to have adjuvant activity (100), it could be that after inoculation with live bacteria, much of the antigen stimulation of the gut immune system takes place after the period when CT is effective as an adjuvant.

Pre-challenge serum samples collected from mice immunized as described in the legend to Figure 9 had no anti-O1 LPS antibody titres above the background levels of unimmunized controls (data not shown). The sera from day 58 were therefore tested for antibodies to membrane preparation (MP) from the O1 El Tor strain JBK70. By ELISA the two groups immunized with fk bacteria (±CT), but not those given CT only, displayed significant serum IgG titres to MP. Like the serum anti-LPS responses, this response was not increased by CT adjuvantation. Interestingly the serum IgG anti-MP titres correlated inversely with the level of colonization observed on day 9 post challenge with *V. cholerae* O1 in Figure 9 ($P = 0.0007$ and $r = - 0.63$), suggesting that immunity directed towards non-LPS, serogroup-shared determinants might contribute to protection against colonization in this model.

The cross-reactive antibody response was therefore analyzed further using immunoblotting of immune sera against a SDS-PAGE-separated crude whole cell lysate prepared from *V. cholerae* O1 El Tor strain H1. Previous immunoblotting experiments had shown that sera collected on day 24 generally react more strongly to the MP than sera from day 58, hence only the former sera were tested against the H1-lysate. Sera collected from mice given fk *V. cholerae* O139 (±CT) were found to react with several protein bands not detected by sera from unimmunized or CT-immunized mice (Fig. 10). Strikingly, the sera reacted most strongly with 18-19 kDa proteins (position c in Figure 10), with the serum from the CT-adjuvanted group giving the strongest reaction. Monoclonal antibodies to TcpA did not react with these preparations, while mannose-sensitive hemagglutinin (MSHA) pilus specific antibodies stained bacterial proteins at ca. 17 kDa (position d in Figure 10), also apparently stained by sera from the CT-adjuvanted group.

The best characterized protective antigens of *V. cholerae* are LPS and CT, although several studies suggest that immunity might also involve antibody responses to TCP and several other membrane proteins (62, 101, 146-148). Our finding that the anti-membrane antibody response correlated significantly with cross-serogroup protection would be consistent with this. Similarly, protection was also significantly improved by co-administration of CT together

with live *V. cholerae,* even in the absence of increased intestinal antibody responses to LPS. Taken together our immunization experiments suggest that both LPS and non-LPS antigens may be targets for protective host responses in the new model.

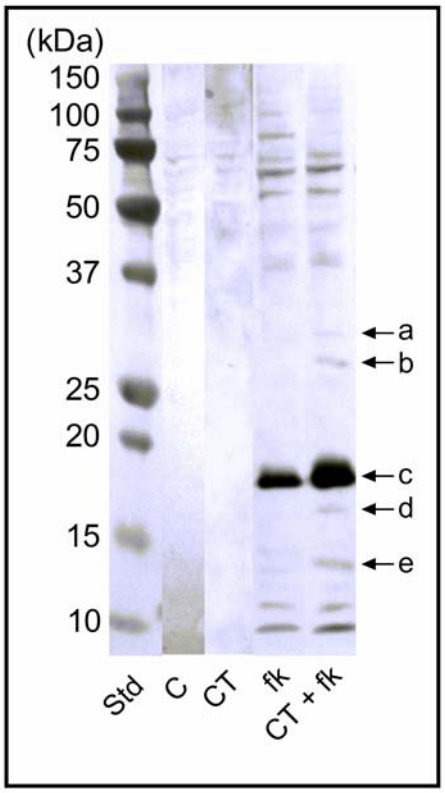

Fig. 10. CT potentiates non-LPS cross-serogroup reactive antibody responses. Serum samples collected from mice immunized in Figure 9 with fk O139, CT and fk O139 or with CT alone were pooled, diluted 200-fold and immunoblotted against immobilized sonicated *V. cholerae* O1 strain H1 cultured in LB-medium over night, as described in Methods. Serum from unimmunized age-matched controls (C) and a standard (Std) with known size was also included. Bands reacting more strongly in the CT + fk group were detected at estimated 30 kDa (position a), 27 kDa (b), 18-19 kDa (c), 17 kDa (d) and 14 kDa (e).

Discussion and conclusions

The main objective of this work has been to examine the feasibility of establishing a model for direct evaluation of the immunogenicity and protective efficacy of cholera vaccine candidates in conventional adult mice. Until now, the inability of pathogenic *V. cholerae* to colonize the conventional adult mouse gut has made it not only difficult to consistently induce strong antibacterial antibody responses, but also impossible to evaluate the impact of vaccination on protection against colonization in such hosts.

The work described in this thesis thus started by delineating the immune response to viable *V. cholerae* administered mucosally or systemically. A simplified two-dose IN immunization schedule with viable *V. cholerae* was shown to be sufficient to induce strong serum anti-LPS responses comparable to those reported by others using a more demanding dosing regime (29, 154). Significantly, these serum responses were accompanied by the induction of consistent intestinal IgA responses, which to our knowledge have not been previously reported following IN vaccination. Using slightly higher doses of viable bacteria we could also generate consistent gut and serum anti-LPS responses by the oral route. *V. cholerae* O1 and O139 strains were similarly immunogenic when administered IN or PO. Comparable serum and intestinal anti-LPS responses could also be generated by mucosal (PO or IN) application of formalin-killed bacteria, although more intensive immunization regimes were then required for consistently strong responses. In contrast to most previous reports, these mucosal immunization schedules do not require co-administration of CT (although later studies revealed the adjuvant potential of this toxin) or any other adjuvant. Further, our immunization schedules also do not require pre-treatment with antibiotics or the use of germ-free animals (24, 37) for induction of significant immune responses.

Our initial work also compared gut IgA responses estimated using two sampling techniques, involving the collection of fresh FPs or the preparation of intestinal tissue extracts. The observed significant correlation between these estimates validates the more convenient approach of measuring intestinal IgA responses using faecal pellet extracts, which offers the added benefit of allowing repeated sampling from the same animals. Our comparison of these two sampling techniques has been restricted to measurement of anti-LPS responses following administration of *V. cholerae*. It is feasible that this conclusion would not apply to antibody

responses to other antigens or organisms, yet preliminary results from a study with OVA as an antigen (U. Yrlid *et al*., unpublished) are in agreement with our findings.

In parallel to our immunization studies, we have also undertaken the more demanding task of establishing a model for intestinal *V. cholerae* colonization using conventional adult mice. Initial experiments showed that pre-treatment of adult mice with oral Sm allowed intestinal colonization by *V. cholerae* strains of either O1 or O139 serogroup. Following Sm treatment and infection, viable *V. cholerae* were recovered from all regions of the gut, but in lower numbers from the small intestine compared with the caecum and large intestine. Importantly the intestinal bacterial loads in all regions of the gut were found to correlate significantly with bacterial excretion in fresh FPs, which thus provide a convenient indicator of the extent and duration of gut colonization.

Later experiments surprisingly showed that mutant strains unable to produce TCP persisted in the intestine at levels no different to those of the WT organism. This is an apparent inconsistency with the human infection (65, 167) and represents a limitation of the model with regard to its usefulness in evaluating the protective capacity of novel cholera vaccines expressing TCP. Our work also revealed that atoxigenic *V. cholerae* colonized mice as efficiently as WT organisms. This observation was however not unexpected considering that CT production is not required for colonization of the infant mouse or human intestine (91, 170). Also the absence of signs of diarrhea in our model is in agreement with several other studies in mice (24, 116, 136) and probably reflects the enormous capacity of the mouse colon to reabsorb fluids rather than a lack of *in vivo* CT expression, since we did observe significant anti-CTB responses following colonization. Further our aim was not to develop a new model for induction of fluid secretion and diarrhea since such models already exists (131, 135, 157, 171), but instead a small animal model for evaluating antibacterial protection against colonization.

When combining the protocols for immunization and colonization (the two ‘phases’ of the *V. cholerae* challenge model) we found that mice vaccinated mucosally or systemically with *V. cholerae* were comparatively refractory to *V. cholerae* colonization following challenge with the immunizing strain. Our challenge experiments indicate that vaccine viability is important for induction of host protective immunity, since six but not two doses of a killed whole cell vaccine induced similar rates of clearance of the challenge organisms as did two

doses of viable *V. cholerae*. Combining a lower inoculum of 10^7 vibrios with a more intensive Sm-dosing regime allowed more consistent and sustained colonization to be established, facilitating detection of protective host immunity.

Despite the strong intestinal antibody responses detected in intranasally immunized mice it is not the authors recommendation that this route should be adopted for human use with cholera vaccines, as has recently been suggested (118). The IN route is however suitable for initial immunogenicity studies of experimental cholera vaccines, especially when access to antigen is a limiting factor. A recent study has further shown that IN immunization of female mice with outer membrane vesicles from *V. cholerae* induces high serum IgA antibody responses which are sufficient for passive protection of suckling mice challenged orally with WT *V. cholerae* (144).

In our final study we expanded our investigations of the oral route of immunization, which is the route of choice for both live attenuated and killed human cholera vaccines (96). Using a compressed and more intensive dosing protocol for oral vaccination we could show that co-administration of CT as an adjuvant potentiates both protection and intestinal antibody responses to LPS and several other bacterial surface proteins/antigens. Importantly, non-LPS responses elicited by a killed whole cell *V. cholerae* O139 vaccine were also shown to correlate with the potential to protect against heterologous *V. cholerae* infection. Further evaluation of the experimental and clinical significance of antibody responses to surface proteins are warranted since such responses may be targets in maximally protective whole cell cholera vaccines.

During the course of these studies Olivier *et al.* (116, 117) described a similar model for *V. cholerae* colonization of Sm-treated juvenile mice. Differences in methods used for establishing colonization, the age and strain of the host, and of the bacterial challenge strains make it difficult to compare the two systems. Yet it is apparent that their system, in contrast to our challenge model, is optimized for lethality, with an inoculum of only 10^8 bacteria representing a LD_{100} (116). This finding also contradicts many reports of investigators feeding tremendous numbers of pathogenic vibrios to adult mice without any adverse effects, and germ-free mice remain healthy following mono-contamination with toxigenic *V. cholerae* (24, 136). From an ethical point of view our model is therefore more acceptable, causing no deaths and only very marginal stress. Although Olivier *et al*. could show protection against

fatal WT *V. cholerae* infections in mice vaccinated with a multi-toxin deficient live cholera vaccine (116), no data showing protection against colonization was described.

The work in this thesis is to our knowledge the first description of a model in conventional adult mice that allows protection against *V. cholerae* colonization to be evaluated directly. Taken together the new adult mouse *V. cholerae* challenge model is useful for studies of specific intestinal-mucosal and serum antibody responses as well as anti-colonization protective immunity after immunization with either live or killed cholera vaccines. The described *V. cholerae* challenge model thus promises to be a useful tool in the preclinical evaluation of novel oral cholera vaccines, and also likely in studies of the capacity of already existing vaccines to protect against new variants of epidemic *V. cholerae* strains.

Acknowledgments

I would like to thank everyone at the department of Microbiology and Immunology that I have had the privilege to work with during my years as a research student, and whose presence have made this project much enjoyable. Especially I would like to express my sincere gratitude to:

Steve Attridge, my main supervisor, for friendship, inspiration, subtle language lessons and for providing an excellent balance between scientific guidance and independence;

Jan Holmgren, my co-supervisor, for guidance, constructive criticism and generous support during my seemingly endless years at the department;

Bin-Ling Li, for enthusiastic and excellent technical assistance with the mice in numerous experiments;

Gun Wallerström, Susanne Källgård and Margareta Fredrikson for high-quality technical assistance and friendly help;

Sara Tengvall, my former room mate and friend, for stimulating discussions and valuable advice in various matters;

Anna-Karin Östberg, Annika Lundquist, Helena Svensson, my present room mates, for nice conversations, laughter, ”kaka på torsdag” and for being very supportive and understanding during this last period;

Gudrun Wiklund and Karin Ahlman for general help in the lab and for morning coffee;

Carl-Fredrik Flach, Anna Lundgren, Anders Janzon and Sukanya Raghavan for friendly advice and discussions;

Ali Harandi, Michael Lebens, Åsa Sjöling, Ulf Yrlid and for very useful comments and eager questioning;

Staffan Nilsson and Miguel Tam for discussions regarding statistical methods;

Ann-Mari Svennerholm and Nils Lycke, head of the department and MIVAC respectively, for creating a friendly atmosphere and an excellent scientific arena;

Susanne Uhlan, for always being very friendly and never complaining when I have forgotten about my duties on the 5th floor;

Pernilla Ahlgren, Ivan Eisner and Lillebil Hagenberg at EBM for very professional handling of my mice;

Ellen Marks, for friendship and revision of the English text;

Clas Lilja, Erik Rehnström, Annie George Chandy, Anna Karlsson and Kristina Eriksson for encouraging me to seek a research career;

All my friends, for still being around even though I at times have neglected you;

Linnea Warger, for giving me laughter on Thursdays;

My parents, brothers and extended family for always having encouraged and supported me;

Jenny, for love, understanding, tremendous patience and support;

This work was supported by grants from the Swedish International Development Agency, the Swedish Research Council for Medicine, the US NIH, the Royal Society of Arts and Sciences in Göteborg and Wilhelm and Martina Lundgrens Foundation.

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