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INFECTION BY ; AND IMMUNITY TO ; VIBRIO CHOLERAE.

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ABSTRACTINFECTION BY , AND IMMUNITY TO, VIBRIO CHOLERAEE.

The studies to be described in this thesis were aimed at assisting the development of a more effective cholera vaccine. Two experimental approaches were adopted. The first of these involved an examination of the adherence phenomenon in Vibrio cholerae, with emphasis on the nature of the bacterial structures responsible for attachment. This work was prompted by studies with other bacterial pathogens which have demonstrated that those structures which facilitate colonization offer great promise as prophylactic immunogens. The second approach entailed a study of the specificity of antibodies which are capable of mediating protection in the infant mouse model of cholera. Particular emphasis was placed upon the possible existence of potentially protective non-lipopolysaccharide (LPS) antigens in V. cholerae. This seemed worthwhile in view of the fact that conventional vaccines contain the LPS antigens, yet are not sufficiently effective to make vaccination a significant measure in cholera control.

The main conclusions which have resulted from this work may be summarized as follows. Firstly, there is a correlation between adherence and virulence in V. cholerae. In addition, motile organisms are able to bind to intestinal surfaces much more efficiently than their non-motile counterparts, both in vitro and in vivo. Secondly, there are at least two factors which can promote the in vitro attachment of V. cholerae; these differ in their stability, location, range of binding substrates and pathogenic relevance. Thirdly, using two independent approaches it has been possible to confirm the existence of non-LPS protective antigens in V. cholerae.

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Subsequent investigation suggested that these determinants may be of greater prophylactic significance than the LPS antigens. Finally, the protective activities of specific antibodies seem to be dependent upon their capacities to directly block vibrio attachment. These findings have been discussed in relation to recent reports from other laboratories, and their implications for the future development of cholera vaccines have also been considered.

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STATEMENT

The material in this thesis has not been previously submitted for a degree in any University, and, to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due reference is made in the text.

(S. R. ATTRIDGE)

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(vi)

TO MY WIFE AND TO MY FAMILY

WITH LOVE AND THANKS

TABLE OF CONTENTS

	<u>Page</u>
TITLE	(i)
ABSTRACT	(ii)
STATEMENT	(iv)
ACKNOWLEDGEMENTS	(v)
 <u>CHAPTER 1</u>	 <u>INTRODUCTION TO CHOLERA</u>
1.1	General Background to the Disease. 1
1.1.1	Epidemiology and etiology 1
1.1.2	Pathogenesis and pathophysiology 4
1.1.3	The treatment of cholera 7
1.1.4	Future management of the disease 8
	(i) Treatment 9
	(ii) Immunization 9
	(iii) Sanitation 10
1.2	The Antigens of <u>V. cholerae</u> . 12
1.2.1	Introduction 12
1.2.2	Heat-stable (O) antigens 12
1.2.3	Heat-labile (H) antigens 14
1.2.4	Other somatic antigens of <u>V. cholerae</u> 16
	(i) Heat-stable protein antigens 17
	(ii) Heat-labile protein antigens 17
1.2.5	Enterotoxin 18
1.3	Immunity to <u>V. cholerae</u> . 20
1.3.1	Introduction 20
1.3.2	Antibacterial vs antitoxic immunity 20
1.3.3	Coproantibodies and the local immune system 22
1.3.4	The nature and significance of coproantibodies 25
	(i) Secretory IgA 25
	(ii) Other coproantibodies 28
1.3.5	Mechanisms of antibacterial immunity to cholera 31
1.3.6	Local vs systemic immunization 34
1.3.7	Vaccination against cholera 38

CHAPTER 2 BACTERIAL ADHERENCE

2.1	Introduction	44
2.2	The Role of Adherence in Pathogenicity	44
2.3	The Selectivity of Bacterial Adherence	46
2.3.1	Introduction	46
2.3.2	Host specificity	47
2.3.3	Tissue specificity	49
2.4	The Nature of Host Receptors	51
2.5	Adherence Mechanisms of Pathogenic Bacteria	53
2.5.1	Introduction	53
2.5.2	<u>E. coli</u>	54
2.5.3	Streptococci	57
2.5.4	Gonococci	59
2.5.5	<u>V. cholerae</u> and other pathogens	61
2.6	Summary, and Aims of this Thesis	62

CHAPTER 3 MATERIALS AND METHODS

3.1	Bacterial Strains	64
3.1.1	Background information	64
3.1.2	Maintenance and growth conditions	64
3.1.3	Selection of motile variants	65
3.1.4	The sloppy-agar overlay method	66
3.2	Animals	67
3.2.1	Adult mice	67
3.2.2	Infant mice	67
3.2.3	Guinea-pigs	67
3.3	Collection and Storage of Serum and Intestinal Fluid	68
3.3.1	Mouse serum	68
3.3.2	Guinea-pig serum	68
3.3.3	Mouse intestinal washings	68
3.3.4	Human intestinal fluid	69
3.4	Isolation of Lipopolysaccharide (LPS) from <u>V. cholerae</u>	69
3.5	<u>In vitro</u> Assays of <u>V. cholerae</u> Adherence	70
3.5.1	Attachment to murine intestinal tissue	70
3.5.2	Attachment to isolated murine intestinal epithelial cells	70

	<u>Page</u>
3.5.3	Bacterial haemagglutination (HA) 71
3.5.4	Attachment to inert particles 71
3.6	<u>In vitro</u> Titration of Antibodies to <u>V. cholerae</u> 72
3.6.1	Haemagglutination 72
	(i) Sensitization of sheep erythrocytes (Srbc) 72
	(ii) Titration of antisera 72
3.6.2	Bacterial agglutination 73
3.6.3	Complement-mediated bacteriolysis 73
3.6.4	Radioimmunoassay (RIA) 74
3.6.5	Inhibition of motility 76
3.6.6	Inhibition of adherence 76
3.7	<u>In vivo</u> Assays with Infant Mice 76
3.7.1	Virulence assays 77
3.7.2	Comparisons of colonization potential 78
3.7.3	Protection assays 78
3.8	Methods of Inactivation of <u>V. cholerae</u> 78
	(i) Boiling 79
	(ii) Glutaraldehyde-fixation 79
	(iii) Formalin-treatment 79
	(iv) Phenol-treatment 79
	(v) Azide-treatment 79
	(vi) Alcohol- and acetone-treatment 79
3.9	Fractionation of Antisera using Protein A-Sepharose 79
3.10	Quantitation of Antibodies to LPS 80

CHAPTER 4 ADHERENCE, MOTILITY AND VIRULENCE OF V. CHOLERA

4.1	Introduction 82
4.2	Establishing an Adherence Assay 83
4.3	Adherence and Virulence of <u>V. cholerae</u> 87
4.3.1	Survey of strains 87
4.3.2	Adherence to infant mouse small intestine 90
4.4	<u>In vitro</u> and <u>in vivo</u> Consequences of Selection for Motility 91
4.4.1	Selection of motile variants 91
4.4.2	Effect of selection for motility on <u>in vitro</u> adherence 93
4.4.3	<u>In vivo</u> consequences of selection for motility 94
4.4.4	The role of motility <u>in vivo</u> - a different approach 98

		<u>Page</u>
4.5	Effects of <u>in vitro</u> Selection for Adherence	101
4.6	Summary	105
4.A	Appendix : Attachment of <u>V. cholerae</u> to Mucosal and Serosal surfaces of adult mouse small intestine	106
<u>CHAPTER 5</u>	<u>THE NATURE OF THE <u>V. CHOLERA</u> ADHERENCE PHENOMENON</u>	
5.1	Introduction	108
5.2	The Specificity of <u>V. cholerae</u> Adherence	108
5.2.1	The range of adherence substrates for <u>V. cholerae</u>	110
5.2.2	Consequences of <u>in vitro</u> selection for adherence to inert substrates	112
5.2.3	The effect of monosaccharides on adherence	116
5.3	The Role of the Flagellum in Adherence	117
5.3.1	Initial attempts to separate motility and adherence	119
5.3.2	Separation of motility and adherence using specific antibodies	122
5.4	The Existence of two Adhesins in <u>V. cholerae</u>	124
5.4.1	Introduction	124
5.4.2	Influence of growth conditions on development of haemagglutinating capacity	126
5.4.3	The role of the slime agglutinin in adherence	130
	(i) Attachment to intestinal tissue	130
	(ii) Attachment to inert particles	132
5.4.4	Other studies within the slime agglutinin	134
5.5	Summary	136
<u>CHAPTER 6</u>	<u>THE PROPHYLACTIC SIGNIFICANCE OF THE NON-LPS</u>	
	<u>ANTIGENS OF <u>V. CHOLERA</u></u>	
6.1	Introduction	137
6.2	Confirmation of the Existence of non-LPS Protective Antigens in <u>V. cholerae</u> . I. The presence of protective antibodies specific for non-LPS determinants in an antiserum prepared against live <u>V. cholerae</u> 569B	139
6.3	Confirmation of the Existence of non-LPS Protective Antigens in <u>V. cholerae</u> . II. Studies with the hybrid vibrio strain 569B/165	143
6.3.1	Initial demonstration of non-LPS protective antigens in 569B/165	144

		<u>Page</u>
6.3.2	Confirmation of the non-LPS, heat-labile nature of the protective antigens of 569B/165	146
6.4	The Distribution of non-LPS Protective Antigens in <u>V. cholerae</u>	148
6.4.1	Absence of non-LPS protective antigens in <u>V. cholerae</u> 017	149
6.4.2	Absence of non-LPS protective antigens in <u>V. cholerae</u> T50	150
6.4.3	Possible bases for the restricted distribution of non-LPS protective antigens in <u>V. cholerae</u>	152
6.5	Further Studies with the non-LPS Protective Antigens of <u>V. cholerae</u> 569B	153
6.5.1	Introduction	153
6.5.2	Suitability as oral immunogens	154
6.5.3	Resistance to various preservatives	159
6.6	Possible Protective Functions of Specific Antibodies	161
6.6.1	Introduction	161
6.6.2	Bacterial agglutination	162
6.6.3	Inhibition of motility	163
6.6.4	Blocking of vibrio adhesins	164
6.7	The Relative Prophylactic Significance of the LPS and non-LPS Antigens of <u>V. cholerae</u> 569B	167
6.7.1	Experimental approach	167
6.7.2	Preparation and characterization of antibody fractions	169
6.7.3	Relative protective efficiencies of antibodies specific for LPS or non-LPS determinants	171
6.8	Summary	173
<u>CHAPTER 7</u>	<u>DISCUSSION</u>	
7.1	Re-iteration of Aims	174
SECTION A	<u>INFECTION BY V. CHOLERA</u>	
	THE NATURE OF THE ADHERENCE PHENOMENON	
7.2	The Correlation between Adherence and Virulence	175
7.3	The Role of the Flagellum in <u>V. cholerae</u> Adherence	176
7.3.1	The significance of motility for <u>in vitro</u> and <u>in vivo</u> adherence	176
7.3.2	Studies in other laboratories	178

		<u>Page</u>
7.3.3	Evidence for a flagellar adhesin	180
7.4	A Two-Adhesin Model of <u>V. cholerae</u> Adherence	183
7.4.1	The slime agglutinin as a second mediator of <u>in vitro</u> attachment	183
7.4.2	Discussion of the results obtained in various <u>in vitro</u> systems of adherence	185
7.4.3	The significance of the flagellar adhesin, the slime agglutinin and motility <u>per se</u> to the <u>in vivo</u> colonization of <u>V. cholerae</u>	188
7.5	The Specificity of <u>V. cholerae</u> Adherence	192
SECTION B	IMMUNITY TO <u>V. CHOLERA</u>	197
	THE PROPHYLACTIC SIGNIFICANCE OF THE NON-LPS ANTIGENS OF <u>V. CHOLERA</u>	
7.6	The Existence of non-LPS Protective Antigens in <u>V. cholerae</u> 569B	197
7.6.1	Initial studies	197
7.6.2	The stability of the non-LPS protective antigens of <u>V. cholerae</u> 569B	199
7.6.3	The relative prophylactic significance of the LPS and non-LPS antigens of <u>V. cholerae</u> 569B	201
7.6.4	Restricted distribution of the non-LPS protective antigens	201
7.7	The Protective Antigens of <u>V. cholerae</u> : studies in other laboratories	202
7.8	The Protective Antibacterial Function of Specific Antibodies	204
SECTION C	CONCLUSIONS	205
BIBLIOGRAPHY		

C H A P T E R 1

INTRODUCTION TO CHOLERA

1.1 General Background to the Disease.

1.1.1 Epidemiology and etiology.

Cholera is a diarrhoeal disease found only in humans, and has classically been regarded as a typically acute and often fatal infection. It has afflicted mankind for hundreds of years, and in recent times has shown the potential to erupt from its endemic home and initiate worldwide epidemics.

The history of the disease is divided into four main periods. Prior to 1817, cholera was almost exclusively confined to the state of Bengal and the neighbouring Indian subcontinent. The delta regions of the Ganges and Brahmaputra rivers quite possibly provided the original home for the disease, and it has remained endemic in this region ever since. Between 1817 and 1923 the world experienced six pandemics, all of which are believed to have been generated by marked exacerbation of the cholera situation in Bengal (see Pollitzer, 1959).

Apart from the widespread incidence and heightened severity of the disease within its endemic foci at the commencement of each pandemic, other factors contributed to the dissemination of the infection. Religious pilgrimages frequently attracted millions of devotees from all regions of India, and sanitary conditions at such assemblies were extremely poor. In a severe cholera season this migration would facilitate passage of the disease around the subcontinent. Further spread might be subject to seasonal interruption, but the disease showed the capacity to re-appear when climatic conditions again became favourable. Another important aid to the establishment of the pandemics was the busy sea trade based around the endemic home of the disease. Finally, the movement of troops during this turbulent era has also been implicated in assisting the transmission of cholera on several occasions (see Pollitzer, 1959).

During this second phase of cholera's history, two important events occurred which greatly advanced man's knowledge of the disease. Firstly, in 1854, Snow demonstrated the important role of water in the transmission of cholera (Snow, 1854-cited by Gangarosa, 1971). The value of a safe water supply is still appreciated today, and contributes to the requirement for adequate standards of sanitation and personal hygiene in general. We shall return to this issue later, in relation to the future control and ultimate eradication of the disease (see Section 1.1.4).

The second major advance was made in 1883-84, when the causative agent of cholera was first isolated and described. Following studies of outbreaks in Egypt and India, Koch showed that cholera was the result of a specific gastro-intestinal infection (Koch, 1883,1884-cited by Pollitzer, 1959). The organism responsible was Vibrio cholerae - a Gram-negative, "comma-shaped" bacterium with a single polar flagellum providing motility.

Subsequently, Gotschlich identified a second type of vibrio, designated El tor after the quarantine station at which it was first isolated (Gotschlich, 1905,1906-cited by Pollitzer, 1959). It differed from the "classical" biotype, which was responsible for the six pandemics of this period, in that it apparently lacked the capacity to cause cholera in man. In addition, vibrios of the El tor biotype displayed haemolytic activity when cultured (see Kamal, 1974). However, haemolysis has not proved to be a consistent property of El tor isolates over the years, and more reliable characteristics are now used in the differentiation of the two biotypes. In particular, the El tor biotype is characterized by an ability to agglutinate chicken red blood cells (Finkelstein and Mukerjee, 1963), and a resistance to polymyxin B (Gan and Tjia, 1963; Roy, Mridha and Mukerjee, 1965) and Mukerjee's Type IV cholera phage (Mukerjee, 1963). The general characteristics of the genus Vibrio as a whole have been recently summarized (Sakazaki, 1970; Gallut, 1974).

From 1923 to 1961 cholera once again became confined almost entirely

to its endemic home in Bengal. Then, in 1961, the fourth and current era of the disease began, with the onset of the seventh pandemic. The outstanding feature of this outbreak was the finding that, for the first time, the organisms responsible were of the El tor biotype (Barua and Cvjetanovic, 1970; Kamal, 1974).

The seventh pandemic is still in progress, and is thought to have arisen in Sulawesi (Kamal, 1974) - an area first described as an endemic focus of the El tor biotype in 1938 (deMoor, 1938-cited by Kamal, 1974). Since 1961 it has spread throughout Asia, the Middle East and Africa, and has even impinged occasionally upon Europe (see Kamal, 1974). As well as involving territories never previously affected in the entire recorded history of the disease, this pandemic has featured the first successful transmission of the disease by air (Barua, 1972). In 1971 alone, over 150,000 cases were reported (Kamal, 1974).

The return of cholera to the world scene has led to a resurgence of interest in the disease. As a result of an intensive research effort over the past fifteen years, significant advances have been made in our understanding and treatment of the infective process. Several studies have suggested that cholera should no longer be regarded as an acute and often fatal disease, by demonstrating that those cases severe enough to warrant treatment represent only a small proportion of the total number infected. The presence of many asymptomatic carriers has been demonstrated, even in areas lacking a single recognized cholera victim (van de Linde and Forbes, 1965; Sinha, Deb, De Abou-Gareeb and Shrivastava, 1967; Sinha, Deb, De, Sirkar, Abou-Gareeb and Shrivastava, 1968; McCormack, Islam, Fahimuddin and Mosley, 1969; also see Gangarosa and Mosley, 1974). These findings may well provide an explanation for the maintenance of the disease during those periods between epidemics (see Gangarosa and Mosley, 1974)

Finally, the current pandemic has provided an opportunity for comparison of the epidemiological characteristics of the two biotypes of

Vibrio cholerae. It now seems that organisms of either biotype are capable of causing cholera in its severest form, but the infection-to-case ratio is approximately ten times higher during El tor outbreaks (Bart, Huq, Khan and Mosley, 1970; Woodward and Mosley, 1972; also see Gangarosa and Mosley, 1974). The El tor vibrios are able to survive longer in the environment (Felsenfeld, 1963, 1965) and have the capacity to rapidly outnumber and eventually eliminate cases caused by organisms of the classical biotype (Barua, Mukherjee and Sack, 1964-cited by Kamal, 1974; Mukerjee and Basu, 1967).

1.1.2 Pathogenesis and pathophysiology.

As a result of many studies in both man and animals, it is now possible to construct a reasonably complete picture of the cholera infective process. The animal models which have proved to be of most value in this research have been recently discussed (Burrows and Sack, 1974).

Following ingestion, most commonly in the form of contaminated drinking water, the Vibrio cholerae eventually reach and colonize the small bowel. During growth the organisms elaborate a toxin, which binds to the cells of the gut epithelium and induces hypersecretion of isotonic body fluid. The resultant copious, watery diarrhoea constitutes the "rice water stool" characteristic of the disease, and is directly responsible for the clinical features which typically ensue. The two main phases of the infective process - the establishment of vibrios in the small bowel, followed by the action of the toxin upon the host - will now be considered in more detail.

In a non-immune individual, the only well-defined line of defence operating against the process of colonization is the natural acidity of the stomach. However, this constitutes quite an effective barrier to infection, as demonstrated by the studies of Hornick and co-workers (Hornick, Music, Wenzel, Cash, Libonati, Snyder and Woodward, 1971). They found that choleraic diarrhoea could be elicited in human volunteers by the oral administration of 10^4 vibrios, provided that sodium bicarbonate was given concomitantly to

neutralize gastric acidity. When the inocula were given without sodium bicarbonate, the minimum effective dose was 10^8 organisms (Hornick et al, 1971). The sensitivity of vibrios to natural gastric acidity has also been inferred from studies in dogs and infant rabbits (see Burrows and Sack, 1974) and in adult mice (Knop and Rowley, 1975). Further evidence suggesting an important role for gastric acidity in the prevention of human cholera has been obtained from studies which show that individuals suffering from achlorhydria are predisposed to the disease (Gitelson, 1971; Sack, Pierce, Hennessey, Mitra, Sack and Masumder, 1972).

Those organisms which survive passage through the stomach reach an environment conducive to growth upon arrival in the small bowel. Provided that their number is sufficient, and subject to other factors which at present remain unknown (see Carpenter, Greenough and Gordon, 1974), they may successfully colonize this new habitat. Throughout this phase ~~the~~^{*} vibrios remain confined to the lumen and mucosal epithelium of the gut. The structural integrity of the epithelium has been demonstrated in both human (Gangarosa, Beisel, Benyajati, Sprinz and Piya-ratn, 1960; Fresh, Versage and Reyes, 1964) and experimental (LaBrec, Sprinz, Schneider and Formal, 1965; Norris and Majno, 1968; Elliott, Carpenter, Sack and Yardley, 1970; Ghosh, 1970) cholera, with ~~no~~^{*} evidence of penetration of organisms into the sub-epithelial tissues (LaBrec et al, 1965; Ghosh, 1970).

^{*}In several animal studies direct attachment of vibrios to the gut epithelium has been observed (LaBrec et al, 1965; Freter, 1969; Ghosh, 1970; Nelson, Clements and Finkelstein, 1976). This adherence might be facilitated by a chemotactic response to attractants associated with the intestinal surface (Allweiss, Dostal, Carey, Edwards and Freter, 1977), and ^{*}could be advantageous in two respects. ^{*}Firstly, it might prevent the organisms from being flushed out of the small intestine - either by peristaltic action prior to the colonization process, or by the copious secretions which are subsequently induced. ^{*}Secondly, it would facilitate the effective delivery of toxin to its site of action. This brings us to the second phase of the disease process - the effect

of the toxin upon the host.

Evidence suggests that the same toxin moiety is produced by all pathogenic strains of Vibrio cholerae (Evans and Richardson, 1968; Finkelstein, 1969; Holmgren, Lonnroth and Ouchterlony, 1971). It has now been purified and characterized (Finkelstein and LoSpalluto, 1970; LoSpalluto and Finkelstein, 1972; Finkelstein, 1972) and its role in the pathogenesis of cholera has been frequently reviewed (Finkelstein, 1969, 1972; Pierce, Greenough and Carpenter, 1971; Carpenter, 1972; Carpenter, Greenough and Gordon, 1974). The structure of the toxin will be discussed later (see Section 1.2.5).

The mechanism by which the toxin exerts its effect upon the host has not been completely elucidated. * The initial step involves the adsorption of the toxin to the entire mucosal surface of the small bowel epithelium (Peterson, LoSpalluto and Finkelstein, 1972; also see Pierce et al, 1971). This binding occurs very rapidly, and the subsequent hypersecretory response induced by the toxin is irreversible (Mosley and Ahmed, 1969; Carpenter and Sack, unpublished data-cited by Pierce et al, 1971). * The epithelial cell membrane component which serves as a receptor for the binding of the toxin has now been identified as a sialidase-resistant monosialosyl ganglioside (van Heyningen, Carpenter, Pierce and Greenough, 1971; King and van Heyningen, 1973; van Heyningen, 1974).

* Following the binding of cholera toxin, investigators have observed an increase in adenylyl cyclase activity in the mucosal epithelial cells. This leads to elevated intracellular levels of cyclic 3'5' adenosine monophosphate (cyclic AMP), which parallel the observed increases in the rate of secretion (see Pierce et al, 1971; Carpenter et al, 1974). To date no intermediate steps have been described.

In both clinical (Greenough, 1965; Banwell, Pierce, Mitra, Brigham, Caranasos, Keimowitz, Fedson, Thomas, Gorbach, Sack and Mondal, 1970) and experimental (Carpenter, Sack, Feeley and Steenberg, 1968) cholera, the only

site of fluid loss is the small bowel. In both instances, the observed electrolyte pattern of the choleraic secretions varies considerably from duodenum to ileum, yet closely approximates that characteristic of each region under normal conditions (Carpenter et al, 1968; Banwell et al, 1970).

Despite the finding that the absorptive capacity of the bowel appears to remain undiminished during cholera (see Pierce et al, 1971), the hypersecretory response elicited by the toxin results in a substantial net loss of isotonic body fluid. This occurs in the absence of any histological evidence of damage to the gut wall, either in man (Gangarosa et al, 1960; Fresh et al, 1964), or in experimental animals (LaBrec et al, 1965; Norris and Majno, 1968; Elliott et al, 1970; Ghosh, 1970). The loss of large volumes of isotonic electrolyte solutions which are rich in bicarbonate is responsible for the characteristic clinical profile of the disease. Hypovolemia and acidosis are regular features of severe cholera, which may last for four to six days even if properly treated. During this time the patient may lose twice his own body weight in liquid stools (see Pierce and Mondal, 1974).

1.1.3 The treatment of cholera.

The finding that the large majority of cholera victims experience only a mild or even inapparent form of the disease led to a new appreciation of the etiology of cholera (see Section 1.1.1). The development and application of highly effective treatment regimes strengthened the realisation that cholera need not necessarily be regarded as a pestilence which inflicts widespread mortality. Indeed, the characteristic symptoms of the disease can now be rapidly reversed by the prompt initiation of fluid replacement therapy, a practice which has been able to reduce the adult mortality rate to less than one per cent (see Hirschhorn, Pierce, Kobari and Carpenter, 1974).

In principle, treatment of cholera victims is based upon the restoration and maintenance of fluid and electrolyte balance. Once the state of hydration of the patient has been assessed, the appropriate volume of

rehydration fluid is promptly administered. The most severe cases require immediate intravenous rehydration, but otherwise oral therapy will suffice (see Nalin and Cash, 1974). The advantages of the latter practice are its low cost and widespread availability. The solutions used do not have to be sterilized and can be made up with ordinary drinking water - making them ideally suited for use in endemic areas where alternative health facilities are frequently poor (Nalin and Cash, 1974).

Intravenous rehydration fluids contain saline and alkali as their main constituents, with usually a low level of potassium (see Hirschhorn et al, 1974). Solutions given orally contain, in addition, glucose to facilitate the uptake of water and electrolytes by the small bowel (see Nalin and Cash, 1974). Either route of administration leads to a rapid restoration of fluid and electrolyte balance, and thereafter these are maintained by further therapy in response to each diarrhoeal episode. As a result of such treatment the clinical features of the disease are reversed, and the infection becomes self-limiting. During this phase the duration and volume of diarrhoea can be reduced by the administration of antibiotics, particularly tetracycline (see Hirschhorn et al 1974).

1.1.4 Future management of the disease.

When formulating programmes for the future control of cholera, scientists have three main measures at their disposal. These are treatment, immunization and sanitation, and the relative contribution to be made by each must be carefully assessed (see Shrivastav, 1974). Cost - benefit analyses may help in this respect, by suggesting the most suitable application of the limited funds available for tackling the disease in developing countries (see Cvjetanovic, 1974). However, such studies are necessarily restricted to economic considerations, and omit the moral and ethical issues which also influence decisions concerning future management. The three available measures will now be briefly

discussed.

(i) Treatment.

Treatment is highly effective from the viewpoint of the alleviation of suffering and prevention of mortality, and hence its presence in future management programmes is vindicated. However, as it is applicable to only a small minority of cholera victims, it is an inappropriate measure with respect to the control and ultimate eradication of the disease. The only contribution it might make in this regard - the disposal of highly contaminated stools, and the surveillance of case contacts - might be more correctly assessed as sanitary measures.

(ii) Immunization.

The role of immunization in the future management of cholera has been recently discussed (Shrivastav, 1974; Cvjetanovic, 1974). With the resurgence of cholera in the current pandemic, considerable effort has been devoted to the preparation of vaccines capable of protecting the inhabitants of cholera-prone areas. The many resulting field trials have been generally disappointing, as will be discussed later in relation to immunity to cholera (see Section 1.3.7). Currently available vaccines can provide significant levels of protection, but the state of immunity which they elicit is transient, and diminishes rapidly after three months. Furthermore, vaccination does not reduce the rate of inapparent infection, nor does it lessen the severity of the disease among those who still succumb (see Shrivastav, 1974).

Thus, in its present status, immunization can have little impact upon the future epidemiology of cholera. In purely economic terms, it is rarely justifiable when compared to the costs of alternative control measures (Mosley, Aziz, Rahman, Chowdhury, Ahmed and Fahimuddin, 1972; Cvjetanovic, 1974). Until recently, the dilemma existed, however, that many endemic regions lack adequate treatment facilities, and so immunization of these areas

would probably lower the mortality rate. The recent advent of oral rehydration therapy, with its widespread applicability, has nevertheless weakened even this argument in favour of immunization.

(iii) Sanitation.

The only known natural reservoir of cholera is man, the disease being maintained by cyclical transmission between man and his environment. Consequently, cholera will only be finally eradicated when those regions which are now endemic are eventually made non-receptive to the disease, as a result of vast improvement in standards of sanitation and personal hygiene. The implementation of basic sanitary measures - including the provision of safe water supplies and facilities for excreta disposal, and the introduction of health education programmes - will eventually break the cycle of transmission at both stages (see Shrivastav, 1974).

Snow's early recognition of the importance of a safe water supply (see Section 1.1.1) still has relevance today. In endemic regions, contaminated surface waters provide a convenient source of water, not only for drinking but also for bathing and the preparation of foodstuffs. Consequently, the provision of a clean supply of drinking water will not have a significant impact on the incidence of cholera if the population continues to bathe in and prepare food with polluted water (see Curlin, Aziz and Khan, 1977). Thus the introduction of any sanitary improvement will need to be accompanied by an effective education programme to ensure that the population derives maximum benefit.

As well as lowering the likelihood of vibrio ingestion, scientists can attack the man-to-environment link of the infective cycle by providing an efficient means of excreta disposal. As vibrios have a limited survival outside the human body, this will eventually lead to a less contaminated environment, which will in turn further reduce the risk of infection (see Shrivastav, 1974). It is obvious that the benefits derived from basic sanitary measures

will be both cumulative and permanent. They will be reflected in an ever decreasing incidence of cholera (and probably of other diseases of similar etiology) and will result in a much improved standard of living.

In conclusion, environmental sanitation offers the only permanent solution to the cholera problem. It compares favourably with alternative control measures from an economic viewpoint, and would provide many other long term benefits for developing communities. The effectiveness of sanitary measures as a sole defence against cholera is attested to by the fact that the disease has consistently failed to gain a foothold in the developed countries, despite frequent chance introductions (Shrivastav, 1974; Kamal, 1974). In fact the United States no longer requires visitors from cholera-prone areas to be vaccinated before entry (see Gangarosa, 1971).

Unfortunately, the provision of an adequate standard of sanitation is such a long term undertaking that cholera will be with us for many years yet. In the meantime, treatment and immunization will continue to be employed for their ability to relieve suffering and reduce mortality. However, they are relatively expensive and contribute little to the control, and nothing to the eventual eradication, of the disease. Scientists continue to search for more effective vaccines which might warrant a re-appraisal of the role of immunization in future management programmes. If a longer lasting and more complete level of immunity could be induced, then immunization could become a significant weapon in the fight against cholera.

1.2 The Antigens of *V. cholerae*.

1.2.1 Introduction.

Soon after Koch's isolation of *V. cholerae*, studies by Pfeiffer, and also by Gruber and Durham, implicated the existence of antigenic determinants on these organisms by demonstrating the capacity of immune sera to lyse or agglutinate vibrios of the homologous strain (Pfeiffer, 1895; Gruber and Durham, 1896-both cited by Pollitzer and Burrows, 1959). Initially the determinants responsible for antigenicity were considered to be totally heat stable, but in 1926 Balteanu convincingly demonstrated the existence of heat-labile antigens (see Pollitzer and Burrows, 1959).^{*} Those determinants destroyed by heat (generally 100°C for 2 hours) were referred to as the H-antigens, whilst the thermostable determinants were called the O-antigens.

The arrival of the seventh cholera pandemic led to an unprecedented interest in the antigenic structure of *V. cholerae*. The hope of producing an effective vaccine resulted in a particular interest in the roles of the various antigenic moieties in immunity to infection. In this section we shall briefly consider the current state of knowledge with respect to the antigens of *V. cholerae*, but leave until later a discussion of their possible immunoprophylactic significance (see Sections 1.3.2 and 2.5.5; Chapters 6 and 7).

1.2.2 Heat-stable (O) antigens.

V. cholerae strains of either biotype are further divisible into three serotypes on the basis of their O-antigenic pattern. These sub-types were first recognized by Kabeshima (1913) and by Nobechi (1923), and were demonstrated by agglutination tests using immune sera before and after absorption with heterologous organisms (see Pollitzer and Burrows, 1959).^{*} They were termed Inaba, Ogawa and Hikojima (which occurs at a much lower frequency) and have the O-antigenic formulae AC, AB, and ABC respectively (see Kauffmann, 1950).

In 1935, Gardner and Venkatraman showed that the heat-stable antigens could also be used to differentiate between true cholera vibrios and a morphologically and biochemically similar group of organisms called "non-cholera vibrios" or NCVs (Gardner and Venkatraman, 1935). These NCVs were able to cause diarrhoea of varying severity in man (McIntyre, Feeley, Greenough, Benenson, Hassan and Saad, 1965; Aldova, Laznickova, Stepankova and Lietava, 1968) and carried the same H-antigens as V. cholerae (see Section 1.2.3). However, Gardner and Venkatraman found that all of the true cholera vibrios examined fell into O-subgroup 1, whereas the NCVs always possessed a different O-antigenic pattern (Gardner and Venkatraman, 1935). The NCVs were consequently also referred to as the NAG (non-agglutinable) vibrios, as they could not be agglutinated by antisera specific for the O-subgroup 1 antigens. This differentiation has now been confirmed by Sakazaki and co-workers, who have classified the NAG vibrios into many O-antigenic subgroups (Sakazaki, Tamura, Gomez and Sen, 1970).

In addition to their stability to heat, the O-antigens are generally resistant to chemical treatment. Early attempts to isolate these antigens from the surfaces of vibrios involved the use of organic solvents, acid hydrolysis, proteolytic enzymes, or trichloroacetic acid extraction (see Pollitzer and Burrows, 1959). More recently, the phenol-water method of Westphal, Luderitz and Bister (1952) has been successfully adopted (Shrivastava, 1965; Watanabe and Verwey, 1965; Neoh and Rowley, 1970). The O-antigens have also been purified from culture supernatants (Kaur and Shrivastav, 1964; Watanabe and Verwey, 1965).

In several studies, the presence of O-antigens has been reported in preparations practically devoid of protein (Kaur and Shrivastav, 1964; Watanabe and Verwey, 1965; Neoh and Rowley, 1970). It is now generally accepted that these determinants reside in the polysaccharide component of the cell wall. The identity of the sugars responsible for the various serotypic determinants remains unknown, although glucose, galactose and glucosamine

have been consistently detected in preparations containing Inaba O-antigens (Takaishi, 1959; Misra, Pant and Shrivastava, 1961; Kaur and Shrivastav, 1964; Jackson and Redmond, 1971).

1.2.3 Heat-labile (H) antigens.

The heat-labile or H-antigens of V. cholerae have classically been regarded as those determinants associated with the flagellum. Serological examinations have suggested that all strains of V. cholerae possess a common H-antigen (Gardner and Venkatraman, 1935; Sakazaki et al, 1970). This same antigen is also present on the NAG vibrios (Gardner and Venkatraman, 1935; Sakazaki et al, 1970), although not on vibrios of other species (Sakazaki et al, 1970), strengthening the proposal that the NAG vibrios should be considered with the true (O-subgroup 1) vibrios as a single species (Sakazaki, Gomez and Sebald, 1967; Sakazaki et al, 1970).

These observations have recently been confirmed and extended by the studies of Bhattacharyya (Bhattacharyya and Mukerjee, 1974; Bhattacharyya, 1975). It now appears that, further than possessing a common determinant, the flagella of all cholera and NAG vibrios are serologically completely identical (Bhattacharyya and Mukerjee, 1974). Furthermore, by demonstrating the existence of essentially different H-antigens on vibrios isolated from fish or birds, Bhattacharyya confirmed the earlier work of Sakazaki's group (Sakazaki et al, 1970), which suggested the feasibility of identifying vibrio species on the basis of H serology (Bhattacharyya, 1975).

Electron microscopic examinations of the vibrio flagellum have revealed the existence of a two-component structure (Follett and Gordon, 1963; Ogasawara and Kuno, 1964). These studies suggested that the flagellum comprised a central core, which was probably proteinaceous (see Follett and Gordon, 1963), surrounded by a tubular sheath structure. The sheath appeared to be an extension of the cell wall, a notion which was eventually strengthened by the demonstration that fluorescein-labelled O-antibodies were able to

bind specifically to the flagella (Zykin, 1968 - cited by Bhattacharyya, 1975). In addition,* antibodies specific for the O-antigens have been found to inhibit the motility of V. cholerae (Williams, Verwey, Schrank and Hurry, 1973-cited by Finkelstein, 1975; Bhattacharyya, 1975; also see Section 6.6.3).

Attempts to isolate and purify flagella from V. cholerae have met with varied success. Balteanu (1926) and White (1940) were apparently able to obtain preparations which yielded relatively pure H-antisera (see Pollitzer and Burrows, 1959), whereas Gallut commented that his preparation, although apparently pure by electron microscopy, was equivalent to live organisms from the viewpoint of stimulating O-antibodies (Gallut, 1965). In addition to the possibility that Gallut's preparation contained contaminating O-somatic antigens (see Gallut, 1965), these contrasting results might also be reconciled on the basis of the integrity of the sheath structures in the various preparations. White recommended the use of rough variants of vibrio, and furthermore used chloroform in the extraction procedure (White, 1940-see Pollitzer and Burrows, 1959). This is known to decrease O- and increase H-agglutinability (Vassiliadis, 1936 - see Pollitzer and Burrows, 1959), possibly by selective destruction of the sheath in a manner similar to that reported by Follett and Gordon after autolysis of vibrios, or treatment with acid or urea (Follett and Gordon, 1963). Thus White's preparations may have consisted entirely of flagellar cores, whereas Gallut probably immunized with flagella possessing sheath structures and their associated O-antigens.

Recently Yang, Schrank and Freeman reported the purification of the flagellar cores of V. cholerae (Yang, Schrank and Freeman, 1977). The polymeric protein nature of this component was confirmed, and the density and molecular weight of the subunits were similar to those reported for the unsheathed flagella of other species. Antibodies specific for the core determinants were found to bind along the entire length of the flagella of intact vibrios, but did not bind to the surfaces of the cells (Yang et al, 1977). This confirms that the antigens of the inner core are normally

exposed, as had been suggested by the demonstration that cholera and NAG vibrios possess identical H-antigens (Bhattacharyya and Mukerjee, 1974) despite the fact that their flagella sheaths bear different O-determinants.

Two studies at variance with the concept of exposed H-determinants have recently been reported. Bhattacharyya observed that purified H-antibodies did not bind to the flagella of homologous vibrios, nor did they immobilize the organisms (Bhattacharyya, 1975). It has also been reported that antisera raised against sheathed vibrio flagella are unable to precipitate the purified flagellar cores (Eubanks, Guentzel and Berry, 1977). Again it is possible that the explanation for these apparently inconsistent findings may lie in the structure of the flagellar sheath material. The studies of Follett and Gordon, and of Yang et al, suggest that the sheath structure is tighter in the organisms of younger cultures, and becomes more permeable (to negative stain or anti-core antibody respectively) with aging of the culture (Follett and Gordon, 1963; Yang et al, 1977). Bhattacharyya used very young (two-hour) cultures for motility inhibition assays, which might explain the failure of H-antibodies to immobilize; and likewise the finding that such antibodies did not bind to the vibrio flagella might also be attributed to the impermeability of the sheaths. Similarly, if the flagella used as immunogens by Eubanks et al were surrounded by very tight sheath structures, one might not expect the production of antibodies specific for the flagellar core material.

1.2.4 Other somatic antigens of *V. cholerae*.

In addition to the O- and H-antigens already discussed, the existence of other somatic antigens has been periodically reported. These determinants have consistently been described as protein in nature, but have varied in their localization and sensitivity to heat. Accordingly the terms "heat stable" and "heat labile" should be recognized as being quite broad descriptive terms, whereas "O-" and "H-antigens" should be restricted to those determinants associated with the polysaccharide and flagellar components

respectively.

(i) Heat-stable protein antigens.

In 1935, White described the existence of an alcohol-soluble, heat-stable Q protein antigen in V. cholerae. Antibodies specific for this antigen were able to agglutinate boiled vibrios, and this agglutination was not abolished by prior absorption of the antibodies with carbohydrate fractions prepared from the homologous organisms (White 1935 -see Pollitzer and Burrows, 1959).

White subsequently reported a second heat-stable somatic protein, termed HSSP (White 1940 - see Pollitzer and Burrows, 1959), which was thought to be deeply buried within the vibrio cell wall. Gallut later described the preparation of thermostable protein fractions which he considered were probably identical to the HSSP antigens of White (Gallut, 1960, 1965). These determinants were detected in many strains of cholera vibrios, as well as in the NAG vibrios (Gallut, 1960).

Most recently, Neoh and Rowley have described a heat-stable protein antigen capable of inducing the formation of bactericidal and agglutinating antibodies (Neoh and Rowley, 1970, 1971, 1972). This protein could be prepared from Boivin antigen by phenol extraction, and could be detected in several vibrio strains (Neoh and Rowley, 1970, 1971).

(ii) Heat-labile protein antigens.

In 1940, White described a heat-labile somatic protein antigen (HLSP) which could be prepared by chloroform extraction of V. cholerae (White 1940 - see Pollitzer and Burrows, 1959). This antigen was apparently deeply buried within the cell wall, and did not elicit the production of agglutinating antibodies. It was detected in all strains of V. cholerae examined.

Lankford and co-workers have reported another thermolabile protein antigen in V. cholerae (Bales and Lankford, 1961; Lankford and Legsomburana, 1965; Chulasamaya and Lankford, 1970). This antigen is exposed on the cell

surface, and was detected in all strains of V. cholerae examined (Bales and Lankford, 1961). It appears to be the moiety responsible for the haemagglutinating activity of vibrio cultures, and can be released from the cells in active form by incubation with buffers (Lankford and Legsomburana, 1965; Chulasamaya and Lankford, 1970). Its possible contribution to virulence will be discussed later (see Chapter 7).

Finally, fimbriae (or pili) have frequently been observed on the surfaces of V. cholerae (Barua and Chatterjee, 1964; Zinnaka, Shimodori and Takeya, 1964; Tweedy, Park and Hodgkiss, 1968; Chulasamaya and Lankford, 1970). The existence of these thermolabile, proteinaceous appendages has been linked with the capacity for haemagglutination in many genera (see Section 2.5). The validity of this correlation for V. cholerae has not been conclusively demonstrated, and remains a contentious issue (Finkelstein and Mukerjee, 1963; Zinnaka et al, 1964; Barua and Mukerjee, 1965; Tweedy et al., 1968).

1.2.5 Enterotoxin.

The diarrhoeagenic factor released by strains of V. cholerae has been purified to varying degrees in several laboratories (see Pierce et al, 1971). Different isolation procedures have been adopted, and the final preparations have been tested in a variety of assay systems. However, the physical similarity of the active materials has led to the general belief that the same moiety is responsible for all of the activities described (Pierce et al, 1971).

The enterotoxin has now been completely purified and well characterized by Finkelstein's group (Finkelstein and LoSpalluto, 1970; LoSpalluto and Finkelstein, 1972; Finkelstein, 1972). It is a heat-labile protein of molecular weight (MW) approximately 84,000 daltons, and has been termed cholera-gen. A spontaneously occurring toxoid, termed cholera-genoid, was simultaneously isolated and described. This has a MW of about 56,000 and immunodiffusion studies have shown that it lacks an antigen present on

the active toxin (Finkelstein, Boesman, Neoh, LaRue and Delaney, 1974; Holmgren and Lonnroth, 1975).

Cholera toxin exists as a complex of two types of subunit (Lonnroth and Holmgren, 1973; Finkelstein et al, 1974; van Heyningen, 1976). The heavier (H) subunit, also referred to as ~~the~~ region A, has a MW of approximately 28,000 daltons, and comprises two covalently-bound polypeptides (Holmgren and Lonnroth, 1975; van Heyningen, 1976; Klapper, Finkelstein and Capra, 1976; Gill, 1976; Kurosky, Markel, Touchstone and Peterson, 1976). The balance of evidence suggests that the lighter (L) subunits, also collectively referred to as region B, each have a MW of about 10,000 daltons (Klapper et al, 1976; Gill, 1976; Kurosky et al, 1976), and accordingly the formulae HL_4 (Jacobs, Niall and Sharp, 1974; van Heyningen, 1976; Kurosky et al, 1976) or HL_5 (Klapper et al, 1976; Gill, 1976) have been proposed for the intact toxin moiety. Choleragenoid lacks the H subunit (Finkelstein et al, 1974; Holmgren and Lonnroth, 1975; Kurosky et al, 1976) and so may be designated L_4 or L_5 (or region B). Immunodiffusion analyses employing purified subunit preparations have confirmed that the toxin-specific determinants reside in the H subunit (Holmgren and Lonnroth, 1975).

Both choleragen and choleragenoid have the capacity to bind to cells of either intestinal or extra-intestinal origin (Peterson et al, 1972; Holmgren, Lindholm and Lonnroth, 1974; also see Finkelstein, 1975). This finding suggested that the ability to bind might be a property of the B region of the active toxin. The fact that the choleragenoid was biologically inactive, however, implicated the A region as the component responsible for stimulation of the adenylate cyclase system. This has recently been confirmed by the demonstration that purified A regions can activate the adenylate cyclase of pigeon erythrocytes in vitro (van Heyningen and King, 1975). Although such regions are inactive in vivo, toxicity is fully restored upon re-association with the B region (Finkelstein et al, 1974).

* Thus it is now generally accepted that the B region of the cholera toxin is responsible for the initial binding of the toxin to the cell,

following which the A region provides the stimulus for the intracellular accumulation of cyclic AMP. This postulated mode of action is remarkably similar to that proposed for diphtheria toxin (Gill, Pappenheimer and Uchida, 1973; Everse, Lappi, Beglau, Lee and Kaplan, 1977).

1.3 Immunity to *V. cholerae*.

1.3.1 Introduction.

The choleraic infective process is confined to the lumen and mucosal epithelium of the small intestine, and accordingly immunity to *V. cholerae* must be mediated by antibodies operating within this environment. In this section, the specificity, origin and nature of such antibodies will be considered, with reference to information obtained from studies of both clinical and experimental cholera. Our current knowledge of the local immune system will be summarized, and the mechanisms of immunity which might be effective at secretory surfaces will be discussed. Finally, a brief history of man's attempts at immuno-prophylaxis in the field will be given, concluding with the present status of research in this area.

1.3.2 Antibacterial vs antitoxic immunity.

The manifestations of cholera arise as a direct result of the action of the enterotoxin upon the small intestinal tissues of the host. Consequently, there has been much speculation as to whether the purified toxin might be a more effective immunogen, from the viewpoint of stimulating immunity to *V. cholerae*, than the vibrio itself. The evidence accumulated to date, although not conclusively resolving the issue, suggests that this is not the case.

Many studies in a variety of experimental systems have demonstrated that immunity to cholera can be effectively mediated by antibodies specific for the cholera toxin (Finkelstein, 1970; Fujita and Finkelstein, 1972; Pierce, Kaniecki and Northrup, 1972; Pierce and Reynolds, 1974; Holmgren, Svennerholm,

Ouchterlony, Andersson, Wallerstrom and Westerberg-Berndtsson, 1975). The protective capacity of antibodies directed against the somatic determinants of the vibrio itself has also been well documented (see Sections 1.3.4 and 1.3.7; also see Chapter 6).

Attempts to evaluate the relative contributions of antitoxic as opposed to antibacterial immunity have suggested that the latter is of greater significance in resistance to cholera, although these studies have not been completely convincing. Firstly, Neoh and Rowley reported that antibodies specific for somatic vibrio antigens were, on a weight basis, much more effective than antibodies directed against the toxin with respect to the protection of infant mice from the otherwise lethal effects of an oral inoculum of V. cholerae (Neoh and Rowley, 1972). This experimental model of cholera, however, is not ideally suited to the monitoring of antitoxic immunity, as the antibodies are only present at the very beginning of the infective process (in contrast to the situation which exists in other models which have been successfully used to demonstrate antitoxic immunity - see references above). A second comparative study was reported by Sinha and Bhaskaran, who demonstrated antibacterial immunity in a rabbit ileal loop assay under conditions where antitoxic immunity could not be observed (Sinha and Bhaskaran, 1973). However, their inability to detect antitoxic immunity in an assay which relies upon a toxin-induced hypersecretory response is surprising, and in contrast to results obtained in similar systems by other workers (Finkelstein, 1970; Pierce and Reynolds, 1974; Holmgren et al, 1975).

The best evidence that antibacterial immunity may be of greater significance than antitoxic immunity is derived from studies of clinical cholera. Cash and co-workers reported that human volunteers who had recovered from an induced choleraic episode were subsequently protected against re-infection with organisms of the homologous serotype only (Cash, Music, Libonati, Craig, Pierce and Hornick, 1974). This suggested that any antitoxic immunity which may have been elicited was of little relevance, and indeed it

was concluded that the observed immunity was due to an antibacterial protective mechanism (Cash et al, 1974).

Furthermore, in contrast to the numerous field trials which have indicated that killed cellular vaccines can provide quite good protection, at least for a short period of time (see Section 1.3.7), recent testing of two preparations of cholera toxoid has been discouraging. The first field trial was held in Bangladesh, and led to the conclusion that there was no consistent relationship between serum antitoxin titres and susceptibility to infection (Curlin, Levine, Aziz, Rahman and Verwey, 1976). The only significant protection observed was inexplicably confined to one age-group, and endured for just three months (Curlin et al, 1976). A second toxoid preparation was tested in a later trial conducted in the Philippines, where it was concluded that immunization had not provided any significant protection (Noriki, 1976).

1.3.3 Coproantibodies and the local immune system.

The term "coproantibody" was introduced by Burrows and co-workers to denote antibody present in the faeces, as distinct from serum antibody (Burrows, Elliott and Havens, 1947). Studying experimental cholera in guinea-pigs, they were able to demonstrate a correlation between the level of agglutinating antibody detected in the faeces, and the rate of elimination of vibrios from the gut. No such correlation could be found in the case of serum antibody activity (Burrows et al, 1947). Coproantibodies could also be detected in the faeces of humans vaccinated against cholera, and once again the temporal pattern of these antibodies differed markedly from that of serum antibodies (Burrows and Havens, 1948). These observations supported the concept of a local immune system - discrete from, and independent of, the central immune apparatus - as had been proposed many years earlier by Besredka (Besredka, 1919, 1927 - cited by Tomasi and Bienenstock, 1968).

The study of local immunity received considerable impetus from the discovery of IgA by Heremans' group in 1959 (Heremans, Heremans and Schultze,

1959). This was found to be the principal immunoglobulin class in a variety of human external secretions, in contrast to its minor contribution to the serum pool of immunoglobulins (Tomasi, Tan, Solomon and Prendergast, 1965; also see Tomasi and Bienenstock, 1968). The concept of local synthesis was strengthened by the realization that the IgA found in secretions differed chemically and antigenically from that present in the serum (Tomasi et al, 1965; also see Section 1.3.4(i)). The former was therefore termed secretory IgA, and has now been detected in the secretions of a wide range of vertebrates (see Tomasi and Grey, 1972).

Studies using radiolabelled serum IgA have shown that the predominance of secretory IgA in the various mucosal secretions of man cannot be attributed to a selective transport of this immunoglobulin class from the serum (Tomasi et al, 1965; Strober, Blaese and Waldmann, 1970). This point received confirmation from the finding that in certain circumstances the local immune response could be restricted to the site of antigenic challenge (Ogra and Karzon, 1969b), thereby strongly implicating a local synthesis of antibody. Further support for the existence of a local immune system was provided by fluorescent antibody studies in humans which demonstrated the presence of large numbers of plasma cells in the submucosal tissues of the gastro-intestinal and respiratory tracts (see Tomasi and Bienenstock, 1968).

Restricting discussion to the mucosal surface of relevance to cholera, the local production of coproantibodies suggests that the gut is a lymphoid organ capable of responding to antigenic stimulation. The relative importance of each of the gut-associated lymphoid tissues (see Parrott, 1976) for the immunological competence of the gut as a whole is not known, although recent work has implicated the Peyer's patches as fulfilling a critical role. Unlike the surrounding submucosal tissues these structures do not contain IgA-producing plasma cells, and do not respond to direct antigenic stimulation (see Parrott, 1976). However, the demonstration that the shielding of a single Peyer's patch preserves immunocompetence in an otherwise lethally irradiated animal (Jacobson, Marks, Simmons and Gaston, 1961)

suggests that each of these structures possesses the capacity to recognize and respond to antigen. This has been supported by several studies which suggest that the development of Peyer's patches appears to depend upon the intensity of the antigenic barrage to which they are exposed (see Parrott, 1976).

A recent study by Robertson and Cebra extended these observations by demonstrating an absolute requirement for Peyer's patches in the generation of a local immune response in the gut (Robertson and Cebra, 1976). Using a rabbit loop model, they showed that the capacity to respond to locally applied antigens was dependent upon the presence of Peyer's patches in the loop. Furthermore, as all loops remained connected to the general circulation, it was possible to detect the local production of coproantibody in all loops - even those lacking any Peyer's patches - following challenge of a single loop possessing these structures (Robertson and Cebra, 1976). This migration of antigen-stimulated lymphoid cells had been reported by other workers (see Parrott, 1976), and is of great relevance to our understanding of the cellular basis of local immunity. Present concepts of the sequence of events involved in the generation of a local immune response in the gut may be summarized as follows.

The Peyer's patches, which form part of the lymphocyte recirculatory system (see Parrott, 1976), are thought to constitute the sites of antigen recognition. The mucosal epithelium overlying these structures - the dome epithelium - appears to be appropriately modified for such a role. This area contains a specialized cell type - the M cell (Owen and Jones, 1974) - which, by virtue of its pinocytotic capacity, effects the uptake of intestinal luminal material into the body of the Peyer's patch (Owen, 1977).

Immediately below the dome epithelium lies an area of mixed cell traffic. Following exposure to antigen, a subpopulation of the lymphoid cells is stimulated to undergo a blastogenic response. The resulting blast cells leave the Peyer's patches and travel via the efferent lymphatics of the gut to the mesenteric lymph nodes. This migration takes several days, after which the cells journey to the thoracic duct, and thence to the

general circulation. During this migration the cells also differentiate, and both processes are completed by their return to the lamina propria of the gut as specific IgA-secreting plasma cells. The mechanism whereby the cells leave their cycle of migration to take up permanent residence in the gut is not understood, although evidence suggests that this "homing" capacity is gradually acquired during the process of differentiation (see Parrott, 1976).

1.3.4 The nature and significance of coproantibodies.

(i) Secretory IgA.

Human secretory IgA (s-IgA) is an 11S dimeric immunoglobulin with a MW of 385,000 daltons (Tomasi and Grey, 1972; Mestecky, Kulhavy and Kraus, 1972). It is assembled and released as a dimer (see Tomasi and Grey, 1972) and therefore comprises two identical monomeric subunits. Electron micrographs have revealed that the intact molecule has a "double-Y" structure, suggesting that the subunits are joined near the terminal regions of their Fc fragments (Parkhouse, Virella and Dourmashkin, 1971; Bloth and Svehag, 1971).

Serum IgA exists predominantly as a 7S monomer in man (Heremans et al, 1959) although polymeric forms have also been detected (Vaerman, Fudenberg, Vaerman and Mandy, 1965). Secretory IgA may be distinguished from the various forms of serum IgA on the basis of an associated polypeptide which bears unique antigenic determinants (Tomasi et al, 1965). This "secretory component" (SC) has a MW of about 60,000 daltons, and is synthesized independently of the 11S IgA dimers (see Tomasi and Grey, 1972). It is attached to the dimer primarily by covalent bonds (Tomasi and Calvanico, 1968; Mestecky et al, 1972).

The SC is thought to be responsible for the marked resistance of s-IgA to proteolysis (Brown, Newcomb and Ishizaka, 1970; Shuster, 1971; Ghetie and Mota, 1973; Underdown and Dorrington, 1974; Lindh, 1975), presumably by masking those regions of the molecule susceptible to enzymic attack. In view of the environment within which s-IgA has to function, this

stability offers obvious advantages. The SC has also been postulated to play an important role in the transport of locally-produced IgA into the lumen of the gut (Brandtzaeg, 1974a). It is produced by the epithelial cells which line the gut mucosa, and is thought to be displayed on the surfaces of these cells as a receptor for the dimeric IgA produced nearby (Brandtzaeg, 1974a,b). Following its binding to such an SC receptor, the 11S IgA (now with its attached SC) is taken up into the cytoplasm of the epithelial cell, and subsequently released into the lumen of the gut (Brandtzaeg, 1974a,b). This model can similarly explain the appearance of IgM with an attached SC in the secretions of both IgA-deficient and normal humans (Brandtzaeg, 1975; Richman and Brown, 1977). An IgM-SC complex had previously been detected in the sera of patients with IgA deficiency (Thompson, 1970), and more recent studies have demonstrated the capacity of IgM to bind SC in vitro (Eskeland and Brandtzaeg, 1974; Weiker and Underdown, 1975).

A second non-immunoglobulin polypeptide associated with the s-IgA complex is the J chain, which has a MW of 23-26,000 daltons (Halpern and Koshland, 1970; Mestecky et al, 1972). This moiety is linked covalently to the heavy chains of the dimeric IgA (Halpern and Koshland, 1970; Mestecky et al, 1972), and is synthesized in the same cells that produce the immunoglobulin (Halpern and Coffman, 1972; Parkhouse, 1972). The same J chain has been found in secretory IgA, myeloma polymeric IgA, and IgM, in the ratio of one chain per immunoglobulin molecule (Morrison and Koshland, 1972; Mestecky, Zikan, Butler and Kulhavy, 1972). It is not found in monomeric immunoglobulins, and has accordingly been considered to play an important role in the linking of monomers during polymerization (Halpern and Koshland, 1973). However there is some evidence that polymerization can occur in the absence of J chains (Abel, 1968; Eskeland and Brandtzaeg, 1974), and recent studies have suggested that the principal function of the J chain may be to mediate the attachment of the SC (Eskeland and Brandtzaeg, 1974; Weicker and Underdown, 1975).

The significance of s-IgA as a protective coproantibody has now

been reported in several experimental models of cholera. Firstly, Burrows and co-workers purified 11S IgA from an intestinal tissue extract prepared from rabbits which had been vaccinated both parenterally and orally with a cell-free culture supernatant of V. cholerae, and showed that this preparation possessed antitoxic activity (Kaur, McGhee and Burrows, 1972). Subsequently Fubara and Freter demonstrated antibacterial immunity mediated by a preparation of s-IgA obtained from the intestinal washings of orally vaccinated germfree mice (Fubara and Freter, 1973). Finally Steele and co-workers have recently reported the findings of a most revealing study using highly purified preparations of IgG, IgM and (11S) IgA obtained from rabbits which had been challenged parenterally or orally with live V. cholerae (Steele, Chaicumpa and Rowley, 1974). Antibodies of the IgA class were, on a weight basis, equally as protective as IgG or IgM antibodies in the infant mouse protection assay (Steele et al, 1974). The relevance of this study to a consideration of possible mechanisms of antibacterial immunity to cholera will be discussed shortly (see Section 1.3.5).

Unfortunately, studies of the role of s-IgA coproantibody in resistance to clinical cholera have been less definitive. The intestinal fluids of convalescent cholera patients have been shown to possess antibody activity residing in the IgA, as well as in the IgG and IgM classes (Northrup and Hossain, 1970). Subsequently Waldman and co-workers reported a dramatic increase in the level of antibacterial antibodies of the IgA class which could be detected in the intestinal secretions of patients recovering from cholera (Waldman, Bencic, Sinha, Deb, Sakazaki, Tamura, Mukerjee and Ganguly, 1972). Obviously these observations require confirmation and considerable extension. However, when considered in conjunction with the results obtained from the studies of experimental cholera, they do suggest that s-IgA is likely to play a significant role in protecting man against this disease (as has been more conclusively demonstrated with several viral infections - see Tomasi and Grey, 1972).

(ii) Other coproantibodies.

Although s-IgA is by far the most prevalent immunoglobulin found in the external secretions of man, there is good evidence to suggest that other classes can also be produced locally at the various mucosal surfaces of the body (see Tomasi and Grey, 1972). IgE in particular seems to be primarily synthesized at secretory sites, and both IgM and IgG are produced by at least some tissues (see Tomasi and Grey, 1972). Although IgM predominates in the secretions of individuals with IgA deficiency (see Section 1.3.4(i)), the appearance of IgG in the normal gut secretions is probably not a reflection of local production (see below; Tomasi and Grey, 1972).

In addition to production by the local immune system, coproantibodies may be derived by passage into the intestine from the circulation (Burrows and Havens, 1948; Mitchison, 1953; Batty and Bullen, 1961). This finding was of great interest to workers in the field of enteric immunity, for it raised the possibility of the serum as a source of protective antibody against infections in the gut. Attempts to demonstrate immunity to experimental cholera which was mediated by serum-derived antibodies, either actively or passively acquired, met with varying success. Frequently the degree of protection observed appeared to be unrelated to the level of serum antibody which had been induced (see Freter, 1974).

The likely explanation for the many apparently inconsistent reports was provided by the work of Feeley (Feeley, 1965). He found that rabbits could be protected against the effects of an oral challenge with V. cholerae by prior parenteral administration of antiserum raised against the homologous strain. However, the degree of protection observed depended upon the nature of the serum antibodies which were administered. Sera rich in 19S antibodies were only poorly protective, whereas sera with a high content of 7S antibodies were much more effective (Feeley, 1965). Feeley postulated that the 7S antibodies had been more readily transmitted to the subsequent site of challenge in the gut, whereas the 19S antibodies had been retained

intravascularly. Such an explanation was suggested by the work of Cohen and Freeman (Cohen and Freeman, 1960), and their study has since been confirmed (Hall, Smith, Edwards and Shooter, 1969). Thus serum antibody levels might only be expected to correlate with enteric immunity in those cases where the activity is concentrated in the 7S immunoglobulin fraction.

Subsequent reports in a variety of experimental models have confirmed the capacity of serum-derived IgG antibodies to mediate antitoxic or antibacterial immunity in the gut. Burrows and co-workers detected antitoxic activity in an IgG preparation obtained from intestinal tissue extracts of rabbits following parenteral immunization with a cell-free culture supernatant (Kaur et al, 1972). Other groups have demonstrated that, following passive (Pierce and Reynolds, 1974) or active (Holmgren et al, 1975) immunization, serum IgG antibodies directed against the cholera toxin can protect intestinal loops against challenge with either toxin or viable V. cholerae. Neoh and Rowley observed antibacterial immunity in infant mice following parenteral administration of antiserum, and commented that this immunity was more efficient when the antibody activity of the serum was present in a mercaptoethanol-resistant form (Neoh and Rowley, 1972). Other demonstrations of antitoxic (Curlin and Carpenter, 1970) and antibacterial (Freter, 1969) immunity mediated by serum antibodies have been reported. In these two studies however, the class of the antibodies responsible for protection was not determined, although the fact that the animals received at least three parenteral injections suggests that IgG antibodies were probably involved. Finally one rather limited study has suggested that 7S antibodies may also be of more significance than 19S antibodies in resistance to clinical cholera (Merritt and Sack, 1970).

The relative significance of the contribution made by serum-derived antibodies to the level of copro-immunity is difficult to assess, since such antibodies are highly susceptible to proteolytic degradation in the gut (Freter, 1962; Brown et al, 1970; Fubara and Freter, 1972). Despite

this disadvantage, however, these antibodies can function as protective coproantibodies, as discussed above. Several studies offer explanations for this apparent paradox.

Firstly, in 1971, as a result of studies of antitoxic immunity in rabbit intestinal loops, Burrow's group introduced the concept of "tissue-contained antibody" (Burrows, Kaur and Cercavski, 1971; Kaur et al, 1972). The levels of such antibody correlated with the levels of serum antibody, and unlike the coproantibody activity present in the loop washings, also correlated with the resistance of the loops to intraluminal toxin challenge (Burrows et al, 1971). Intact IgG possessing antitoxic activity was subsequently purified from a crude intestinal tissue extract (Kaur et al, 1972). This work suggests that the "tissue-contained antibody" was serum derived, and in the process of transmission into the gut. It also suggests that, until it reaches the lumen of the gut, such antibody may effectively function as coproantibody in an environment in which it is protected from proteolysis.

Secondly, serum antibodies specific for V. cholerae have been found to retain their capacity to bind antigen following intra-intestinal incubation in vivo, despite their loss of bactericidal potential (Fubara and Freter, 1972). This raised the possibility that antibodies derived from the serum may retain some functional activity even after exposure to the proteolytic environment of the gut. This point has been recently confirmed and extended by Steele, Chaicumpa and Rowley (1975). They found that F(ab)₂ fragments prepared from rabbit IgG antibodies to V. cholerae retained the full protective capacity of the parent molecules in an infant mouse protection assay (Steele et al, 1975). This work will be discussed shortly in relation to the possible mechanisms of antibacterial immunity to cholera (see Section 1.3.5).

The final potential source of coproantibody is the maternal colostrum and milk. Following vaccination of the mother, such antibodies have been

found to protect infant rabbits (Panse and Dutta, 1964) and mice (Ujiye and Kobari, 1970; Pitkin and Actor, 1972; Chaicumpa and Rowley, 1972; also see Section 6.3) from experimental cholera, and piglets (Rutter, Jones, Brown, Burrows and Luther, 1976) and calves (Wilson and Jutila, 1976) from enteritis caused by enteropathogenic Escherichia coli.

The nature of the antibodies responsible for this type of passive copro-immunity will depend upon the route of immunization of the mother. In the majority of the above studies systemic vaccination was employed, and accordingly the protective antibodies were probably serum-derived. However when the antigen is presented locally, specific s-IgA can be detected in the mammary secretions (Eddie, Schulkind and Robbins, 1971; McClelland, Samson, Parkin and Shearman, 1972; Allardyce, Shearman, McClelland, Marwick, Simpson and Laidlaw, 1974; Steele et al, 1974; Holmgren, Hanson, Carlson, Lindblad and Rahimtoola, 1976; Stoliar, Pelley, Kaniecki-Green, Klaus and Carpenter, 1976). The finding that human colostrum contains s-IgA specific for organisms found in the gastrointestinal tract (McClelland et al, 1972; Allardyce et al, 1974) suggests that transmission of passive immunity from mother to young might be of relevance to resistance to cholera in endemic regions (also see Holmgren et al, 1976; Stoliar et al, 1976). This is supported by the fact that cholera is rarely observed in infants less than a year old (Curlin, 1977).

1.3.5 Mechanisms of antibacterial immunity to cholera.

Following the realisation that s-IgA was the predominant immunoglobulin in the external secretions, much effort was devoted to defining the properties of this class of antibody in the hope that such research might elucidate the nature of the antibacterial mechanisms which operate in the gut. Although other classes of immunoglobulin can contribute to the defence of the intestinal surface (see 1.3.4(ii)), thereby expanding the biological potential of the coproantibody pool, nevertheless it seems likely that immunity to cholera involves mechanisms which can also be mediated by s-IgA. The capacity of this class to display antitoxic and antibacterial

activities has already been mentioned (see 1.3.4(i)), and in at least one experimental model of cholera IgA was shown to be as protective as either IgG or IgM on a weight basis (Steele et al, 1974).

Antibodies of the s-IgA class are unable to activate the complement system, either by the classical or the alternate pathway (Eddie et al, 1971; Fubara and Freter, 1972; Steele et al, 1974; Colten and Bienenstock, 1974), nor can they function as opsonins for phagocytosis (Eddie et al, 1971; Wilson, 1972; Zipursky, Brown and Bienenstock, 1973; Reynolds and Thompson, 1973; Steele et al, 1974). Although complement-fixing antibodies of other classes may be present in the gut, the anti-complementary nature of the intestinal contents (Jenkin and Rowley, 1960) and the rapid loss of bactericidal potential within such an environment (Fubara and Freter, 1972) argue against the significance of this mechanism. Furthermore, a recent report has demonstrated the irrelevance of complement-mediated bacteriolysis or phagocytosis in at least one model of experimental cholera. Steele and co-workers showed that $F(ab)_2$ fragments prepared by proteolysis of specific rabbit IgG retained full protective activity in the infant mouse protection assay, despite the complete loss of complement-fixing and opsonic functions (Steele et al, 1975).

In 1969 Freter postulated that coproantibodies might mediate anti-bacterial immunity by inhibiting the adherence of invading organisms (Freter, 1969). If the bacteria are unable to attach to the mucosal surfaces of the gut, but are instead confined to the lumen, they will be subjected to the effects of peristalsis and antagonism by the resident enteric flora (see Freter, 1974). Consequently, bacterial growth in the small intestine will be severely restricted, as will, in the case of vibrios, the associated release of toxin. Furthermore, the toxin that is produced will not be efficiently delivered to its site of action, but rather may be susceptible to inactivation in the proteolytic environment of the gut lumen (see Section 1.3.6).

Thus, the prevention of adherence may be a powerful antibacterial

mechanism in the gut, and one which can be mediated by both locally produced and serum derived antibodies. Freter's initial study demonstrated the capacity of (either actively or passively acquired) serum derived antibodies to inhibit the attachment of vibrios to the surfaces of rabbit loops (Freter, 1969), and this has been recently confirmed (Schrunk and Verwey, 1976). Subsequently Freter reported that purified s-IgA also possessed this capacity (Fubara and Freter, 1973), in confirmation of a study by Williams and Gibbons which demonstrated that salivary s-IgA could inhibit the attachment of streptococci to human buccal epithelial cells (Williams and Gibbons, 1972).

The means by which coproantibodies are able to inhibit the attachment of organisms to the intestinal surface is not known. Agglutination is one possible mechanism - both serum derived antibodies and the tetravalent s-IgA antibodies which are locally produced would be able to display such activity (Eddie et al, 1971; Newcomb and Sutoris, 1974; Bellamy, Knop, Steele, Chaicumpa and Rowley, 1975). Indeed, the capacities of purified rabbit IgG and s-IgA antibodies to protect infant mice from experimental cholera could be directly correlated with their abilities to agglutinate the challenge organisms (Bellamy et al, 1975). Agglutinating activity was in turn directly related to the capacity of the antibodies to inhibit attachment of vibrios to isolated intestinal epithelial cells (Bellamy et al, 1975). Thus agglutination, followed by the peristaltic removal of the clumped organisms, might be one means of minimizing colonization of the intestinal surface.

Inhibition of adherence, however, can also be demonstrated in the absence of bacterial agglutination (Freter, 1969; Freter and Jones, 1976) and so at least one other mechanism must exist. Such a mechanism would be essential to protect against challenge with sub-agglutinating levels of organisms, and similarly, to guard against colonization by those bacteria escaping agglutination even in situations where clumping can occur. Several

recent reports have suggested that bacterial motility might assist the colonization process (Guentzel and Berry, 1975; Jones and Freter, 1976), which might also involve the participation of cell-bound adhesion factors (Jones and Freter, 1976; Freter and Jones, 1976). Whether coproantibodies might therefore owe some of their protective function to a capacity to inhibit vibrio motility, or to mask structures responsible for mediating bacterial attachment, is not known. These points will be discussed more fully in Chapter 6.

1.3.6 Local vs systemic immunization.

"Since coproantibodies may be of diverse origin, consideration of prophylactic immunization involves the question of which type of copro-antibody should be stimulated" (Freter, 1974). That is, should cholera vaccines be administered orally in an attempt to stimulate local antibody production, or should they be given parenterally, to elicit serum antibodies which can subsequently be transported into the gut?

It is now generally acknowledged that the production of s-IgA at the various secretory surfaces is most effectively induced by local antigenic challenge (see Tomasi and Bienenstock, 1968; Ogra, Karzon, Righthand and MacGillivray, 1968; Genco and Taubman, 1969; Eddie et al, 1971). Such exposure results in an immune response which is mediated primarily by the submucosal lymphoid tissue, and thus the host defence is concentrated at the potential site of infection. Moreover, of particular relevance to enteric immunity, the s-IgA antibodies which are locally produced are relatively resistant to inactivation by the proteolytic environment of the gut.

There are, however, several disadvantages associated with the practice of oral immunization against enteric infection. Firstly, the primary local immune response is generally of relatively short duration (Burrows et al, 1947; Burrows and Havens, 1948; see Tomasi and Bienenstock, 1968), and may be difficult to elicit with non-replicating antigens (Freter and Gangarosa,

1963; Pierce and Reynolds, 1975; see below). Secondly, an anamnestic response is by no means assured following oral boosting. Although Pierce and co-workers have demonstrated the capacity of the local immune system to display immunological memory (Pierce and Gowans, 1975; Pierce and Reynolds, 1975), several other groups have been unable to detect any enhancement of the primary response, despite repeated antigenic challenge (Ogra and Karzon, 1969a; Andre Bazin and Heremans, 1973; Porter, Kenworthy, Noakes and Allen, 1974; also see Freter, 1974). In addition to the difficulties associated with its induction, the local secondary response is also very transient (Pierce and Gowans, 1975; Pierce and Reynolds, 1975).

Finally, the immunoprophylactic potential of killed or subcellular vaccines may be diminished as a direct consequence of their oral administration. Exposure to the environment of the gastrointestinal tract could lead to both quantitative and qualitative losses of immunogenicity. The former refers to the reduction in effective antigen dose which might result from general antigenic degradation (see discussion by Kaur et al, 1972; Fubara and Freter, 1972; also see below), while the latter could result from the selective destruction of some immunogenic components of the vaccine (see Section 6.5).

These difficulties suggest that the relative merits of systemic immunization should be considered. The serum antibody response persists longer than the local immune response (Burrows et al, 1947; Burrows and Havens, 1948; see Tomasi and Bienenstock, 1968) and furthermore a second dose of vaccine will evoke a strong and enduring anamnestic response. Accordingly, there is no requirement for repeated immunization, and also the problem of vaccine destruction does not arise. Although serum antibodies transmitted to the gut will not possess the resistance to proteolysis that is a feature of s-IgA, many studies have demonstrated that they can nevertheless effectively mediate both antitoxic and antibacterial immunity to V. cholerae (see 1.3.4(ii)).

Several studies have directly compared the relative efficiencies

of the local and systemic routes of immunization in the generation of antitoxic immunity to experimental cholera (Kaur et al, 1972; Fujita and Finkelstein, 1972; Holmgren et al, 1975; Pierce, Sack and Sircar, 1977). Together, these reports provide conclusive evidence that parenteral immunization is far superior to oral or intra-intestinal administration of toxin. For example, two doses of antigen given by the former route conferred significant protection in rabbits (Holmgren et al, 1975) and dogs (Pierce et al, 1977), whereas even twenty oral doses of antigen elicited only marginal (Holmgren et al, 1975) or insignificant (Pierce et al, 1977) levels of immunity. The low level of protection observed by Holmgren's group was probably a consequence of the massive dose of antigen administered, as each rabbit received a total of 4-8 g of lyophilized culture filtrate (cf. total dose of 1 mg of cholera toxoid per dog, as used by Pierce et al, 1977). Even so, the protection observed was only one-tenth of that elicited by a total of 25 mg of antigen given sub-cutaneously (Holmgren et al, 1975).

The above studies demonstrate the difficulty of establishing and maintaining an effective level of coproantibodies when antigen is administered by the oral route. In this regard, several studies appear to emphasize the significance of antigen destruction by the gut. The work of Pierce's group suggests that direct local application of antigen is more efficient than (indirect) oral administration from the viewpoint of generating local immunity. Thus, a total of 0.5 mg of cholera toxoid was found to prime dogs for a subsequent anamnestic response when given intrajejunally (Pierce and Reynolds, 1975) but not when given orally (Pierce et al, 1977). Similarly, Holmgren and co-workers reported that two intrainestinal depositions of crude cholera toxoid (totalling 125 mg) were just as effective as twenty oral doses of lyophilized culture filtrate (totalling 4-8 g) with respect to eliciting local antitoxic immunity (Holmgren et al, 1975). Neither direct intrainestinal immunization, nor a vaccination programme requiring repeated oral administration of antigen, would be a feasible protective measure in the field.

Recently Pierce and co-workers demonstrated that antitoxic immunity in dogs could be most effectively induced following a combination of parenteral and oral immunization (Pierce et al, 1977). They found that subcutaneous (s.c.) priming with cholera toxoid, followed about one month later with a series of ten oral doses, resulted in a highly significant level of antitoxic immunity which persisted for at least nine months. This contrasted with the protection generated by parenteral immunization alone, which had completely disappeared after five months, and the finding that immunization confined to the oral route (two series of ten daily doses of toxoid) was "strikingly ineffective" (Pierce et al, 1977). Furthermore, whereas the s.c. - s.c. regime elicited high titres of serum antitoxic activity which correlated well with the observed level of protection, the s.c. - oral schedule apparently generated an immunity which was mediated by locally produced antibodies. The persistence of the latter state of protection offers great promise, especially in view of the massive dose of V. cholerae used to challenge the dogs ($0.8 - 3.0 \times 10^{11}$ - Pierce et al, 1977).

Copro-immunity mediated by serum-derived antibodies depends upon the maintenance of high levels of circulating antibody, which in turn requires periodic vaccination. The above study suggests, however, that it may be possible to confer a more permanent state of immunity by vaccinating in such a way as to also stimulate the local immune system of the gut. This is supported by the finding that, following induced clinical cholera, volunteers remained immune from subsequent re-infection by organisms of the homologous serotype for at least a year (Cash et al, 1974). If immunization is to become a significant weapon in the fight against cholera, the delineation of the optimal vaccination regime may well be of greater importance than the development of the vaccines themselves.

1.3.7 Vaccination against cholera.

The arrival of the seventh cholera pandemic initiated a resurgence of interest in the area of vaccine production for prophylactic use in the field. The majority of the vaccines produced have been killed whole-cell preparations, although cell-free extracts have also been tested. Generally, these preparations have been administered parenterally, although more recently the possibility of oral vaccination has also been investigated. At present, however, immunization is not a significant measure from the viewpoint of cholera control.

It is now generally accepted that the only means of accurately assessing the immunoprophylactic value of a vaccine is by strictly controlled testing in the field. Such trials constitute a major undertaking however, and so various laboratory assays have been examined with the hope of finding a correlate of field protection which may be used as a guide in the development of vaccines. Several serological surveys have suggested that, in clinical cholera, the serum level of bactericidal antibodies generally reflects an individual's susceptibility to infection (see Joo, 1974). Whether naturally acquired or induced by vaccination, increasing antibody titres correlated with decreasing likelihood of attack. There are some reports however, particularly in relation to serotype-specific immunity (see below), which show that the bactericidal assay is not an infallible predictive tool (Mosley, Woodward, Aziz, Rahman, Chowdhury, Ahmed and Feeley, 1970; Cash et al, 1974), and therefore suggest that additional assays should also be employed during the preparation of vaccines. The most promising supplementary assay would be the active mouse protection test (see Joo, 1974). This test accurately reflected the exceptional protective capacity of the vaccine used in the first Bangladesh field trial (Feeley and Pittman, 1965), and could have also predicted the serotype-specific nature of the immunity observed in the fourth Bangladesh trial (Mosley et al, 1970). It thus appears to be the most reliable indicator of a vaccine's prophylactic potential in the field, and could prove to be of great value in the future.

The protection afforded by parenteral administration of either killed cellular or subcellular vaccines has been variable, ranging from 4-93% in the first three months (see Joo, 1974). The effectiveness of the immunization has generally waned rapidly thereafter, although one vaccine - a phenol-inactivated preparation comprising classical vibrios of both serotypes - did confer a longer lasting state of protection (see Joo, 1974).

Vaccination tends to be most effective when applied to older children and adults living in cholera-endemic areas. In such situations, the vaccine probably acts as a booster rather than a primary immunization, as suggested by the data collected during the third Bangladesh field trial (Mosley, McCormack, Fahimuddin, Aziz, Rahman, Chowdhury, Martin, Feeley and Phillips, 1969a; Mosley, McCormack, Ahmed, Chowdhury and Barui, 1969b). It was observed that older children appeared to possess a degree of natural immunity to cholera, and that a single dose of vaccine was sufficient to confer upon them a maximal level of protection. Among children less than five years of age, however, a single dose of vaccine was virtually ineffective, but following a second immunization the level of protection was dramatically increased (Mosley et al, 1969 a,b).

The results obtained from the field trials conducted to date enable us to draw several conclusions with respect to the nature of future cholera vaccines. Firstly, several field trials have established that there is no biotype specificity associated with the state of protection provided by conventional cellular vaccines (Philippines Cholera Committee, 1965; Mosley et al, 1970; Philippines Cholera Committee, 1973). The choice of biotype should perhaps be governed by contemporary epidemiological studies, which would suggest the use of El Tor strains during the present pandemic. Secondly, and in contrast to the observed lack of biotype specificity, there is evidence to suggest that serotype-specific immunity can be induced. One field trial showed that an Ogawa vaccine was unable to confer protection against Inaba cholera (Mosley et al, 1970), and similarly human volunteers

who had recovered from Inaba cholera were not protected against the effects of an Ogawa challenge (Cash et al, 1974). Despite the existence of conflicting reports (Benenson, Mosley, Fahimuddin and Oseasohn, 1968; Philippines Cholera Committee, 1973), these findings suggest that, until the issue of serotype specificity has been resolved, cholera vaccines should possess vibrios of both serotypes.

The organisms which comprise the conventional cellular vaccines are usually killed by phenol, formalin, or heat treatment (see Joo, 1974). Although they have not been directly compared in the field, studies using the active mouse protection test have suggested that the various methods of inactivation preserve the immunogenicity of the vaccine to a similar extent (Joo, Pusztai and Juhasz, 1969). This point will be discussed further in Section 6.5.3. It is worth noting here, however, that several experimental studies have suggested that live vaccines are superior to inactivated ones (Panse, Jhala and Dutta, 1964; Panse and Dutta, 1964; Feeley, 1970; also see Chapter 6). The finding that live organisms are the most effective in the active mouse protection test (Feeley, 1970) indicates that a loss of immunoprophylactic potential invariably accompanies vaccine inactivation. Furthermore, live vaccines might also provide a less transient state of protection, for there is some evidence that killed organisms have a greatly reduced capacity to elicit the production of IgG antibodies, at least in rabbits (see Feeley, 1965, and the implications of this study for the work of Panse et al, 1964, and Panse and Dutta, 1964). With respect to clinical cholera, one report has demonstrated that patients recovering from natural infection do indeed possess markedly elevated serum levels of IgG antibodies (Waldman et al, 1972) but the comparative response stimulated by conventional killed vaccines has, unfortunately, not been investigated. Historically, the first vaccines ever used in the fight against cholera were live parenteral vaccines, but although these still appear to offer the above advantages, ethical considerations are likely to preclude their re-introduction.

Two more recent areas of vaccine development appear to offer hope of improving the significance of immunization as a cholera control measure. The first of these - the preparation of subcellular vaccines - appears particularly promising. Two such vaccines have already been tested in the field, and one of these was as effective as the corresponding whole-cell vaccine (see Joo, 1974). The latter type of preparation sometimes suffers the disadvantage of causing painful local reactions in its recipients, a consideration which limits the amount of vaccine which can be given. By purifying and detoxifying the most immunogenic fractions of the organisms, it should be possible to administer high doses of the most relevant antigens, and thus increase the effectiveness of immunization.

Accordingly, much effort is currently being applied to a more precise delineation of the infective process, with the aim of identifying the most significant antigens from an immunoprophylactic viewpoint. The virulence determinants of pathogenic bacteria are frequently protein in nature (see Section 2.5), and might therefore be ideally suited to the generation of an enduring state of protection. This could apply whether the vaccine was administered parenterally, to elicit the production of IgG antibodies which may subsequently provide copro-immunity (see Section 1.3.4(ii)); or according to the immunization regime devised by Pierce's group, to directly stimulate the local immune system to provide long-lasting immunity (see Section 1.3.6).

The final approach to the problem of immunization against cholera has been the use of oral vaccines (reviewed by Joo, 1974). These have generally been killed whole-cell preparations, although more recently the concept of a live attenuated oral vaccine has been developed. Theoretically, by establishing an attenuated strain of V. cholerae as a constituent of the normal intestinal flora, it should be possible to evoke the enduring state of protection provided by the natural infection (as reported by Cash et al, 1974).

Such vaccines appear to offer many advantages (see Finkelstein, 1976), including the capacity to overcome the difficulties associated with the oral administration of killed preparations (see Section 1.3.6). Thus, by successfully colonizing the gut, the organisms would provide a constant antigenic stimulus, which would compensate for the relatively transient nature of local immune responses. Furthermore, the continued synthesis of surface determinants would minimize the effects of antigenic degradation.

Recent testing of an atoxigenic strain of V. cholerae yielded encouraging results, in that the level of protection observed was comparable to that provided by conventional parenteral vaccines (Woodward, Gilman, Hornick, Libonati and Cash, 1976). However, this trial demonstrated that the two major difficulties associated with the use of attenuated strains - their inability to permanently colonize the gut, and their tendency to revert to pathogenicity - have yet to be completely overcome. The capacity of the vaccine to persist in vivo is likely to be the prime determinant of its effectiveness, and indeed strains which are unable to colonize have been shown to be non-protective (Cash, Music, Libonati, Schwartz and Hornick, 1974). Finally, it is of paramount importance that the vaccine lacks the potential to revert to pathogenicity. One of the advantages of a live oral vaccine is that conditions in the field would be likely to favour its dissemination throughout the population (see Finkelstein, 1976) and the implications of an unstable state of attenuation are obvious.

To conclude this section, it is worth mentioning a recent report which suggests that a toxoid preparation should be added to conventional cholera vaccines (Holmgren, Svennerholm, Lonnroth, Fall-Persson, Markman and Lundbeck, 1977). Unfortunately, the results are confined to animal studies, and it could be argued that despite numerous demonstrations of antitoxic immunity in a variety of experimental models, there is still no evidence that such immunity plays any role in protection against cholera in

man (see Section 1.3.2). Nevertheless the data appear most promising, and warrant careful consideration, especially in view of the fact that the authors are not proposing that the toxoid be given alone, but rather that its main value may be to increase the effectiveness of current bacterial vaccines.

Holmgren's group has reported the development of a cholera toxoid which is much more immunogenic than the glutaraldehyde - fixed preparation which yielded such discouraging results in a recent field trial (Curlin et al, 1976). The nature of the toxoid precludes any risk of reversion to toxicity, as it is comprised solely of the L (binding) subunits of the toxin molecule (Holmgren et al, 1977). When given parenterally to rabbits, together with killed V. cholerae or purified vibrio lipopolysaccharide, the antitoxic and antibacterial immunities induced acted synergistically, providing a dramatically improved level of protection. This protection was more complete and longer-lasting than that provided by either type of immunity alone, and prompted the suggestion of a combined bacterial-toxoid vaccine (Holmgren et al, 1977).

A similar effect might possibly be achieved by the administration of an oral vaccine which owes its attenuated status to the production of an inactive yet immunogenic toxin molecule, and the isolation of such strains is currently being pursued (Finkelstein, 1976). In addition to its potentiating effect on resistance to cholera, the generation of antitoxic immunity might also provide a degree of protection against infections of similar etiology (see Finkelstein, 1976; Holmgren et al, 1976). There is evidence to suggest that antibodies specific for the cholera toxin might be able to neutralise the toxins released by certain strains of NAG vibrio (Ohashi, Shimada and Fukumi, 1972; Zinnaka and Carpenter, 1972) and also the heat-labile toxin produced by many strains of Escherichia coli (Kwan and Wishnow, 1974; Donta and Viner, 1975; Gill, Evans and Evans, 1976).

C H A P T E R 2

BACTERIAL ADHERENCE

2.1 Introduction.

This chapter will deal with the phenomenon of bacterial adherence, and has direct relevance to much of the work to be described in this thesis. Following a brief consideration of the role of adherence in bacterial pathogenicity, the host and tissue specificity of this process will be discussed. This will lead to a review of the known mechanisms of bacterial attachment, and an examination of the concept of specific host receptor structures. The possible immunoprophylactic significance of the various adherence moieties will also be considered, and the chapter will be completed by a brief discussion of the aims of this thesis.

2.2 The Role of Adherence in Pathogenicity.

Bacterial infections generally require an initial colonization of one or other of the host's mucosal surfaces (be it alimentary, respiratory or urogenital - see Smith, 1977). The disease process will then follow one of three well-defined patterns (see Savage, 1972; Smith, 1977). Firstly, the invading organisms may remain confined to the mucosal surface, multiplying there but not entering the underlying tissue. Such pathogens (eg. V. cholerae and E. coli) damage the host by liberating diarrhoea-inducing toxins. Secondly, the process of attachment may be followed by penetration into, and proliferation within, the mucosal epithelial cells. No further dissemination occurs, however, and the damage to the host is reasonably localized in such diseases (eg. Shigellosis, diphtheria). Finally, colonization of the mucosal surface may lead to penetration through the epithelial layer and into the sub-mucosal tissue, resulting in widespread damage to the host (eg. Streptococcal infections and Salmonellosis).

It is apparent that all three modes of infection are dependent upon the successful colonization of a host mucosal surface. Since V. cholerae is non-invasive (see Section 1.1.2), our discussion of bacterial pathogenicity will be restricted to this one aspect of the host-parasite relationship. It should be remembered, however, that the potential for colonization might be only one of a number of factors which are essential for pathogenicity. Thus, depending upon the nature of the infection, some or all of the following properties might also be required - the capacity to multiply in vivo, the capacity to release a functional toxin, the possession of factors to assist penetration, and mechanisms of resisting or avoiding host defences (see Smith, 1977).

The host mucosal surfaces are bathed by a continuous stream of secretions, the flow of which is assisted by the regular beating of the cilia (in the lungs) or the villi (in the gut) of the underlying epithelium. Since potential pathogens are unlikely to be able to multiply sufficiently rapidly in vivo to compensate for the cleansing activity of this secretory flow (see Gibbons, 1977), successful enduring colonization will be dependent upon the capacity of the organisms to attach firmly to the cells comprising the epithelial lining. In the intestine, this process will be further inhibited by the flushing effects of the peristaltic contractions, which will rapidly remove any bacteria which remain free in the lumen (Dixon, 1960). The significance of peristalsis is evidenced by the fact that, in several animal models of infectious disease, the establishment of the infective process has depended upon the prior administration of drugs to inhibit such intestinal movements (Freter, 1956; see Gibbons, 1977).

Those bacteria which successfully attach to the epithelial lining are still subjected to the continuous process of desquamation which occurs at mucosal surfaces. The rate of the epithelial cell turnover appears to be related to the size of the microbial burden, prompting the suggestion that

desquamation acts as a host defence mechanism to further limit the extent of colonization (Gibbons and van Houte, 1975). The potential pathogens must not only compensate for their continuous depletion as a result of the sloughing process, but must also be able to repeatedly colonize newly exposed cell surfaces. This might occur as a result of contiguous growth from neighbouring cells, but, at least in the oral cavity, the observed bacterial densities are "probably not sufficient to make direct spread of bacteria an event of high frequency" (Gibbons, 1977). In such circumstances it is possible that newly exposed surfaces might be colonized by unattached organisms transiently present in the secretions, since there is evidence to suggest that some bacterial-host associations are reversible (see Gibbons, 1977). Indeed, it has been speculated that persistent colonization of desquamating surfaces may require a balance between bacterial attachment and spontaneous desorption (Gibbons, 1977).

2.3 The Selectivity of Bacterial Adherence.

2.3.1 Introduction.

Pathogenic bacteria generally display a very limited natural host range, and this has been reflected by the difficulty associated with the establishment of suitable animal models for many human diseases. Even within a susceptible host, the infective process is initiated by the selective colonization of a particular region of only one of the available mucosal surfaces. The complex situation which exists in vivo makes it impossible at present to account for the host and tissue specificity of bacterial colonization, but several explanations can be put forward.

Firstly, the inability of an organism to colonize resistant tissues may be due to an inability to attach to the cells comprising those tissues (Gibbons and van Houte, 1975; Rutter, 1976- cited by Smith, 1977; Gibbons, 1977). Secondly, the colonization of potentially adherent bacteria might be prevented by the antagonistic effects of the resident microbial flora

(Freter, 1970; Savage, 1972; Gibbons, Spinell and Skobe, 1976). Finally, the restricted distribution of bacteria may have a nutritional basis (Pearce and Lowrie, 1972), or perhaps be attributable to host immunological defence mechanisms (Smith, 1972; Pearce and Lowrie, 1972; Shedlofsky and Freter, 1974).

Recent studies have indicated that indigenous bacteria also display considerable selectivity in their colonization of host surfaces. Several reports have demonstrated that the capacities of commensal organisms to attach to the cells of different tissues or from various hosts directly correlate with the observed natural distribution of these organisms (see below). Such findings have led Gibbons to propose that the capacity of commensal or pathogenic bacteria to attach to a given surface is likely to be the most important determinant of the potential to colonize that surface (Gibbons et al, 1976; Gibbons, 1977). Let us now examine the evidence in support of this explanation of the host and tissue specificity of bacterial colonization.

2.3.2 Host specificity.

Several studies support Gibbons' thesis that selective adherence may be responsible for the host tropisms of both commensal and pathogenic bacteria. With respect to the former, Fuller related the natural distribution of indigenous lactobacilli to their in vitro adherence capacities (Fuller, 1973). Whereas 14 out of 15 strains isolated from the crop epithelia of birds were able to attach to chicken crop epithelial cells, none of the 13 strains of mammalian origin displayed this capacity (Fuller, 1973). This observation was subsequently confirmed by the demonstration that commensal lactobacilli isolated from rats or chickens could bind in vitro only to those cells prepared from the tissues of their natural hosts (Suegara, Morotomi, Watanabe, Kawai and Mutai, 1974).

More recently, Gibbons' group reported that Streptococcus salivarius, a member of the indigenous human oral flora, attached much better to human than rat buccal epithelial cells in vitro, and also colonized human tongues

more efficiently than rat tongues in vivo (Gibbons et al, 1976). Conversely, a serum-requiring diphtheroid isolated from the tongues of rats preferentially adhered to rat tissues in both situations (Gibbons et al, 1976).

Similar observations have been made with respect to the host specificity of pathogenic bacteria. Two strains of Streptococcus pyogenes pathogenic for humans were found to attach more efficiently to human buccal epithelial cells than to similar cells of rat origin (Gibbons et al, 1976). A strain of E. coli enteropathogenic for rabbits was likewise reported to bind to brush borders prepared from rabbit intestinal epithelial cells, but not to those prepared from human or guinea-pig cells (Boedeker, Higgins, Formal and Takeuchi, 1977).

Two further studies have confirmed that the capacity of potential pathogens or commensals to colonize a particular host surface may be critically dependent upon their ability to attach to that surface. During a study of the in vitro adherence of enteropathogenic E. coli to brush borders prepared from porcine epithelial cells, it was noted that the bacteria attached much more readily to some preparations than to others (Sellwood, Gibbons, Jones and Rutter, 1975). This was of interest because of the observed differences in the innate susceptibilities of piglets from the same herd to infection by such pathogens. It was subsequently shown that the bacteria were unable to attach to the epithelial cells of resistant piglets, and it was suggested that such cells lacked receptor structures essential for colonization (Rutter, 1976 - cited by Smith, 1977).

Similarly, the lack of indigenous streptococci in the mouths of newborn human infants has been attributed to the absence of functional receptors on the buccal epithelial cells (Ofek, Beachey, Eyal and Morrison, 1977). The capacity of such cells to serve as substrates for streptococcal attachment was shown to increase markedly within the first 48 hours after birth, and paralleled the acquisition of these organisms as part of the oral microbial flora (Ofek et al, 1977).

It is therefore apparent that selective adherence may often underlie selective colonization, although it should be stressed that other factors can also influence host range. For example, cholera naturally affects only humans, but the causative organisms can also attach to the small intestinal tissue of rabbits (Freter, 1969) and mice (Bellamy et al, 1975; also see Chapter 4), and, under appropriate conditions, establish a lethal infection in guinea-pigs (Freter, 1956) and dogs (Pierce et al, 1972). The natural resistance of adult mice seems likely to be due to the antagonistic effects of their normal gut flora (Shedlofsky and Freter, 1974; also see footnote 3 in Chapter 4), and such microbial antagonism would appear to be another common determinant of host resistance (see Savage, 1972; Gibbons et al, 1976).

2.3.3 Tissue specificity.

The concept of tissue-specific colonization arose from the studies of Savage's group on the composition of the indigenous flora of the rodent intestinal tract (summarized by Savage, 1972). It was found that the different members of the natural flora were not randomly distributed throughout the gut, but consistently colonized specific regions. For example, the commensal lactobacilli were normally restricted to the non-secreting areas of the stomach epithelium in both rats and mice. These organisms disappeared following administration of penicillin, but when this treatment was discontinued they re-colonized the same epithelium (Savage, 1972).

Unfortunately, the underlying basis of the selective colonization of the rodent intestinal tract remains obscure, although bacterial antagonism is likely to be one important determinant (see Savage, 1972; Morotomi, Watanabe, Suegara, Kawai and Mutai, 1975). However the commensal lactobacilli show a similarly restricted distribution in the guts of germfree rodents (Savage, 1972; Morotomi et al, 1975), and so it is conceivable that in this instance selective adherence might be the primary regulator of colonization.

More convincing evidence along these lines has been derived from studies of the distribution of the various commensals within the human mouth (reviewed by Gibbons and van Houte, 1975). This environment comprises a variety of surfaces, and again it became apparent that the different members of the indigenous flora selectively colonized certain sites. Thus, Streptococcus mitis preferentially colonized the cells of the buccal mucosa, S. salivarius the dorsal surface of the tongue, and S. mutans the surfaces of the teeth (see Gibbons and van Houte, 1975). The importance of adherence as a determinant of such tissue-specific colonization was suggested by a series of in vitro and in vivo studies which compared the affinities of the different commensals for the various oral surfaces. It was found that there was a consistent and direct correlation between the capacity of a given organism to attach to a particular surface, and the extent to which the organism naturally colonized that surface (see Gibbons and van Houte, 1975).

There is little direct evidence to suggest that selective adherence may similarly underlie the tissue tropisms of pathogenic bacteria. Gibbons' group did show that, when mixtures of S. pyogenes and E. coli were applied to various mucosal surfaces of the rat, the former attached more efficiently to the tongue while the latter bound more readily to the urinary bladder (Ellen and Gibbons, 1974). Neither of these organisms, however, were natural pathogens for the rat, and the strain of S. pyogenes employed actually attaches much better to human cells (see Gibbons et al, 1976).

The tissue-specificity of the colonization potential of pathogenic bacteria has generally been inferred from studies of the in vivo distribution of the organisms during the course of the disease (see Savage, 1972). Such studies cannot disclose the bases of the restricted colonization patterns observed however, and it is probable that these reflect the net result of host regulatory influences acting upon the natural adherence capacities of the pathogens. Thus, although certain strains of enteropathogenic E. coli attach equally well to porcine jejunal or ileal epithelial cells in vitro,

these strains preferentially colonize the ileal region in vivo (see Isaacson, Fusco, Brinton and Moon, 1978). Obviously selective attachment cannot underlie the tissue specificity of these organisms, but before we can assess the generality of such a conclusion, more in vitro studies are required to determine the capacities of other pathogens to attach to a variety of tissues from their appropriate hosts.

2.4 The Nature of Host Receptors.

The preceding discussion has suggested that initial colonization may depend upon a successful interaction between bacterial and host cell surface structures, and that the absence of the latter can underlie the observed selectivity of the attachment process. Before considering the various adherence structures of pathogenic bacteria, let us briefly review our knowledge of the receptor moieties with which they must interact.

The exact nature of host receptors is not known, but evidence is accumulating to suggest that the surface sugars of epithelial cells may be important components of the structures "recognized" by potential pathogens (see Gibbons, 1977; Ofek, Beachey and Sharon, 1978). For example, many bacteria can effect mannose¹-sensitive haemagglutination (see Duguid and Campbell, 1969), and it is probable that this is a reflection of their capacity to bind to mannose residues generally - including those present on the surfaces of host epithelial cells (Duguid and Gillies, 1957; Ofek, Mirelman and Sharon, 1977; Ofek and Beachey, 1978). Thus, Ofek's group has reported that, of a range of sugars tested, only mannose and its derivatives were able to inhibit the binding of E. coli to human buccal mucosal cells (Ofek et al, 1977; Ofek and Beachey, 1978). Similarly, concanavalin A -

¹ Unless otherwise stated, sugars referred to in the text were studied in their D (dextro)-rotatory forms.

a lectin which binds specifically to mannose and glucose residues - prevented bacterial attachment by masking the receptor sites on the epithelial cells, whereas lectins of other specificities were ineffective (Ofek et al, 1977). The mannose-binding activity of the bacteria was demonstrated by their capacity to aggregate mannan-bearing yeast cells (Ofek et al, 1977; Ofek and Beachey, 1978).

Other examples of mannose-sensitive adherence have been reported (see Ofek and Beachey, 1978), and recent work has indicated that, even in vivo, such attachment can be inhibited by free sugar. Thus the administration of mannose has been successful in preventing the colonization of rabbit gastrointestinal and mouse urinary surfaces by E. coli (see Ofek and Beachey, 1978).

Freter's group has reported that the attachment of V. cholerae to human group O erythrocytes, or to brush borders prepared from rabbit intestinal epithelial cells, can be partially inhibited by L-fucose (Jones and Freter, 1976). Adherence to intact ileal slices, however, could not be blocked by free fucose (Freter and Jones, 1976). In an attempt to elucidate the nature of the tissue receptor involved in the latter interaction, mucosal scrapings were examined as a possible source of attachment-inhibiting factors. Such a moiety has been partially purified, and its small size (MW < 30,000) and resistance to proteolysis are compatible with a carbohydrate structure (Freter and Jones, 1976).

Host cell glycoproteins have been implicated in the adherence of enteropathogenic E. coli strains of porcine origin, and the fact that pretreatment of epithelial cells with periodate destroys their receptor capacity supports the involvement of sugar residues (see Ofek et al, 1978). Similarly, streptococci indigenous to the human oral cavity attach to the (carbohydrate) blood-group substances present on the surfaces of buccal epithelial cells (Williams and Gibbons, 1975). This adherence can be blocked by pretreatment of the epithelial cells with antisera directed

against certain of the blood-group antigens, or by pre-incubation of the bacteria with preparations of salivary glycoproteins. The inhibitory activity of the latter was attributed to their possession of determinants antigenically similar to those functioning as receptors on the underlying epithelial cells (Williams and Gibbons, 1975). It was postulated that such antigenic mimicry might be a general mechanism by which the constituents of mucosal secretions reduce the extent of microbial colonization (Williams and Gibbons, 1975) , and this concept was subsequently supported by the above-mentioned work with V. cholerae (Freter and Jones, 1976).

2.5 Adherence Mechanisms of Pathogenic Bacteria.

2.5.1 Introduction.

We shall now briefly review our knowledge of some of the better-elucidated mechanisms by which pathogenic bacteria attach to host surfaces, with emphasis upon the significance of the various adherence structures as both virulence determinants and protective antigens. The former may be defined as those components of an organism which contribute to the infective capacity of that organism, and, for an invasive pathogen, these will be many and varied (see Section 2.2). Protective antigens are those determinants which, when injected into a potential host, will induce in that host the production of specific antibodies capable of preventing the establishment of infection by the homologous organisms. Obviously these two concepts are closely related, since virulence determinants would themselves be prime candidates for use as protective immunogens. Thus, if a bacterial component performs a vital pathogenic function, then the presence of antibodies to that component may so diminish the infectivity of the organism that it is unable to establish as infection.

As V. cholerae is a non-invasive organism, our discussion of pathogenicity has been confined to the process of colonization of the mucosal surface. Similarly, our consideration of virulence determinants will be

restricted to those involved in colonization, and of protective antigens to those which can elicit antibodies capable of mediating protection during this event. Thus, components which may fulfil subsequent roles in the infective processes of invasive pathogens will not be discussed. Such determinants are likely to induce the production of antibodies, the protective mechanisms of which (eg. complement-mediated lysis, or opsonization) would be of little significance at mucosal surfaces, especially those of the gastrointestinal tract (see Section 1.3.5).

2.5.2 E. coli.

Most of the pathogenic strains of E. coli establish an infection in the small bowel of their hosts. Such enteropathogenic E. coli (EPEC) are commonly responsible for neonatal diarrhoea in man and his domestic animals, and the economic significance of their effects upon the latter provided the stimulus for an intensive research effort over the past decade. Those strains of animal origin have received the most attention, but fortunately the fruits of this research are assisting investigation into the human pathogens.

As with V. cholerae (see Section 1.1.2), the infective processes of EPEC are confined to the small intestine, and involve the action of toxin molecules upon the epithelial cells of the mucosal surface (Smith and Halls, 1967; Arbuckle, 1970). It soon became apparent, however, that toxigenicity was not a sufficient condition for pathogenicity, which also depended upon a capacity to proliferate in the anterior small intestine (Smith and Halls, 1967; Smith and Linggood, 1971). In those EPEC of porcine origin, this capacity is conferred by the plasmid-encoded K88 antigen (Smith and Linggood, 1971), which facilitates colonization by mediating attachment to the mucosal epithelial cells (Jones and Rutter, 1972; Wilson and Hohmann, 1974; Sellwood et al, 1975).

K88 is a protein antigen (Stirm, Orskov, Orskov and Mansa, 1967a) which appears under the electron microscope as a fringe of thread-like

structures around the periphery of the cell (Stirm, Orskov, Orskov and Birch-Andersen, 1967b). Its significance as a virulence determinant for porcine EPEC has been clearly established. Firstly, Smith and Linggood (1971) examined the pathogenic potential of related E. coli strains possessing various combinations of the plasmids encoding enterotoxin, haemolysin and K88 antigen, and concluded that the capacity to proliferate in vivo depended solely on the presence of the K88 plasmid. Subsequently Jones and Rutter (1972) demonstrated that the in vivo synthesis of K88 antigen enabled bacteria to colonize the mucosal surface of the small intestine, and that, whereas a K88-positive strain killed 50% of conventionally reared piglets, a K88-negative strain derived from it was virtually avirulent. Finally, several studies have confirmed that the attachment of EPEC to porcine small intestinal epithelial cells in vitro is mediated by K88 antigen (Jones and Rutter, 1972; Wilson and Hohmann, 1974; Sellwood et al, 1975).

Following their appreciation of the critical role of K88 in colonization, Rutter and Jones (1973) attempted to protect suckling piglets from infection by EPEC by the immunization of pregnant gilts with a cell-free preparation of this antigen. This approach proved most successful, achieving a five-fold reduction in piglet mortality (Rutter and Jones, 1973). Subsequent investigation suggested that antibodies passively transmitted in the colostrum and milk had protected the piglets by neutralizing the adherence capacity of the K88 antigen present on the challenge organisms, thus inhibiting their colonization (Rutter et al, 1976). This work offered a new approach to the problem of immunizing against disease, for it was the first to explore the feasibility of isolating virulence determinants from the surfaces of pathogenic organisms and using them as protective immunogens.

EPEC isolated from calves and lambs share a different K antigen, K99, which is analogous to the K88 of porcine pathogens (Smith and Linggood, 1972; Orskov, Orskov, Smith and Sojka, 1975). Like K88, K99 is a plasmid-

encoded, proteinaceous, pilus-like structure (Smith and Linggood, 1972; Isaacson, 1977), which appears as a fringe of fine fibrils on the surface of the cell (Burrows, Sellwood and Gibbons, 1976). It, too, functions as a virulence determinant by facilitating colonization (Smith and Linggood, 1972; Moon, Nagy, Isaacson and Orskov, 1977), presumably by mediating attachment to intestinal epithelial cells (Burrows et al, 1976; Isaacson et al, 1978). K99 has also been successfully employed as a protective immunogen, and again neonatal resistance was conferred by antibodies which diminished the colonization potential of the challenge organisms (Morgan, Isaacson, Moon, Brinton and To, 1978).

Many human isolates of EPEC possess either of two "colonization factor antigens", termed CFA/I and CFA/II, both of which are again fimbriate in nature (Evans, Silver, Evans, Chase and Gorbach, 1975; Evans and Evans, 1978). These structures have been found to play a critical role in colonization in an infant rabbit model (Evans et al, 1975; Evans and Evans, 1978), and recently the significance of CFA/I as a virulence determinant in human infection was confirmed (Evans, Satterwhite, Evans and Dupont, 1978). The prophylactic potential of these antigens has yet to be evaluated, but their prevalence in human EPEC (see Evans and Evans, 1978), and the success obtained with similar fimbriate virulence antigens, is encouraging.

Before concluding this discussion, three further points should be made. Firstly, although antigenically distinct (Evans et al, 1978; Isaacson et al, 1978; Morgan et al, 1978), the K88, K99 and CFA determinants are all fimbriate structures which provide a mannose-resistant haemagglutinating capacity (Jones and Rutter, 1974; Burrows et al, 1976; Evans and Evans, 1978). This contrasts with the mannose-sensitive haemagglutination mediated by type-1 fimbriae² (see Duguid and Campbell, 1969), and suggests that

² These fimbriae are produced by many bacterial genera, but their role in host-parasite relationships remains obscure (see Duguid, Anderson and Campbell, 1966; Duguid, Darekar and Wheeler, 1976).

different types of bacterial fibril may interact with different host receptor structures. Indeed, a recent report demonstrated that each of three such fimbriate components attached to a separate receptor on the surfaces of porcine small intestinal epithelial cells (Isaacson et al, 1978).

Secondly, the K88, K99 and CFA structures are not the only mechanisms of attachment displayed by EPEC. For example, some porcine EPEC lack the K88 antigen, but are nevertheless able to colonize the small intestine in vivo, and to adhere to isolated epithelial cells in vitro (Isaacson, Nagy and Moon, 1977; Nagy, Moon and Isaacson, 1977). In addition to the recently-recognised capacity of K99 to mediate attachment to porcine epithelial cells (Moon et al, 1977; Isaacson et al, 1978), a new type of fibril - the 987 pilus - was shown to be capable of promoting the colonization of K88-negative E. coli (Nagy et al, 1977; Isaacson et al, 1978). Following its delineation as a virulence determinant, the 987 antigen was also successfully employed as a protective immunogen (Nagy, Moon, Isaacson, To and Brinton, 1978; Morgan et al, 1978).

Finally, E. coli strains can also establish infections in the human urinary tract. Such organisms have not been as intensively studied as the EPEC, but recently it has been reported that their capacity to attach to uroepithelial cells can similarly be related to their possession of a fimbrial fringe (Svanborg-Eden and Hansson, 1978).

2.5.3 Streptococci.

Those streptococci commonly responsible for human infections share a specific, serologically detectable carbohydrate moiety, and have been collectively designated the group A streptococci. Within this group, however, the strains are serotyped on the basis of their M protein antigens, since it is these components which elicit the production of type-specific antibodies (see Lancefield, 1962).

In 1969 Swanson and co-workers demonstrated that the M proteins were associated with a fimbrial fringe on the surface of the organisms (Swanson,

Hsu and Gotschlich, 1969). Several strains which were serologically positive for M antigens were found to possess fimbriae when viewed under the electron microscope, while their related M-negative strains did not. Furthermore, trypsin treatment of M-positive cells resulted in the loss of M-antigen and the fimbrial fringe, both of which re-appeared following incubation in trypsin-free medium (Swanson et al, 1969).

Subsequently, Gibbons' group reported that a similar fibrillar component appeared to be responsible for the adherence of several species of Streptococcus to human buccal mucosal cells (see Gibbons and van Houte, 1975). For example, virulent strains of *S. pyogenes* possessing a "fuzzy surface structure" attached efficiently to such cells in vitro, but following trypsin treatment to remove this component they could no longer adhere (Ellen and Gibbons, 1972). Since the adherent organisms were M-positive and the non-adherent ones M-negative, and since antibodies against M protein could inhibit attachment, it seemed that the M proteins associated with the surface fibrils provided the capacity to adhere (Ellen and Gibbons, 1972).

However in 1976 Beachey and Ofek conclusively demonstrated that this was not the case. Using mild pepsin digestion they were able to selectively remove M protein from the surfaces of streptococci, but leave the fimbrial fringes intact. Such M-negative fimbriated organisms displayed an undiminished capacity to attach to human buccal epithelial cells in vitro, in contrast to streptococci which had been treated with trypsin or hydrochloric acid, and which had lost both their M protein and their fimbrial fringe (Beachey and Ofek, 1976). Thus it was the fimbriae themselves, and not their associated M proteins, which were essential for adherence.

Beachey and Ofek went on to identify the lipoteichoic acid (LTA) associated with the fimbriae as the moiety which mediated the adherence function of these structures. It was found that both homologous and heterologous M-type-specific antisera were able to inhibit the attachment of fimbriated streptococci, and that this activity could be removed from both sera by

absorption of those antibodies specific for LTA (Beachey and Ofek, 1976). Furthermore, an antiserum prepared against an LTA-protein conjugate possessed no group-specific or type-specific activities, yet displayed the highest anti-LTA activity of the sera examined, and was the most efficient inhibitor of streptococcal binding (Beachey and Ofek, 1976).

Although the prophylactic potential of the fimbrial structures has yet to be assessed, there seems reason to be optimistic in this regard. The demonstration that antisera specific for homologous or heterologous M protein were each able to inhibit the attachment of group A streptococci in vitro, and the finding that the inhibitory activities of both sera were conferred by anti-LTA antibodies (Beachey and Ofek, 1976), suggests the feasibility of immunization with isolated fimbriae, or with an LTA-protein conjugate, as a means of protecting against streptococcal infection.

S. mutans preferentially colonizes the tooth surfaces of the human mouth, and has evolved a mechanism of attachment which seems uniquely adapted for adhesion to such a substrate (see Gibbons and van Houte, 1975). This mechanism will not be discussed here, however, since there is no evidence that it has any relevance to the attachment of bacteria to mucosal surfaces (and S. mutans itself is unable to colonize the oral epithelia - Gibbons and van Houte, 1975).

2.5.4 Gonococci.

The virulence of different strains of Neisseria gonorrhoeae was shown by Kellogg to correlate with colonial morphology; those organisms which produce acute infection display colonial types 1 or 2, while those unable to establish disease are associated with types 3 or 4 (Kellogg, Cohen, Norins, Schroeter and Reising, 1968). Subsequently Swanson and co-workers isolated all four colonial types from each of several strains, and demonstrated that type 1 and type 2 gonococci always possessed surface pili, whereas types 3

and 4 never did (Swanson, Kraus and Gotschlich, 1971). Thus the presence of surface pili correlated with pathogenic potential, prompting an investigation of the possible significance of these structures as virulence determinants.

It was soon reported that the surface pili facilitated the attachment of gonococci to human amnion (Swanson, 1973) and buccal mucosal (Punsalang and Sawyer, 1973) cells, and also provided the organisms with a haemagglutinating capacity (Punsalang and Sawyer, 1973; Buchanan and Pearce, 1976). The structures were found to be proteinaceous, being destroyed by trypsin treatment; depiliated organisms were unable to attach to buccal epithelial cells or to erythrocytes, but these capacities returned with the synthesis of new pili (Punsalang and Sawyer, 1973).

Recently Buchanan and Pearce isolated surface pili from four different gonococcal strains, and confirmed the capacity of these structures to agglutinate erythrocytes (Buchanan and Pearce, 1976), and to bind to human buccal and vaginal epithelial cells (Pearce and Buchanan, 1978). The four pilus preparations were antigenically distinct (Buchanan and Pearce, 1976), but apparently bound to the same epithelial cell receptors (Pearce and Buchanan, 1978). Antibodies to pili inhibited their attachment, and were much more efficient in this respect when assayed against the homologous pilus type (Buchanan and Pearce, 1976; Pearce and Buchanan, 1978).

In view of the antigenic heterogeneity of the gonococcal pili (Buchanan and Pearce, 1976; also see Buchanan, Pearce, Schoolnik and Arko, 1977), one might reasonably hold reservations as to the feasibility of an effective pilus vaccine against gonorrhoea³. Nevertheless it is tempting to speculate that, like the streptococcal fimbriae (see above), the gonococcal

³ Nagy et al (1978) refer to a successful trial with a gonococcal pilus vaccine, but the author was unable to obtain the cited reference. If the challenge organisms possessed pili which were antigenically distinct from those comprising the vaccine, the trial might provide support for the concept of a common attachment moiety (see above).

pili may display an antigenic heterogeneity which has no significance from the viewpoint of adherence. The finding that antigenically distinct pili attach to the same epithelial cell receptor (Pearce and Buchanan, 1978) suggests that they may share a common adherence moiety analogous to the LTA of the streptococcal fimbriae. If so, immunization with this moiety might provide resistance against gonococci bearing a range of pilus types, by eliciting the production of antibodies which prevent pilus-mediated attachment.

2.5.5 V. cholerae and other pathogens.

V. cholerae adheres to the small intestinal tissue of various animals, both in vivo (LaBrec et al, 1965; Freter, 1969; Ghosh, 1970; Nelson et al, 1976) and in vitro (Freter, 1969; Freter and Jones, 1976; also see Chapter 4). At the time of commencing these studies, however, the mechanisms of vibrio attachment remained undefined. Fimbriae had been observed on the surfaces of these organisms, and a surface haemagglutinin had also been described (see Section 1.2.4(ii)), but neither structure had been implicated as a virulence determinant.

In 1969 Freter reported that antibodies to V. cholerae could inhibit the attachment of these organisms to the surfaces of rabbit loops in vivo, and to strips of rabbit ileum in vitro (Freter, 1969). Subsequently it was reported that the capacities of various antibody preparations to protect infant mice from experimental cholera could be correlated with their capacities to inhibit the in vitro attachment of vibrios to isolated murine epithelial cells (Bellamy et al, 1975). It therefore seems reasonable to suppose that, when the adherence structures of V. cholerae have been identified and isolated, they might constitute effective prophylactic immunogens. Since the recent work in this area is closely related to the studies to be described in this thesis, it will be discussed in relation to the same in Chapter 7.

Salmonella typhimurium is pathogenic for mice, and attaches to

murine intestinal tissue both in vivo (Tannock, Blumershire and Savage, 1975) and in vitro (Hohmann, Schmidt and Rowley, 1978). The surface components which mediate this adherence remain undefined. Duguid's group reported that *Salmonella* frequently bear surface fimbriae (Duguid et al, 1966), but subsequently concluded that these structures were unlikely to mediate colonization in vivo (Duguid et al, 1976).

Surface pili have been shown to mediate the attachment of *Proteus mirabilis* to the epithelial cells of the renal pelvis in a rat model of pyelonephritis (Silverblatt, 1974). *Corynebacterium renale* causes a similar infection in cattle, and also possesses pili with adhesive properties (Honda and Yanagawa, 1974). Finally, surface fibrils have also been implicated in the attachment of *Actinomyces naeslundii* to oral epithelial cells (Ellen and Walker, 1977).

2.6 Summary, and Aims of this Thesis.

Our brief review of the nature of bacterial adherence can be conveniently summarized by drawing the following conclusions -

- (i) Adherence is likely to be an essential step in the pathogenesis of bacterial infections of mucosal surfaces. The observed selectivity of this phenomenon suggests that attachment results from interactions between bacterial surface components and specific receptor structures present on the surfaces of host epithelial cells.
- (ii) The precise nature of the host receptors is not known, although carbohydrate moieties have frequently been implicated. In those instances where the bacterial adherence mechanisms have been defined, attachment has been mediated by proteinaceous, fibrillar structures which radiate out from the cell surface.

- (iii) The adherence structures of pathogenic bacteria have been firmly established as virulence determinants. As such, at least three have been successfully employed as prophylactic immunogens, and in each case immunity was conferred by antibodies which inhibited the colonization of the potential pathogen.

These conclusions relate directly to one of the two major aims of this thesis - an investigation of the adherence capacity of V. cholerae. It is of particular interest to determine whether the capacity of these organisms to adhere can be correlated with their capacity to establish an infection (using the infant mouse model of cholera). Secondly, does the attachment process display substrate specificity, and, if so, what is the nature of the host receptor structure? Finally, attempts will be made to identify the bacterial components which mediate adherence. Such studies must necessarily precede the isolation of these moieties, and an evaluation of their prophylactic significance (see Section 1.3.7).

The second, related aim of this thesis is to examine the protective antigens of V. cholerae. As discussed previously (see Section 1.1.4) immunization is not at present a significant weapon against cholera, and one way of increasing its effectiveness lies in the development of improved vaccines. Those currently available generally comprise inactivated suspensions of whole cells. Little regard is paid to the consequences of the inactivation procedures employed with respect to the possible destruction of potentially protective immunogens, because of the known stability of the lipopolysaccharide (LPS) structure. However these vaccines have not proved very effective, and, in view of the prophylactic significance of proteinaceous components of other bacterial pathogens, it seemed important to determine whether non-LPS protective antigens might also exist in V. cholerae. If so, it would be of great interest to establish the relative prophylactic significance of the LPS and non-LPS determinants, by comparing the protective efficiencies of their respective antibodies.

C H A P T E R 3

MATERIALS AND METHODS

3.1 Bacterial Strains.

3.1.1 Background information.

The bacterial strains used in these studies were obtained from the departmental culture collection. They are listed in Table 4.2, which also gives the biotype and serotype of each. All are resistant to streptomycin (100µg/ml); when necessary, such resistance was developed by selection on streptomycin-containing (100µg/ml) nutrient agar plates (Difco, Michigan).

The most commonly used strains were V. cholerae 569B (originally obtained from Mr. I Huq, Cholera Research Laboratories, Dacca), V. cholerae 017 (from Dr. W.F. Verwey, Department of Microbiology, University of Texas, Galveston), and the non-cholera vibrio (NCV) 569B/165 (from Dr. K. Bhaskaran, Central Drug Research Institute, Lucknow).

In 1971, Dr. S. Neoh had serially passaged both V. cholerae 569B and V. cholerae 017 through infant mice (see below) to increase their virulence. None of the other strains have been passaged. NCV 569B/165 and NCV T50/165 are hybrid vibrio strains, constructed by genetic conjugation by Dr. K. Bhaskaran. The nature of these strains is discussed in detail in Chapter 6.

3.1.2 Maintenance and growth conditions.

All strains were stored lyophilized in skimmed milk at 6C or -20C. Fresh ampoules were opened at regular intervals (6-8 weeks), streaked onto nutrient agar plates to ensure purity and onto streptomycin-containing plates to check resistance, and stored in nutrient agar stabs at room temperature. For routine use, the stabs were streaked onto nutrient agar plates which were then incubated at 37C overnight. Several of the resulting colonies were used to inoculate 10-ml aliquots of nutrient broth (Difco, Michigan), which were then

shaken at 37C. Such cultures typically attained a concentration of $1-2 \times 10^9$ bacteria per ml after 3-4 hours. For the preparation of lipopolysaccharide, large scale cultures were employed. One-litre volumes of nutrient broth were dispensed into 5-1 flasks, autoclaved, inoculated with 10^{11} organisms per flask (in the form of a 100-ml overnight shaking culture) and grown as before.

3.1.3 Selection of motile variants.

Some of the strains listed in Table 4.2 were found to be only very weakly (of the order of 0.1%) motile upon microscopic examination (using the hanging drop method), and so attempts were made to select highly motile variants. Initially this was a painstaking process which involved repeated passaging through Craigie tubes (see Cruickshank, Duguid, Marmion and Swain, 1975), but subsequently the sloppy-agar overlay method (see below) proved to be of great assistance.

Craigie tubes were prepared by dropping 5-cm lengths of glass tubing (internal diameter approx. 3mm) into 10-ml aliquots of molten sloppy agar (1 part nutrient agar to 2 parts nutrient broth) contained in 25-ml screw-capped bottles. After autoclaving, the medium was allowed to cool and set before use. 0.2mls of bacterial suspension (containing $1-2 \times 10^9$ organisms) was carefully layered onto the surface of the medium inside the glass tube, and the bottles stood at 37C until growth appeared on the surface of the medium surrounding the tube. This growth was then seeded into nutrient broth and shaken at 37C, and the resulting culture inoculated into agar stabs and streaked onto nutrient agar plates.

After overnight incubation at 37C, the streak plates were inspected to check purity. If satisfactory, the growth on the surface of the agar stabs was harvested into nutrient broth and shaken at 37C to provide the source of organisms for the next passage. This procedure was adopted because it was found that small gains in overall motility were more reliably preserved in agar stabs than on the surface of nutrient agar plates.

When a reasonable (about 20%) level of motility had been achieved, individual colonies were picked from the purity plates and grown up in separate broths. In this manner it was possible to obtain highly motile cultures, which were then lyophilized and stored at 6C. Each time a new ampoule was opened, the level of motility was inspected, and, if necessary, improved by the selection and growth of single colonies. Motile organisms were routinely stored in agar stabs, and harvested directly into nutrient broth as required.

3.1.4 The sloppy-agar overlay method.

During the course of these studies the author became familiar with the sloppy-agar overlay method devised by Stocker as a means of identifying flagellar phase variants in Salmonellae (Stocker, 1949). This technique was subsequently employed as a means of obtaining an objective, quantitative estimate of the level of motility of bacterial cultures.

The culture of interest was diluted and spread onto the surfaces of nutrient agar plates (in triplicate), such that 100-200 colonies would develop per plate. After overnight incubation at 37C, each plate was overlaid with 10-12 mls of sloppy agar (see above) at about 45C. The plates were left at room temperature for 15 minutes to allow the overlay to set, and then incubated at 37C. Those colonies comprised of motile bacteria gradually developed a halo during this latter incubation period, as the organisms swam into the sloppy agar. Microscopic observation of broth cultures seeded with "haloed" or "non-haloed" colonies confirmed the reliability of this technique, which was routinely used to provide estimates of percentage motility.

The value of this method for enrichment of motility is readily appreciated. It removes the necessity for repeated passaging through Craigie tubes and the chance selection of individual colonies, for, by spreading replicate plates with a dilution of a weakly-motile culture, the overlay method makes it possible to immediately identify the one colony in a thousand

which contains motile organisms. This one-step selection procedure not only saves much time and effort, but also avoids the dangers of contamination or attenuation which are inherent in lengthy motility-enrichment programmes.

3.2 Animals.

3.2.1 Adult mice.

Adult mice of the outbred LAC (substrain A) variety were reared under specific-pathogen free conditions by the Central Animal House of this university. They were housed in the departmental animal rooms for several weeks before use, to permit "conventionalization". This strain was used for the preparation of all antisera, employing immunization schedules described in the text.

Whilst establishing an in vitro bacterial adherence assay (see Section 4.2), several other strains were also used. These were the outbred SWISS mice, and the inbred C57B1, BALB/c and CBA strains, all of which were bred and maintained in this department.

3.2.2 Infant mice.

A breeding colony of conventionalized LAC mice was maintained in this department in association with Dr. D. Horsfall. Each breeding cage contained four female mice and one male, and the breeding stock was replaced at six-monthly intervals. The infant mice were used at 5-6 days of age (weight range restricted to 2.4-2.7g).

3.2.3 Guinea-pigs.

Guinea-pig serum was used as a source of complement for bactericidal assays, and erythrocytes were used to assess bacterial haemagglutinating capacity. Animals of various ages were obtained from the Central Animal House, kept in the department until required, and then bled as described below (see Section 3.3.2).

3.3 Collection and Storage of Serum and Intestinal Fluid.

3.3.1 Mouse serum.

Blood was collected from the retro-orbital plexus using a shortened Pasteur pipette. The blood taken from all mice within a particular immunization group was pooled, and allowed to clot at 37C for 60 minutes. The serum was then separated by centrifugation, and either stored at 4C in the presence of 0.1% (w/v) sodium azide, or frozen at -20C in aliquots without preservative.

3.3.2 Guinea-pig serum.

Animals were bled by cardiac puncture under ether anaesthesia, the blood from each animal being kept separate during the clotting (60 minutes at room temperature) and spinning procedures. The resulting sera were then pooled, and stored in aliquots at -20C without preservative. Fresh stocks were prepared every 6-8 weeks.

Such serum would usually lyse haemolysin (CSL, Australia)- sensitized sheep erythrocytes out to a dilution of about 1 in 100. For bactericidal assays, it was used at a final dilution of 1 in 20, at which concentration there was no detectable natural activity against V. cholerae.

3.3.3 Mouse intestinal washings.

Adult mice were killed by cervical dislocation, and their small intestines excised and cooled by brief immersion in a saline-ice bath. Intestinal fluid was collected by flushing each length of tissue with 1ml of cold saline. This was introduced by means of a syringe and a smooth-tipped hypodermic needle (19g), and its passage through the intestine facilitated by gentle massage. The washings from 20-25 mice were pooled by collection into a 50-ml plastic tube on ice. They were then homogenized (Ultraturrax, Janke and Kunkel KG. West Germany; full speed for 20 seconds)

to disperse gut contents, and clarified by centrifugation (10,000g for 15 minutes at 4C). The supernatant (pH 7.0-7.4) was then dispensed into aliquots and stored at -20C without preservative.

3.3.4 Human intestinal fluid.

Frozen aliquots of human intestinal fluid were kindly provided by Dr. J. LaBrooy of this department. These had been obtained from healthy volunteers by aspiration of fluid from the proximal small intestine, and each sample had a pH>6.

Fluids collected from three individuals were thawed and mixed in equal volumes to obtain a pool of material. This was dispensed into aliquots and re-frozen at -20C without preservative.

3.4 Isolation of Lipopolysaccharide (LPS) from *V. cholerae*.

LPS was isolated from several strains during the course of these studies, primarily for the production and absorption of antisera, and for sensitizing erythrocytes for haemagglutination assays. The extraction procedure was based upon the original method of Westphal et al (1952), and has been described elsewhere (Kabat and Mayer, 1971). A minor modification was the use of nucleases (Calbiochem, California) in preference to alcohol precipitation for the removal of nucleic acids. LPS preparations obtained in this manner typically contained 5-20% contaminating protein (Neoh and Rowley, 1970), and were therefore subjected to a second phenol extraction. In this way the level of contaminating protein could routinely be reduced to less than 1%. All preparations were stored at 4C in the presence of 0.1% (w/v) sodium azide.

For efficient sensitization of sheep erythrocytes, an aliquot of LPS was alkali-treated. LPS was diluted in saline to a final concentration of 2mg/ml, and made 0.02N with respect to NaOH (using 1N NaOH). After overnight

stirring at room temperature the pH was restored to neutrality (by addition of IN HCl), and the material stored at 4C with 0.1% (w/v) sodium azide.

3.5 In vitro Assays of *V. cholerae* Adherence.

3.5.1 Attachment to murine intestinal tissue.

The development of a standard in vitro assay of adherence, using adult mouse small intestine as the substrate for attachment, is described in detail in Section 4.2.

3.5.2 Attachment to isolated murine intestinal epithelial cells.

Epithelial cells were isolated from adult mouse small intestine according to the method of Reiser and Christiansen (1971). Briefly, this involved the incubation of closed intestinal loops with a medium containing hyaluronidase (Fisons, England), and the subsequent dislodgement of the epithelial cells by gentle massaging of the external surfaces of the loops.

The cells collected from several (inbred) mice were pooled, and deposited by low speed centrifugation at 4C. After resuspension in cold collection medium (see Reiser and Christiansen, 1971), the preparation was clarified by passage through a fine gauze mesh. The filtrate was then layered onto a small volume of foetal calf serum (CSL, Australia), and left standing on ice for 10 minutes to allow the sedimentation of clumped cells. The uppermost layers of the suspension were then comprised almost exclusively of single cells. Typically, the cell concentration was $\approx 5 \times 10^6$ per ml, with a viability of $\approx 75\%$ (as determined by trypan blue exclusion).

To assay adherence, $\approx 10^6$ *V. cholerae* (in 0.05ml phosphate-buffered saline (PBS), pH 7.4) were added to 0.45ml of epithelial cell suspension (containing $4-5 \times 10^6$ cells), and the mixture incubated at 37C for 15 minutes with occasional shaking. The epithelial cells were then subjected to three

10-ml PBS washes to remove unattached bacteria. The resulting wash fluids and the resuspended cell pellet were then diluted and plated onto streptomycin-agar to determine vibrio recoveries.

3.5.3 Bacterial haemagglutination (HA).

The capacity of V. cholerae to agglutinate vertebrate red blood cells (rbc), usually of guinea-pig origin (see Section 5.2.1), was routinely assayed as follows. The organisms were grown in 10-ml shaking cultures - initially in nutrient broth at 37C (Section 5.2.1) but subsequently under a variety of growth conditions (Section 5.4.2) - to a concentration of $1-2 \times 10^9$ per ml. Meanwhile the rbc were washed three times and made up to a 1.5% (v/v - after Bales and Lankford, 1961) suspension in PBS.

The bacteria were subsequently deposited by centrifugation (4,000g at 4C for 5 minutes) and resuspended in 1 ml of PBS. Falling two-fold dilutions of the resulting suspension were prepared in a perspex HA tray (0.1 ml volumes, in PBS), with control wells receiving PBS alone. An equal volume of rbc was added to each well, and after brief mixing the tray was incubated at room temperature (or 37C - see Section 5.4.2) for 45 minutes. The endpoint of HA was taken as the last well to show a definite agglutination pattern, and was expressed as the reciprocal of the dilution present in that well.

3.5.4 Attachment to inert particles.

The capacity of V. cholerae to bind to non-physiological substrates was examined by assaying for adherence to three types of inert particle. These were glass Ballotini beads (Selby, Australia), polyvinyl chloride Pevikon beads (ICI, Australia) and polystyrene Dowex particles (Sigma, Missouri). The first of these had to be heated at 100C for 30 minutes to expel air, and all were washed several times in saline before use.

For the adherence assays, 0.2 mls of V. cholerae culture (containing

$\approx 2 \times 10^8$ organisms) was added to duplicate 2-ml suspensions ($\approx 25\%$ v/v in PBS, pH 7.4) of each of the various inert particles, and the resulting mixtures incubated with continuous rolling at room temperature (15-25C, for 15 minutes). The particles were then subjected to three 20-ml saline washes to remove unattached bacteria; although the Ballotini and Pevikon beads were sufficiently dense to rapidly settle out of suspension, the Dowex particles required low-speed centrifugation. Following resuspension in saline, the particles were then diluted and spread across the surfaces of streptomycin-agar plates. The resulting mean vibrio recoveries were expressed as percentages of the numbers of organisms originally added.

3.6 In vitro Titration of Antibodies to *V. cholerae*.

3.6.1 Haemagglutination.

(i) Sensitization of sheep erythrocytes (Srbc).

Srbc were washed three times and resuspended to a concentration of 2.5% (v/v) in saline. Alkali-treated LPS (see Section 3.4) was added to a final concentration of 50 μ g/ml, and the mixture incubated with continuous rolling at 37C for 90 minutes. The Srbc were again washed three times, and finally resuspended to 1% (v/v) in saline. Sensitized erythrocytes were always freshly prepared on the day of use.

(ii) Titration of antisera.

Serial two-fold dilutions of the test sera were made in saline in plastic microtitre HA trays (Cooke Eng. Co., Virginia). A standard serum of known HA capacity was included to check the efficiency of erythrocyte sensitization. Following the addition of an equal volume (25 μ l) of a 1% (v/v) suspension of sensitized Srbc to each well, the trays were shaken briefly and incubated at 37C for 60 minutes. The HA titre was expressed as the reciprocal of the highest serum dilution effecting an erythrocyte settling pattern clearly different from that seen in control wells (lacking serum). Additional

controls checked the background HA capacities of the test sera against normal Srbc.

3.6.2 Bacterial agglutination.

0.4 ml volumes of PBS were dispensed into glass tubes and used to prepare serial two-fold dilutions of the test sera. An equal volume of a PBS-suspension of either live (grown in nutrient broth at 37C, then washed once in PBS) or boiled (see Section 3.8) V.cholerae was added to each tube, to give a final bacterial concentration of $2-3 \times 10^9$ per ml. After brief mixing, the tubes were incubated at 37C for 2 hours and then at 4C overnight. The agglutination titre was expressed as the reciprocal of the highest dilution of serum capable of macroscopic bacterial agglutination, in contrast to the "buttoning" of organisms which occurred in control tubes lacking serum.

3.6.3 Complement-mediated bacteriolysis.

Serial two-fold dilutions of the test sera were prepared using sterile 0.1% (w/v) proteose-peptone (Difco, Michigan) in saline as diluent. 0.4ml volumes were used, and the dilutions performed in glass tubes kept in an ice-water bath until commencement of the assay. A suspension of the V. cholerae indicator strain (at $\approx 5 \times 10^3$ per ml) was prepared using the same diluent, and to this was added guinea-pig serum to a final dilution of 1 in 10 as a source of complement. 0.4mls of this mixture was added to each of the tubes, which were then briefly mixed, incubated at 37C for 60 minutes, and finally returned to an ice-water bath to terminate the reaction.

To determine the number of bacteria surviving within each tube, three drops of each reaction mixture were "spotted" onto one half of a nutrient agar plate with a sterile Pasteur pipette. Following overnight incubation at 37C, the mean percentage survival was calculated by comparing the mean vibrio recovery from each reaction tube with that obtained from control tubes which lacked antiserum. For each test serum, percentage survival was plotted

against $\log_{10}$ reciprocal dilution, and the bactericidal titre obtained by interpolation as the reciprocal of that (theoretical) dilution capable of effecting a 50% loss of viability.

A standard antiserum of known bactericidal potential was included in each assay to check the complement activity of the guinea-pig serum used. Additional controls consistently demonstrated that, at the final dilution of 1 in 20 employed in this test, this serum possessed no natural bactericidal activity against V. cholerae.

Occasionally it was of interest to ascertain the effect of mercapto-ethanol (ME; Sigma, Missouri) pretreatment on the bactericidal potential of an antiserum. A convenient dilution of the test serum was incubated with an equal volume of 0.2M ME (freshly prepared, and diluted in PBS) at 37C for 45 minutes. The serum was then titrated in the normal manner, with extra control tubes to check that the ME itself had no effect on vibrio viability.

3.6.4 Radioimmunoassay (RIA).

This assay was performed in disposable 5-ml plastic tubes, using 0.05% (w/v) bovine serum albumen in saline as diluent. Serial two-fold dilutions of the test sera were prepared, and incubated with an equal volume (0.1mls) of a suspension of V. cholerae (either live or glutaraldehyde-fixed- see Note (i) below - at a concentration of $\approx 10^{10}$ organisms per ml). After 30 minutes in an ice-water bath, all tubes received 2 mls of diluent, and were then spun at 6,000g for 5 minutes at 4C. The resulting supernatants were discarded, and the bacteria washed twice more in a similar volume of diluent.

The final bacterial pellets (with attached specific antibodies) were then resuspended in 0.05 mls of I^{125} - radiolabelled goat anti-mouse immunoglobulin (see Note (ii)). Approximately 10ng of developing antibody - with an associated 6-8,000 cpm - were added to each tube. After brief mixing, the tubes were incubated for 30 minutes on ice, and then the organisms washed

in the same manner as before. The final pellets were assayed for radioactivity (Packard Autogamma counter - settings for I^{125} were 70% gain, 50-250 window).

For each test serum, the cpm associated with each bacterial pellet was plotted against $\log_{10}$ dilution, and the endpoint obtained by interpolation as that (theoretical) dilution resulting in the binding of 10% of the added radio-activity. Control tubes which lacked serum, and received bacteria and developing antibodies only, generally displayed only a low level of background binding (<0.5% - See Note (iii)). The RIA titre - in terms of serum binding units per ml - was obtained by multiplying the serum binding endpoint by 10. This was necessary because only 0.1 ml dilutions were incubated with the bacteria; had larger volumes been used, the attachment of primary antibodies would have been greater, thus increasing the binding endpoint. None of the other methods of antibody titration used in this thesis require a similar correction factor, since in all other assays it is only the serum dilution, and not the absolute volume of serum, which is important in the calculation of endpoints.

Notes

(i) Preliminary experiments demonstrated that glutaraldehyde-fixed organisms (see Section 3.8) were equivalent to live organisms with respect to their capacity to bind antibodies specific for either LPS or "non-LPS" determinants. Accordingly the former were adopted for routine use in this assay, since they were more convenient to use, and provided a constant antigenic substrate for several weeks.

(ii) This material was kindly supplied by Dr. D. Horsfall of this department, and its preparation has been described (Horsfall and Rowley, 1979). It is directed against the light chain determinants of mouse immunoglobulins, and detects antibodies of the IgM and IgG classes with similar efficiency (Horsfall and Rowley, 1979). However this serum is not a sensitive indicator of IgA antibodies, and so a goat antiserum raised against mouse IgA was used to detect antibodies of this class (also supplied and described by Horsfall and Rowley, 1979).

(iii) To obtain this low level of background binding, it was necessary to pre-absorb the developing antiserum to reduce the tendency for non-specific binding of radiolabelled antibodies (both to the bacteria and to the inner surfaces of the plastic tubes). This was conveniently achieved by absorption of the goat antiserum with boiled (see Section 3.8) *V. cholerae* 569B (10^{10} bacteria per ml; the serum was incubated on ice for 30 minutes and then spun to remove the bacteria) prior to its use as a developing serum. Without this step, the background binding in the absence of primary antibodies was typically

≈5% of the total radioactivity added, which was unacceptably high in view of the arbitrary binding endpoint adopted (10% of the total cpm added).

3.6.5 Inhibition of motility.

Serial two-fold dilutions of the test sera were prepared in a perspex HA tray using PBS (pH 7.4). The indicator strain of V. cholerae was grown to a concentration of $\approx 10^9$ organisms per ml, checked microscopically for motility, and then diluted 100-fold in warm nutrient broth. An equal volume (0.1 ml) of this suspension was added to each well, and the tray incubated at 37C for 15 minutes. The low bacterial concentration precluded agglutination during this period. Each reaction mixture was then microscopically examined for residual motility, and compared to that observed in control suspensions which lacked serum (or contained normal mouse serum). The inhibition-of-motility titre was expressed as the reciprocal of the greatest serum dilution which effected a severe ($\approx 90\%$) inhibition of motility.

3.6.6 Inhibition of adherence.

This assay is based upon the standard in vitro system of adherence to segments of everted mouse intestine (see Section 4.2), and is described and discussed in Section 6.6.4.

3.7 In vivo Assays with Infant Mice.

The infant mouse model of cholera was first described by Ujiye and co-workers, who reported that, following their oral administration to 6-8 day old mice, V. cholerae were able to multiply within the gastrointestinal tract and establish a lethal diarrhoeal infection (Ujiye, Nakatomi, Utsunomiya, Mitsui, Sogame, Iwanaga and Kobari, 1968). This report was subsequently confirmed by Chaicumpa and Rowley (1972), who extended the model by demonstrating that specific antisera could protect mice from experimental cholera by preventing the in vivo multiplication of the challenge organisms.

The studies to be described in this thesis have variously employed this model to assess the virulence of different strains of V. cholerae, to compare the colonization potential of related strains, and to determine the efficiencies with which various antisera are able to provide protection against experimental cholera (see below).

The infant mice used in these assays (see Section 3.2.2) were removed from their parents about 6 hours before use, to permit emptying of stomach contents. The challenge strain was grown in nutrient broth at 37C to a concentration of $\approx 10^9$ bacteria per ml, at which stage the culture was examined microscopically to guard against auto-agglutination (see footnote 2 in Chapter 4). The organisms were then spun out of growth medium and resuspended in peptone saline (PS, a 0.1% (w/v) solution of proteose-peptone in saline), which was used as the bacterial and serum diluent in all experiments with infant mice. After challenge (see below), the mice were not returned to their mothers but were kept in tissue-lined plastic containers in the laboratory.

3.7.1 Virulence assays.

Serial ten-fold dilutions were prepared from a PS suspension of the test strain of V. cholerae, and each was used to feed one group of 8-12 mice. Each mouse received 0.1 mls of bacterial suspension, administered by means of a smooth-tipped hypodermic needle (22g). 48 hours after challenge, the survival of mice within each group was noted, and these data used to construct a plot of cumulative percentage mortality versus $\log_{10}$ challenge dose (using the method of Reed and Muench, 1938). By interpolation it was then possible to determine the (48 hour) LD_{50} dose for a given strain - that is, the number of organisms capable of killing (within 48 hours) 50% of the mice to which it is administered. The most virulent strain examined was V. cholerae 017, with an LD_{50} of 1.2×10^5 organisms; many other strains had LD_{50} values of $> 10^9$ organisms, and were considered to be non-pathogenic in this model (see Table 4.2).

3.7.2 Comparisons of colonization potential.

Comparisons of the in vivo colonization capacities of related motile and non-motile variants of V. cholerae strains 111 and SC198 were performed as described in Sections 4.4.3 and 4.4.4. Whenever viability counts were performed on intestinal homogenates, the resulting raw data were logarithmically transformed (see Best, 1970).

3.7.3 Protection assays.

Five- or ten-fold dilutions of the test serum were used to pretreat separate aliquots of a PS suspension of the challenge organisms (usually V. cholerae 569B). After incubation at 37C for 15 minutes, these mixtures were fed to different groups of infant mice (8-12 per group). The bacterial concentration in the original suspension had been arranged such that each mouse would receive 20-100 LD₅₀ doses in the 0.1 ml challenge inoculum. Control mice received organisms which had been incubated in PS alone, or in the presence of normal mouse serum. The survival of all groups was then followed until $\geq$ 90% of the control mice had died; generally this occurred 42-52 hours after challenge (whereas mice given PS alone consistently survived for 72 hours).

Following the plotting of cumulative percentage mortality against log₁₀ serum dilution (after Reed and Muench, 1938), the protective endpoint of the serum was obtained by interpolation as that (theoretical) dilution which could protect 50% of the mice from death. The reciprocal of this dilution was referred to as the protective titre of the serum, expressed in PD₅₀ units per ml.

3.8 Methods of Inactivation of V. cholerae.

Boiled and glutaraldehyde-fixed organisms were frequently used during the course of these studies, and in one particular experiment (see Section 6.5.3) several other preservation procedures were employed. The

various methods of inactivation were performed as follows.

(i) Boiling. Bacteria were grown in nutrient broth, washed twice in saline, then resuspended to a concentration of $\approx 10^{10}$ per ml. A viable count was performed, following which the suspension was heated to 100C for 2 hours. The organisms were then washed three times in saline, resuspended to 5×10^{10} per ml in saline containing 0.1% (w/v) sodium azide, and stored at 4C.

(ii) Glutaraldehyde-fixation. A saline suspension of V. cholerae was prepared and counted as in (i), and then incubated with glutaraldehyde (final concentration of 0.2% (v/v)) at 37C for 60 minutes. The organisms were then washed 6 times in saline, and resuspended and stored as above.

(iii) Formalin-treatment. An equal volume of 1% (v/v) formalin was added to a suspension of V. cholerae (at $\approx 10^{10}$ per ml in PBS pH 7.4). After incubation at 37C for 60 minutes the bacteria were spun and resuspended in saline to the concentration required for injection.

(iv) Phenol-treatment. As for (iii), except that the organisms were inactivated by incubation with an equal volume of 2% (v/v) phenol.

(v) Azide-treatment. As for (iii), except that an equal volume of 16% (w/v) sodium azide was used for inactivation.

(vi) Alcohol- and acetone-treatment. Five volumes of 70% (v/v) alcohol or acetone were added to one volume of V. cholerae suspension (as in (iii)), and incubated at 4C overnight. The bacteria were then spun, washed once, and resuspended in saline for injection.

3.9 Fractionation of Antisera using Protein A-Sepharose.

IgG fractions were isolated from several mouse antisera using the methodology of Ey, Prowse and Jenkin (1978), which is based upon the natural affinity of mouse IgG for staphylococcal protein A. Briefly, a protein A-

Sepharose column (Pharmacia, Sweden; bed volume approx. 5 mls), kindly supplied by Mr. G. Russell-Jones of this department, was equilibrated at 6°C with phosphate buffer (PB; 0.14M, pH 8.0). A 3-ml aliquot of antiserum was adjusted to pH 8.5 and applied to the column, which was then washed with PB (flow rate approx. 25 mls per hour). 3.8 ml fractions were collected, and assayed for protein by measurement of optical density at 280nm (Hitachi, Japan). Peak fractions were pooled, dialysed against saline, and concentrated using Aquacide (Calbiochem, California). This material was referred to as Fraction I.

Meanwhile, the IgG which had bound to the column was eluted using 0.1M citrate buffer, pH 3.0 (see Ey et al, 1978). 3.8 ml fractions were collected into tubes containing 1.7 ml 1M Tris-HCl buffer (pH 9.0) to ensure rapid pH neutralization. Peak fractions were pooled, dialysed and concentrated as above, and constituted Fraction II. The column was then re-equilibrated with PB.

Both fractions were subsequently re-applied to the column - to remove any remaining IgG from Fraction I, and to ensure that Fraction II was totally comprised of material which could bind to protein A. Both were collected and processed as before, and finally stored at 4°C with preservative.

3.10 Quantitation of Antibodies to LPS.

Following the isolation of IgG from a mouse anti-LPS serum by fractionation on protein A - Sepharose, it was of interest to quantitate the amount of specific antibody present in both the IgG fraction and the residual serum (see Section 6.7.2). This was achieved by the adsorption of antibodies onto, and their subsequent elution from, highly purified (homologous) V. cholerae LPS.

Prior to adsorption, both antibody fractions were spun at high speed (100,000g at 4°C for 60 minutes) to remove any macromolecular complexes. The LPS preparation (see Section 3.4) was similarly deposited, then resuspended in saline and spun again; this would remove any material of smaller size

which might otherwise adsorb antibodies but be subsequently lost during the washing procedure. Then 2 mg LPS was added to 1 ml aliquots of each antibody fraction, and to 2 1 ml volumes of saline in control tubes. The resulting suspensions were incubated at 37C for 60 minutes, and then spun at high speed as above. The LPS pellets were subjected to 3 10-ml saline washes, and finally resuspended in 1 ml 0.1N NaOH. The protein concentrations were then assayed by absorption at 280nm, using extinction coefficients of 12.8 for IgM and 13.6 for IgG (see Eddie et al, 1971).

C H A P T E R 4

ADHERENCE, MOTILITY AND VIRULENCE OF V. CHOLERAЕ.

4.1 Introduction.

The capacity of bacteria to adhere to host surfaces has been linked with virulence for a wide variety of pathogens (see Section 2.5), although at the time of commencement of these studies no such relationship had been reported for V. cholerae. The author's interest in this area developed during an examination of the bactericidal potential of adult mouse small intestine in vitro. Freter had proposed the existence of an antibody-dependent killing mechanism associated with the ileal mucosa (Freter, 1969, 1970), and subsequent work from this group had indicated that the intestinal flora may contribute to the effectiveness of such a mechanism, either by increasing the availability of serum-derived coproantibodies (Fubara and Freter, 1973), or by reducing the growth rate of the invading vibrios (Shedlofsky and Freter, 1974). More recently, Knop and Rowley have also reported the existence of an antibody-independent bactericidal capacity associated with the surfaces of murine intestinal loops in vivo (Knop and Rowley, 1975).

On the basis of such studies, we hoped to clarify the role of the small intestine in the resistance of the adult mouse to cholera, and to use in vitro manipulations to dissect the nature of any underlying antibacterial mechanisms. However, we were unable to demonstrate any killing potential associated with the adult mouse small intestine, either independent of, or dependent upon, specific antibody. Using a system similar to that of Freter, we did observe a reduction in the number of vibrios which could be recovered from the intestinal slices in the presence of specific antibody, but this was found to be completely explicable on the basis of agglutination of organisms in the fluid phase. Indeed, a similar reduction could be observed in the presence of boiled segments of intestine.

One interesting observation did arise from the above study, however -

that the two strains of V. cholerae employed differed in their capacities to attach to the slices of intestine. One strain (V. cholerae 017 - El Tor biotype, Ogawa serotype) consistently adhered better than the other (V. cholerae 569B - Classical biotype, Inaba serotype). In terms of the percentage of the added vibrios which attached to the intestinal segments, the difference was approximately forty-fold. A similar but less significant difference had previously been observed between these strains in vivo (Knop and Rowley, 1975).

The fact that the more adherent strain was also of much greater virulence in the infant mouse assay (see below) suggested that the capacity to attach might be a significant determinant of pathogenicity for V. cholerae. This possibility merited further investigation, and it was therefore decided to use the 017 strain to establish a standard assay of adherence, with which we might compare a number of strains of V. cholerae. It would then be of interest to assess the virulence of these same strains to determine whether the link between adherence and virulence proved to be a consistent one.

4.2 Establishing an Adherence Assay.

In 1969, Freter devised a system for studying the effects of specific antibody upon the in vitro adsorption of V. cholerae to slices of rabbit ileum (Freter, 1969). Essentially the same methodology had been adopted in the author's above-mentioned attempts to demonstrate killing of vibrios by adult mouse small intestine in vitro, and it also provided a convenient starting point for the development of an optimal adherence assay. Briefly, this procedure was as follows.

Mice (see Section 3.2.1) were killed by cervical dislocation, and their small intestine - from just below the duodenum to the caecum - were excised. These were flushed through with 5 mls of cold saline to remove faecal material, and were then slit longitudinally. Following a further rinse in saline, 5-cm lengths were prepared, and suspended in phosphate-

buffered saline (PBS; pH 7.4) at 37C. Bacteria which had been grown to late log-phase were added to the intestinal segments, which were then incubated for 15 minutes. This period was selected in order to minimize bacterial multiplication and fragmentation of the intestinal slices, yet also provide an opportunity to perform viable counts on the bacterial cultures being tested. The mixtures were not shaken, as this was found to accelerate the breakdown of the intestine, and did not increase the observed level of attachment.

At the end of the incubation period, the intestinal segments were taken from the flask and washed thoroughly to remove any vibrios which were not firmly attached. This involved passing each slice sequentially through three 250-ml saline baths, a procedure which reduced the carryover of unattached bacteria to an insignificant level (less than 0.1% of the total number of vibrios bound in the case of the most adherent strains, up to a maximum of about 2% for the least adherent strains). Each segment was then individually homogenized (ULTRA-TURRAX, Janke and Kunkel, West Germany - full speed for 15 secs), as was a dilution of the vibrio suspension remaining in the flask. The homogenization process did not affect vibrio viability. The resulting homogenates were diluted and plated onto nutrient agar containing streptomycin (100µg/ml) to inhibit the growth of intestinal commensals. The percentage attachment was calculated by expressing the total number of vibrios bound to the tissue slices as a percentage of the number of organisms added initially (which in turn was generally of the order of 80% of the total number of vibrios recovered).

The first modification to this protocol was introduced during the attempted demonstration of the bactericidal potential of the intestinal surface. The above method of preparation of the intestinal segments resulted in both surfaces of the tissue being in contact with the vibrio suspension. It therefore seemed important to assess the likely contribution of the serosal surface - with its possible complement of adherent peritoneal macrophages - to any bactericidal capacity associated with the intestinal tissue as a whole.

Accordingly, rather than being sliced longitudinally following flushing, normal or everted small intestines were tied off into 5-cm segments, which were then cut and incubated with bacteria as before (see Figure 4.1). In this way it was possible to separately examine the capacities of the two tissue surfaces. Again, however, there was no evidence of any bactericidal effect associated with either surface. Nevertheless, it was found that similar numbers of V. cholerae 017 adhered to each surface - an unexpected result which has since been confirmed, and which will be discussed again later (see Section 4.A). From the viewpoint of an adherence assay however, this observation meant that the intestinal tissue would have to be routinely everted and tied off, in order to ensure that the bacterial-intestinal interactions would be restricted to that surface of pathophysiological relevance.

Subsequent experiments indicated that the strain of mouse used as a source of intestinal tissue was not an important consideration. Thus, V. cholerae 017 adhered to a similar extent to the (everted) intestine of all strains examined, both inbred (C57B1, BALB/c, CBA) and outbred (SWISS, LAC). Accordingly, inbred mice were used in all future adherence assays, in the hope of minimizing the variation in the attachment observed to the individual intestinal segments by providing genetically homogeneous substrates for binding. The variation between segments could be further reduced by preparing no more than two lengths of intestine from each mouse. Segments prepared from the lower ileum had a smaller cross-sectional area, and consistently bound fewer vibrios than those prepared from the jejunum or upper ileum. By eliminating the use of such segments, the variation could be typically limited to the level shown in Table 4.1.

Finally, the overall percentage adherence observed with V. cholerae 017 appeared to be critically dependent upon the ratio between the number of intestinal strips available for attachment and the volume of the medium in which they were suspended. Table 4.1 shows the results of one experiment designed to examine the significance of this ratio. By comparing groups A

TABLE 4.1

Importance of the ratio between the number of intestinal segments and the volume of suspending medium to the observed level of attachment of *V. cholerae* 017.

	<u>Group A</u>	<u>Group B</u>	<u>Group C</u>
No. of vibrios added	1.62×10^8	1.62×10^8	1.62×10^8
No of intestinal segments	8	4	4
Volume of PBS ^a (mls)	10	10	20
No. of vibrios recovered :			
Segment # 1	3.00 ^b	3.71	3.01
2	3.32	4.51	3.39
3	3.94	4.75	3.70
4	5.35	5.24	4.02
5	5.65		
6	5.76		
7	5.85		
8	7.12		
Mean $\pm$ standard error	5.00 ± 0.50^b	4.55 ± 0.32	3.53 ± 0.22
Total no. vibrios attached	4.00×10^7	1.82×10^7	1.41×10^7
<u>Percentage adherence</u>	<u>24.7</u>	<u>11.2</u>	<u>8.70</u>

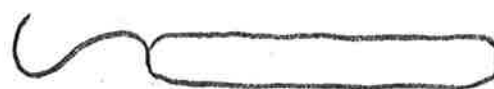
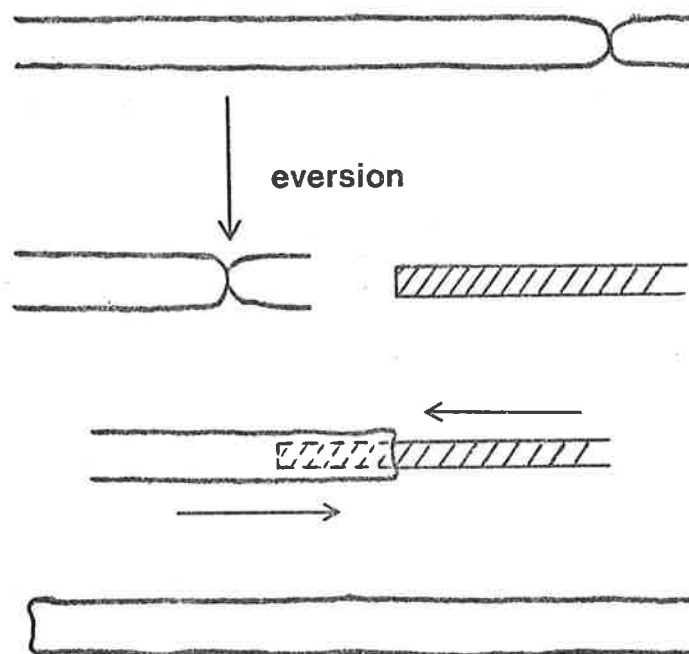
a PBS : phosphate buffered saline, pH 7.4

b These figures have been $\times 10^{-6}$

FIGURE 4.1

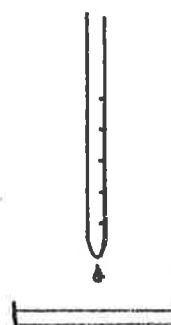
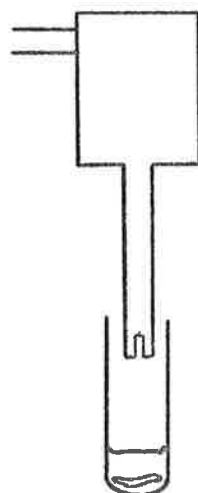
The standard in vitro assay of adherence.

1. The adult mouse small intestine is excised, chilled, and flushed with cold saline.
2. The length of intestine is tied off at one end, then everted using a thin brass rod.
3. The everted intestine is tied off and cut into two 5-cm segments, leaving thread to facilitate handling.
4. After incubation with V. cholerae (see text), the segments are washed, homogenized and processed, to allow calculation of the percentage adherence.



incubation
with
V. cholerae

rinsing,
homogenisation
and processing



and B, it is apparent that, for a given volume of suspending medium - and hence a given concentration of vibrios in the fluid phase - the total number of organisms bound is directly proportional to the number of segments present. Similarly, the results from groups B and C indicate that, for a given number of intestinal strips and added vibrios, the level of attachment depends upon the volume of the suspending medium. As a compromise between the desire to use as many strips as possible, to maximize the observed level of adherence, and the time involved in their preparation, a ratio of 6 strips per 10 mls of suspending medium was adopted for routine future use.

Under these conditions, the percentage adherence observed did not vary despite tenfold changes in the number of organisms incubated with the intestinal strips. In a typical assay, $1 - 4 \times 10^8$ vibrios were added, and each segment would bind $0.3 - 1.2 \times 10^7$ organisms if these were of an adherent strain. At this level each strip was far from saturated with vibrios, as subsequent experiments demonstrated a binding capacity of at least $1 - 2 \times 10^8$ organisms per segment.

The adherence assay in its final form is summarized in Figure 4.1.

4.3 Adherence and Virulence of *V. cholerae*.

4.3.1 Survey of strains.

Having ascertained the optimal parameters of an in vitro adherence assay, several strains of *V. cholerae* were examined for their capacities to attach to strips of everted adult mouse small intestine. The results of these experiments are given in Table 4.2, together with estimates of the virulence of each strain in infant mice (see Section 3.7.1).

These data can be most conveniently discussed by grouping the various strains into four categories. The first of these comprises the three El Tor strains, and vibrios within this group are highly adherent in vitro and extremely virulent for infant mice. *V. cholerae* KUKI and *V. cholerae* 0162

TABLE 4.2

Adherence and Virulence of V. cholerae

<u>Strain : biotype , serotype</u>		<u>Adherence</u> ^a	<u>Virulence</u> ^b
017	: El Tor, Ogawa	17.2 ± 1.2	1.2 ± 0.2 x 10 ⁵
V86	: " , Inaba	12.1 ± 1.5	2.7 ± 0.6 x 10 ⁵
T50	: " , Ogawa	13.2 ± 1.2	2.3 ± 0.5 x 10 ⁶
KUKI	: Classical, Inaba	11.4 ± 0.76	>10 ⁹
0162	: " , Ogawa	21.8 ± 0.31	>10 ⁹
111	: Classical, Ogawa	0.28 ± 0.02	>10 ⁹
SC198	: " , Ogawa	0.37 ± 0.06	>10 ⁹
569B	: " , Inaba	0.37 ± 0.04	2.0 ± 0.1 x 10 ⁷
KB155	: Non-cholera vibrio	4.47 ± 0.74	>10 ⁹
KB156	: " "	13.1 ± 1.4	<10 ⁶
T50/165	: " "	13.7 ± 0.58	>10 ⁹
569B/165	: " "	0.38 ± 0.02	>10 ⁹

- a Data in this column show percentage adherence of each strain, as determined in the standard in vitro assay. Each set of figures represents the mean ± standard error of 3-5 experiments (using tissue obtained from C57 B1 mice of both sexes, and 3-6 months of age).
- b Virulence is expressed in terms of the (48-hour) LD₅₀ in infant mice (see Section 3.7.1). Figures represent mean ± standard error of 3 determinations.

form the second group, which is characterized by a capacity to attach efficiently to intestinal tissue in vitro, but a complete lack of virulence in vivo. The existence of such organisms indicates that adherence is not a sufficient condition for pathogenicity, which is not surprising in view of the nature of the infection. For example, an inability to produce a functional toxin could underlie the avirulence of these vibrios, or alternatively they may suffer some nutritional deficiency which is not apparent in vitro because of the rich growth media routinely employed. Indeed, evidence to be presented later suggests that some organisms are able to initially colonize quite effectively in vivo, but are avirulent because of an inability to multiply there (see Section 4.4.3).

The strains which constitute the third category are those which are unable to attach to intestinal tissue in vitro. Two of these - V. cholerae 111 and V. cholerae SC198 - are unable to persist in vivo (see Sections 4.4.3 and 4.4.4) and are consequently avirulent, but the third - V. cholerae 569B - is pathogenic for infant mice. This strain had been repeatedly passaged in vivo upon receipt, and was subsequently shown to be able to colonize and multiply within the infant mouse small intestine (Chaicumpa and Rowley, 1972). Thus, although unable to attach in vitro, V. cholerae 569B can apparently express an adherence capacity in vivo, hence maintaining the relationship between adherence and pathogenicity. Furthermore, by passaging this strain such that it acquired the capacity to adhere to intestinal tissue in vitro, it proved possible to increase its virulence ten-fold (see Sections 4.4.3 and 4.5).

The final group of strains comprises two non-cholera vibrios (NCVs), and two hybrid strains derived by genetically conjugating V. cholerae T50 or V. cholerae 569B with a third NCV strain, NCV-165¹. The properties of the organisms within this group are similar to those displayed by the true cholera vibrios, and it is interesting to note that the hybrid strains have retained the adherence characteristics of their respective V. cholerae parents.

¹ The production of these hybrid strains is discussed in Section 6.3.1.

To summarize, this brief survey has confirmed the correlation between adherence and virulence which was suggested by our earlier observations with V. cholerae 017 and V. cholerae 569B. Those strains which are pathogenic for infant mice all possess the capacity to adhere, and for all but one of these this adherence potential could be demonstrated using our standard in vitro assay. Conversely, organisms unable to bind to intestinal tissue in vitro were generally found to be avirulent.

Whilst investigating the possible link between adherence and virulence, the author became interested in the contribution of bacterial motility to each of these parameters. Before testing for either adherence in vitro or virulence in vivo, each culture was routinely examined by phase contrast microscopy to ensure that no clumping of vibrios had occurred.² It soon became apparent that the degree of motility observed microscopically permitted one to infallibly predict the outcome of the subsequent adherence assay. Highly motile cultures were also highly adherent, and it was a property of vibrios of the El tor biotype in particular to rapidly and irreversibly attach to the coverslip during microscopic examination. In contrast, motile bacteria were scarce in cultures which were later found to be only weakly adherent. It therefore seemed possible that motility might be a prime determinant of adherence, and in this way also contribute to virulence.

Before further investigation along these lines however, it seemed necessary to first validate the use of an adherence assay employing adult mouse small intestine. The adult mouse is completely resistant to repeated feedings of massive doses of live vibrios, and it might therefore be considered

² This would obscure interpretation of the adherence data, as each clump would represent a single entity with respect to attachment, yet would be scored many times as a result of the homogenization process. Similarly, clumping could well affect (lower) the estimate of virulence, as agglutination of vibrios has been postulated to be important in the protection of infant mice by specific antibody (Bellamy et al, 1975; Steele et al, 1975).

invalid to compare adherence to adult mouse intestine with virulence in infant mice. Although it seemed unlikely that the adult mouse owed its resistance to cholera to an intestinal surface that was, per se, significantly less susceptible to colonization than that of the infant mouse³, it was nevertheless decided to examine adherence to infant mouse small intestine.

4.3.2 Adherence to infant mouse small intestine.

The adherence system used in these experiments represented only a slight departure from that described above. The infant mouse was sacrificed, and its small intestine excised and flushed. The delicate nature of the tissue, however, made eversion impractical, and consequently the intestine were merely slit longitudinally. Six such intestinal lengths were suspended in 10 mls of PBS (pH 7.4) and the percentage adherence determined in the normal manner.

In addition, in order to be able to compare the capacities of motility and attachment more objectively, the former was quantitated using a sloppy-agar overlay method (described in Section 3.1.4). The results of these experiments are shown in Table 4.3a, together with the corresponding adherence figures for adult mouse small intestine for purposes of comparison.

The first conclusion to be drawn from these data is that the value of the adherence index does not depend upon the origin of the intestinal tissue. Each strain attached to infant mouse intestine to a degree which reflected both qualitatively and quantitatively its capacity to adhere to adult

³ Adult germ-free mice can be successfully mono-contaminated with V. cholerae (Sack and Miller, 1969; Shedlofsky and Freter, 1974), and the persistence of these organisms suggests that the adult mouse gut is not per se refractory to colonization by V. cholerae. Such colonization can be dramatically inhibited, however, by the prior implantation of an artificial gut flora (Shedlofsky and Freter, 1974; also see Freter, 1956), suggesting that the adult mouse may owe its resistance to cholera to the antagonistic effects of its normal gut flora.

TABLE 4.3

Adherence of *V. cholerae* to infant mouse small intestine in vitro.

Table 4.3a Comparison of strains.

Strain	Percentage adherence to infant mouse intestine	Percentage motility ^a	Percentage adherence to adult mouse intestine
017	14.6	100	17.2
V86	8.83	100	12.1
569B	0.28	<0.12	0.37
111	0.055	<0.16	0.28

Table 4.3b Effect of selection for motility on capacity to adhere.

Strain of <i>V. cholerae</i>	Percentage adherence	Percentage motility ^a
Original 569B	0.28	<0.12
Motile ^b 569B	7.0	82.5
Original 111	0.055	<0.16
Motile ^b 1111	6.5	99.6

a Determined using the sloppy-agar overlay method

b The development of these strains is discussed in the text.

mouse tissue. Accordingly the latter may be used exclusively henceforth⁴, and the adherence indices thus obtained may be validly discussed in relation to virulence in infant mice.

Secondly, the results shown in Table 4.3a confirm the direct relationship between bacterial motility and the capacity to attach. At this stage it was not known whether this was merely a chance correlation, with the adherence phenomenon being mediated by surface structures completely independent of those responsible for bacterial motility, or whether motility was indeed a necessary condition for adherence in our system. The remainder of the work to be described in this chapter was designed to examine the likely significance of this relationship, and the possible contributions of both motility and adherence to virulence.

4.4 In vitro and in vivo Consequences of Selection for Motility.

4.4.1 Selection of motile variants.

A comparison of two strains of V. cholerae, one motile and one non-motile, from the viewpoint of attachment, could lead to no meaningful conclusion with respect to the likely contribution of motility to this capacity, since the strains would also differ in many other respects. The greater the number of strains upon which such a relationship is based, the greater the likelihood that motility is the common property which confers the capacity to adhere. However, the ideal approach would involve comparing the adherence properties of two strains which were known to be identical in every respect except for motility. Our "non-motile" strains provided a unique opportunity to perform such comparisons.

⁴ Strips can be more conveniently prepared from adult tissue, and furthermore eversion makes it possible to exclude attachment to the serosal surface (see Appendix to this chapter). The infant tissue is considerably more delicate, and visible fragmentation occurs during the incubation period.

It should be stressed that the term "non-motile" will be used strictly in a quantitative sense, and will be applied to cultures displaying a level of motility more than three orders of magnitude below that of "motile" cultures. For example, the sloppy-agar overlay method indicated that a typically "non-motile" culture of V. cholerae 569B would possess fewer than 0.05% motile organisms. Nevertheless, microscopic examination of such cultures regularly led to the observation of an occasional motile vibrio, thus suggesting the feasibility of a motility enrichment programme.

The methodology involved in the development of motile variants from our original "non-motile" strains has been described (see Sections 3.1.3 and 3.1.4). Initially, the author was not familiar with the sloppy-agar overlay technique, and relied heavily on the use of Craigie tubes. The number of in vitro passages was kept to the minimum possible, through fear of attenuation. During the selection process agar stabs provided a valuable means of short-term preservation of gains in overall motility, which tended to be less stable and easily lost on normal agar plates. The final motile variant was lyophilized, and henceforth maintained as previously described ⁵(see Section 3.1.3).

Before testing, an attempt was made to establish the identity of each motile variant with its corresponding starting strain. This was done by determining the relative sensitivities of the two forms of each strain to lysis by antibody and complement. On no occasion could any difference be detected, whether the antibody was specific for LPS determinants, or directed against "non-LPS" antigens (see Section 6.2).

⁵ In this manner it proved possible to obtain highly motile variants of V. cholerae 569B, V. cholerae 111, and the Classical hybrid strain 569B/165. However despite repeated attempts, no increases in motility could be obtained with the last of our "non-motile" strains, V. cholerae SC198. It was only much later during the course of this work that a motile variant of this strain was fortuitously obtained. A single broth culture displayed a low level of motility, and this provided a starting point for a successful series of in vitro passages.

4.4.2 Effect of selection for motility on in vitro adherence.

The related motile and non-motile variants of several V. cholerae strains were compared for their capacities to adhere to adult mouse small intestine in the standard in vitro assay. Related variants were tested on the same day using the same mice, and the results of this series of experiments are shown in Table 4.4.

The data indicate that the development of motility has been accompanied by the development of the capacity to adhere, thus considerably strengthening the relationship between the two parameters. Similar results were obtained when infant mouse intestine was used as the substrate for attachment, as shown in Table 4.3b.

It seems unlikely that the in vitro motility selection procedure would have simultaneously led to the acquisition of a non-flagellar adherence structure, the production of which is independent of the production of the flagellum, which might nevertheless be responsible for the increased capacity for attachment. Apart from the fact that there would appear to have been no selection pressure for the development of such a moiety, it soon became evident that the loss of motility, whether spontaneous or induced, resulted in the loss of the enhanced adherence potential. This is illustrated by the data shown in Table 4.5.

This experiment was designed to assess the contribution made by motility to the enhanced adherence capacity of the motile variant of V. cholerae 111, by determining the effect of homogenization on the level of attachment observed in vitro. One part of a culture of this variant was homogenized (ULTRA-TURRAX, full speed for 30 seconds), spun and resuspended, and then compared in an in vitro adherence assay with both the (untreated) motile and non-motile forms. Microscopic examination confirmed that the vast majority of the organisms had been immobilized, and Table 4.5 shows that their subsequent adherence capacity was dramatically reduced. Similar results were

TABLE 4.4

Effect of selection for motility on in vitro adherence capacity of *V. cholerae*.

Strain of <i>V. cholerae</i>	Percentage adherence of original form ^a	Percentage adherence of motile variant ^b
569B	0.35 ± 0.02 ^c	11.4 ± 1.2
111	0.28 ± 0.02	18.8 ± 1.3
SC198	0.37 ± 0.06	16.3 ± 1.0
569B/165	0.38 ± 0.02	12.2 ± 1.6

a Percentage motility of original strains <0.05%

b " " " motile variants >90%

c Figures represent mean ± standard error of 4 assays.
Intestinal segments were prepared from C57B1 mice.

TABLE 4.5

Effect of homogenization on adherence capacity of motile *V. cholerae* 111.

<u>Variant of <i>V. cholerae</i> 111</u>			
	Motile	Homogenized Motile	Original non-motile
No. of vibrios added	1.23×10^8	0.88×10^8	0.97×10^8
No. of vibrios recovered :			
Segment # 1	2.22×10^6	0.97×10^5	2.00×10^4
2	3.74×10^6	1.28×10^5	3.18×10^4
3	4.20×10^6	1.31×10^5	4.02×10^4
4	4.82×10^6	1.49×10^5	4.68×10^4
5	5.17×10^6	1.50×10^5	4.72×10^4
6	5.63×10^6	1.69×10^5	5.31×10^4
Mean vibrio recovery	4.30×10^6	1.37×10^5	3.99×10^4
Total no. vibrios bound	2.58×10^7	8.24×10^5	2.39×10^5
<u>Percentage adherence</u>	<u>21</u>	<u>0.94</u>	<u>0.25</u>

obtained with other motile variants, and throughout these experiments the residual, post-homogenization adherence capacity varied inversely with the effectiveness of the immobilization.

Concomitant losses of the capacities for motility and adherence were also occasionally observed naturally, with cultures seeded from older agar stabs. This problem was mainly confined to the motile variant of the Classical hybrid strain 569B/165, and tended to reinforce the correlation between the two parameters. Before further examination of this relationship (see Section 4.5), it was of interest to compare the behaviour of motile and non-motile variants in vivo.

4.4.3 In vivo consequences of selection for motility.

Unfortunately, only one of the four strains for which paired motile and non-motile variants were available was pathogenic for infant mice, and so only in this one case could the relative virulence of the two forms be compared. Over four determinations, in which both variants were assayed in parallel using the same batches of mice, the motile variant of V. cholerae 569B proved to be ten times more virulent than the non-motile form. Its LD₅₀ at 48 hours was $3.1 \pm 0.4 \times 10^6$, compared to $3.4 \pm 1.1 \times 10^7$ for the non-motile V. cholerae 569B. This finding - that an in vitro motility selection procedure had led to a ten-fold increase in virulence - strongly implicated motility as an important contributing factor in the pathogenesis of cholera.

The virulence of the relatively non-motile form of V. cholerae 569B - albeit rather weak - indicates that motility is not a necessary condition for pathogenicity⁶. Nor is it a sufficient condition, as attested to by the

⁶ It seems extremely unlikely that the limited virulence of the non-motile form of V. cholerae 569B could be attributed to the small percentage (<0.05%) of motile organisms typically present. An LD₅₀ dose of $\approx 3 \times 10^7$ non-motile V. cholerae 569B would contain at most $\approx 3 \times 10^4$ motile bacteria, yet the LD₅₀ of the fully motile variant of this strain is $\approx 3 \times 10^6$. Furthermore, using the sloppy-agar overlay method (in a manner to be described below), it was found that those organisms recovered from the small intestines of infant mice dying from the effects of an oral inoculum of non-motile V. cholerae 569B retained their non-motile status.

fact that the motile variants of V. cholerae 111, V. cholerae SC198, and the Classical hybrid vibrio 569B/165 were found to be completely avirulent in infant mice (LD_{50} at 48 hours $>10^9$)⁷. Motility should therefore perhaps be most appropriately considered as an accessory determinant of virulence.

The results of the comparative adherence assays previously described (see Section 4.4.2) indicated that the motile variants of each strain were better able to attach in vitro. In the present context, therefore, it seemed likely that the role of motility in the infective process might be to promote the initial colonization of the host tissue, and thereafter to facilitate the persistence of the organisms in spite of the copious diarrhoeal secretions. It was decided to explore this possibility by examining the in vivo persistence of the motile and non-motile variants of V. cholerae 111.

Two groups of infant mice were fed either motile or non-motile V. cholerae 111, with inocula of $1-2 \times 10^8$ bacteria per mouse. For purposes of comparison, a third group received a similar challenge dose of V. cholerae 017, in order to delineate the progress of the infection established by a highly virulent strain. At various intervals from 6 - 48 hours after challenge, mice were sacrificed and their small intestines excised. These were then homogenized, diluted, and plated onto streptomycin-agar in the normal manner. The vibrio recovery data (see Section 3.7.2) are displayed in Figure 4.2.

Before considering the pattern of recovery of motile V. cholerae 111, it is helpful to compare the behaviour of the highly virulent V. cholerae 017 and the avirulent non-motile form of V. cholerae 111. Firstly it is apparent that, regardless of pathogenicity, the great bulk of the challenge inoculum is cleared from the small intestine within six hours after feeding. At this time, only $\approx 2.5\%$ of the V. cholerae 017 inoculum remains, and $\approx 0.2\%$ in the case

⁷ Although these variants possess the capacity to adhere in vitro, there could be several explanations for their lack of pathogenicity (see Section 4.3.1). The avirulence of the hybrid vibrio 569B/165 is understandable in view of the fact that the parental V. cholerae 569B strain had not been passaged in vivo.

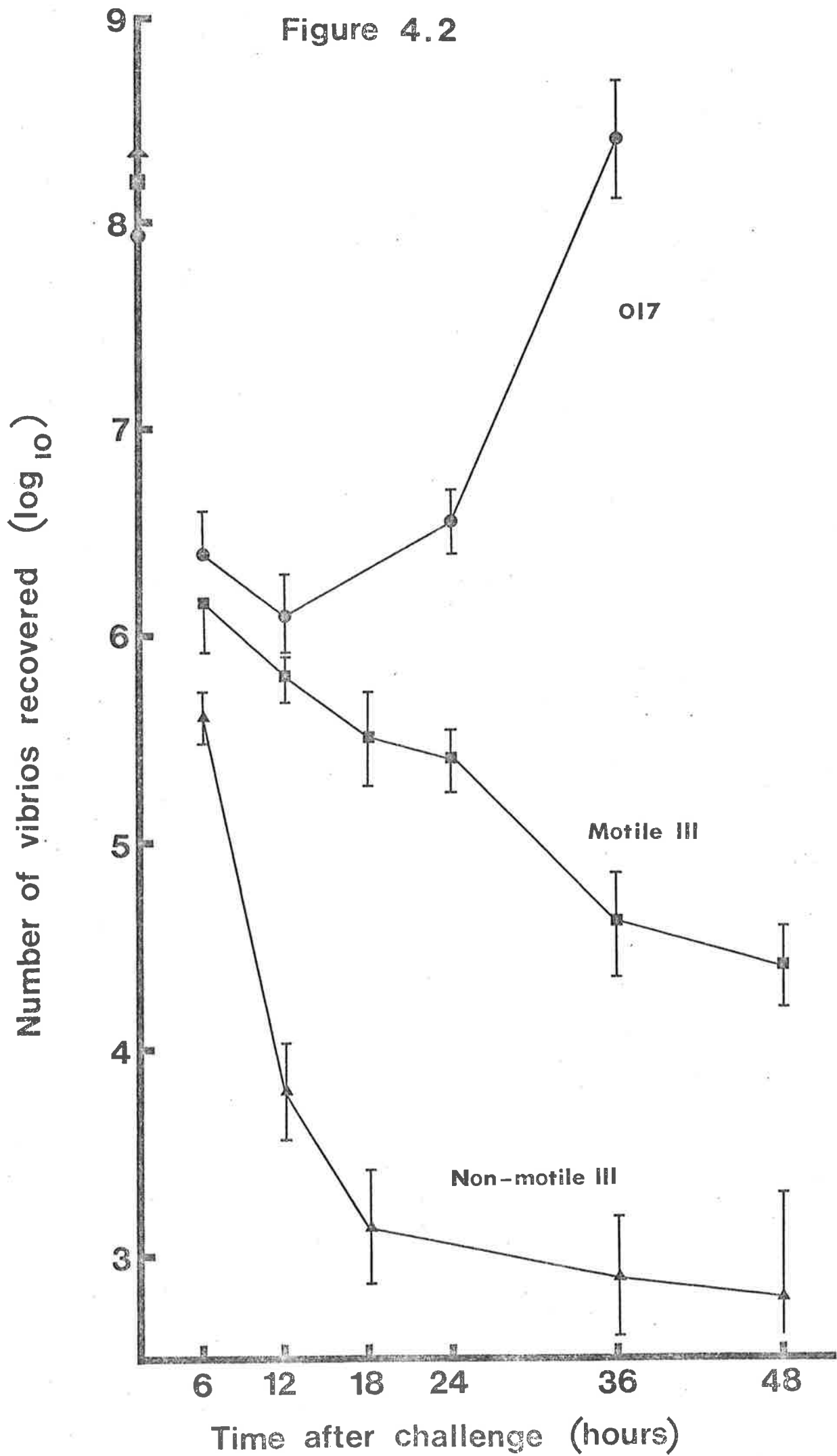
FIGURE 4.2

Persistence of *V. cholerae* in small intestines of infant mice.

This experiment was performed as described in the text.

The raw vibrio recovery data were logarithmically-transformed (after Best, 1970) before plotting; each point represents the mean $\pm$ standard error of 4-6 mice.

Figure 4.2



of non-motile V. cholerae 111. During the next 30 hours, the recovery patterns for these two strains directly reflects the qualitative difference in their pathogenicity. Thus, the V. cholerae 017 population remains relatively constant for approximately 18 hours, before entering a phase of extremely rapid growth, during which it increases almost 100-fold in 12 hours. The infant mice begin to succumb shortly afterwards. In contrast, the numbers of vibrios recoverable from the small intestines of infants given non-motile V. cholerae 111 continue to fall dramatically; 18 hours after challenge, only $\approx 0.001\%$ of the challenge inoculum remains.⁸

Against this background one can readily assess the importance of motility to the in vivo processes of colonization and persistence. It is apparent that, during the first 12 hours after challenge, the numbers of vibrios recovered from the small intestines of mice given motile V. cholerae 111 or (motile) V. cholerae 017 are very similar. At the end of this period, the residual population of motile V. cholerae 111 is 100-fold greater than that recoverable from mice given the non-motile form of this strain. The fact that the property of motility can so influence the initial pattern of vibrio recovery - conferring upon an avirulent strain the colonization potential of a highly virulent one - attests to the significance of this parameter in the establishment of choleraic infection.

Following the similarity of their earlier patterns of persistence however, the subsequent behaviour of V. cholerae 017 and motile V. cholerae 111 differs markedly. Whereas the former strain multiplies and eventually kills its host, the latter appears to be unable to replicate in vivo, and is gradually cleared from the small intestine. The recovery of the motile V. cholerae 111 continues to remain much higher than that of the non-motile

⁸ This experiment was performed during winter, with ambient daytime temperatures of $\approx 15^{\circ}\text{C}$. In warmer weather, the growth of V. cholerae 017 is accelerated (such that all mice receiving the above challenge dose would succumb within 24 hours of feeding), as is the elimination of non-motile V. cholerae 111.

form of this strain (a minimum of 30-fold higher), but this seems entirely attributable to its enhanced initial colonization. Thereafter the variants are eliminated at a similar rate.

Similar evidence of the importance of motility to colonization in vivo was obtained from a comparison of the relative persistence of the motile and non-motile forms of V. cholerae SC198 (for example see 24-hour vibrio recovery data in Table 4.6b).

4.4.4 The role of motility in vivo - a different approach.

An alternative approach to the in vivo comparison of motile and non-motile variants would be to examine the relative persistence of the two forms in the same mice. Such a protocol would offer two appealing advantages. Firstly, it would provide an opportunity to compare the behaviour of the two variants in the same in vivo environment. Secondly, working at the level of each individual mouse would mean that the biological variation inherent in experiments such as the one described above would be of little consequence. The absolute vibrio recovery would be of only a secondary interest, the key issue being the proportion of each variant in the bacterial population recovered from each mouse. Such data might provide a powerful insight into the role of motility in the pathogenesis of V. cholerae.

The implementation of such an approach requires a means of performing differential counts of the numbers of motile and non-motile organisms within a mixed population. The most convenient method of achieving this appeared to lie in the selection of a second antibiotic resistance marker in one of the variants (both of which were already resistant to streptomycin), with subsequent plating of intestinal homogenates on both types of agar plates. However all efforts in this direction proved unsuccessful, as bacteria selected for resistance to low levels of tetracycline, ampicillin or nalidixic acid consistently reverted to sensitivity when grown in the absence of antibiotic. As similar reversion would probably occur in vivo, an alternative

means of discrimination was sought. The sloppy-agar overlay method (see Section 3.1.4) appeared to offer promise in this respect.

Preliminary experiments using this technique indicated that motility or non-motility were stable properties in vivo. Thus, infant mice fed with either variant subsequently yielded intestinal homogenates which, when plated and overlaid with sloppy agar, reliably reflected the motility characteristics of the challenge inocula. Therefore the question of immediate interest could now be tackled - if mice are challenged with a mixture of motile and non-motile forms of the same strain, does either variant display a greater capacity to persist in vivo? Any significant differences in this respect would be reflected by an altered level of motility in the bacterial population recovered from the small intestine.

Four groups of infant mice were used in this experiment, which again compared the in vivo behaviour of the two forms of V. cholerae 111. Two of the groups received either variant alone, whilst the challenge inocula for the other groups were designed to possess approximately 1% or 10% motile organisms. All mice were sacrificed 24 hours after feeding, and their small intestines excised and processed in the normal manner. After overnight incubation at 30C, those plates bearing a convenient number of colonies (up to 500) were overlaid with sloppy agar, and the percentage motility subsequently determined. The raw data from this experiment are shown in Table 4.6a, and the summarized results in Table 4.6b. The latter also gives the results of a similar experiment using the motile and non-motile variants of V. cholerae SC198.

Several points can be made on the basis of these data. Firstly, as mentioned above, the motility or non-motility of the two variants of each strain remain stable characteristics in vivo (see groups D, Z and A, X respectively in Table 4.6b). Secondly, in every case in which a mouse received a mixture of both motile and non-motile variants (groups B,C and Y, totalling 15 mice), the vibrio population recovered from the small intestine at 24 hours displayed a higher level of motility than the challenge inoculum.

TABLE 4.6

Table 4.6a The relative persistence of motile and non-motile variants
of *V. cholerae* 111 within individual infant mice : raw data.

Challenge group : % motility of inoculum		Log ₁₀ vibrio recovery ^a	% Motility of vibrios recovered	
A : < 0.05% (2061) ^c	Mouse #	1	ND ^b	
		2	ND	
		3	< 0.69	(145) ^c
		4	< 0.28	(363)
		5	< 0.21	(468)
		6	< 0.12	(850)
B : 1.09% (1656)	Mouse #	1	19.3	(140)
		2	76.4	(144)
		3	9.6	(690)
		4	31.5	(1095)
		5	29.9	(542)
		6	ND	
C : 10.3 % (1119)	Mouse #	1	48.9	(438)
		2	70.4	(409)
		3	96.5	(346)
		4	82.7	(376)
		5	92.8	(567)
		6	ND	
D : 98.7 % (911)	Mouse #	1	99.7	(355)
		2	93.7	(773)
		3	99.9	(667)
		4	98.5	(540)
		5	98.9	(892)
		6	99.8	(488)

a 24 hours after challenge - see text.

b ND - not determined.

c Figures in parentheses indicate the numbers of colonies upon which motility estimates were based.

TABLE 4.6 (contd.)

Table 4.6b The relative persistence of motile and non-motile variants of V. cholerae within individual infant mice : summarized data.

Challenge group : % motility of inoculum	Log ₁₀ vibrio recovery ^a	% Motility of vibrios recovered
A ^b : < 0.05%	3.46 ± 0.32	< 0.33 ± 0.13
B : 1.09%	4.24 ± 0.33	33.3 ± 11.5
C : 10.3 %	5.12 ± 0.35	78.3 ± 8.6
D : 98.7 %	5.19 ± 0.24	98.4 ± 1.0
X ^c : < 0.03%	2.21 ± 0.30	ND ^d
Y : 0.90%	4.22 ± 0.41	62.3 ± 17.7
Z : 99.1 %	4.51 ± 0.24	98.5 ± 1.0

a At 24 hours - see text.

b Groups A - D refer to the experiment shown in Table 4.6a, using the motile and non-motile variants of V. cholerae 111. Vibrio recovery data give mean ± standard error (6 mice per group), as do the % motility data (4-6 mice - see Table 4.6a).

c Groups X - Z refer to a similar experiment performed with the motile and non-motile variants of V. cholerae SC198 (5 mice per group).

d ND - not determined; vibrio recoveries were too low to permit meaningful estimates of percentage motility.

The enrichment for motility resulting from this "in vivo passage" was most dramatic in those mice receiving a lower proportion of motile organisms (30-fold enrichment in group B, and 70-fold in group Y), and strongly suggests that motility provides a great selective advantage in terms of in vivo persistence.

Thirdly, the vibrio recovery data obtained from these experiments confirm the results shown in Figure 4.2, in that the numbers of bacteria recoverable from those mice receiving the motile variants were much greater than those recoverable from mice receiving the corresponding non-motile forms. Thus, the recovery of motile V. cholerae 111 was about 70-fold greater than that of non-motile V. cholerae 111 (see groups A and D in Table 4.6b), while for V. cholerae SC198 the corresponding difference was approximately 200-fold (Groups X and Z). Whilst considering absolute vibrio recoveries, it is interesting to note that similar numbers of organisms were present in the mice of groups Y and Z, despite the fact that the animals in the latter group had received a 100-fold greater dose of motile organisms. This seeming paradox is explicable upon consideration of the results shown in Figure 4.2. It must be inferred, however, that the majority of the organisms which successfully colonized the intestines of the mice in group Y were derived from the small (0.90%) subpopulation of motile bacteria initially present in the challenge inoculum. In contrast, the vast majority of the non-motile bacteria - which initially comprised 99% of the challenge dose - were rapidly eliminated. Thus once again the data indicate that motile vibrios possess a tremendous advantage during the process of colonization in vivo.

Finally, the results obtained in these experiments have confirmed the promise of this particular experimental approach. Although the variation in vibrio recoveries ranges from 50-fold in group D to 240-fold in group C (see Table 4.6a), this does not detract from the significance of the data with respect to the role of motility in vivo.

4.5 Effects of in vitro Selection for Adherence.

The in vitro comparisons of related motile and non-motile variants described in Section 4.4.2 demonstrated that, following their in vitro passaging for motility, the former displayed a dramatically increased capacity to adhere. The origin and nature of the structure responsible for this attachment (which might also underlie the enhanced persistence of motile variants in vivo) remains obscure, although two possibilities exist. Firstly, the "adhesin" may have been selected during the motility enrichment process, in which case it probably resides on the flagellum. It is possible that the adhesin might exist as a separate surface structure, but if so it nevertheless appears probable that the production of this second moiety would be linked to the production of the flagellum. This would explain the co-selection of the two structures. (It seemed unlikely that the enhanced adherence capacity of the motile variants would be attributable to the selection of an adhesin completely independent of, and unrelated to, the flagellum - see Section 4.4.2).

The second possibility would be that the structure mediating in vitro adherence was already present on each of the non-motile variants. In this event, selection for motility might merely have provided a means for expression of the adhesin, presumably by increasing its frequency of interaction with the intestinal segments.

In an attempt to distinguish between the above alternatives, it was decided to passage the non-motile form of V. cholerae 569B on everted intestinal strips in vitro, to determine whether selection for adherence would lead to the parallel development of motility. The methodology adopted for this passaging for adherence was based essentially upon the standard in vitro adherence assay. However, in an attempt to minimize selection for motility, should the second alternative discussed above prove correct, the concentration of bacteria in the fluid phase was increased approximately 50-fold. In this way it was hoped to increase the frequency of interactions between the non-motile organisms and the intestinal surfaces

so that, should the vibrios indeed possess (non-flagellar) adherence structures, they would have a better opportunity to attach and be selected.

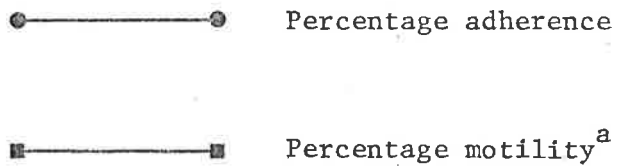
Thus each passage would involve the incubation of $1-2 \times 10^{10}$ organisms with each of two groups of six everted intestinal strips. One group of strips would be used to monitor the adherence capacity at that particular stage of the selection procedure (calculating percentage attachment in the normal manner), while the other would be used as a source of organisms for the next passage. Those strips in the latter group were washed thoroughly to remove any vibrios which were not firmly bound, and then dropped into nutrient broth containing streptomycin and shaken at 37C. When this culture reached late log-phase, it was used to inoculate nutrient agar stabs, which were then incubated at 37C overnight. The resulting surface growth was harvested directly into nutrient broth (containing streptomycin) and shaken at 37C to provide the source of organisms for the next passage on intestinal segments. At the end of the selection procedure, the "strip-passaged" variant of V. cholerae 569B was lyophilized.

In addition to monitoring the adherence capacity at each stage of the selection procedure, the motility of the vibrio population was assessed just prior to each passage using the sloppy-agar overlay method. The results of this work are presented in Figure 4.3.

These graphs reveal a striking parallel between the development of the capacities for adherence and motility in response to in vitro passaging on intestinal strips. Those passages which were most effective in increasing adherence were also those which led to the most dramatic rises in overall motility, whereas passages 5 and 7 were relatively ineffective in both respects. The fact that the motility determinations were performed prior to the adherence assays suggests that the observed gains in adherence were a direct reflection of preceding gains in motility. Indeed, in addition to the data obtained thus far, these results suggest that the relationship between motility and adherence may well be one of cause and effect. Although the second explanation discussed above with respect to the role of motility in the adherence phenomenon cannot

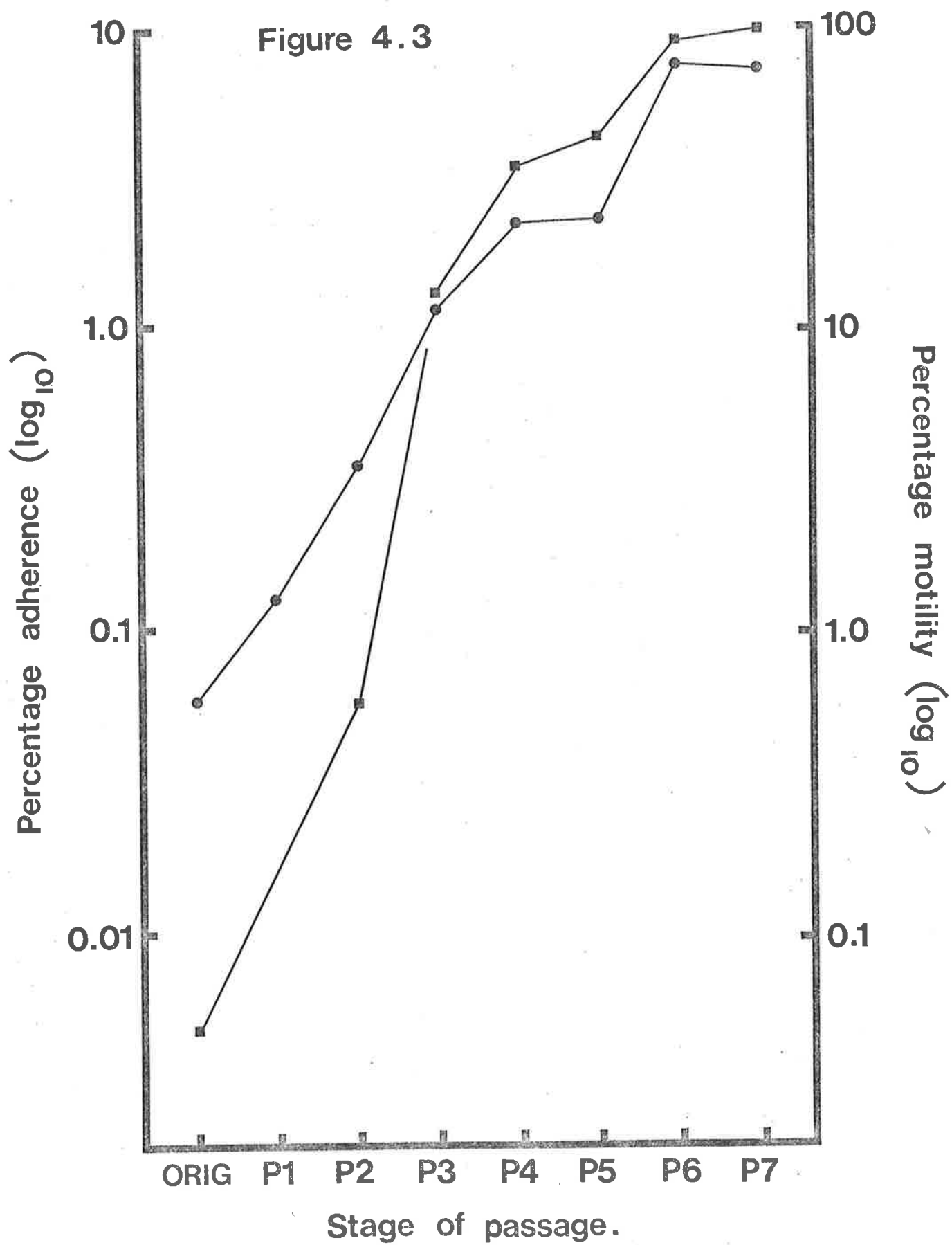
FIGURE 4.3

Parallel development of adherence and motility during
serial in vitro passaging of non-motile V. cholerae 569B
on everted intestinal segments.



^a Motility determinations performed on the original culture (ORIG), and that obtained after one passage (P1), yielded estimates of <0.1%.

Figure 4.3



be completely eliminated⁹, the present data would appear to support the concept of the flagellum as the carrier of the vibrio adhesin.

The maximum adherence capacity observed during the in vitro selection procedure represented a percentage attachment of only 7.5%, and may have been limited by the binding capacity of the intestinal strips. At this level, $1-2 \times 10^8$ bacteria remained associated with each strip after rinsing, and this could represent a state of saturation. (The residual vibrio suspension was not depleted of organisms with the capacity to attach, since a similar level of binding could again be observed upon the addition of more intestinal strips). When the passaging for adherence was repeated using the normal concentration of organisms in the fluid phase, the maximal level of adherence was 13.9%, but this represented the binding of less than 10^7 vibrios per strip. In this latter experiment, the capacities of adherence and motility again developed in parallel, but reached their maxima after only four passages (suggesting that motility was of greater relevance to the selection process?)

Finally, it was of interest to compare the virulence of our strip-passaged variant of V. cholerae 569B with that of the non-motile starting form. Repeated simultaneous comparisons yielded the following (48-hour) LD_{50} estimates : $2.0 \pm 0.1 \times 10^7$ organisms before passage, and $1.7 \pm 0.3 \times 10^6$ organisms after passage. Thus the link between motility and virulence was again demonstrated, and once again the data are consistent with the notion that motility contributed to virulence by facilitating adherence. The increase in virulence resulting from selection for adherence is strikingly similar to that observed following in vitro passaging for motility. Indeed, no difference

⁹ It could still be argued that, for non-motile organisms, the interaction characteristics of the in vitro adherence assay are so inefficient that motility is necessary for the expression of pre-existing adhesins. However, using a different selection procedure which would appear to be much more conducive to the binding of non-motile bacteria, the development of adherence was again accompanied by the parallel development of motility. (see Section 5.2.2).

between the two variants could be detected using a variety of assays¹⁰, and it seems likely that the two are, in fact, identical. This in itself provides strong evidence that the properties of motility and adherence are closely associated.

4.6 Summary.

Following the development of a standard in vitro adherence assay, it proved possible to demonstrate a correlation between the capacity of vibrios to attach to intestinal tissue in vitro, and their pathogenic potential in infant mice. Motility appeared to be essential for in vitro adherence and the evidence suggested that this was a reflection of the fact that the adhesin mediating attachment was located on the vibrio flagellum. Thus non-motile strains which were passaged for motility also acquired the capacity to bind to intestinal segments in vitro, and similarly when V. cholerae 569B was selected for adherence it simultaneously developed motility.

A comparison of the in vivo behaviour of the non-motile strains and their corresponding motile derivatives suggested that, by facilitating adherence, motility was also likely to be important for the virulence of V. cholerae. Using two different experimental approaches, it was found that the motile variants of V. cholerae 111 and V. cholerae SC198 were able to colonize the infant mouse small intestine far more effectively than their related non-motile forms, and consequently were able to persist longer in vivo. In addition, the in vitro selection of V. cholerae 569B for motility (or adherence) led to a ten-fold increase in virulence, and this was presumably also a reflection of the enhanced colonization potential of motile organisms.

¹⁰ In addition to their similar capacities for motility and adherence, these variants are also identical with respect to their sensitivities to lysis or agglutination by specific antibodies, regardless of the specificity of the latter. The difference in their LD₅₀ estimates results from the fact that these were determined under different climatic conditions. Nevertheless their virulence relative to that of the non-motile variant was practically identical, and similar LD₅₀ values could be obtained if the two were simultaneously compared.

4.A Appendix : Attachment of *V. cholerae* to mucosal and
serosal surfaces of adult mouse small intestine.

The work to be described in this section was prompted by the unexpected finding (briefly discussed in Section 4.2) that *V. cholerae* 017 adhered to the mucosal and serosal surfaces of adult mouse small intestinal tissue with similar efficiency, despite the much greater surface area of the former. A recent publication has suggested that such is not the case in a similar adherence system using slices of rabbit ileum (Freter and Jones, 1976), and it therefore seemed worthwhile to re-examine the above finding using our standard in vitro adherence assay. Our interest in this matter was not merely academic, however, as this work would provide a starting point for a study of the nature of the adherence substrate (see following Chapter). In addition, it seemed essential to demonstrate the physiological relevance of the adherence index being used.

Accordingly six adherent strains of *V. cholerae* were examined for their capacities to attach to the serosal or mucosal surfaces of adult mouse small intestine. This was achieved by incubating bacteria from the same culture with lengths of intestine which had been tied off either before or after eversion. The levels of adherence were determined in the normal manner, and the results of these experiments are shown in Table 4.A.1.

These data confirm that, at least for the three strains of El Tor biotype, the two intestinal surfaces provide similar opportunities for attachment. Before considering the implications of this finding, it is interesting to note that such is not the case for organisms belonging to the Classical biotype, which consistently adhered in greater numbers to the mucosal surface of the intestinal tissue. These results suggest that the vibrios of the two biotypes may possess different mechanisms of attachment¹¹ - a point which will be discussed again in the next chapter (Sections 5.2.1 and 5.4.2). The data also resolve the apparent disagreement between our work and the recent findings

TABLE 4.A.1

Attachment of *V. cholerae* to mucosal and serosal
surfaces of adult mouse^a small intestine in vitro.

Strain : biotype		% adherence to mucosal surface	% adherence to serosal surface
017	: El Tor	23.9	17.2
V86	: "	10.9	12.7
T50	: "	13.6	14.5
Motile 569B	: Classical	9.52	2.78
" 111	: "	13.2	2.37
" SC198	: :	17.7	1.98

a Mice used for these experiments were C57B1 males, 5-6 months of age.

of Freter and Jones. The latter workers observed that "vibrio association with the serosal surface (of rabbit ileal slices) was noted only very infrequently", but their examinations were restricted to a single strain of Classical biotype (Freter and Jones, 1976).

The finding that strains of El Tor biotype could attach equally well to either surface of the mouse intestine suggested that, under the conditions of the in vitro adherence assay, the "adherent" organisms might merely have become associated with the mucous layer covering the epithelial surface. If this was the case, the much greater surface area of the underlying epithelium would be of no relevance to the overall level of adherence observed. Furthermore, the adherence indices obtained might therefore be of little relevance with respect to the capacity of "adherent" organisms to interact with the epithelial surface of the intestine.

It was decided to examine this possibility by determining the capacity of V. cholerae to attach to isolated intestinal epithelial cells. Suspensions of these cells were prepared from adult mouse small intestine as previously described (see Section 3.5.2). Following incubation with bacteria, the epithelial cells were washed to remove non-adherent organisms, and then resuspended, diluted and plated onto streptomycin-agar (see Section 3.5.2). The results of such an experiment are shown in Table 4.A.2. It is clear that the capacities of the three strains to bind to isolated epithelial cells reflect both qualitatively and quantitatively their capacities to attach to everted intestinal segments. Accordingly the author felt confident that adherence indices obtained using the latter substrates were meaningful and of physiological significance.

¹¹ An alternative explanation might be that organisms of both biotypes are able to attach to the serosal surface with equal efficiency, but that the Classical vibrios are subsequently killed by some bactericidal mechanism associated with this surface (such as phagocytosis by adherent peritoneal macrophages). However, similar results were obtained using serosal strips prepared from mice which had been subjected to peritoneal washouts before sacrifice, and with serosal strips which had been pretreated with sodium azide (1% w/v) before use in the adherence assay.

TABLE 4.A.2

Adherence of *V. cholerae* to isolated murine intestinal epithelial cells.

Strain	Ratio of vibrios :		% of total vibrio recovery found in :			
	epithelial cells ^a		WF ^b # 1	WF # 2	WF # 3	Epi. cell pellet
017	1 : 3		46	16	10	25
Non-motile 569B	1 : 5		86	9.4	2.8	0.45
Strip-passaged ^c 569B	1 : 1		60	17	14	8.8

a Collected from BALB/c female mice; concentration $\approx 10^7$ per ml.

b WF - wash fluid.

c See Section 4.5.

C H A P T E R 5

THE NATURE OF THE *V. CHOLERAE* ADHERENCE PHENOMENON

5.1 Introduction.

Following the demonstration that many strains of *V. cholerae* are able to attach to mouse intestinal tissue in vitro, it was of interest to further investigate the nature of the interaction between bacterium and substrate. The experiments to be described in this chapter represent our attempts to delineate the bacterial and host surface structures involved in the adherence phenomenon, and were designed with several particular questions in mind.

Firstly, does attachment result from the interaction of the vibrios with specific receptors on the host tissue, or, alternatively, is the association lacking in substrate specificity? Secondly, at the level of the bacterium, is it possible to separate the capacities of motility and adherence, or are these both mediated by the same surface structure? Finally, in view of the fact that different systems have yielded conflicting descriptions of the nature of *V. cholerae* attachment (Jones, Abrams and Freter, 1976; Jones and Freter, 1976; Freter and Jones, 1976), it seems of considerable importance to examine the possibility that these organisms might possess more than one mechanism of adherence.

5.2 The Specificity of *V. cholerae* Adherence.

There are many reports to suggest that one of the factors governing bacterial distribution in vivo is the capacity of organisms to attach to the cells which comprise the various host tissues (see Section 2.3). Such studies have led to the concept of specific adherence, which is thought to result from the interaction of bacteria with special "receptor" structures present

on the surfaces of only a restricted range of host cells.¹ It was of interest to determine whether the V. cholerae attachment process might also display substrate specificity. If so, we could attempt to characterize the host structure involved, and thus begin to dissect the adherence phenomenon.

The results of some previous experiments are of relevance here. Firstly, although some strains of V. cholerae appeared to bind preferentially to the mucosal surface of adult mouse small intestine, others were found to adhere equally well to the serosal surface (see Section 4.A). This indicates that, if specific receptors are involved in the binding event, these structures are not restricted to the cells comprising the intestinal epithelium. Secondly, other experiments have demonstrated that the capacity of mouse intestinal tissue to function as an adherence substrate for V. cholerae is not diminished by boiling, suggesting that any putative receptors must also be completely heat stable (Attridge and Rowley, unpublished data). Alternatively, it could be argued that these findings raise the possibility that, at least for some strains of V. cholerae, the adherence event may be non-specific.

It was decided to continue this investigation by comparing two forms of V. cholerae 569B - the original non-motile strain and the variant obtained from it by in vitro selection for adherence - for their capacities to attach to a variety of physiological and non-physiological substrates. Such an

¹ Obviously the selectivity of bacterial-substrate interactions can also be considered in terms of differences at the bacterial level. Thus the experiments described in the previous chapter demonstrated that even organisms of the same strain can differ markedly in their capacity to adhere to a given substrate (see Section 4.4.2). Nevertheless it was found that the variant forms of any strain could be reliably considered as either adherent or non-adherent, and that within each type of culture all organisms possessed a similar capacity to attach. Accordingly, differences between individual bacteria will not be relevant to the above discussion, and the term "specificity of adherence" can be exclusively applied to those situations in which the success or failure of the attachment process is governed by differences at the substrate level.

approach would indicate whether, as a result of passaging upon intestinal tissue, the organisms had also acquired a capacity to bind to "irrelevant substrates".² If this proved to be the case, it would argue against the concept of specific receptor structures for the binding of V. cholerae. Alternatively, such a theory would receive considerable support from the finding that only a restricted range of materials could function as substrates for adherence.

5.2.1 The range of adherence substrates for V. cholerae.

A previous experiment had demonstrated that the increased capacity of the strip-passaged variant of V. cholerae 569B to adhere to adult mouse small intestine was paralleled by an increased capacity to bind to isolated intestinal epithelial cells (see Table 4.A.2). As such cells constitute the most likely in vivo source of any specific receptor structures, this finding in a sense provides a positive control for the experiments which follow.

Firstly, several species of vertebrate erythrocytes were tested as adherence substrates for the two forms of V. cholerae 569B. The strip-passaged variant was found to actually agglutinate³ guinea-pig, mouse, chicken and human (but not sheep) red blood cells (rbc), whereas organisms of the non-motile phenotype were quantitatively lacking in this respect (see Table 5.1a). Guinea-pig cells were consistently the most sensitive indicators of this capacity, and were adopted for all subsequent experiments. Typically, about 5×10^7 bacteria of the motile variant were present at the endpoint of

² V. cholerae 569B was one of the strains found to attach more efficiently to the mucosal surface of the intestinal tissue (see Table 4.A.1), and therefore seemed suitable for the search for the putative receptor structures.

³ This haemagglutinating capacity was limited, in the sense that it was much weaker than that typically displayed by organisms of El Tor biotype, and could not be observed at 37C. Later experiments suggested that these features were a consequence of the growth of the vibrios in nutrient broth at 37C (see Sections 5.4.1 and 5.4.2).

TABLE 5.1

The range of adherence substrates for V. cholerae.

Table 5.1a Agglutination of vertebrate erythrocytes (rbc)^a

Reciprocal agglutination titre following
incubation of rbc with -

Source of rbc	Non-motile <u>V. cholerae</u> 569B	Strip-passaged <u>V. cholerae</u> 569B	Control (rbc alone)
Guinea-pig	2	32	< 2
Mouse	< 2	16	< 2
Chicken	< 2	8	< 2
Human	< 2	8	< 2
Sheep	< 2	< 2	< 2

Table 5.1b Adherence to inert particles.^b

Percentage adherence to -

Strains of <u>V. cholerae</u>	Ballotini beads (glass)	Pevikon beads (polyvinyl)	Dowex beads (polystyrene)
Non-motile 569B	0.020	0.095	0.33
Strip-passaged 569B	1.48	20.5	2.72
017	12.6	35.7	13.1

a These assays were performed at room temperature (15-20C), using the methodology described in Section 3.5.3.

b These assays were performed exactly as described in Section 3.5.4.

haemagglutination (together with approximately 2×10^7 rbc), whereas at least 1×10^9 non-motile organisms were required.

Phase-contrast microscopy of such suspensions was most revealing. Under oil immersion, motile organisms could be observed to collide with and attach to the erythrocytes, and their capacity to effect clumping was readily appreciated. In contrast, rbc incubated with non-motile V. cholerae 569B remained as a single-cell suspension, with no obvious bacterial adherence. These interpretations received support from an experiment performed to assess the binding capacities of the two variants. Using the methodology described for determining the level of attachment to epithelial cells (see Section 3.5.2), organisms of either phenotype were incubated with a suspension of guinea-pig rbc at a ratio of approximately 1 : 2. After centrifugation, and washing to remove unattached bacteria, 49% of the strip-passaged organisms remained associated with the final rbc pellet, compared to only 4.4% of the non-motile organisms.

Thus, in addition to intestinal epithelial cells, vertebrate rbc provide another (physiological) adherence substrate for V. cholerae. In an attempt to determine whether the attachment process depended upon the presence of a (heat-stable) receptor structure which is widely distributed among mammalian cells, or alternatively whether this phenomenon might be completely lacking in substrate specificity, it was decided to next examine adherence to non-physiological materials.

Once again the procedure adopted was simply to incubate bacteria with suspensions of the various (particulate) test substrates (which are described in Section 3.5.4); the organisms were then washed, diluted and plated to permit calculation of the percentage adherence (see Section 3.5.4). In addition to comparing the two forms of V. cholerae 569B, a third sample of each substrate was incubated with V. cholerae 017. There is some evidence to suggest that strains of El Tor biotype might display different binding

characteristics⁴, and so it was of interest to contrast the binding capacities of one such strain. The results of these experiments are shown in Table 5.1b.

It is readily apparent that the two variants of V. cholerae 569B differ markedly in their capacities to attach to non-physiological substrates. Evidently, in selecting for adherence to intestinal tissue we have simultaneously selected for the capacity to bind to various inert particles. As these substrates are unlikely to display any structures which resemble physiological cell surface receptors, these data argue against the concept of specific adherence in V. cholerae.

A comparison of the binding properties of V. cholerae 017 and the adherent variant of V. cholerae 569B is also of interest. Although both strains adhered to Pevikon beads to a similar degree, the different binding efficiencies observed with the other substrates are similar to those seen when El Tor and Classical strains were previously compared for their capacities to adhere to the serosal surface of mouse intestine (see Table 4.A.1). Thus the results confirm that vibrios of El Tor biotype display a superior capacity to bind to certain substrates, and raise the possibility that these organisms possess an additional adhesive structure. This point will be discussed again later (see Section 5.4).

5.2.2 Consequences of in vitro selection for adherence to inert substrates.

The experiments described above indicated that, as a result of passaging on intestinal tissue, vibrios could also acquire the capacity to bind to several non-physiological substrates. If the V. cholerae attachment

⁴ Firstly, unlike Classical vibrios, organisms of El Tor biotype bind equally well to both surfaces of adult mouse small intestine (see Table 4.A.1). Secondly, El Tor strains are known to possess a cell bound haemagglutinin (see Section 1.2.4(ii)), and it has been our experience that haemagglutination mediated by such organisms is much stronger and more rapid than that observed with motile bacteria of Classical biotype.

process is indeed non-specific, then the reverse situation should also apply. That is, it should be possible to passage a non-adherent strain on an inert substrate, and so select a variant which is also capable of attaching to intestinal tissue. To test this prediction, it was decided to serially passage the original, non-motile form of V. cholerae 569B on both Ballotini and Pevikon beads, and to test the resulting variants for their capacities to adhere to everted mouse intestine in our standard in vitro assay system. These substrates were chosen because they were the most discriminatory with respect to the binding capacities of the original and strip-passaged variants of V. cholerae 569B (see Table 5.1b), and thus seemed to offer the most promise for achieving the desired selection.

Briefly, the selection procedure was performed as follows. Non-motile V. cholerae 569B were added to suspensions of either substrate and incubated, with continuous rolling, for 15 minutes at room temperature (15-20C). The particles were then allowed to settle out of suspension, washed to remove unattached organisms (see Section 3.5.4), and finally resuspended in nutrient broth containing streptomycin (0.1mg/ml). An aliquot of this suspension was kept aside for determining the percentage adherence, while the remainder was shaken at 37C. When the cultures became turbid they were inoculated into agar stabs and also plated to check their purity. The stabs were incubated at 37C overnight, and the resulting growth washed off into nutrient broth and shaken at 37C to provide the population of vibrios for the next passage.

It was also of interest to determine whether the development of adherence to these substrates would be accompanied by the development of motility, since the contact parameters of the selection system would appear to minimize the significance of motility to the selection process.⁵

⁵ Both types of particles are sufficiently dense to settle out of suspension. The continuous agitation of the mixtures of bacteria and beads should therefore ensure ample opportunities for interaction and binding, and it is difficult to envisage a significant role for motility per se. In contrast, it could be argued that motile organisms might enjoy a considerable advantage in the stationary system used to select for adherence to strips of mouse intestine (see discussion in Section 4.5).

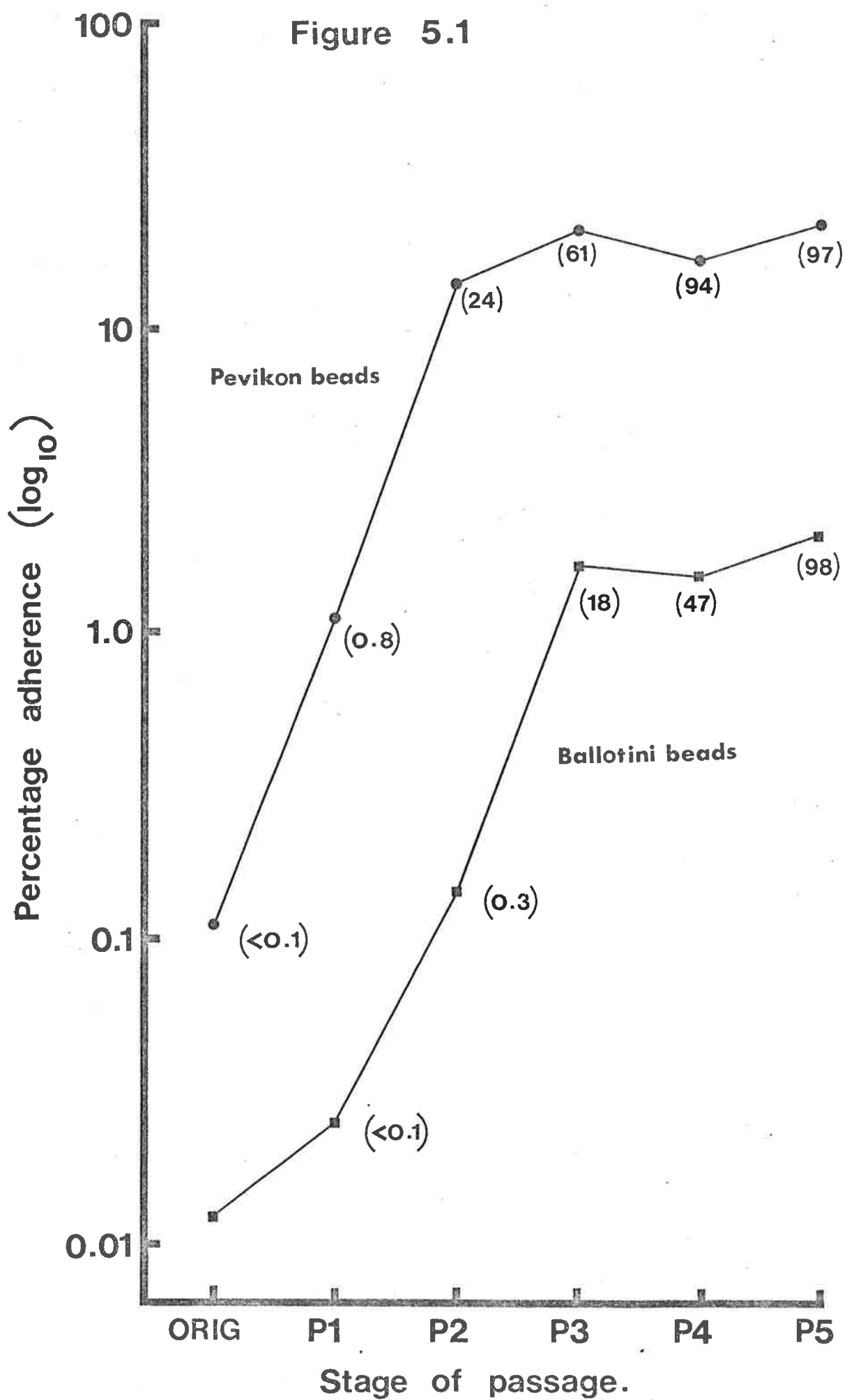
FIGURE 5.1

Development of the capacities of adherence and motility
during repeated in vitro passaging of non-motile
V. cholerae 569B on inert particles.

Graphs show development of adherence capacities during passaging
on Pevikon (● — ●) or Ballotini (■ — ■) beads.

The figures in parentheses give the estimated percentage
motility at each stage of the passaging procedure (see Text).

Figure 5.1



Accordingly, the sloppy-agar overlay method was used to provide an estimate of percentage motility after each passage, and these data are included in Figure 5.1, which shows the development of the capacities to bind to Ballotini and Pevikon beads as a result of repeated in vitro passage.

The graphs show that the organisms rapidly acquired the capacity to bind to either type of particle - after only three passages each population had attained near-maximal binding efficiency. The variants finally selected were lyophilized and stored at 6C. They were approximately 200 times more adherent than the original strain, and displayed binding capacities very similar to those observed previously with the strip-passaged form of V. cholerae 569B (see Table 5.1b). In terms of the binding ratios between bacteria and beads, the passaging procedure increased the level of attachment to Ballotini beads from one organism per 330 beads to one organism per 2 beads. With Pevikon beads, the corresponding ratios were 1 vibrio per 1.5 beads increasing to 130 vibrios per bead (all ratios standardized with respect to a given concentration of organisms).

The figures in parentheses in Figure 5.1 show the estimated percentage motility of both populations at each stage of the passaging procedure, and indicate that for both substrates the development of the capacity to adhere was accompanied by the development of motility. The nature of the selection system would seem to have diminished the importance of motility per se, suggesting perhaps that the selection pressure responsible for the enrichment of motility reflects the fact that the flagellum is the carrier of the vibrio adhesin. A similar conclusion was tentatively drawn in the previous chapter (see Section 4.5), and this point will be discussed again shortly (see Section 5.3).

Having selected variant strains of V. cholerae 569B on the basis of their capacities to bind to non-physiological substrates, it was possible to re-examine the concept of specific adherence in V. cholerae. Both variants

were compared to the original strain with respect to their capacities to attach to mouse intestinal segments, using our standard in vitro assay of adherence. The results of this experiment are shown in Table 5.2. Just as the strip-passaged variant was previously found to bind efficiently to non-physiological substrates (see Table 5.1b), so these data indicate that both variants passaged on inert particles have also acquired the potential to attach to intestinal tissue. Thus once again the evidence argues against the concept that specific receptor structures are necessary for the successful adherence of V. cholerae.

Finally, it was of interest to determine the virulence of the Ballotini- and Pevikon-passaged forms of V. cholerae 569B. As both were fully motile and highly adherent, it was anticipated that they would display the increased virulence characteristic of those variants previously selected for motility or adherence. This was indeed found to be the case, as shown by the following estimates of LD₅₀ doses in infant mice: original strain - 1.3×10^7 vibrios, Ballotini-passaged variant - 1.2×10^6 vibrios, and Pevikon-passaged variant - 3.3×10^6 vibrios. Such increases in virulence following in vitro passaging on inert substrates again argue strongly against the concept of specific adherence in V. cholerae.

5.2.3 The effect of monosaccharides on adherence.

During the course of these studies, Freter's group described the characteristics of V. cholerae attachment in two other in vitro systems of adherence (Jones et al, 1976; Jones and Freter, 1976; Freter and Jones, 1976). These reports will be discussed more fully in the next Section, but the point of relevance here was the claim that, in one of these systems, the attachment of a Classical strain of V. cholerae could be partially inhibited by the presence of L-fucose in the incubation medium. Such a finding would argue against our evidence in favour of a non-specific adherence phenomenon, and

TABLE 5.2

Adherence of bead-passaged variants of V. cholerae 569B
to adult mouse small intestine in vitro

Variant of <u>V. cholerae 569B</u>			
	Original	Ballotini -	Pevikon -
	non-motile	passaged	passaged
No. of vibrios added	1.95×10^8	1.59×10^8	1.80×10^8
No. of vibrios recovered :			
Segment # 1	0.79×10^5	2.16×10^6	2.78×10^6
2	0.90 "	2.53 "	3.16 "
3	0.91 "	2.55 "	3.38 "
4	1.11 "	3.10 "	3.64 "
5	1.51 "	3.68 "	3.73 "
6	1.70 "	4.04 "	4.51 "
Total no. vibrios attached	6.92×10^5	1.81×10^7	2.12×10^7
<u>Percentage adherence</u>	<u>0.35</u>	<u>11.4</u>	<u>11.8</u>

so seemed worthy of investigation.

Accordingly several monosaccharides were examined for their capacities to inhibit the binding of three variants of V. cholerae 569B to their respective substrates. In addition to L-fucose, the other sugars tested were D-mannose (because of its known association with at least one mechanism of attachment - see Section 2.4), D-glucose and D-galactose. Each was added to the incubation medium at either of two concentrations (0.1mg/ml and 10mg/ml, after Jones and Freter, 1976) and the resulting levels of adherence were expressed as percentages of those obtained with controls (PBS alone). The results of these assays are shown in Table 5.3.

It is apparent that none of the sugars tested could effect a consistent inhibition of attachment in our assay systems. A slight (but reproducible) reduction in adherence to Ballotini beads was noted in the presence of D-mannose, but this observation did not extend to binding to the other substrates. Viability counts of supernatant fluids indicated that the presence of the sugars did not cause significant increases in bacterial concentration during the incubation period, and hence the figures in Table 5.3 are unlikely to under-estimate the inhibitory capacities of the monosaccharides. Thus again our data fail to support the concept of a specific receptor structure essential for the binding of V. cholerae.

5.3 The Role of the Flagellum in Adherence.

The finding that, in our systems of adherence at least, the V. cholerae attachment process was completely lacking in substrate specificity, meant that a better understanding of the mechanism of attachment could only result from further investigation into the nature of the bacterial adhesin. In particular, our interest returned to the problem of determining the exact contribution of the flagellum to the process of adherence.

In all of the experiments performed thus far, the properties of

TABLE 5.3

The effect of monosaccharides on the in vitro adherence of three variants of V. cholerae 569B to their respective substrates.^a

		Variant of <u>V. cholerae</u> 569B		
Monosaccharide		Ballotini - passed	Pevikon - passed	Strip - passed
L - fucose :	0.1 mg/ml	92 ^b	ND ^c	110 ^d
	10 "	78	150	110
D - mannose :	0.1 "	64	ND	120
	10 "	75	94	95
D - glucose :	0.1 "	88	ND	ND
	10 "	99	87	ND
D - galactose :	0.1 "	110	ND	ND
	10 "	160	130	ND
Control		100	100	100

a Adherence to inert particles or intestinal segments was assayed using the standard procedures (but see d below).

b Figures show percentage attachment, relative to that observed with control organisms in the absence of monosaccharide.

c ND - not determined.

d Intestinal strips prepared from outbred LAC mice (4 strips per assay).

motility and adherence have remained inseparable. As a result of in vitro enrichment for motility, the original strain of V. cholerae 569B also acquired the capacity to adhere (see Section 4.4.2), and similarly non-motile organisms selected for adherence to either physiological (Section 4.5) or non-physiological (Section 5.2.2) substrates simultaneously developed motility. Conversely, motile variants which had suffered either a spontaneous or an induced loss of motility were also found to have lost the potential to adhere (Section 4.4.2).

As discussed previously (see Section 4.5), there are at least two hypotheses which can satisfactorily explain these observations, and these differ with respect to the significance attributed to the presence of the flagellum on adherent vibrios. The first proposes that the flagellum is solely an agent of motility, and as such merely provides the means of expression for a surface adhesin which is present even on non-motile organisms. However this theory would predict that non-motile vibrios should have the potential to adhere, but no such capacity can be detected in vitro - either by incubation with intestinal slices or isolated epithelial cells, or by continuous rolling with various inert particles, or even by centrifugation onto slices of rabbit ileum (as performed by Freter and Jones, 1976).

The second hypothesis therefore seems more appealing. According to this theory, the flagellum not only provides motility, but is also the carrier of the vibrio adhesin. This would explain the constant association of motility and adherence, and also the development of the former during adherence selection procedures in which the significance of motility per se would appear to be minimal.

The work to be described in this section was performed in the hope of distinguishing between the above hypotheses. The initial experiments (Section 5.3.1) were prompted by the recent demonstration that, at least in one system of V. cholerae attachment, motility and adherence are separable parameters (Jones et al, 1976). It was obviously of interest to determine whether a similar separation might prove possible in our systems, and, if so,

to consider the implications of such a finding with respect to the role of the flagellum in adherence. The later work (Section 5.3.2) represents our own approach to examining the possibility that the flagellum might fulfil a dual role in attachment, and involves the use of specific antibodies in an attempt to independently demonstrate both motility and binding capacities associated with this structure.

5.3.1 Initial attempts to separate motility and adherence.

Freter and co-workers have recently described the characteristics of V. cholerae attachment in two in vitro systems of adherence. One of these involves attachment to brush border membranes prepared from rabbit intestinal epithelial cells (Jones et al, 1976; Jones and Freter, 1976), while the other uses slices of rabbit ileum as the substrate for adherence (Freter and Jones, 1976). The latter system was originally described in 1969 (Freter, 1969), and it was this earlier report which provided the starting point for the development of the in vitro intestinal slice adherence system used throughout this thesis.

The studies of Freter's group demonstrated that, in the brush border system, the parameters of motility and adherence could be dissociated in several ways (Jones et al, 1976). The standard adherence assay involved growth of the organisms in Trypticase Soy broth (TSB) at 37C, followed by their centrifugation, resuspension in buffer, and incubation with formalin-preserved brush borders. However, if the vibrios were pre-incubated in buffer at 37C for 45 minutes, they were subsequently unable to display any binding capacity, although retaining full motility (Jones et al, 1976). Similarly, organisms which had been allowed to bind to brush borders in the normal manner were found to spontaneously detach with continued incubation (in buffer) at 37C. This phenomenon was termed "elution" and was again an apparent reflection of the lability of the vibrio adhesin. Elution was not observed at 22C, indicating that the denaturation of the adhesive structure

was a consequence of both temperature and suspending medium (since organisms grown in TSB at 37C were adherent). Finally, it was found that vibrios harvested directly from Trypticase Soy agar (TSA) showed an undiminished level of motility but were also unable to attach to brush borders (Jones et al, 1976).

Although these findings could not be extended to the rabbit ileum adherence assay (Freter and Jones, 1976), their implications were of sufficient interest to merit investigation on our part, particularly as the brush-border-adhesin was reported to be associated with the flagellum (Jones and Freter, 1976). Accordingly we examined the above procedures, to determine whether motility and attachment might also be separable in either or both of our adherence systems.

Several strains of V. cholerae were used to study attachment to everted mouse intestinal slices, while adherence to inert particles was examined with the variants obtained by in vitro passage on Ballotini or Pevikon beads (see Section 5.2.2). Control organisms were grown in nutrient broth at 37C and then tested for adherence, while others were spun out of this medium, washed and incubated in PBS at 37C for 45 minutes prior to addition to substrate. The effect of growth on TSA was also investigated, with vibrios being harvested from such plates into PBS, inspected for motility, and then immediately assayed for adherence. The results of these experiments are shown in Table 5.4.

With the exception of the original V. cholerae 569B, all of the strains used in these experiments were normally highly motile. Neither pre-incubation in buffer nor growth on TSA diminished motility, and the data in Table 5.4 indicate that both procedures also failed to reduce the capacity to adhere. Thus in both of our systems of attachment, the association between the properties of motility and adherence has been maintained.

The third situation in which Freter's group was able to observe motile yet non-adherent organisms arose as a result of the spontaneous detachment of bound vibrios in the brush border system (Jones et al, 1976). This elution was attributed to the denaturation of the bacterial adhesin as

TABLE 5.4

Effects of pre-incubation in buffer, or growth on TSA, on adherence of V. cholerae.

Strain of <u>V. cholerae</u>	Adherence substrate	<u>Percentage Adherence</u>		
		(i) Following pre-incubation	(ii) Following growth on TSA	(iii) Controls
Non-motile 569B	Intestinal strips	0.26	0.54	0.37
Strip-passaged 569B	" "	12.1	8.62	13.9
Motile 111	" "	19.2	17.0	18.8
017	" "	15.2	13.0	17.2
Ballotini-passaged 569B	Ballotini beads	1.21	1.27	1.42
Pevikon-passaged 569B	Pevikon beads	12.2	22.2	23.8

a consequence of prolonged incubation in buffer at 37C (see Jones et al, 1976). It therefore seemed unlikely that spontaneous detachment would be observed in our binding assays, since the bacterial structures responsible for attachment were able to resist such treatment (see Table 5.4). The following experiments confirmed that elution did not occur, whether intestinal strips or inert particles were used as the substrates for adherence.

In the former system, vibrios of several strains were incubated at 37C with twelve everted segments of intestinal tissue. At 15-minute intervals, three strips were removed, washed, and processed in the usual manner, and the subsequent vibrio recoveries were expressed as percentages of those obtained after the normal incubation period of 15 minutes. The possibility of elution from inert particles was examined in a similar fashion. The selected variants of V. cholerae 569B were added to each of six replicate suspensions of the appropriate substrate, and incubated with rolling at 37C⁶. At 15, 30 and 45 minutes, two suspensions of each type of bead were washed, diluted and plated, and once again the mean vibrio recoveries were expressed as percentages of the 15-minute values. The results of these experiments are shown in Table 5.5.

It is apparent that no overall detachment was observed in either system of adherence. The recoveries of vibrios from the intestinal segments actually increased with time (see Table 5.5), paralleling an increasing concentration of organisms in the fluid phase (data not shown). In view of this bacterial multiplication, however, and the fact that the substrates were incubated in the continued presence of vibrios, it seemed necessary to eliminate the possibility that elution did occur, but that detached organisms were replaced by others from the fluid phase. An alteration to the experimental design showed that this was an unlikely explanation for the data. Thus, if after an initial period of contact with vibrios (15 minutes at 37C) the

⁶ The attachment of vibrios to inert particles is normally assayed at room temperature (15-25C), but the extent of elution from brush borders was found to depend upon the incubation temperature, and was maximal at 37C (see Jones et al, 1976).

TABLE 5.5

Lack of spontaneous detachment of bound *V. cholerae* in two systems of adherence.

Percentage adherence

Strain of <i>V. cholerae</i>	Adherence substrate	Incubation period (minutes)			
		15	30	45	60
Non-motile 569B	Intestinal strips	100 ^a	162	217	250
Strip-passaged 569B	" "	100	173	250	331
Motile 111	" "	100	166	187	257
017	" "	100	216	272	231
Ballotini-passaged 569B	Ballotini beads	100	88	81	ND ^b
Pevikon-passaged 569B	Pevikon beads	100	117	113	ND

a Figures show vibrio recoveries, expressed as percentages of those obtained after 15 minutes.

b ND - not determined.

substrates were washed and transferred to fresh medium, there was still no evidence of significant detachment.

Accordingly, none of the procedures which successfully separated motility from adherence in Freter's brush border assay have proved effective in our systems, the characteristics of which therefore more closely resemble those of his rabbit ileum model (Freter and Jones, 1976). A possible explanation for the differences between the various adherence systems will be discussed shortly (see Section 5.4).

5.3.2 Separation of motility and adherence using specific antibodies.

Other studies in progress at this time suggested another approach with which to attempt the separation of the properties of motility and adherence. It was found that, whereas antibodies specific for the LPS determinants of the vibrio were able to completely inhibit motility, those directed against the other somatic antigens qualitatively lacked this capacity (see Section 6.6.3). Accordingly it seemed potentially rewarding to examine the effects of both types of antibody on in vitro adherence. Should either permit a dissociation of the two parameters, it might prove possible to comment on the likelihood that both are properties of the flagellum, and thus approach the problem of defining the exact contribution of this structure to the process of attachment.

The biological activities of the antisera mentioned above will be discussed at length in Chapter 6. Here it is sufficient to note that one of the sera was raised against highly purified LPS prepared from V. cholerae 569B, and has an inhibition-of-motility titre of 1 in 1,600 against the same strain (this assay is described in Section 3.6.5). The other serum was prepared by immunizing mice with a specially constructed hybrid vibrio, such that the resulting antibody activity is directed only against the battery of non-LPS antigens of V. cholerae 569B. This serum is unable to immobilize V. cholerae 569B, even at a dilution of 1 in 10.

The inhibition-of-adherence assay is also described in detail later (see Section 6.6.4). Briefly, motile vibrios are suspended in PBS at low concentration ($\approx 10^6$ per ml - to avoid agglutination), and incubated with either normal mouse serum (NMS) or various dilutions of the test serum. After 5 minutes at 37C, four everted intestinal strips are added, and after a further 15 minutes at 37C the percentage adherence is determined in the normal manner. In this way the effects of the antisera described above on the in vitro adherence capacity of motile V.cholerae 569B were determined, and selected data from two such experiments are shown in Table 5.6.

The data from Experiment 1 show the result of pre-incubating the vibrios with antibodies directed against their "non-LPS" determinants. Although this treatment had no effect on the motility of the organisms, it is clear that it significantly reduced their subsequent capacity to adhere to the intestinal slices, and thus achieved the first (limited⁷) separation of the two parameters. Evidently, motility per se is not sufficient for attachment.

The fact that antibodies of this specificity were able to inhibit the expression of the vibrio adhesin suggests that this moiety might be non-LPS in nature, but its cellular location remains uncertain. Although it has been possible to restrict adherence without affecting motility, the antibody-blocked adhesin could still be sited either on the flagellum or on the cell wall.

In this respect the results obtained with the anti-LPS serum were more instructive. Initially it seemed that this serum would be of little assistance, since it inhibited both motility and adherence (see Sections 6.6.3 and 6.6.4). However it became apparent that the capacity of such antibodies to restrict adherence could be titrated out more quickly than their capacity

⁷ The residual adherence capacity of the antibody-treated vibrios is still 5 to 6 times greater than that characteristic of "non-adherent" strains. The degree of inhibition observed in this assay is discussed further in Section 6.6.4.

TABLE 5.6

The use of specific antibodies to separate the properties of motility and adherence in V. cholerae 569B.

Antibody pretreatment :	<u>Experiment 1</u>		<u>Experiment 2</u>	
	Anti - "non-LPS" 1 in 50 final	NMS 1 in 50 final	Anti - LPS 1 in 1,000 final	NMS 1 in 50 final
No. of vibrios added :	0.97×10^7	0.97×10^7	1.74×10^7	1.74×10^7
Vibrio recoveries :				
Segment # 1	0.27×10^5	1.6×10^5	2.74×10^5	2.74×10^5
2	0.33 "	1.9 "	3.07 "	3.85 "
3	0.39 "	2.0 "	3.63 "	4.05 "
4	0.65 "	2.2 "	5.03 "	5.11 "
Total no. vibrios attached	1.6×10^5	7.7×10^5	1.45×10^6	1.58×10^6
<u>Percentage adherence</u> :	<u>1.7</u>	<u>8.0</u>	<u>8.3</u>	<u>9.1</u>
Inhibition of motility ?	NO	NO	YES	NO
Inhibition of adherence ?	YES	NO	NO	NO

to prevent motility. Accordingly, it proved possible to add sufficient antiserum to completely immobilize the vibrios, without reducing their adherence potential, as is demonstrated by the data from Experiment 2 in Table 5.6. Thus antibodies directed against the LPS antigens also permitted a dissociation of the properties of motility and adherence, but in this case the former was prevented while the latter remained unaffected.

The implications of this latter separation are most interesting. In all previous experiments adherence has appeared to be dependent upon the simultaneous expression of motility, but in this situation immobilized vibrios have displayed an undiminished capacity to attach. The only difference between these organisms and the non-adherent non-motile vibrios encountered previously is the presence of antibody-immobilized flagella on the former. Thus, even when it can no longer function as an agent of motility, the flagellar structure appears to be necessary for adherence. This finding is consistent with the theory that the moiety which mediates attachment to murine intestinal tissue is located on the vibrio flagellum.

Finally, it is interesting to note that, in addition to their capacities to inhibit the attachment of V. cholerae 569B in vitro, both of the sera used in these experiments are also able to protect infant mice from experimental cholera caused by this strain (see Chapter 6). The possibility that antibodies might be able to protect mucosal surfaces from colonization by inhibiting the attachment of potential pathogens has been mentioned previously (Section 1.3.5), and will be discussed in more detail later (see Section 6.6).

5.4 The Existence of two Adhesins in V. cholerae.

5.4.1 Introduction.

The experiments described in Section 5.3.1 suggested that the moiety responsible for attachment to intestinal tissue or inert particles differs from that which mediates binding in Freter's brush border system. Although

both structures appear to be in some way associated with the flagellum (Sections 4.5 and 5.3.2; Jones and Freter (1976)), there nevertheless seem several reasons for supposing that they are indeed different, and for postulating the existence of two adhesins in V. cholerae.

Firstly, none of the procedures reported to denature the brush-border-adhesin (BBA) had any effect on the capacity of vibrios to attach to a mucosal substrate (either in our system (see Section 5.3.1) or in Freter's (see Freter and Jones, 1976)) or to inert particles (Section 5.3.1). Apparently then, adherence in these systems is mediated by some other mechanism. This conclusion receives support from the demonstration that a suspension of TSA-grown vibrios, which were unable to bind to brush borders and thus apparently lacked the BBA, displayed an undiminished capacity to attach to an intact mucosal surface (see Freter and Jones, 1976).

Secondly, the BBA confers upon bacteria the capacity to strongly agglutinate human erythrocytes (Jones et al, 1976), whereas the motile vibrios which are highly adherent in our system lack this property. The latter organisms do possess some haemagglutinating activity (see Section 5.2.1), but the resulting agglutination patterns are incomplete, and the reaction cannot be observed at 37C. In contrast, the vibrios which adhere to brush borders produce strong clumping reactions - of a type which in our experience is only observed with strains of the El Tor biotype. The haemagglutinating potential associated with the BBA also suggests that this adhesin is discrete from that which mediates attachment in our systems.

Finally, data published by Freter's group in relation to their brush border system also support the concept of a two-adhesin mechanism of attachment. Thus, although denaturation of the BBA resulted in a five-fold reduction in the capacity of motile vibrios to bind to brush borders (Jones et al, 1976), their residual adherence potential was still six times greater than that of non-motile mutants of the same strain (approximately 2.2 organisms per brush border (Jones et al, 1976), compared to an average of 0.36 organisms per brush

border for six non-motile mutants under the same conditions (Jones and Freter, 1976)) .

Thus the evidence suggests that there might be two different structures capable of mediating the attachment of V. cholerae, and it seemed important for our better understanding of the adherence phenomenon to investigate this possibility. If the existence of two discrete adhesins could indeed be confirmed, and if their relative significance could perhaps be linked to the nature of the substrate, then it might be possible to explain the different observations arising from the various adherence systems.

5.4.2 Influence of growth conditions on development of haemagglutinating capacity.

The properties of the BBA reminded the author of the slime agglutinin (SA) described by Lankford's group (see Section 1.2.4 (ii)), and further inspection revealed a close similarity between the two moieties. Both confer upon vibrios a capacity for haemagglutination (HA), and they are produced under similar cultural conditions. Neither structure is associated with organisms harvested from solid growth media, and both are denatured upon prolonged incubation at 37C - resulting in the detachment of the vibrios from their substrates (Lankford and Legsomburana, 1965; Jones et al, 1976).

Thus it seemed possible that the BBA and the SA are actually different descriptions of the same moiety. This being the case, the "exacting" cultural conditions required for the development of the SA (Bales and Lankford, 1961) offer an explanation for our inability to detect an adhesin with the characteristics of the BBA (Section 5.3.1). Our strains are routinely grown in nutrient broth (NB), whereas TSB was used for the production of the SA (Bales and Lankford, 1961) and the BBA (Jones et al, 1976).

It was therefore decided to compare the capacities of these two media to support the development of the SA by V. cholerae 569B, using the bacterial HA assay (see Section 3.5.3) to monitor the presence of this moiety.

The effect of incubation temperature was also examined, since 25C was recommended for production of the SA (Bales and Lankford, 1961), whereas the BBA was expressed by vibrios grown at 37C (Jones et al, 1976). Finally, it was of interest to use both motile and non-motile forms of V. cholerae 569B, in an attempt to resolve a more significant inconsistency arising from the studies of the two groups. Thus, whereas the BBA was reported to be associated with the vibrio flagellum (Jones and Freter, 1976), the SA appeared to cover the vibrio surface (Lankford and Legsomburana, 1965; Chulasamaya and Lankford, 1970) and furthermore "aflagellate mutants of V. cholerae and El Tor retained their haemagglutininability" (Sweet, 1963 - cited by Lankford and Legsomburana, 1965).

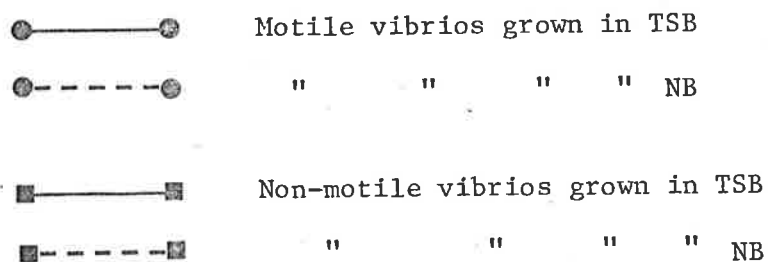
Accordingly, the motile and non-motile forms of V. cholerae 569B were grown in shaking cultures in NB or TSB at 25C or 37C. At various stages of the growth cycle, cultures of each type were tested for their capacities to agglutinate a suspension of guinea-pig erythrocytes (see Section 3.5.3). For those vibrios grown at 37C, the HA assay was also performed at this temperature; for those grown at 25C, the assay was performed at room temperature (20-25C). The development of HA capacity under the various cultural conditions is shown in Figure 5.2, which represents the combined data from three experiments.

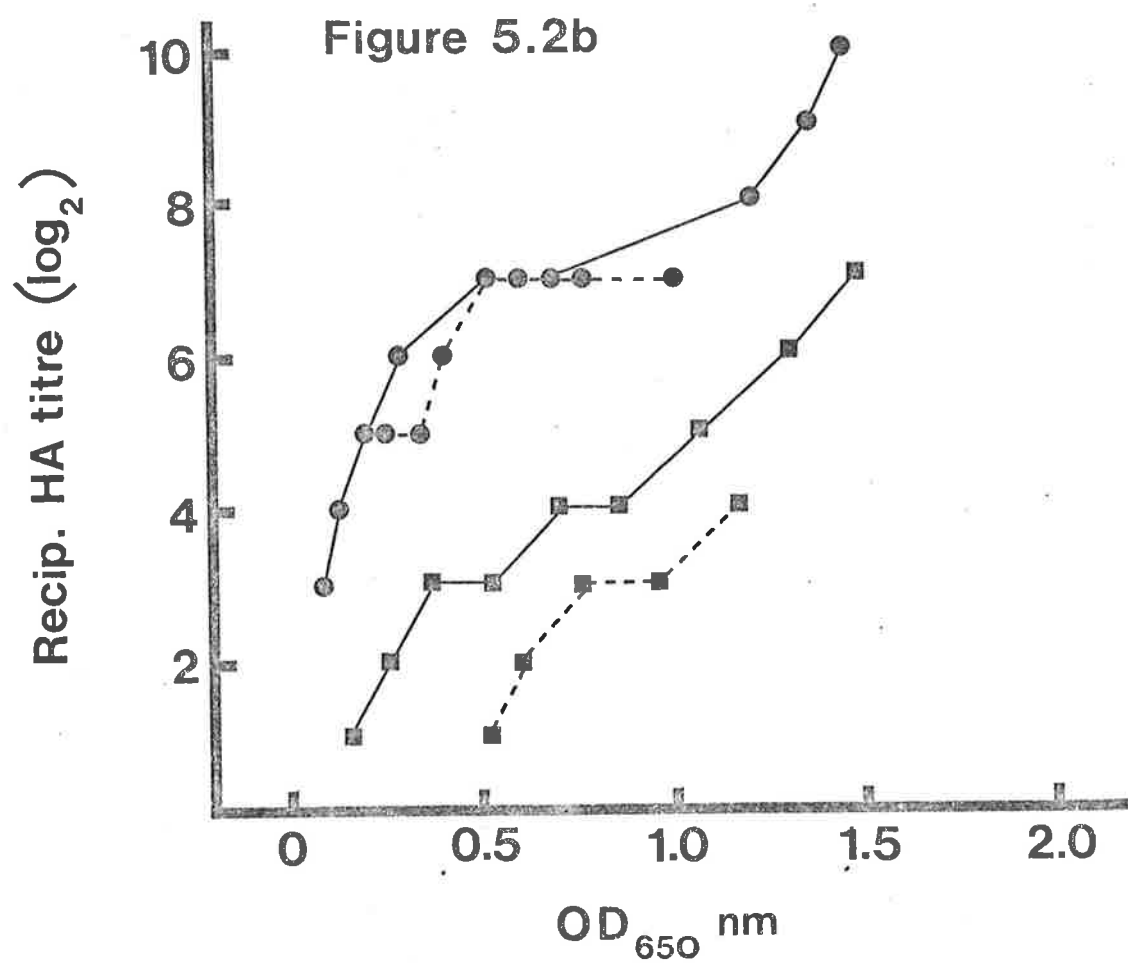
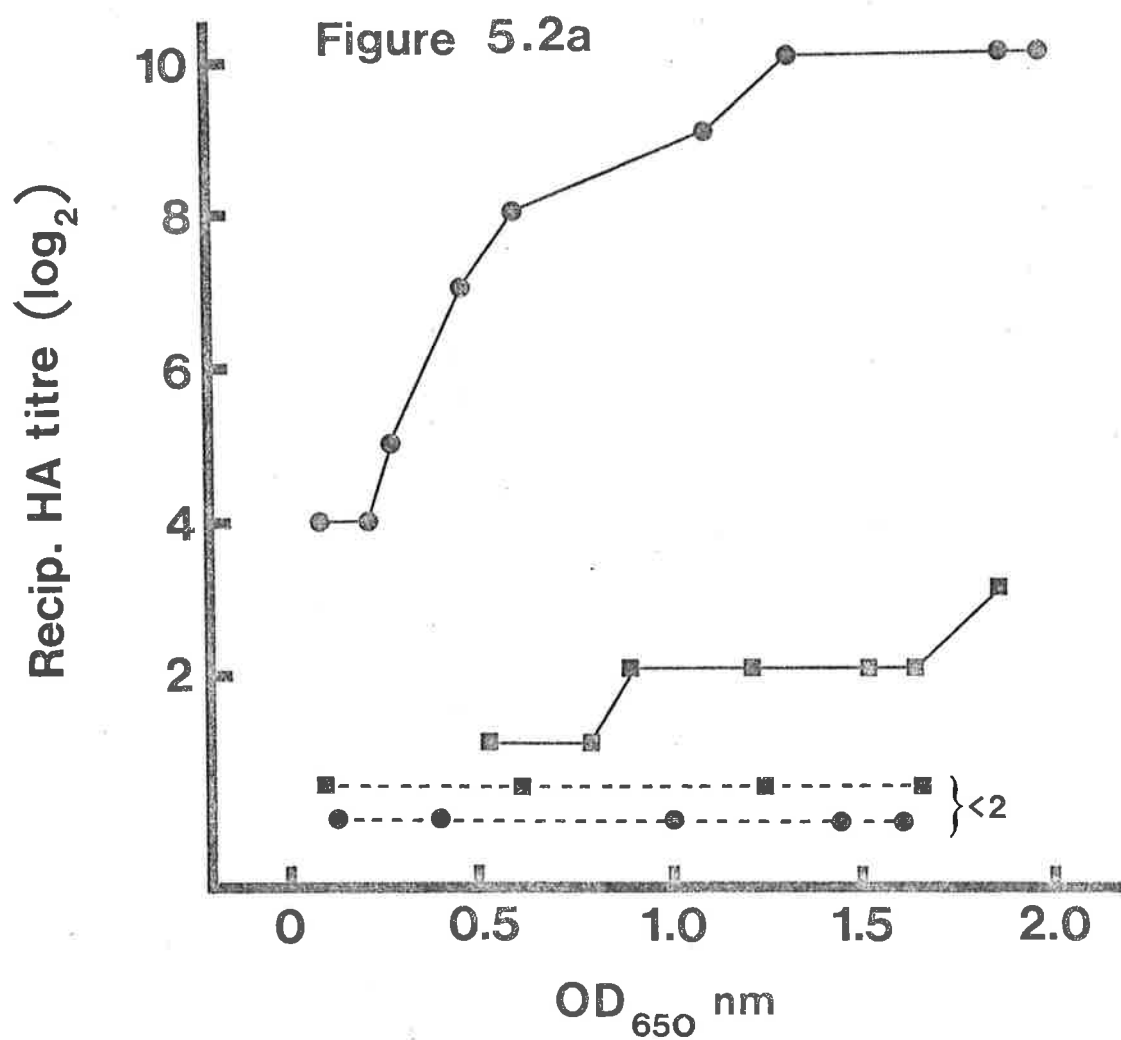
First let us consider the results obtained with the motile variant of V. cholerae 569B. Those organisms grown in TSB - whether at 25C (after Bales and Lankford, 1961) or at 37C (after Jones et al, 1976) - subsequently displayed a capacity to strongly agglutinate guinea-pig erythrocytes. The HA reactions observed were clearly of a different type from those previously obtained with the strip-passaged variant of this same strain (see Section 5.2.1), in that they were more rapid, more complete, stable at 37C, and could be titrated an additional five wells. Apparently then, the vibrios have produced an additional adherence factor as a result of their growth in TSB, and this moiety is presumably the same as Lankford's SA and Freter's BBA. It is clear that

FIGURE 5.2

The influence of cultural conditions upon the development of
a haemagglutinating capacity by motile and non-motile
variants of *V. cholerae* 569B.

Figure 5.2a shows the results of growth in either
Trypticase soy broth (TSB) or nutrient broth (NB) at 37C,
while Figure 5.2b shows the results obtained using
the same media at 25C. For both figures -





even our limited study has confirmed Lankford's observation that cultural conditions are of paramount importance for the production of the cell-bound haemagglutinin (Bales and Lankford, 1961). Thus, although at 25C NB was also able to support the development of the SA (Figure 5.2b); this moiety was evidently denatured (or lost from the cell surface) at 37C, so that organisms grown under the latter conditions possessed no HA potential (Figure 5.2a). These results therefore strengthened the suspicion that our inability to detect a BBA-like moiety in our systems of adherence was due to the fact that the vibrios being studied were grown under unsuitable cultural conditions.

The development of HA activity by non-motile V. cholerae 569B was also investigated, in the hope of determining whether the SA is associated with the vibrio flagellum, as postulated by Freter's group, or whether these two structures can be synthesized independently, as suggested by Lankford's work (see above). The former group routinely grew their strains in TSB at 37C (Jones and Freter, 1976), and it is apparent from Figure 5.2a that under these conditions there is indeed almost a qualitative difference in the capacities of motile and non-motile organisms to produce the SA. However the results shown in Figure 5.2b indicate that at 25C this moiety can also be produced by non-motile vibrios⁸, and is therefore not dependent upon the presence of a flagellum. Nevertheless it seems likely that the latter structure in some way stabilizes the SA on the surface of the cell. The HA capacities of the non-motile vibrios grown at 25C were consistently 3-4 wells weaker than those of motile organisms grown under the same conditions, and, when both variants were grown in TSB at 37C, this difference was 7-8 wells (see Figure 5.2).

Subsequent experiments demonstrated that neither the motile nor the non-motile forms of V. cholerae 111 and V. cholerae SC198 were able to produce the SA under any of the growth conditions described above. Thus our results

⁸ These haemagglutinating vibrios retained their non-motile status.

have not provided unqualified support for either Freter's or Lankford's findings (see above), but suggest that the vibrio flagellum is neither necessary nor sufficient for the production of the SA.

5.4.3 The role of the slime agglutinin in adherence.

The experiments described above demonstrated that V. cholerae 569B can develop the SA under suitable cultural conditions, and it was then of interest to determine whether this moiety would provide the organisms with an additional binding capacity. Previous experience (Section 5.3.1; also see Section 5.4.1) suggested that the characteristics of the attachment process might depend upon the nature of the substrate, and so it seemed necessary to evaluate the significance of the SA in both of our systems of adherence. In principle, this would involve growing motile and non-motile V. cholerae 569B under conditions which would either favour or inhibit the production of the SA, and comparing the adherence capacities of the resulting cultures.

For convenience, motile and non-motile organisms will be referred to as F+ and F- in the following discussion. These terms will denote the presence or absence of the flagellar structure and its associated adhesin. Similarly, the notations SA+ and SA- will signify the presence or absence of the slime agglutinin.

(i) Attachment to intestinal tissue.

Non-motile V. cholerae 569B was grown either in NB at 37C (to obtain F- SA- organisms - see Figure 5.2) or in TSB at 25C (F- SA+), while the strip-passaged variant of this strain was grown in NB at 37C (F+ SA-) or in TSB at 37C (F+ SA+). The motility of the resulting cultures was examined microscopically, and HA assays were used to ascertain the presence of the slime agglutinin. All cultures were then tested for their capacities to attach to everted intestinal segments in the standard in vitro assay - with the single modification that the incubation was performed at 25C instead of 37C. This departure from

the normal procedure was necessitated by the finding that the SA produced by non-motile V. cholerae 569B is apparently destroyed at 37C (see Figure 5.2). However the lower incubation temperature might also stabilize the SA associated with the motile vibrios, since Freter's group reported that their BBA was also denatured upon prolonged incubation in buffer at 37C (Jones et al, 1976).

The results of these adherence assays are shown in Table 5.7, and suggest that the SA plays no significant role in the process of attachment to intestinal tissue. Although the HA assays confirmed that both motile and non-motile organisms developed the SA when grown under suitable conditions, the presence of this moiety did not significantly increase the adherence capacity of either variant. These findings are consistent with the report from Freter's group that denaturation of the BBA did not diminish the capacity of vibrios to bind to slices of rabbit ileum (Freter and Jones, 1976). In contrast, the presence of a functional flagellum provided a 30-fold increase in binding potential, as previously demonstrated (Section 4.4.2).

It is interesting to note that, following growth in NB at 37C, the HA capacity displayed by motile V. cholerae 569B depends upon the temperature at which the assay is performed. Although these organisms are unable to effect HA at 37C (see Figure 5.2), they can do so at 25C (see Table 5.6), suggesting that during incubation at the lower temperature they have been able to develop the SA. The fact that these vibrios have little pre-existing SA at the time of harvesting probably underlies the finding that, by comparison with organisms grown in TSB, they subsequently display a relatively weak HA capacity (HA titres of 32 (Table 5.1a) or 64 (Table 5.7) compared to 1024 observed following growth in TSB (Figure 5.2)).

It seemed equally likely that the F+ SA- organisms originally added to the intestinal segments would also have developed some SA during their subsequent incubation at 25C. This might have resulted in an underestimation of the contribution of this moiety to attachment, and so it seemed necessary

TABLE 5.7

The role of the slime agglutinin (SA)
in adherence to intestinal tissue.

Variant of V. cholerae 569B

Growth conditions^a Non-motile : F- Strip-passaged : F+

Inhibitory for SA : SA-	0.35 ^b	(8) ^c	10.3	(64)
Conducive to SA : SA+	0.41	(64)	12.8	(512)

a See text.

b Figures show percentage adherence, calculated in the normal manner.

c Figures in parentheses show haemagglutination titres of the various cultures (see Text). These were determined at room temperature ($\approx 25^{\circ}\text{C}$).

to repeat the adherence assay in such a way as to ensure the continued SA- or SA+ status of the motile vibrios⁹. Accordingly, motile organisms grown in NB at 37C were subsequently incubated with intestinal segments in NB at 37C, to prevent the development of SA during the course of the assay. Similarly, the vibrios grown in TSB at 37C were tested for adherence under the same conditions, to prevent any denaturation of the SA (as might previously have occurred during incubation in buffer). Despite these modifications however, the SA- and SA+ organisms still displayed similar adherence capacities, confirming the notion that this factor plays no significant role in attachment to intestinal tissue in vitro.

(ii) Attachment to inert particles.

It was then of interest to determine whether the slime agglutinin might have more significance with respect to attachment to inert substrates. Once again the baseline for adherence was provided by non-motile V.cholerae 569B grown in NB at 37C (F- SA-), and the binding capacity of these organisms was compared with that displayed following growth in TSB at 25C (F- SA+). The bead-passaged variants of V. cholerae 569B were also examined, and these were grown at 37C in either NB (F+ SA-) or TSB (F+ SA+). All cultures were tested for adherence in the normal manner (see Section 3.5.4), and the results of these assays are shown in Table 5.8.

It soon became apparent that the SA dramatically improved the capacity of organisms to adhere in this system, and it therefore seemed important to ensure that the F+ SA- vibrios retained their SA- status during the course of the assay (see discussion above). Accordingly the motile variants were also tested for adherence using the modified incubation conditions described above. Instead of incubating at 25C in buffer, the F+ SA- vibrios were assayed

⁹ Similar precautions did not seem justified for non-motile V. cholerae 569B, however, since these organisms produce much less SA at 25C than the motile vibrios (see Figure 5.2 and Table 5.7).

TABLE 5.8

The role of the slime agglutinin (SA)
in adherence in inert particles.

Table 5.8a Adherence to Ballotini beads.

Variant of V. cholerae 569B

Growth conditions^a Non-motile : F- Ballotini-passaged : F+

Inhibitory for SA : SA-	0.012 ^b	0.40	(0.036) ^c
Conducive to SA : SA+	0.27	3.1	(1.6)

Table 5.8b Adherence to Pevikon beads.

Variant of V. cholerae 569B

Growth conditions^a Non-motile : F- Pevikon-passaged : F+

Inhibitory for SA : SA-	0.037 ^b	7.1	(3.6) ^c
Conducive to SA : SA+	2.6	17	(15)

a See text.

b Figures show percentage adherence, calculated in the normal manner.

c Figures in parentheses show percentage adherence under modified incubation conditions - see text.

in NB at 37C, while the F+ SA+ organisms were assayed in TSB at 37C. The estimates of adherence obtained under such conditions are given in parentheses in Table 5.8.

Before considering the implications of these data, it should be stated that in all cases HA tests confirmed the anticipated SA status of the cultures. It is also apparent that the capacities of the bead-passaged variants to bind to their respective substrates do indeed depend upon the incubation conditions employed (see Table 5.8). In particular, the estimate of adherence obtained with the F+ SA- organisms of the Ballotini-passaged variant was ten-fold higher at 25C than at 37C, confirming our suspicion that incubation at the lower temperature permits the development of the SA. When evaluating the contribution of this moiety to attachment, it would therefore be more appropriate to use the estimates shown in parentheses in Table 5.8.

The results obtained with both types of inert particle suggest that the slime agglutinin plays an important role in attachment to such substrates. Indeed, in contrast to the data obtained with intestinal tissue, the evidence indicates that the SA can be of much greater significance than the adhesin carried by the vibrio flagellum. Thus, with respect to attachment to Ballotini beads, the presence of the latter moiety provided a 3-fold increase in the adherence potential of SA- vibrios, and a 6-fold increase in the case of SA+ organisms. The development of the SA, however, resulted in a 22-fold greater adherence capacity for F- vibrios, and a 44-fold increase for F+ organisms (see Table 5.8a).

Slightly different results were obtained with Pevikon beads, for the data indicate that both adhesins are of similar significance in attachment to this substrate. The addition of either the flagellar adhesin or the SA to F- SA- organisms dramatically improved their binding capacity (100-fold or 70-fold respectively - see Table 5.8b). The additional presence of the second adhesin then provided a smaller, although significant, increase in adherence (4-fold or 6-fold - see Table 5.8b).

Thus our studies with erythrocytes and inert particles, in addition to previous work with isolated brush borders (Jones and Freter, 1976), suggest that the slime agglutinin plays an important role in the attachment of V. cholerae to non-mucosal substrates. The possible pathogenic relevance of this moiety will be discussed in Chapter 7.

5.4.4 Other studies with the slime agglutinin.

Before completing our study of the slime agglutinin, several other properties of this structure were of interest. Firstly, it seemed worthwhile to examine the sensitivity of SA-mediated attachment to inhibition by L-fucose. Jones and Freter (1976) reported that the (BBA- or SA-mediated) binding of V. cholerae to brush borders could be partially (60%) inhibited by the presence of L-fucose in the suspending medium, and suggested that this sugar might constitute part of a natural host receptor structure.

Accordingly, it was decided to test the effect of L-fucose (at 10mg/ml suspending medium, after Jones and Freter, 1976) in two systems where SA-mediated attachment is thought to be important - bacterial haemagglutination (see Section 5.4.2) and adherence to inert particles (see Section 5.4.3(ii)). In the former assay, the presence of L-fucose had no effect upon the capacity of F+ SA+ V. cholerae 569B to agglutinate either guinea-pig or human (group O) erythrocytes. Similarly, L-fucose did not inhibit the binding of F-SA+ V. cholerae 569B to Pevikon particles, and had only a slight (30%) inhibitory effect on attachment of the same organisms to Ballotini beads.

Secondly, it was of interest to determine whether the SA could mediate attachment to the serosal surface of adult mouse small intestine. Previous experiments have demonstrated that V. cholerae strains of El Tor biotype attach more efficiently to a variety of non-mucosal substrates, including serosal intestinal tissue (see Table 4.A.1), isolated epithelial cells (Table 4.A.2), inert particles (Table 5.1.b) and erythrocytes (see footnotes 3 and 4 of this chapter, and Section 5.4.1). It seemed probable

that this increased binding capacity was a reflection of the constitutive expression of SA by the El Tor strains - despite their routine growth in NB at 37C¹⁰. This view is supported by the findings that the presence of the SA conferred upon Classical vibrios the superior haemagglutinating and inert particle-binding capacities characteristic of El Tor organisms (see Sections 5.4.2 and 5.4.3(ii)).

Thus motile V. cholerae 569B was grown in TSB at 37C and then tested for attachment to both the mucosal and serosal surfaces of murine intestinal tissue. The procedure employed has been previously described (see Section 4.A), with the single modification that the intestinal segments were suspended in TSB rather than buffer, to ensure that the vibrios retained their SA+ status. It was found that 12.0% of the organisms attached to the mucosal surfaces, and 14.7% to the serosal surfaces, thus again demonstrating the significance of the SA in adherence to non-mucosal substrates.

Finally, in an attempt to assess the likely in vivo significance of the SA, it was decided to compare the virulence of the SA+ and SA- forms of V. cholerae 569B in infant mice. The non-motile form of this strain was used for this comparison, as it seemed likely that any contribution of the SA to virulence might be more readily detectable in the absence of the flagellar adhesin (since the latter has itself been found to significantly increase the virulence of V. cholerae 569B - see Section 4.4.3). However no difference in virulence was observed when the F- SA+ and F- SA- organisms were compared in the same batch of infant mice (48-hour LD₅₀ values of 1.0×10^7 and 1.5×10^7 respectively). This result is consistent with the finding that the SA is unable to promote attachment to intestinal tissue in vitro (see Section 5.4.3(i)).

¹⁰ Lankford and Legsomburana (1965) reported that the SA is much more stable on the surfaces of El Tor vibrios than on Classical vibrios, and is also less susceptible to denaturation by temperatures above 30C when associated with the former.

5.5 Summary.

The experiments described in this chapter were aimed at improving our understanding of the adherence phenomenon in V. cholerae. The data obtained do not support the theory that these organisms attach to specific host receptor structures, but rather suggest that their adherence mechanisms may lack substrate specificity. Thus, vibrios passaged on inert particles in vitro also acquired the capacity to attach to intestinal tissue, and vice versa.

With respect to the bacterial factors which mediate adherence, these experiments have confirmed the involvement of the vibrio flagellum. Using specific antibodies it has proved possible to independently demonstrate both motility and attachment capacities associated with this structure. Since motility per se was found to be neither necessary nor sufficient for in vitro adherence, this work has reinforced our concept of the flagellum as the carrier of an important vibrio adhesin.

Finally, our studies have confirmed previous reports that, under appropriate cultural conditions, V. cholerae can produce a labile surface haemagglutinin. In contrast to the flagellar adhesin, this factor did not promote adherence to intestinal tissue, but could mediate attachment to several non-mucosal substrates. Its possible pathogenic significance remains uncertain.

C H A P T E R 6

THE PROPHYLACTIC SIGNIFICANCE OF THE NON-LPS

ANTIGENS OF V. CHOLERAEE.

6.1 Introduction.

Most of the cholera vaccines tested to date have consisted of bacterial suspensions which have been inactivated by phenol-, formalin- or heat-treatment (see Joo, 1974). Despite the resistance of LPS antigens to such procedures, these vaccines have failed to provide an effective level of protection in the field, and immunization is not at present a significant measure in cholera control. However studies with human volunteers who had recovered from an induced choleraic infection demonstrated that it is possible to stimulate the immune system to provide complete immunity to this disease for at least a year (Cash et al, 1974). If this could be achieved with a non-viable vaccine in the field, immunization might then significantly accelerate the eradication of cholera, by preventing the infection and multiplication of V. cholerae within its natural host (see Section 1.1.4).

Many bacterial pathogens possess proteinaceous virulence determinants (see Section 2.5), and several of these have been isolated and successfully employed as prophylactic immunogens (Section 2.5.2, and footnote 3 in Chapter 2; also see Chandler and Gulasekharam, 1974). Since such structures would not possess the chemical stability of LPS antigens, it is likely that they would be denatured by the procedures used to inactivate cholera vaccines. In view of the disappointing results obtained with the latter, it seemed worthwhile to investigate the prophylactic potential of the non-LPS determinants of V. cholerae. There seemed several reasons to be optimistic in this regard.

Firstly, the finding that live vibrios were more effective than heated ones in the active mouse protection test (Feeley, 1970; also see Section 1.3.7) is consistent with the notion that the former possess heat-labile

determinants capable of eliciting the production of protective antibodies. Secondly, previous work in this department has also suggested the existence of non-LPS protective antigens in V. cholerae. Neoh and Rowley (1972) reported that a mouse antiserum prepared against V. cholerae 569B contained protective antibodies which could not be removed by repeated absorption with highly purified homologous LPS, and subsequently an IgG fraction from a similar rabbit antiserum was shown to contain protective antibodies specific for heat-labile, non-LPS determinants (Bellamy et al, 1975). Finally, the experiments described in the preceding chapters suggest that V. cholerae possesses at least two factors which mediate adherence; both of these seem likely to be non-LPS in nature¹, and at least one - the flagellar adhesin - contributes to virulence. By analogy with the adherence structures of other pathogenic bacteria, it seems possible that this moiety may prove to be of prophylactic significance by virtue of its capacity to elicit antibodies capable of inhibiting colonization (see Section 5.3.2).

Thus the non-LPS antigens of V. cholerae would seem to warrant more detailed investigation. However before scientists attempt to define and isolate these moieties it is important to ascertain whether this is likely to be a sufficiently rewarding line of research. Accordingly, the aim of this chapter is to assess the prophylactic potential of the non-LPS determinants. Initially, based on the work of our previous colleagues, two experimental approaches will be adopted in an attempt to confirm the existence of non-LPS protective antigens in V. cholerae. Should this prove successful, it would be of interest to examine the nature and distribution of such determinants, and to consider the mechanisms by which their specific antibodies might mediate protection. Our ultimate aim would then be to evaluate the relative prophylactic significance

¹ The experiments described in Section 5.3.2 suggest that the flagellar adhesin is more likely to be a non-LPS moiety, while the slime agglutinin is primarily (if not completely) proteinaceous (Lankford and Legsomburana, 1965).

of the LPS and non-LPS antigens of V. cholerae, by comparing the protective efficiencies of their specific antibodies. All of these studies will be performed using highly motile strains.

6.2 Confirmation of the Existence of non-LPS Protective Antigens in V. cholerae. I. The presence of protective antibodies specific for non-LPS determinants in an antiserum prepared against live V. cholerae 569B.

Our first approach involved the repeated absorption of a mouse antiserum prepared against live V. cholerae 569B with highly purified (see Section 3.4) homologous LPS (after Neoh and Rowley, 1972). Each of 8 absorptions was performed by the addition of LPS to a final concentration of 100µg/ml, incubation at 37C for 60 minutes (except the 4th and 8th absorptions, which were incubated at 4C overnight to remove less avid antibodies), and then ultracentrifugation (100,000g at 4C for 60 minutes) to deposit the LPS. Aliquots of serum were set aside after each stage, and the effects of this absorption programme on various antibacterial capacities of the original serum are summarized in Table 6.1a.

It is apparent that, despite the rapid and complete loss of haemagglutinating activity, the serum has retained a significant bactericidal potential at the end of the absorption programme (≈5% of the original activity, since the last two absorptions appear to have been superfluous). In addition, the residual serum possesses about 20% of the original protective capacity. Thus these data confirm previous reports that the non-LPS antigens of V. cholerae 569B are able to elicit the production of bactericidal (Neoh and Rowley, 1970) and protective (Neoh and Rowley, 1972) antibodies.

It is interesting to note that the ratio between the bactericidal and protective capacities of the serum fell 4-fold as a result of the absorption programme (see Table 6.1a). This disproportionate loss of bactericidal activity might be a consequence of the selective removal of antibodies which mediate

TABLE 6.1

The effects of repeated absorption with purified 569B-LPS on various antibacterial capacities of a mouse antiserum prepared against live V. cholerae 569B.

- a Haemagglutination (HA), bactericidal (Bact.), and protection (Prot.) assays were performed as described in Sections 3.6.1, 3.6.3 and 3.7.3. Each endpoint represents the mean of two determinations.
- b ND - not determined.
- c ME - mercaptoethanol; see Section 3.6.3.
- d These preparations were kindly supplied by Dr. D. Horsfall of this department; their purification has been described (Horsfall and Rowley, 1979).

Table 6.1a Removal of antibacterial capacities.

Activities^a vs V. cholerae 569B

No. of LPS absorptions	HA	Bact.	Prot.	<u>Bact.</u> <u>Prot.</u>
0 (original serum)	256	104,000	1,300	80
2	<2	28,000	ND ^b	ND
4	<2	10,000	ND	ND
6	<2	5,000	ND	ND
8 (residual serum)	<2	4,600	250	18

Table 6.1b Selective removal of ME^c-sensitive bactericidal activity.

Bactericidal activity vs. V. cholerae 569B

Source of antibody	- ME	+ ME
Original anti-569B serum	104,000	1,900
Residual anti-569B serum	4,600	2,000
IgM vs 569B-LPS ^d	600,000	<100
IgG vs 569B-LPS ^d	2,500	1,000

bacteriolysis more effectively than protection (i.e. IgM antibodies - see Steele et al, 1974; Horsfall and Rowley, 1979; Section 6.7.2). Alternatively, the lower bactericidal:protection ratio observed with the residual serum might reflect the fact that the antibodies in this serum are directed against surface determinants which are not effective substrates for a lytic reaction (e.g. bacterial appendages).

It was decided to examine the likelihood of the former explanation by comparing the mercaptoethanol (ME - see Section 3.6.3) sensitivities of the bactericidal antibodies present in the original and residual sera. Purified IgM and IgG antibodies, specific for 569B-LPS², were included as controls for the specificity of ME-reduction, and the results of this assay are given in Table 6.1b.

These data clearly show that the LPS absorptions have selectively removed the ME-sensitive (IgM) antibodies, while the ME-resistant (IgG) activity has been completely retained. They readily explain the fall in bactericidal:protection ratio, and suggest that it is meaningless to compare sera on such a basis unless the antibodies responsible for the antibacterial capacities of the different sera are known to be of the same class (also see Neoh and Rowley, 1972). In the present instance, the bactericidal capacity of the unabsorbed serum is mainly attributable to its content of IgM antibodies, whereas that of the residual serum is primarily (and, by comparison with the effect of ME-treatment on the control preparation of IgG, perhaps completely) mediated by antibodies of the IgG class.

The results also suggest a correlation between antibody specificity and class, since the LPS absorptions have removed most if not all of the IgM antibodies, while the IgG antibodies appear to be exclusively directed against non-LPS antigens (see Table 6.1b). If confirmed, such a correlation would have important implications for the practice of parenteral immunization against

² These preparations were kindly supplied by Dr. D. Horsfall of this department.

enteric disease. As discussed previously (Section 1.3.7), those parenteral vaccines which elicit the highest levels of IgG protective antibodies are likely to be the most effective, since immunoglobulins of this class are selectively transmitted to the gut.

To further investigate the possible correlation between antibody specificity and class, it was decided to examine the effects of ME on the bactericidal capacities of several other mouse antisera. These included three hyperimmune sera collected following the repeated intraperitoneal injection of either live V. cholerae 569B (with mice receiving doses which gradually increased from 10^7 to 10^9 organisms), or LPS preparations obtained from either this strain or V. cholerae 017 (doses from 5-50 μ g). All sera were assayed for bactericidal activity against organisms of the homologous strain, both in the presence and absence of ME. The resulting titres are shown in Table 6.2, which includes the antiserum used in the previous experiments for purposes of comparison. Each figure represents the mean of two determinations, and IgM and IgG controls again confirmed the specificity of ME-reduction.

Two main conclusions can be drawn from these results. Firstly, it is clear that purified LPS can elicit (bactericidal) antibodies of the IgG class. Indeed, allowing for the facts that (i) mouse IgM antibodies specific for V. cholerae LPS mediate bacteriolysis about 120 times more efficiently than similar IgG antibodies (Horsfall and Rowley, 1979; Table 6.11b); and that (ii) 0.2M ME has a slight denaturing effect on IgG antibodies which is likely to lead to a 2-fold underestimation of their actual concentration (see Tables 6.1b and 6.11a); then the average weight ratio of IgM:IgG antibodies in the two sera prepared against isolated LPS is 1.6:1.

Secondly, those sera raised against live vibrios contain much higher levels of ME-resistant antibodies than those prepared against LPS (see Table 6.2). The proportion of bactericidal activity which resides in the IgG class appears to increase with the intensity of immunization, and reaches 6.7% in the hyperimmune anti-live 569B serum. Allowing for (i) and (ii)

TABLE 6.2

Mercaptoethanol - resistance of the bactericidal
antibodies present in several mouse antisera.

Bactericidal activity^a

Specificity of antiserum	- ME ^b	+ ME	% ME - resistant
Live <u>V. cholerae</u> 569B	1.0×10^5	1.9×10^3	1.8
Live <u>V. cholerae</u> 017	3.9×10^5	9.0×10^3	2.3
Live <u>V. cholerae</u> 569B (hyperimmune)	2.4×10^6	1.6×10^5	6.7
Purified 569B-LPS	5.2×10^6	1.2×10^4	0.23
Purified 017-LPS	6.4×10^6	1.9×10^4	0.30

a Each serum was assayed for bactericidal activity against organisms of the homologous (immunizing) strain.

b ME - mercaptoethanol.

above, this figure suggests an IgM:IgG weight ratio of 1:16. As mentioned above, the relative concentration of IgG antibodies evoked by a parenteral vaccine is likely to be a prime determinant of its effectiveness, and the possible implications of these results will be discussed later (see Chapter 7).

6.3 Confirmation of the Existence of non-LPS Protective Antigens in *V. cholerae*. II. Studies with the hybrid vibrio strain 569B/165.

The second approach employed in our attempt to confirm the existence of non-LPS protective antigens in *V. cholerae* involved the use of a hybrid bacterial strain 569B/165. This strain was one of several hybrids constructed by Bhaskaran by conjugating true *V. cholerae* strains with a non-cholera vibrio, NCV-165 (see Bhaskaran, 1971). The conjugation process was arranged such that the NCV-165 genes encoding LPS production were used to replace those in the corresponding region of the *V. cholerae* genome. Consequently, each hybrid strain possesses the LPS determinants of NCV-165, but retains all of the non-LPS antigens of its respective *V. cholerae* parent.

These hybrids were originally constructed in order to be able to evaluate the relative significance of antibacterial and antitoxic immunities to *V. cholerae* (see Sinha and Bhaskaran, 1973). However it is apparent that the capacity of a given hybrid to elicit the production of antibodies capable of mediating protection against its *V. cholerae* parent would offer a means of assessing the prophylactic potential of their common non-LPS antigens. This approach was prompted by an earlier finding that absorptions with live 569B/165 organisms could remove the remaining protective activity from a residual anti-live 569B serum similar to the LPS-absorbed serum discussed in the previous section³ (see Bellamy et al, 1975).

³ In contrast, Sinha and Bhaskaran (1973) had been unable to demonstrate any immunity to *V. cholerae* (using a rabbit ileal loop model) following immunization with the related hybrid strain. The likely explanation for the difference between these reports became apparent later (see Section 6.4.2, and discussion in Chapter 7).

6.3.1 Initial demonstration of non-LPS protective antigens in 569B/165.

Several groups have demonstrated that immunity to experimental cholera can be passively transferred from immunized mothers to their suckling young (Ujjiye and Kobari, 1970; Pitkin and Actor, 1971; Chaicumpa and Rowley, 1972; Guentzel and Berry, 1974), and a similar experimental design was adopted for our⁴ initial studies with the hybrid vibrio 569B/165. Four groups of female LAC mice were immunized with either live or boiled (see Bellamy et al, 1975) suspensions of V. cholerae 569B or 569B/165, while a fifth group received injections of saline and served as controls. During the course of the immunization programme - which is detailed in the legend to Table 6.3 - each group was divided into lots of 4 mice, which were then separately mated by the introduction of a single LAC male mouse. The resulting offspring were collected at 5-6 days of age, and challenged with 10-20 LD₅₀ doses of V. cholerae 569B (see Section 3.7). The survival data from these assays are given in Table 6.3.

Initially, each female mouse was given 6 or 7 injections of vibrios (Expt. 1 in Table 6.3), but a repeat experiment demonstrated that similar results could be obtained following only 3 immunizations (Expt. 2 in Table 6.3). Accordingly the data from the two experiments have been pooled, to obtain a mean estimate of the percentage survival of offspring within each group (see Table 6.3). In addition, to provide some indication as to the responses evoked by the immunization regimes, the maternal sera from Expt. 1 were assayed for various antibacterial activities against the V. cholerae 569B challenge strain, and some of these data are also given.

Several conclusions can be drawn from these results. Firstly, it is evident that the passive transfer of immunity from mother to young has again been demonstrated. Those females immunized with live V. cholerae 569B

⁴ The experiments described in Section 6.3.1 were performed in association with Dr. E. Steele, formerly of this department.

TABLE 6.3

Passive transfer of immunity to *V. cholerae* 569B.

- a Sera were collected from the mothers used in Exp't. 1, and individually assayed for their bactericidal (Bact.) and agglutinating (Aggl.) capacities against (live) *V. cholerae* 569B (see Sections 3.6.3 and 3.6.2). Each figure represents the geometric mean titre of 6 - 10 samples.
- b The mice in groups 1 - 4 received intraperitoneal injections of saline suspensions of live or boiled vibrios, while those in group 5 received saline alone and served as controls. The immunization schedules were as follows (mice were mated on day 0) -
- Expt. 1 : mice received 5×10^7 vibrios on days -23 and -16;
 10^8 vibrios on days -9 and -3; and 2×10^8
vibrios on days +7 and +14 (and also +25 for a few mice who were still to litter).
- Expt. 2 : mice received 5×10^7 vibrios on day -7;
 10^8 vibrios on day 0; and 2×10^8 vibrios on day +7.
- c Ratios show the no. of surviving mice/total no. of mice challenged (with 10-20 LD₅₀ doses of *V. cholerae* 569B).

TABLE 6.3

Passive transfer of immunity to *V. cholerae* 569B : protection of suckling mice
by maternal antibodies directed against heat-stable or heat-labile antigens.

Immunization group ^b	Activities of maternal sera ^a		Survival of suckling mice			
	Bact.	Aggl.	Expt. 1	Expt. 2	Total	%
# 1 : Live <i>V. cholerae</i> 569B	1.8×10^6	3,900	7/8 ^c	33/36	40/44	91
# 2 : Boiled <i>V. cholerae</i> 569B	1.6×10^6	420	34/34	23/29	57/63	90
# 3 : Live 569B/165	1.4×10^4	1,300	54/59	32/38	86/97	89
# 4 : Boiled 569B/165	<240	<40	0/15	3/22	3/37	8
# 5 : Saline	<200	<40	3/50	17/149	20/199	10

developed high serum activities against this strain, and obviously supplied protective antibodies to their suckling infants. The data obtained from the mice in Group 2 are similar, and indicate that antibodies specific for the heat stable determinants of 569B are alone sufficient to confer protection against challenge with this strain. The young of the control mothers were not protected, and showed a normal susceptibility to infection (see Table 6.3).

The survival data from Groups 3 and 4 are of particular interest. It is clear that immunization with live 569B/165 organisms has enabled the mice in the former group to supply highly protective antibodies to their suckling young. The sera of these mothers displayed strong agglutinating activities against live V. cholerae 569B, but relatively weak bactericidal capacities (see Table 6.3). In confirmation of the reported relationship between the hybrid strain and its V. cholerae parent, none of the ten maternal sera tested showed any agglutinating capacity for either 569B-LPS-sensitized rbc, or boiled 569B organisms (data not shown, but see Section 6.3.2). Collectively these results demonstrate that the 569B/165 vibrios bear non-LPS antigens able to stimulate the production of antibodies which can mediate protection against infection by organisms of the parental strain.

A comparison of the survival data obtained from groups 3 and 4 suggests that these protective antigens are heat-labile, thus confirming their non-LPS nature (and also the results of Bellamy et al, 1975). The sera of those mice which received injections of boiled 569B/165 vibrios displayed no antibacterial activities against V. cholerae 569B, and the infants reared by these mice were as susceptible to the 569B challenge as the young suckled by control mothers.

6.3.2 Confirmation of the non-LPS, heat-labile nature of the protective antigens of 569B/165.

Although the experiments described above yielded some encouraging data, they did suffer two major disadvantages. Firstly, the measure of

protection provided by such a protocol is necessarily a qualitative one, but it would also be of interest to compare the relative protective capacities of the antibodies elicited by live 569B and live 569B/165. Secondly, it had proved necessary in the above work to relate the protection conferred by the antibodies in the milk with the agglutinating and bactericidal capacities of the serum antibodies, whereas it would be preferable to compare the various anti-bacterial activities of the one source of material.

Therefore, in an attempt to rectify these shortcomings and confirm the conclusions made above, it was decided to prepare hyperimmune antisera to live 569B/165 and live V. cholerae 569B. These sera were raised in parallel by the repeated immunization of adult mice (with doses increasing from 10^7 to 10^9 organisms, given intraperitoneally). After collection, each serum was tested for various anti-bacterial capacities, using both of the immunizing strains as indicators. The protective activities of the two sera were also titrated, using the infant mouse protection assay (see Section 3.7.3) with V. cholerae 569B as the challenge strain. The results of these tests are given in Table 6.4, which, for purposes of comparison, also shows the characteristics of a typical antiserum prepared against boiled 569B/165 vibrios.

Firstly, these data confirm the suspected antigenic relationship between the hybrid and its V. cholerae parent. The two sera prepared against live organisms were able to agglutinate vibrios of homologous or heterologous type with similar efficiency, attesting to the close relationship between these strains. However the haemagglutination data, and the inability of either serum to agglutinate boiled heterologous organisms, again indicate that the shared antigens are neither LPS in particular nor heat-stable in general. These points are supported by the finding that sera raised against boiled 569B/165 vibrios do not display any activities against the V. cholerae 569B parent strain (see Table 6.4).

Secondly, the results of the in vivo protection assays confirm that the non-LPS antigens of V. cholerae 569B which are present on the live 569B/165

TABLE 6.4 Antibacterial capacities of mouse antisera prepared against
live *V. cholerae* 569B, or live or boiled 569B/165.

Antibacterial capacities^a

Immunizing strain	Indicator Strain			Agglutination of :		Protection
		HA	Bact.	boiled vibrios	live vibrios	
Live <u>V. cholerae</u> 569B	Homologous	12,800	2.0×10^6	800	12,800	32,000
	Heterologous	<10	800	<40	25,600	—
Live 569B/165	Homologous	6,400	6.0×10^5	1,600	25,600	—
	Heterologous	<10	1,300	<40	12,800	26,000
Boiled 569B/165	Homologous	3,200	1.1×10^5	4,800	1,200	—
	Heterologous	<10	<40	<40	<40	<10

a Haemagglutination (HA), bactericidal (Bact.) and agglutination assays were performed as described in Section 3.6, using both homologous and heterologous strains as indicators. Protective capacities (see Section 3.7.3) could only be assessed against *V. cholerae* 569B however, as 569B/165 is not pathogenic for infant mice (see Table 4.2).

vibrios have enabled the latter to elicit high levels of protective antibodies against the parental strain. Indeed, although it lacks antibodies specific for the LPS determinants of the challenge organisms, the protective capacity of the anti-live 569B/165 serum is almost equivalent to that of the anti-live 569B serum. This finding strongly suggests that further investigation of the non-LPS antigens of V. cholerae is warranted.

Of the in vitro assays performed, the agglutination of live vibrios provided the best correlate of in vivo protective activity. Previous studies in this department have demonstrated an important role for agglutination in the protection of infant mice from experimental cholera (Bellamy et al, 1975; Steele et al, 1975), and it seems likely that such a mechanism at least partly underlies the protective capacities of the sera described in Table 6.4. This point will be discussed again shortly (see Section 6.6).

Finally, the experiments described in the previous section suggested that the shared protective antigens were heat-labile, and this is supported by the present finding that an antiserum prepared against boiled hybrid vibrios displayed no protective activity against V. cholerae 569B (see Table 6.4). Still further confirmation of this point was subsequently derived from the demonstration that the protective capacity of the anti-live 569B/165 serum could not be removed by absorption with purified 569B-LPS or boiled V. cholerae 569B. The protective titre of this serum remained essentially undiminished after six absorptions with 569B-LPS (100µg/ml; 37C for 60 minutes) and three with boiled 569B (5×10^{10} organisms/ml; 37C for 60 minutes), whereas the anti-live 569B serum lost over 90% of its protective activity under the same conditions (titre fell from 32,000 to 2,400 PD_{50} 's/ml).

6.4 The Distribution of non-LPS Protective Antigens in V. cholerae.

The experiments described thus far have confirmed the existence of non-LPS protective antigens in V. cholerae 569B, and it was next of obvious importance to assess the generality of this finding. Since 569B is a Classical

strain of Inaba serotype, it was decided to examine two El Tor, Ogawa strains for the presence of similar non-LPS protective determinants. These strains - V. cholerae 017 and V. cholerae T50 - were studied using the two approaches which had successfully demonstrated such antigens in V. cholerae 569B.

6.4.1 Absence of non-LPS protective antigens in V. cholerae 017.

Following the procedure described in Section 6.2, a mouse antiserum prepared against live V. cholerae 017 was absorbed six times with highly purified 017-LPS. The removal of haemagglutinating and bactericidal activities was followed as before, with the latter being assayed both in the presence and absence of ME (see Table 6.1b). In addition, the original and residual sera were compared for their capacities to protect infant mice from oral challenge with V. cholerae 017. The results of this work are shown in Table 6.5, and differ significantly from those obtained in the corresponding study with V. cholerae 569B.

It is apparent that the haemagglutinating activity has once again been rapidly removed, but in this instance the LPS absorptions have also removed practically all of the bactericidal potential. The residual serum has retained only 0.3% of the original bactericidal capacity, and there is no evidence of preferential retention of ME-resistant antibodies. In contrast, after a similar number of LPS absorptions in the 569B system, the antiserum retained $\approx 5\%$ of its original ME-sensitive, and 100% of its ME-resistant bactericidal capacity. Finally, and most significantly, the LPS absorptions removed all ($\approx 99\%$) of the protective activity from the anti-017 serum, suggesting the absence of non-LPS protective antigens in V. cholerae 017. This would indicate that the prophylactic significance of the non-LPS determinants may vary markedly between different strains of V. cholerae.

TABLE 6.5

The effects of repeated absorption with purified 017-LPS
on various antibacterial capacities of a mouse antiserum
prepared against live *V. cholerae* 017.

Activities^a vs. *V. cholerae* 017

No. of LPS absorptions	HA	Bact. - ME ^b	Bact. + ME	Prot.
0 (original serum)	256	560,000	14,000	450
2	<2	38,000	ND ^c	ND
4	<2	2,500	ND	ND
6 (residual serum)	<2	1,700	<100	<5

a See footnote a to Table 6.1.

b ME - mercaptoethanol; see Section 3.6.3.

c ND - not determined.

6.4.2 Absence of non-LPS protective antigens in *V. cholerae* T50.

The examination of a second El Tor, Ogawa strain - *V. cholerae* T50 - for the presence of non-LPS protective antigens was performed using another hybrid vibrio, T50/165. Like 569B/165, this hybrid strain had been produced by inserting the NCV-165 genes for LPS production into the corresponding region of a parental *V. cholerae* genome. Thus, the capacity of T50/165 to elicit antibodies capable of mediating protection against *V. cholerae* T50 would again permit an evaluation of the prophylactic significance of their common (non-LPS) determinants.

Accordingly, antisera to live T50/165 and to live *V. cholerae* T50 were raised in parallel by repeated intraperitoneal immunization of adult mice. The resulting sera were then assayed for various antibacterial activities against the parental T50 strain, and these results are shown in Table 6.6 (sera 1 and 2). When it became apparent that the anti-T50/165 serum was unable to mediate protection against *V. cholerae* T50, a second group of mice was more intensively immunized with the hybrid strain, to produce a hyperimmune antiserum with the properties shown in Table 6.6 (serum 3). Finally, the capacity of antibodies specific for LPS antigens to provide protection in this system was also examined, by characterizing a serum raised against Ogawa (*V. cholerae* 017) LPS (see serum 4 in Table 6.6).

The results shown in Table 6.6 again confirm the reported antigenic relationship between the hybrid strains and their *V. cholerae* parents. Neither serum prepared against live T50/165 displayed any reactivity towards the heat stable antigens of *V. cholerae* T50, and hence their bactericidal and (live) agglutinating capacities against this strain are presumably a reflection of shared heat-labile determinants. Despite these anti-T50 capacities, however, neither serum was able to protect infant mice from the effects of an oral challenge with this strain. This finding again indicates that the non-LPS antigens of an El Tor, Ogawa strain are unable to

TABLE 6.6

Absence of non-LPS protective antigens from T50/165.

Antibacterial activities^a vs. V. cholerae T50.

Specificity of antiserum	Haemagglutination	Bacteriolysis	Agglutination of		Protection
			boiled vibrios	live vibrios	
# 1 : Live <u>V. cholerae</u> T50	400	9.0×10^5	800	600	750
# 2 : Live T50/165	<2	2.6×10^3	<10	40	<10
# 3 : Live T50/165	<2	8.2×10^3	<10	800	<10
- hyperimmune	(12,800) ^b	(5.1×10^6)	(2,400)	(4,800)	
# 4 : Purified 017-LPS	200	8.0×10^5	1,600	800	1,000

a All assays were performed as described in Sections 3.6 and 3.7.3.
Each figure represents the mean of at least two determinations.

b Figures in parentheses show activities of antiserum # 3 vs T50/165.

elicit the production of antibodies which are alone sufficient to provide protection against homologous infection, thus confirming the results obtained with V. cholerae 017.

The homologous antibacterial capacities of the second anti-T50/165 serum (shown in parentheses in Table 6.6) indicate that this is indeed a hyperimmune antiserum. Although its capacity to agglutinate live V. cholerae T50 is at least equivalent to that of the anti-T50 serum itself, there is nevertheless a qualitative difference in the protective activities of the two sera. Evidently the capacity to agglutinate organisms of the challenge strain is not sufficient to enable a serum to mediate protection against that strain.

The protective activity of the anti-017 LPS serum (see Table 6.6) demonstrates that antibodies directed against LPS determinants are sufficient to mediate protection in this system. The fortuitous similarity between the antibacterial activities of this serum and the antiserum prepared against live T50 organisms (see Table 6.6) is consistent with the notion that the protective capacity of the latter is solely due to its possession of anti-LPS antibodies. A similar conclusion was made in the 017 system, following the demonstration that all of the protective activity could be removed from an anti-live 017 serum by repeated absorption with homologous LPS (see Table 6.5). However, neither of these observations can eliminate the possibility that, although not sufficient for protection, antibodies directed against the non-LPS antigens of El Tor, Ogawa vibrios can contribute to the protective capacity of an antiserum raised against such organisms, by increasing the protective efficiency of those antibodies specific for LPS determinants. Such synergy between antibodies of different specificity has been reported in the V. cholerae 569B system (Bellamy et al, 1975), and also in the protection of rabbit loops against challenge with live vibrios or cholera toxin (Holmgren et al, 1977).

To test this possibility, anti-LPS antibodies were titrated for protective activity against V. cholerae T50 in the presence or absence of

antibodies specific for the non-LPS determinants of this strain. This was achieved by diluting the anti-017 LPS serum in diluent containing either anti-T50/165 serum (serum 3 in Table 6.6) or normal mouse serum at a final dilution of 1 in 20. However the presence of antibodies specific for the non-LPS antigens did not alter the protective capacity of the anti-LPS antibodies, and so even an accessory protective role for the former can be excluded. We therefore conclude that the non-LPS antigens of V. cholerae T50 are of no prophylactic significance.

6.4.3 Possible bases for the restricted distribution of non-LPS protective antigens in V. cholerae.

The experiments performed thus far have confirmed the existence of non-LPS protective antigens in V. cholerae 569B, but suggest that such determinants are not present in the 017 and T50 strains. The underlying basis for this restricted distribution remains undefined at the time of writing, but is obviously of great relevance with respect to the likely prophylactic significance of these antigens. Our experimental approach and preliminary results in this regard have been as follows.

V. cholerae 569B is a Classical strain of Inaba serotype, whereas both 017 and T50 are El Tor strains of Ogawa serotype. The simplest explanation for the restricted distribution of non-LPS protective antigens would be that the presence of such antigens is a characteristic of either the Classical biotype or the Inaba serotype. A convenient means of testing these possibilities would be to examine the capacity of antibodies specific for these non-LPS protective antigens to mediate protection against a variety of V. cholerae strains - including strains with the four possible biotype/serotype combinations.

Unfortunately we have been unable to perform such a survey, as our departmental culture collection contains only four V. cholerae strains which are pathogenic for infant mice (see Table 4.2). Assays performed with these strains have indicated that a hyperimmune antiserum prepared against live

569B/165 vibrios can mediate protection against the parental V. cholerae 569B strain, but not against 017, T50 or V86 (El Tor, Inaba). Obviously these findings need confirmation and considerable extension before any firm conclusion can be drawn; we have recently received four more pathogenic strains from Dr. L.J. Berry with which to continue this investigation.

6.5 Further Studies with the non-LPS Protective Antigens of V. cholerae 569B.

6.5.1 Introduction.

In view of our inability to demonstrate the presence of non-LPS protective antigens in the two El Tor, Ogawa strains, our attention returned to the further study of such determinants in V. cholerae 569B. Of particular interest were the likely consequences of either oral administration or vaccine inactivation for the prophylactic potential of these relatively labile antigens. The hybrid vibrio strain 569B/165 offered a unique opportunity to study the stability of the non-LPS determinants of V. cholerae 569B under these conditions, in the absence of the highly resistant LPS antigens.

The optimal route of vaccine administration has long been a contentious issue among cholera immunologists (see Section 1.3.6). One of the disadvantages of oral immunization is the possibility of antigen degradation in the proteolytic environment of the gut (see Section 1.3.6). Thus, although previous experiments have demonstrated the potential of the non-LPS determinants of V. cholerae 569B to elicit protective antibodies when presented systemically (Sections 6.2 and 6.3), the relative lability of these determinants (Sections 6.3.1 and 6.3.2) made it of interest to assess their suitability as oral immunogens.

In principle, this study involved the oral administration of live 569B/165 to adult mice, followed by the collection of intestinal washings, and assay of this material for protective activity against V. cholerae 569B. Since the immunizing strain shares only non-LPS antigens with the challenge strain, this approach will provide a clear indication as to the prophylactic

potential of such determinants when given by the oral route.

Several animal studies have suggested that live cholera vaccines are more effective than killed ones (Panse et al, 1964; Panse and Dutta, 1964; Feeley, 1970), indicating a loss of prophylactic potential as a consequence of the inactivation process. Once again, the availability of the 569B/165 strain provided a means of investigating the possible denaturing effects of the commonly-used preservatives on the relatively labile non-LPS protective antigens of V. cholerae 569B, in the absence of the highly resistant LPS determinants.

The approach adopted was similar to the one outlined above. Suspensions of live 569B/165 were inactivated by various procedures, and then used to systemically immunize different groups of adult mice. Positive and negative control groups received live or boiled 569B/165 respectively (see Table 6.4). After collection, each serum was assayed for protective activity against V. cholerae 569B, and thus provided an indication of the resistance of the common non-LPS antigens to denaturation by a given preservative.

These two studies of the non-LPS protective antigens of V. cholerae 569B - their suitability as oral immunogens, and their resistance to various preservatives - will now be discussed in more detail.

6.5.2 Suitability as oral immunogens.

Recently Bloom and Rowley (1979) compared various immunization regimes to ascertain the most effective means of generating local immunity to V. cholerae in mice. In support of the work of Pierce's group (see Section 1.3.6), they found that a combined oral-parenteral schedule stimulated the greatest response, in terms of the numbers of IgA-producing cells which developed in the submucosal tissue (see Bloom and Rowley, 1979). Accordingly such a schedule was employed to assess the capacity of the non-LPS antigens of V. cholerae 569B to elicit the production of protective antibodies when orally administered.

Four groups of 20 mice were immunized with either live or boiled suspensions of V. cholerae 569B or 569B/165, while a fifth group received only saline and served as controls. Each animal was given four oral doses of 10^{10} organisms (or saline) on days 1, 5, 9 and 13⁴, followed by a single intravenous injection of 10^7 vibrios (of the same type) on day 27 (after Bloom and Rowley, 1979). On day 33, at the anticipated peak of the antibody response in the small intestine⁵, a pool of intestinal washings was collected from each group (see Section 3.3.3). These were subsequently assayed for protective activity against V. cholerae 569B.

The intestinal washings obtained from the mice immunized with live V. cholerae 569B possessed a protective titre of 155 PD_{50} 's per ml (mean of 4 assays; range 80-250), but the other samples displayed no protective activity (<5 PD_{50} 's per ml). Apparently then, only in those animals receiving live V. cholerae 569B was the immune system primed by the series of oral feedings, so that only in these mice was a secondary response evoked by the subsequent intravenous immunization (which alone is not sufficient to induce a local immune response - see Bloom and Rowley, 1979). This indicates that the non-LPS antigens common to the 569B/165 and V. cholerae 569B strains were unable to stimulate the local immune system, and suggests that such determinants would not be effective as oral immunogens⁶. This in turn suggests that it was the LPS antigens present on the live V. cholerae 569B which were responsible for the priming capacity of these organisms.

⁴ 0.2mls of bacterial suspension (or saline) was given to each mouse, about 5 minutes after the administration of 0.2mls of saturated sodium bicarbonate to neutralize gastric acidity.

⁵ Using this immunization schedule, the numbers of IgA-producing cells in the submucosal tissues of the small intestine reach a peak on day 32 (see Bloom and Rowley, 1979).

⁶ This finding confirms the results of a previous study by Horsfall and Rowley (unpublished data). Using an immunization schedule which comprised six oral feedings (each of 10^{11} live vibrios), these workers were unable to detect any protective activity in the intestinal washings of mice which had received the 569B/165 strain (<5 PD_{50} 's per ml, compared to 70 PD_{50} 's per ml in the material collected from mice immunized with live V. cholerae 569B).

The most likely explanation for the failure of potentially protective non-LPS antigens to elicit protective antibodies when given orally would seem to be that the relative lability of these determinants results in their denaturation by the intestinal environment. However the inability of boiled 569B vibrios to prime the local immune system - despite their possession of resistant LPS antigens - suggests that an alternative explanation also merits consideration. It has been reported that boiled vibrios adhere less efficiently to the intestinal surface in vivo than the corresponding live organisms (Freter, 1972), and this presumably underlies their failure as oral immunogens. By analogy, it could be argued that the non-LPS determinants present on the live 569B/165 vibrios retained their antigenicity, but were unable to stimulate the local immune system because these organisms did not attach to the intestinal surface as efficiently as V. cholerae 569B. This would seem to be a less likely explanation for the data however, as 569B/165 adheres as efficiently as V. cholerae 569B, at least in vitro (see Table 4.4). Furthermore, although non-pathogenic in infant mice, the motile status of the 569B/165 organisms would be likely to ensure their efficient colonization in vivo (see Sections 4.4.3 and 4.4.4).

Accordingly an experiment was designed to test the first of the explanations offered above - that the non-LPS antigens present on the 569B/165 vibrios were denatured as a consequence of their oral administration. It was decided to pretreat live 569B/165 organisms with a preparation of mouse intestinal washings in vitro, and then to assess their residual prophylactic potential when presented systemically. If the treated vibrios are no longer able to elicit the production of protective antibodies, then it is likely that their non-LPS protective antigens would be similarly denatured by the intestinal environment in vivo, making them unsuitable for use as oral immunogens.

Using the injection schedule described in the legend to Table 6.7, five groups of 20 mice were immunized as follows. Two groups received either live or boiled 569B/165 vibrios, and served as positive and negative controls

for the stimulation of protective antibodies (see Table 6.4). A third group was immunized with organisms of the same strain which had been pretreated in vitro⁷ with an aliquot from a pool of intestinal washings collected from normal adult mice (see Section 3.3.3). To check whether any denaturation of the non-LPS antigens which might occur would also be likely following oral immunization in humans, another group of mice received live 569B/165 which had been similarly pretreated with normal human small intestinal fluid (see Section 3.3.4). The final group was immunized with live 569B/165 which had been pretreated with trypsin, to assess the likely contribution of proteolytic enzymes to any denaturing effects associated with the intestinal washings.

The antisera subsequently collected from these five groups were assayed for various antibacterial capacities against the 569B/165 and V. cholerae 569B strains, and some of the resulting data are shown in Table 6.7. This also includes the results of viability tests which were routinely performed on the various immunizing suspensions; these are expressed as percentages of the counts obtained from the control suspensions of live vibrios.

Several conclusions can be drawn from these data. Firstly, the viability of the immunizing suspension fell dramatically during pretreatment with either intestinal fluid (see Table 6.7). Since this effect was much less pronounced during incubation with trypsin, it is probably attributable to some non-proteolytic component of the fluid (eg. bile salts). Secondly, all sera possessed similar haemagglutinating, bactericidal and agglutinating capacities for organisms of the immunizing strain, suggesting that the intensity of immunization was the same for all groups. Only the haemagglutination data are shown in Table 6.7, and these indicate that the LPS antigens were not denatured by any of the pretreatment procedures.

⁷ The pretreatment conditions for the organisms given to the three experimental groups were as follows. One volume of mouse intestinal washings (or human intestinal fluid, or a PBS solution of trypsin at 20mg/ml) was added to one volume of a PBS (pH 7.4) suspension of live 569B/165 (at a concentration of 10^{10} organisms/ml). After incubation at 37C for 60 minutes, the bacteria were deposited by centrifugation and then resuspended in saline to the appropriate concentration for injection.

TABLE 6.7

Denaturation of non-LPS protective antigens by intestinal fluids.

Antibacterial capacities^a

Pretreatment of 569B/165 prior to immunization ^b	Vaccine viability ^c	HA vs 165-LPS	Bact. vs 569B	Prot. vs 569B
None (positive control)	100	1,600	780	95
Boiling (negative control)	0	1,600	<20	<5
Mouse intestinal washings ^d	7.0 ± 4.6 ^e	3,200	40	<5
Human intestinal fluid ^d	9.6 ± 2.7	1,600	20	<5
Trypsin ^d	50 ± 18	1,600	340	70

a See footnote a to Table 6.1.

b Mice received 10^7 organisms (in saline, given intraperitoneally) on day 1; 3×10^7 on day 5; 10^8 on day 9; 3×10^8 on day 13; and 10^9 on days 16 and 20. Sera were collected on day 24.

c Figures show percentage viability, relative to that of the control suspension of live organisms.

d The organisms given to these three groups were pretreated just prior to injection - see footnote 7 to main text.

e Figures give mean ± standard error (6 determinations).

Since the vaccines induced similar levels of anti-LPS activity, it is valid to compare the extents to which they elicited antibodies to their non-LPS determinants.

As expected, none of the sera displayed any activity against the heat-stable antigens of the parental strain, V. cholerae 569B. More significantly, only those prepared against live or trypsin-treated 569B/165 were able to mediate protection against V. cholerae 569B, indicating that the non-LPS, potentially protective antigens shared by these strains were destroyed as a result of pre-incubation with intestinal fluids (and boiling). The denaturing potential of these fluids would also appear to be due to some component other than proteolytic enzymes, since trypsin-treated and live 569B/165 induced similar levels of protective activity⁸. These results readily explain the inability of the non-LPS determinants to elicit protective antibodies when given orally to mice, particularly in view of the fact that the intestinal washings used in this study probably represent a 1 in 5 dilution of normal intestinal fluid (see Section 3.3.3). The data also suggest that such antigens would be similarly ineffective if used as oral immunogens in humans.

Finally, it is evident that the immunization schedule employed for this study was not very suitable for the production of protective antibodies to the 569B/165 non-LPS determinants. The protective activity of the serum raised against live vibrios was less than 1% of that typically obtained using more intense immunization programmes (for example, see Table 6.4). In view of the potent immunogenicity of LPS determinants - note that the homologous haemagglutinating capacity is very high - it seems likely that these non-LPS antigens will be more reliable and effective immunogens when isolated and injected separately. It is interesting to note that the live agglutinating

⁸ Although it is possible that the non-LPS antigens were destroyed during the trypsin pretreatment, but were able to be re-synthesized by the vibrios following their removal from this environment.

capacities for V. cholerae 569B were very low in all sera (<40, data not shown). Although agglutination is not sufficient for protection in the infant mouse model of cholera (see Section 6.6.2), it would nevertheless appear to be an important accessory mechanism by which specific antibodies mediate protection against V. cholerae 569B (Bellamy et al, 1975; Steele et al, 1975).

6.5.3 Resistance to various preservatives.

As discussed above (see Section 6.5.1), the purpose of the following study was to examine the consequences of various inactivation procedures for the prophylactic potential of non-LPS protective antigens. The experimental approach was the same as that described above; suspensions of live 569B/165 were inactivated by various means, and then used to systemically immunize different groups of adult mice. The capacities of the resulting sera to mediate protection against V. cholerae 569B then provided indications as to the resistance of the shared non-LPS antigens to the various preservatives employed.

This study was performed in conjunction with the previous experiment, using the same immunization schedule and the same groups of mice as positive and negative controls for the antigenic integrity of the non-LPS determinants. These groups received live or boiled 569B/165 respectively, while five other groups (also each of 20 mice) were immunized with suspensions of 569B/165 which had been inactivated by treatment with phenol, formalin, alcohol, acetone or sodium azide. All but the last of these have been commonly employed for the preservation of bacterial vaccines; sodium azide is a metabolic inhibitor, and it seemed possible that bactericidal concentrations of such an agent might inactivate the vibrios yet leave their surface antigens relatively intact.

The actual conditions used for the various inactivation procedures have been described elsewhere (see Section 3.8). Preliminary experiments were performed to determine the minimal killing concentrations of the various

preservatives⁹, and these were used to pretreat different aliquots of a suspension of live 569B/165. With the exception of the boiled vibrios, the various immunizing suspensions were inactivated just prior to each injection, so that each of the resulting sera would represent an "average response" to a series of six independently-preserved doses of a given vaccine. All sera were collected after the immunization programme described in the legend to Table 6.7, and some of their antibacterial capacities are shown in Table 6.8.

These data bear several similarities to those shown in Table 6.7. Firstly, the high homologous haemagglutination titres again show that the LPS antigens were resistant to all of the preservatives employed. Secondly, the similarity of these titres, and those obtained from the agglutination assays with boiled 569B/165, again suggest that the intensity of immunization was the same in all groups. Thirdly, none of the sera were able to display any reactivity towards the heat-stable determinants of the V. cholerae 569B parental strain (data not shown), and once again their capacities to agglutinate or mediate protection against such organisms were disappointingly low (see previous discussion).

Nevertheless it is clear that the non-LPS protective antigens present on the live 569B/165 vibrios were able to withstand pretreatment with some preservatives more successfully than with others. Boiling, alcohol and acetone were most deleterious, completely destroying the potential of these determinants to elicit bactericidal and protective antibodies against the parent strain (see Table 6.8). The data are not strong enough to allow selection of the "best" preservative. Sodium azide seemed to have no denaturing effect upon the non-LPS protective antigens, but the very high concentration required

⁹ The minimal killing concentration of each agent was the minimal concentration which could effect an absolute loss of viability in a standard suspension of live 569B/165 (10^{10} organisms per ml, in PBS pH 7.4) during an incubation period of 60 minutes at 37C (except for alcohol and acetone - see Section 3.8).

TABLE 6.8

Stability of non-LPS protective antigens to various preservatives.

Antibacterial capacities of resulting sera^a

Method of vaccine inactivation ^b	vs 569B/165		vs <u>V. cholerae</u> 569B	
	HA	Aggl.	Bact.	Prot.
None (+ve control)	1,600	1,280	780	95
Phenol	3,200	1,280	120	75
Formalin	3,200	1,280	300	40
Sodium azide	1,600	1,280	250	105
Alcohol	3,200	1,280	<20	<5
Acetone	6,400	2,560	<20	<5
Boiling (-ve control)	1,600	2,560	<20	<5

a Sera were collected after the immunization programme described in footnote b to Table 6.7. Their various antibacterial capacities were assayed as described in Sections 3.6 and 3.7.3.

b Live 569B/165 vibrios were inactivated just prior to injection, using the procedures outlined in Section 3.8.

to inactivate the organisms (final 8%, w/v) would probably preclude this from being a feasible alternative to the conventional preservatives. Phenol-treatment also seems reasonably safe, although it should be noted that this is sometimes used in conjunction with other inactivation procedures (see Joo, 1974), in which case any deleterious effects are likely to be cumulative. Obviously these results need confirmation and considerable extension.

6.6 Possible Protective Functions of Specific Antibodies.

6.6.1 Introduction.

Having briefly examined two of the more topical aspects of the non-LPS protective antigens themselves, it was of interest to next examine some of the properties of the antibodies which they elicit. Initially this investigation formed part of a study of the protective mechanisms of specific antibodies in general (see below); subsequently the protective efficiencies of antibodies directed against either LPS or non-LPS determinants were compared (see Section 6.7).

The functions of specific antibodies which are involved in mediating protection against clinical or experimental cholera remain obscure (see Section 1.3.5), and it seemed important to devote some attention to this issue. The studies described thus far suggested several ways of assessing the likely significance of the various antibacterial properties of specific antibodies with respect to the overall protective potential of these molecules. Firstly, it would be of interest to compare the various antibacterial capacities of antibodies directed against the LPS or non-LPS determinants of V. cholerae 569B. Since antibodies of either specificity are highly protective, any function displayed by one but not the other could not be necessary for protection. Secondly, as antibodies specific for the non-LPS antigens of the El Tor Ogawa strains 017 and T50 are unable to mediate protection against the homologous organisms, it is obvious that the antibacterial capacities of these antibodies

are collectively not sufficient for protection in this system. Finally, it would be of interest to ascertain whether the latter antibodies lack any capacity which is displayed by protective antibodies generally (i.e. those directed against LPS determinants in the El Tor, Ogawa system or against either LPS or non-LPS antigens in the 569B system).

With such comparisons in mind, it was decided to attempt an evaluation of the protective significance of the different antibacterial capacities of specific antibodies. As discussed previously, complement-mediated bacteriolysis and phagocytosis would appear to have been eliminated as possible protective functions, at least in the infant mouse model of cholera (see Section 1.3.5). Several studies with enteropathogenic E. coli have demonstrated that the protective capacities of specific antibodies correlate with their ability to inhibit the attachment of the potential pathogens (see Section 2.5.2). In view of the similarity between the infective processes of enteropathogenic E. coli and V. cholerae, and of the relationship between adherence and virulence in the latter (see Chapter 4), it seemed likely that a similar explanation might also underlie the capacity of antibodies to mediate protection against cholera. However the inhibition of bacterial attachment is a complex process which might involve several different antibody functions, including agglutination, inhibition of motility, and the blocking of adherence moieties (see Section 1.3.5). It was therefore decided to examine the possible protective significance of each of these antibacterial functions.

6.6.2 Bacterial agglutination.

Previous studies in this department demonstrated that the capacities of several antibody preparations to protect infant mice from experimental cholera could be correlated with their capacities to agglutinate organisms of the challenge strain (Bellamy et al, 1975; Steele et al, 1975). Agglutinating activity also correlated with the capacity of a given preparation to inhibit vibrio attachment to murine intestinal epithelial cells in vitro (Bellamy et al,

1975), an observation which was subsequently supported by the demonstration that agglutinated organisms were less able to adhere to rabbit ileal slices (Freter and Jones, 1976). These findings suggest that agglutination is one function of specific antibodies which contributes to their capacity to inhibit adherence, thereby also suggesting an explanation for the apparent protective significance of this function.

Our own studies with antisera prepared against some or all of the antigens of V. cholerae 569B have confirmed that agglutination provides an excellent in vitro correlate of protective potential (see Table 6.4). However similar experiments with V. cholerae T50 have shown that agglutination is not sufficient for protection, at least in this system. Thus there was found to be a qualitative difference in the protective capacities of two sera which displayed similar agglutinating activities against organisms of the T50 challenge strain (see Table 6.6).

Therefore, even if agglutination is necessary for the efficient (see Steele et al, 1975) mediation of protection, it is clear that, when considering the nature of immunity to V. cholerae strains in general, specific antibodies must possess at least one other antibacterial¹⁰ function. Teleologically, one might suspect the existence of an additional protective mechanism - to safeguard against infection with sub-agglutinating concentrations of V. cholerae (see discussion in Section 1.3.7). The most likely additional protective functions of specific antibodies would be the inhibition of vibrio motility, or the blocking of those factors which mediate adherence.

6.6.3 Inhibition of motility.

In view of the accumulating evidence to suggest that the flagellum plays an important role in the in vitro attachment and in vivo colonization of V. cholerae (see discussion in Chapter 7), it seemed possible that

¹⁰ It is worth reiterating here that antibodies directed against the cholera toxin are unlikely to be of any protective significance in this model (see Neoh and Rowley, 1972).

the inhibition of bacterial motility might be another protective function of specific antibodies¹¹. Therefore an inhibition-of-motility assay (see Section 3.6.5) was used to assess the capacities of various antisera to immobilize homologous V. cholerae, and these were then related to their known protective activities.

In confirmation of previous reports (see Section 1.2.3), antibodies directed against the LPS determinants were found to strongly inhibit vibrio motility. Thus an IgM fraction obtained from a hyperimmune anti-569B LPS serum (see Section 6.7.2) was able to immobilize V. cholerae 569B out to a dilution of 1 in 1600, while an anti-017 LPS serum (see Table 6.6) similarly immobilized V. cholerae T50 out to 1 in 800. Both sera had protective titres of approximately 1,000 PD₅₀'s per ml against the homologous strains (Tables 6.12 and 6.6).

Antibodies specific for the non-LPS antigens of V. cholerae were, in contrast, completely unable to inhibit motility. Hyperimmune antisera prepared against the live hybrid strains - 569B/165 and T50/165 - did not immobilize organisms of the corresponding parental strains - 569B and T50 - even at a dilution of 1 in 10. Although antibodies of this specificity are not protective in the T50 system (see Table 6.6), they are extremely protective against 569B (see Table 6.4), and so in the latter situation the correlation between immobilizing and protective capacities breaks down. Thus it is evident that the capacity to inhibit vibrio motility is not necessary for the mediation of protection by specific antibodies.

6.6.4 Blocking of vibrio adhesins.

Our discussion thus far has suggested that, of the various anti-bacterial capacities of specific antibodies, agglutination is not sufficient

¹¹ This work was performed before the experiments described in Section 5.3.2, which subsequently suggested that motility per se was neither necessary nor sufficient for in vitro attachment.

for protection and immobilization is not necessary. Apparently, then, some other antibacterial function is essential for the mediation of protection; the finding that specific antibodies can inhibit the attachment of V. cholerae under conditions where agglutination does not occur (Freter and Jones, 1976) suggests that such a function might involve the blocking or masking of the bacterial moieties which promote adherence. Compared to agglutination, this would represent a more direct mechanism by which antibodies could inhibit adherence, and, unlike the former, its effectiveness would not depend upon bacterial concentration.

Accordingly various antisera (of known protective potential) were tested for their capacities to inhibit the in vitro attachment of homologous vibrios to everted mouse intestinal strips. The assay used was based upon the standard in vitro adherence system developed previously (see Section 4.2), except that the concentration of organisms in the fluid phase was reduced from $\approx 2 \times 10^7$ per ml to $\approx 10^6$ per ml. This was necessary to prevent bacterial agglutination, which, by increasing the degree of inhibition observed (see Freter and Jones, 1976), would otherwise obscure the significance of any adhesin-blocking mechanism.

Thus, in a typical assay, 10ml suspensions of V. cholerae (at $\approx 10^6$ per ml, in PBS pH 7.4) were pretreated with various dilutions of the test serum, or with normal mouse serum (NMS), for 5 minutes at 37C. The residual adherence capacity of the vibrios within each suspension was then determined by the addition of 4 everted intestinal strips. After a further 15 minutes at 37C each group of strips was washed and processed in the normal manner, and the resulting level of attachment expressed as a percentage of that displayed by the organisms in the control suspension (containing NMS). This procedure was used to determine the capacities of antibodies specific for either LPS or non-LPS determinants to inhibit the in vitro adherence of either V. cholerae 569B or V. cholerae T50. Representative data from these experiments are shown in Table 6.9.

TABLE 6.9

Antibody - mediated inhibition of V. cholerae adherence in vitro.

Table 6.9a Effect of anti - LPS antibodies on adherence of 569B

Serum :	Anti - 569B-LPS			NMS ^a
Concentration ^b :	40	200	1,000	40
% Adherence ^c	44	59	92	100

Table 6.9b Effect of anti - "non-LPS" antibodies on adherence of 569B.

Serum :	Anti - live 569B/165		IgG vs. "non-O"		NMS ^a
Concentration ^b :	40	200	40	200	40
% Adherence ^c	21	53	37	65	100

Table 6.9c Effects of specific antibodies on adherence of T50.

Serum :	Anti - 017-LPS		Anti - live T50/165		NMS ^a
Specificity :	Anti - LPS		Anti - "non-LPS"		—
Concentration ^b :	30	100	30	100	30
% Adherence ^c	32	53	198	245	100

a NMS - normal mouse serum.

b Figures show reciprocal serum dilutions used to pretreat vibrios (see text).

c Figures show percentage adherence relative to that displayed by control organisms pre-incubated in NMS.

Before discussing these results, several comments should be made with respect to the assay itself. Firstly, microscopic examination of the vibrio suspensions at the end of each experiment confirmed that agglutination had not occurred, and so any inhibition of adherence observed must have resulted from the direct action of antibodies on individual organisms. Secondly, in those cases where the antibodies did achieve an inhibition of vibrio adherence, the degree of inhibition observed in repeat experiments generally varied from 50-70%. This is similar to the reduction observed by Freter and Jones (1976), although the latter workers required much lower concentrations of antiserum (final dilution of 1 in 1,000, compared to 1 in 30 or 1 in 40 used in our experiments). Finally, no attempts were made to compare the inhibitory capacities of different sera, since the insensitivity of this assay made the definition of endpoints impractical. Rather, the assay was used solely to provide a qualitative assessment of the capacity of a given serum to inhibit vibrio attachment - presumably under conditions of great antibody excess - and as such it proved a reliable and reproducible test.

Returning to the data obtained from these experiments, it is clear that the adherence of V. cholerae 569B could be inhibited by antibodies of either specificity. Thus, an IgM fraction from a hyperimmune antiserum raised against 569B-LPS (see Section 6.7.2) inhibited attachment (Table 6.9a), as did two independent sources of "non-LPS" antibodies (Table 6.9b). The first of these was a hyperimmune serum prepared against live vibrios of the 569B/165 hybrid strain (described in Table 6.4); the second was an IgG fraction isolated from a hyperimmune anti-live V. cholerae 569B serum, and then absorbed three times with 569B-LPS (see Section 6.7.2). All three sera used in these experiments were also able to protect infant mice from challenge with V. cholerae 569B (see Tables 6.4 and 6.12).

Studies performed with V. cholerae T50, however, indicated that only antibodies directed against the LPS determinants could inhibit adherence in this system. Table 6.9c shows that an anti-017 LPS serum (see Table 6.6)

inhibited attachment, but a hyperimmune serum prepared against live T50/165 vibrios (Table 6.6) did not. Once again, the capacity to inhibit adherence correlates with the capacity to mediate protection, since only antibodies specific for the LPS antigens of V. cholerae T50 can protect infant mice from challenge with this strain (see Table 6.6).

So, unlike the functions of agglutination and immobilization, the capacity of antibodies to directly inhibit attachment at the single-cell level provides a consistent in vitro correlate of protective activity. It therefore seems possible that the antibacterial capacity being monitored in this assay - which is presumably the blocking of vibrio adhesins - may be the antibody function which is essential for the mediation of protection.

Table 6.10 summarizes, in qualitative form, the results obtained from the studies described in this section.

6.7 The Relative Prophylactic Significance of the LPS and non-LPS Antigens of V. cholerae 569B.

Before concluding this work it seemed important to gain some indication as to the likely immunoprophylactic potential of the non-LPS protective antigens of V. cholerae. Accordingly this section describes an assessment of the relative prophylactic significance of the LPS and non-LPS determinants of V. cholerae 569B.

6.7.1 Experimental approach.

The fact that both types of antigen were not available in a purified form precluded the direct approach of comparing, on a weight basis, their capacities to generate protective antibodies upon immunization. It was therefore decided to gauge the relative significance of the two by comparing the protective efficiencies of their respective antibodies. Again, however, the lack of a pure preparation of non-LPS antigens meant that antibodies specific for these determinants could not be quantitated, and so it would not

TABLE 6.10

The antibacterial capacities of antibodies specific
for the LPS or non-LPS determinants of *V. cholerae*.

Indicator strain : Specificity of antibodies :	<u><i>V. cholerae</i> 569B</u>		<u><i>V. cholerae</i> T50</u>	
	LPS	non-LPS	LPS	non-LPS
<u>Antibacterial function</u>				
Agglutination	+	+	+	+
Inhibition of motility	+	-	+	-
Blocking of adhesins	+	+	+	-
Protection	+	+	+	-

be possible to compare protective activities per unit weight of specific antibody. Some other basis for comparison was required - one which assessed the relative concentrations of specific antibodies by titrating one or other of their antibacterial capacities.

Since antibodies of different classes differ in the efficiencies with which they mediate various antibacterial functions (for examples in the present context see Steele et al, 1974; Horsfall and Rowley, 1979), it seemed essential that our comparison of protective efficiencies should be performed using antibodies of the same class. Even then the choice of the most suitable assay for comparing antibody concentrations was important; as the antibodies would be of different specificity, an assay which relied solely on the inherent antigen-binding capacities of these molecules seemed preferable¹². In this respect the radioimmunoassay (RIA) appeared suitable; and as this also offered a means of assessing the total antibody content of a given serum, regardless of specificity, it was adopted as the basis for expression and comparison of protective efficiencies.

The RIA is described in detail in Section 3.6.4. In principle, aliquots of a suspension of V. cholerae 569B are incubated with various dilutions of the test (mouse) serum, and the vibrios then washed to remove unbound immunoglobulins. The organisms - with their attached primary antibodies - are then incubated with a radiolabelled developing serum specific for mouse immunoglobulins. The level of binding of the developing antibodies is then a reflection of the binding capacity of the primary (test) serum, which is subsequently expressed in terms of RIA binding units per ml (see Section 3.6.4).

¹² This would ensure that the efficiency of antibody detection could not be influenced by antibody specificity. For example, if complement-mediated bacteriolysis was used as the basis for comparisons of antibody concentration it could be argued that, because LPS determinants would probably constitute more effective substrates for this reaction than non-LPS antigens (which would include bacterial appendages), the assay would preferentially detect anti-LPS antibodies.

The protective efficiency of a given serum could then be calculated by dividing its protective activity (PD_{50} units per ml) by its RIA binding capacity (RIA units per ml).

6.7.2 Preparation and characterization of antibody fractions.

Hyperimmune antisera prepared against purified 569B-LPS or live 569B/165 vibrios (by repeated intraperitoneal immunization of groups of 50 mice) were used as sources of antibodies specific for the LPS or non-LPS determinants of V. cholerae 569B. In order to be able to compare antibodies of the same class (see above), an IgG fraction was isolated from each serum using the recently-described methodology of Ey et al (1978; see Section 3.9). The residual sera, conveniently referred to as IgM fractions, would also be compared.

The experiments described in Section 6.2 suggested an independent source of "non-LPS" antibodies which might also be included in our study - the ME-resistant component of a hyperimmune serum raised against live V. cholerae 569B (see Table 6.2). Although this would also contain antibodies specific for LPS determinants, these could be removed by absorption. Thus the IgG fraction was isolated from this serum as above, and absorbed three times with purified 569B-LPS (as described in Section 6.2). This successfully removed all haemagglutinating activity (titre fell from 128 to <2), and also the majority of the bactericidal activity (see Table 6.11a). The absorbed preparation, which for convenience is referred to in Tables 6.11 and 6.12 as "IgG vs non-O" (as distinct from "IgG vs 569B/165"), was then included in our study of the protective efficiencies of antibodies of different specificity.

Before proceeding to such comparisons however, it was necessary to check that the specific antibodies present within each of our various fractions were indeed confined to the one class. As the original sera were prepared by parenteral immunization, it seemed likely that the vast majority of their

specific antibodies would be either IgM or IgG in nature (although the possibility of IgA serum antibodies is discussed in the next section). Accordingly it would be possible to gauge the "class purity" of the antibodies within each serum fraction by assessing the effectiveness of the attempted separation of IgG from IgM. This was conveniently achieved by determining the bactericidal capacity of each fraction in the presence or absence of ME. These assays were performed against organisms of the homologous strains, and the results are given in Table 6.11a.

It was also of interest to quantitate the specific antibody content of each of the anti-LPS fractions. This was achieved by absorption of antibodies onto, and their subsequent elution from, purified 569B-LPS (as described in Section 3.10). The resulting estimates of antibody concentration (120 μ g/ml in the IgM fraction, 128 μ g/ml in the IgG fraction) were then used to calculate the bactericidal, RIA-binding, and protective capacities of each class per μ g of specific antibody. These data are shown in Table 6.11b.

Several points can be made on the basis of these results. Firstly, the bactericidal capacities of the two IgM fractions are completely susceptible to ME, whereas those of the three IgG fractions are highly resistant. Thus the separation of IgM and IgG antibodies has been successful¹³, and fractions of the same class can be used with confidence to compare the protective efficiencies of antibodies of different specificity. Secondly, it is interesting to note that, unlike the antisera studied previously (see Table 6.2), the majority of the bactericidal activity of the original anti-live 569B/165 serum

¹³ Applying the figures shown in Table 6.11b to the bactericidal titres in Table 6.11a, the maximum possible contamination of any of the antibody fractions would be an approximate 10% (w/w) content of IgG antibodies in the IgM vs 569B/165. Even if this was the case however, the over-estimation of the protective efficiency of this fraction would be insignificant ($\approx 6\%$).

TABLE 6.11 Characterization of antibody fractions.

Table 6.11a ME^a - resistance of bactericidal capacities.^b

Antibody fraction	Bact. - ME	Bact. + ME
IgM vs 569B-LPS	1,000,000	<100
IgM vs 569B/165	47,000	< 50
IgG vs 569B-LPS	11,000	5,500
IgG vs 569B/165	120,000	80,000
IgG vs 569B	7,400	3,500
IgG vs "non-O"	1,200	950

Table 6.11b Specific antibacterial activities of IgM and IgG
antibodies directed against 569B-LPS.

Antibacterial capacities^b per µg specific antibody

Antibody fraction	Bact.(- ME ^a)	RIA	Protection
IgM vs 569B-LPS	8700	50	10
IgG vs 569B-LPS	86	20	9

a ME - mercaptoethanol ; see Section 3.6.3.

b All assays were performed against organisms of the homologous strain, using methods described in Sections 3.6 and 3.7.3.

was ME-resistant¹⁴. Finally, the data in Table 6.11b support previous findings in this model of cholera, by again demonstrating that anti-V. cholerae LPS antibodies of different classes mediate protection with similar efficiency (see Steele et al, 1974; Horsfall and Rowley, 1979). The much greater bactericidal efficiency of IgM has also been confirmed, and in general the specific activities of each class are very similar to those previously reported for mouse antibodies to LPS (Horsfall and Rowley, 1979)¹⁵.

6.7.3 Relative protective efficiencies of antibodies specific for LPS or non-LPS determinants.

Having separated our sources of LPS and "non-LPS" antibodies into IgM and IgG fractions, it was now possible to determine the protective efficiencies of the antibodies in these fractions, and to compare the latter in relation to antibody specificity. Thus each fraction was tested for protective activity and also titrated in a RIA (both against V. cholerae 569B); the protective efficiency was then expressed in terms of PD₅₀ units per RIA binding unit.

In the protection tests, antibodies of different specificity but of the same class were assayed concurrently in the same batches of mice. Thus the two IgM fractions were tested together, as were the IgG fractions specific for 569B-LPS or 569B/165; the "IgG vs non-0", however, was assayed separately at a later date. Each fraction was assayed 2-4 times, and the mean and range of the protective titres obtained are shown in Table 6.12.

¹⁴ Using the approach discussed in Section 6.2, the weight ratio of IgG:IgM antibodies in this serum can be calculated as >200! It might not be valid to apply this approach to a 569B/165 antiserum, however, as Bhaskaran (1971) originally described this strain as being resistant to lysis by antibody and complement. This point will be further discussed in Chapter 7.

¹⁵ The protective activities cited by these authors are ten-fold higher than those shown in Table 6.11b, but this is merely a consequence of the different methods adopted for the calculation of protective endpoints (see Horsfall and Rowley, 1979 cf. Section 3.6.3).

TABLE 6.12

Relative protective efficiencies of antibodies directed against
the LPS or non-LPS determinants of *V. cholerae* 569B.

Antibody fraction	Protection ^a	RIA ^b	Prot. RIA
IgM vs 569B-LPS	1,250 (3; 900- 1,500)	6,000	0.21
IgM vs 569B/165	6,070 (3; 5,000- 7,400)	600	10
IgG vs 569B-LPS	1,180 (4; 770- 1,400)	2,500	0.47
IgG vs 569B/165	25,000 (4; 12,500 -44,000)	4,000	6.3
IgG vs "non-O"	1,900 (2; 1,600- 2,200)	230	8.3

a Figures in this column show mean protective titre against *V. cholerae* 569B (in PD₅₀ units per ml - see Section 3.7.3), and, in parentheses, the number of determinations and the range of titres obtained.

b RIA - radioimmunoassay. Figures represent mean of 2-3 determinations, and are expressed in binding units per ml (see Section 3.6.4).

The RIA binding capacities of the various fractions were determined as previously described, using as the developing serum goat antibodies directed against the light chain determinants of mouse immunoglobulins (see Section 3.6.4). Each fraction was titrated at least twice, with a standard antiserum being included in each assay to enable correction of the binding endpoints obtained. The mean RIA titre of each fraction is shown in Table 6.12.

Although the developing serum used in these assays detects IgM and IgG antibodies with satisfactory efficiency, it is not a sensitive monitor of IgA antibodies (see Section 3.6.4). Therefore, to test for the presence of such antibodies, RIAs were also performed on each fraction using goat antibodies specific for the mouse α chain as the developing serum (see Section 3.6.4). However, no IgA antibodies could be detected (all binding endpoints were <50 per ml), and so their contributions to the protective capacities of the various fractions would not be significant (<5 PD₅₀'s per ml - see Horsfall and Rowley, 1979, and footnote 15).

The data in Table 6.12 are most encouraging, for they indicate that the protective efficiency of antibodies directed against the non-LPS antigens of V. cholerae 569B is much greater than that of antibodies specific for LPS determinants. Comparing the IgM fractions, the difference in protective efficiency was almost 50-fold; with the IgG fractions, the two sources of non-LPS antibodies were an average of 15 times more efficient in mediating protection. This study therefore suggests that the non-LPS antigens of V. cholerae may be of great prophylactic significance, and that their detailed identification and careful isolation might prove to be a most rewarding line of research (see Chapter 7).

6.8 Summary

Two experimental approaches were successful in confirming the existence of non-LPS protective antigens in V. cholerae 569B. Further investigation showed that, by comparison with LPS determinants, these antigens were relatively labile, and as a consequence were unable to elicit protective antibodies when administered by the oral route. Similar antigens could not be detected on either of two El Tor, Ogawa strains; the underlying basis for this restricted distribution remains undefined.

An attempt was made to evaluate the relative prophylactic significance of the LPS and non-LPS antigens of V. cholerae 569B, by comparing the protective efficiencies of their respective antibodies. It was found that antibodies specific for the non-LPS determinants were much more protective than others of the same class but directed against the LPS antigens. A consideration of the likely protective significance of the various antibacterial functions of specific antibodies in general suggested that the capacity to directly inhibit attachment might be the prime determinant of protective activity.

CHAPTER 7

DISCUSSION

7.1 Re-iteration of Aims.

At this stage it seems appropriate to briefly re-iterate the main objectives of this thesis, before proceeding to discuss the findings which have been made. Our overall aim at the commencement of these studies was to assist the development of more effective cholera vaccines, by extending our knowledge of the protective antigens of V. cholerae. In conjunction with continuing research into the optimal route of vaccine administration (see Section 1.3.6), such an approach will hopefully increase the effectiveness of immunization, and thereby facilitate the control and eventual eradication of this disease (see Section 1.1.4).

Two lines of investigation were pursued, and these are discussed in Sections A and B below. The first comprised an attempt to further elucidate the nature of the choleraic infection, using the infant mouse model of this disease (Section 3.7). Since V. cholerae is non-invasive, a study of its colonization process is tantamount to a study of its pathogenicity, if one ignores the release and action of the toxin. This does not seem a major omission however, as the evidence available to date suggests that antibacterial immunity is of much greater significance than antitoxic immunity in the mediation of resistance to clinical cholera (see Section 1.3.2). Furthermore, we were encouraged in this respect by studies with other bacterial pathogens - particularly those involving the enteropathogenic E. coli (see Section 2.5.2), which establish an infection very similar to cholera - which demonstrated that those structures which promote colonization offer great promise as prophylactic immunogens. We therefore aimed to identify the adherence structures of V. cholerae in the hope that these might eventually be similarly employed (Section 1.3.7).

Our second experimental approach involved an evaluation of the prophylactic significance of the non-LPS antigens of V. cholerae. As previously discussed (Sections 1.1.4 and 1.3.7), conventional cholera vaccines have proved inadequate in the field, despite their content of LPS antigens. It therefore seemed worthwhile to investigate the collective prophylactic potential of the battery of non-LPS antigenic determinants of V. cholerae. Once again, we were prompted by reports of non-LPS protective antigens on a variety of bacterial pathogens, including some encouraging preliminary work with one strain of V. cholerae (see Section 6.1).

SECTION A

INFECTION BY V. CHOLERA

THE NATURE OF THE ADHERENCE PHENOMENON

7.2 The Correlation between Adherence and Virulence.

Colonization of a host mucosal surface is a necessary prerequisite for the establishment of infection by bacterial pathogens in general (Section 2.2), and it is therefore not surprising that the structures which mediate attachment have frequently been implicated as virulence determinants (see Section 2.5). In an attempt to establish a similar relationship between adherence and virulence in V. cholerae, an in vitro adherence assay was developed, using strips of everted adult mouse small intestine as the substrates for attachment (Section 4.2). Subsequent work indicated that the capacities of various strains to adhere to such tissue segments did indeed correlate with their pathogenic potential in infant mice (Section 4.3). Later studies repeatedly confirmed this correlation, by showing that, following several in vitro passaging procedures, originally non-adherent vibrios not only acquired the capacity to attach to intestinal strips, but also displayed greater virulence in vivo (Sections 4.4, 4.5 and 5.2.2).

In view of our more recent appreciation of the slime agglutinin (SA) as a mediator of V. cholerae attachment, it should be noted that this relationship between adherence and virulence has only been demonstrated in respect of binding to everted intestinal strips, which is mediated by a flagellar adhesin (see below). The SA can promote attachment to a range of non-mucosal substrates in vitro (see Section 7.4.1), but no role for this moiety in the establishment of choleraic infection has yet been demonstrated. Thus, although the SA+ form of non-motile 569B binds to Ballotini beads much more efficiently than the SA- form (Section 5.4.3(ii)), the two variants are equally virulent (Section 5.4.4).

7.3 The Role of the Flagellum in V. cholerae Adherence.

7.3.1 The significance of motility for in vitro and in vivo adherence.

Whilst investigating the possible link between adherence and virulence in V. cholerae, it became apparent that bacterial motility correlated directly with the capacity to attach to intestinal tissue in vitro (see Section 4.3.1). In an attempt to further examine this relationship, it was decided (Section 4.4.1) to select motile variants from the four (quantitatively) non-motile strains available and to then compare the adherence capacities of the related forms of each strain. This approach seemed preferable to the generation of non-motile variants by mutagenesis of motile strains (see discussion in next Section).

Motility enrichment initially involved repeated passage through Craigie tubes (Section 3.1.3), but was subsequently much more conveniently achieved using a sloppy-agar overlay method (Section 3.1.4). Comparisons of related motility variants consistently confirmed the significance of the vibrio flagellum to the process of in vitro attachment; on average, motile organisms were 40-fold more adherent than their non-motile counterparts (Table 4.4). Furthermore, spontaneous or induced losses of motility were accompanied by losses of the capacity to adhere (Section 4.4.2).

Subsequent studies confirmed the close association of these parameters. Thus, when non-motile, non-adherent V. cholerae 569B were selected for adherence - by repeated in vitro passaging on either intestinal tissue (Section 4.5) or inert particles (Section 5.2.2) - the organisms simultaneously acquired the property of motility. Indeed, the two parameters developed in close parallel (see Figures 4.3 and 5.1), and the data were consistent with the interpretation that each increase in adherence potential resulted from a preceding increase in the level of motility.

Following our appreciation of the close relationship between motility and adherence in vitro, it was of interest to ascertain whether motility might similarly promote attachment in vivo, and perhaps thereby contribute to the virulence of V. cholerae. Accordingly, the motile and non-motile forms of V. cholerae 111 and V. cholerae SC198 were compared for their capacities to colonize and persist in the infant mouse small intestine. Initially this was achieved using the standard experimental approach, in which the related motility variants were fed to different groups of infant mice, and their subsequent in vivo persistence determined at various intervals after challenge. The resulting vibrio recovery data (see Figure 4.2) indicated that the presence of a functional flagellum could confer upon non-pathogenic organisms the colonization potential of a highly virulent strain. Consequently, although apparently unable to replicate in vivo, the motile forms of the 111 and SC198 strains were able to persist longer than the non-motile forms (Section 4.4.3).

A second, less conventional approach provided an even more convincing demonstration of the in vivo significance of the vibrio flagellum. Mixed suspensions of related motile and non-motile vibrios were prepared, and used to feed different groups of infant mice; it was then possible, using the sloppy-agar overlay method, to subsequently determine the relative persistence of the two variants within the same intestinal environments (see Section 4.4.4). The results of two such experiments are summarized in Table 4.6b; it is apparent that the levels of motility in the vibrio populations recovered from the

small intestines 24 hours after feeding were much higher than those of the challenge inocula (an average of 30-fold higher in group B, and 70-fold higher in group Y - see Table 4.6b).

The extent of this "in vivo selection" for motility attests to the significance of the flagellar structure with respect to the colonization and in vivo persistence of V. cholerae. Presumably this underlies the finding that, following in vitro enrichment for motility, the 569B strain displayed a ten-fold greater virulence (Section 4.4.3; also see Sections 4.5 and 5.2.2). Nevertheless motility is neither necessary nor sufficient for the pathogenicity of V. cholerae (Section 4.4.3), and is therefore perhaps most appropriately considered as an accessory determinant of virulence.

7.3.2 Studies in other laboratories.

Our findings concerning the significance of the flagellum to the in vitro and in vivo adherence of V. cholerae confirm and extend similar observations reported from other laboratories over the past few years. Berry's group was the first to suggest a role for motility in the virulence of V. cholerae, following their demonstration that motile strains were more virulent for infant (Guentzel and Berry, 1975) and adult (Eubanks, Guentzel and Berry, 1976) mice than the non-motile variants derived from them by mutagenesis. Since the decreased virulence of the latter correlated with their reduced capacity to bind to the mucosal surface of infant mouse small intestine in vitro, it was suggested that motility contributed to virulence by facilitating in vivo colonization (Guentzel and Berry, 1975).

It should be noted here however, that the use of bacterial mutagens suffers the disadvantage that the mutational effects induced cannot be restricted to the one characteristic of interest. Thus, although the mutant strains used in the above studies were selected on the basis of their non-motility, it seems likely that they differed from their corresponding parent strains in more than this one respect. Different mutant strains prepared from the same

V. cholerae parent were found to retain the adherence and virulence capacities of the latter to different extents, and one such series of mutants also displayed altered patterns of phage sensitivity (see Guentzel and Berry, 1975). Nevertheless, in view of the large number of mutants examined, it seems highly likely that the common defect underlying their general inability to adhere was their lack of motility (see discussion by Guentzel, Field, Eubanks and Berry, 1977, and Section 4.4.1).

Subsequently Freter and Jones confirmed the findings of Berry's group by similarly demonstrating that non-motile variants derived from motile strains of V. cholerae lacked the capacities of the parent strains to attach to isolated brush border membranes or rabbit ileal slices in vitro (Jones and Freter, 1976; Freter and Jones, 1976). However these workers obtained their non-motile variants not only by mutagenesis but also by counterselection with anti-flagellar antibodies; furthermore they exploited the fact that the non-motile forms frequently reverted to motility by showing that such revertants had regained the potential to adhere (see Jones and Freter, 1976). In the latter situation in particular, it seems highly likely that the strains being compared for their adherence capacities would be identical in all respects but motility, and so no problems in the interpretation of results would arise.

By selecting motile variants from the non-motile strains available, we were able to adopt a similar approach for our examination of the contribution of vibrio motility to in vitro attachment. Our findings have confirmed the observations discussed above, but we have also shown that non-motile organisms selected for adherence in vitro also acquire the property of motility. The sloppy-agar overlay method enabled us to demonstrate the close parallel development of the two parameters, and hence the probable involvement of the vibrio flagellum in the process of in vitro attachment.

To the author's knowledge, comparison of the in vivo behaviour of motile and non-motile strains of V. cholerae has hitherto been restricted to a recent report from Berry's group (Guentzel et al, 1977). Using a fluorescent antibody technique to study the distribution of related motility variants in the small intestines of infant mice, these workers recorded that, whereas motile organisms were able to penetrate the intervillous spaces and enter the intestinal crypts, the non-motile vibrios were mainly confined to the lumen (Guentzel et al, 1977; also see Schrank and Verwey, 1976). Presumably as a consequence of this more effective colonization, vibrio recovery experiments demonstrated that much greater numbers of organisms could be recovered from the mice challenged with motile bacteria (Guentzel et al, 1977).

Our own investigation of the in vivo significance of the vibrio flagellum has confirmed the greater colonization capacity of motile organisms. In particular, when mixed suspensions of motile and non-motile vibrios were used to feed infant mice - in order to be able to compare the behaviour of the two variants within the same intestinal environments - the motile organisms were able to colonize much more effectively, and as a consequence, persisted longer in vivo (see Section 4.4.4). Similar instances of "in vivo selection" for the more adherent members of a bacterial population have been reported in studies with enteropathogenic E. coli (Nagy et al, 1977; Morgan et al, 1978) and S. pyogenes (Ellen and Gibbons, 1972). Presumably this also underlies the varying tendency for non-motile mutant strains to revert to motility in vivo, despite being completely stable in vitro (see Guentzel and Berry, 1975).

7.3.3 Evidence for a flagellar adhesin.

Having demonstrated the significance of motility to the in vitro and in vivo adherence capacities of V. cholerae, it was of interest to more accurately define the role of the flagellar structure in these processes. As discussed previously (see Sections 4.5 and 5.3), it is possible that the flagellum is the carrier of the vibrio adhesin, in which case it would provide

both motility and attachment functions for the bacterium. Alternatively, this structure might be solely an agent of motility, serving merely to facilitate the interaction between a surface adhesin and the binding substrate. Several lines of evidence suggested that the attachment moiety was located on the vibrio flagellum, and hence that the former explanation was likely to underlie the close association of the properties of motility and adherence.

Firstly, the fact that non-motile vibrios are unable to display any binding capacity in a variety of experimental systems (see Tables 4.4. and 5.1, and Jones and Freter, 1976) argues against the concept of non-flagellar surface adhesins. Secondly, our studies suggest that non-motile organisms are unable to acquire the potential for in vitro adherence without simultaneously developing motility. Thus, using two in vitro passaging procedures designed to minimize the significance of motility per se, it was found that the properties of motility and adherence nevertheless developed in close parallel (see Sections 4.5 and 5.2.2).

Finally, and most conclusively, inhibition-of-adherence assays using antibodies of different specificities enabled us to independently demonstrate both motility and adherence capacities associated with the flagellar structure. Thus it was possible to observe motile yet non-adherent organisms, and also immobilized yet adherent organisms; the latter demonstration strongly suggested that the attachment moiety was located on the vibrio flagellum (see Section 5.3.2). Furthermore, these experiments showed that motility per se was neither necessary nor sufficient for in vitro adherence, confirming our hypothesis that the involvement of the flagellum in attachment extended beyond its capacity as an agent of motility.

Recently Freter's group has also put forward the concept of the vibrio flagellum as the carrier of an adhesin, but to date their evidence in support of such a theory has been only indirect. Furthermore, the experiments which prompted this conclusion were performed in their brush border system of adherence (Jones and Freter, 1976). Other studies suggest however, that the

factor responsible for attachment to these substrates is actually the slime agglutinin, and further, that this moiety is not strictly associated with the flagellar structure (see Section 7.4.2). In the ileal slice system described by Freter and Jones (1976) - which probably monitors the same adhesin as our intestinal strip system (Section 7.4.2) - it was reported that non-motile variants derived from a highly motile strain lacked the adherence capacity associated with the latter. Although this indirectly suggests that the adhesin is located on the vibrio flagellum, it is clear that our studies have considerably improved our understanding of the underlying basis for the superior adherence potential of motile vibrios.

Although the nature of the proposed flagellar adhesin remains uncertain, it seems likely that this moiety is non-LPS in nature, since antibodies of this specificity were able to block attachment without inhibiting motility (see Table 5.6). Antibodies directed against LPS determinants could also inhibit adherence (Table 6.9a), but the finding that this capacity could be titrated out much more quickly than the capacity to inhibit motility (Section 5.3.2) suggested that the former might be due to a steric hindrance effect rather than a specific masking of attachment moieties. The vibrio flagellum is known to be surrounded by an LPS-containing sheath structure (Section 1.2.3), and it is not difficult to imagine that, under conditions of anti-LPS antibody excess, the expression of non-LPS adhesins associated with the flagellum might be inhibited. Furthermore, the finding that non-motile vibrios consistently lack the capacity to attach in vitro is in itself evidence that the adhesins are not LPS in nature (especially in view of the fact that, under certain conditions, antibody-immobilized flagellated organisms can display an undiminished binding capacity - see Section 5.3.2).

7.4 A Two-Adhesin Model of *V. cholerae* Adherence.

7.4.1 The slime agglutinin as a second mediator of *in vitro* attachment.

As part of our investigation of the contribution of the flagellum to the in vitro binding potential of motile vibrios, we attempted to dissociate the parameters of motility and adherence using several procedures which had achieved such a separation in a brush border system of attachment (see Section 5.3.1). Although unsuccessful in this respect, these experiments confirmed the findings of Freter's group that the characteristics of *V. cholerae* attachment can differ markedly in vitro, depending upon the nature of the adherence substrate (Jones et al, 1976; Freter and Jones, 1976; also see Section 5.3.1). Thus, whereas attachment to brush borders or erythrocytes is mediated by a labile, fucose-sensitive, Ca^{++} - dependent adhesin (Jones et al, 1976), the moiety responsible for adherence to intact intestinal surfaces is relatively stable, fucose-resistant, and (possibly) Ca^{++} - independent (Freter and Jones, 1976; Section 5.3.1).

There seemed several reasons to suppose that these observations reflected the existence of two discrete attachment moieties (see Section 5.4.1). Our studies to this stage had been concerned with various properties of the adhesin responsible for attachment to murine intestinal tissue - its in vitro and in vivo significance, its binding specificity and its localization. Since these issues had been satisfactorily resolved, our attention turned to an examination of the brush-border-adhesin (BBA) described by Freter's group (Jones et al, 1976).

In many respects the properties of the BBA were similar to those of the slime agglutinin (SA) described by Lankford's group (see Section 5.4.2). Indeed, it seemed possible that the two were actually different descriptions of the same entity, in which case the fact that the SA is only expressed under certain cultural conditions (Bales and Lankford, 1961) offered an explanation for our inability to detect a labile vibrio adhesin in our systems of attachment (Section 5.3.1). Subsequent investigation confirmed that the

growth conditions routinely employed in this laboratory were indeed unsuitable for development of the SA (see Section 5.4.2). However this factor was produced by V. cholerae 569B during growth in Trypticase soy broth (TSB; see Section 5.4.2), and so it was possible to examine its role in the in vitro attachment of these organisms. This was achieved by growing the motile and non-motile variants of the 569B strain under conditions which either favoured or inhibited the development of the SA, and then comparing the adherence capacities of the resulting organisms (see Section 5.4.3).

Using this approach we were unable to demonstrate any role for the SA in attachment to everted intestinal segments (Section 5.4.3(i)), thus confirming the findings of Freter and Jones (1976). Furthermore, non-motile 569B organisms grown under conditions conducive to SA production were no more virulent for infant mice than those grown under conditions inhibitory for the development of SA (Section 5.4.4), suggesting that this factor is similarly unable to facilitate adherence to an intact mucosal surface in vivo.

In contrast, studies in several laboratories have demonstrated that the SA is able to promote attachment to a wide range of non-mucosal substrates in vitro. These include mammalian erythrocytes (Lankford and Legsomburana, 1965; Jones et al, 1976; Section 5.4.2), brush border membranes isolated from rabbit intestinal epithelial cells (Jones et al, 1976), the serosal surface of adult mouse small intestine (Section 5.4.4), and at least two types of inert particles (Section 5.4.3(ii); also see Lankford and Legsomburana, 1965). It is interesting to note that the superior binding potential of El Tor vibrios for such substrates may well be a reflection of the capacity of these organisms to constitutively express the SA (see Section 5.4.4).

The nature of the slime agglutinin remains undefined. This factor was so-termed because of its general association with a slime layer on the vibrio surface (Lankford and Legsomburana, 1965; Chulasamaya and Lankford, 1970); however the finding that the latter could be retained by organisms which had lost their haemagglutinating capacity suggested that the agglutinin itself

is a discrete entity which is normally associated with the slime envelope (Lankford and Legsomburana, 1965). Although fimbriae have been observed on the surfaces of V. cholerae, their possible involvement in haemagglutination remains unresolved (see Section 1.2.4(ii)).

Finally, although Lankford's group concluded that the development of the SA was independent of the production of the vibrio flagellum (Lankford and Legsomburana, 1965), Jones and Freter (1976) postulated that their BBA was actually located on this structure. Since we considered it likely that both groups had studied the same adhesin, we decided to briefly examine this point. Our studies suggested that the flagellar structure was neither necessary nor sufficient for development of the SA; nevertheless the presence of the flagellum appeared to stabilize this factor on the surface of the cell (see Section 5.4.2).

7.4.2 Discussion of the results obtained in various in vitro systems of adherence.

In view of our better understanding of the nature of V. cholerae attachment in vitro, it was of interest to briefly reconsider the results obtained in the various systems of adherence, and to attempt to explain the different observations which have been made. As discussed previously (see Section 2.3), there is good evidence to suggest that, in some situations at least, bacterial attachment involves an interaction between bacterial adherence factors and specific host receptor structures. Accordingly, differences in the characteristics of the binding process might reflect differences at the level of the bacterium, or the substrate, or both.

The studies of Freter's group, using isolated brush borders or rabbit ileal slices as substrates for attachment, have led them to propose that "there are at least two specific mucosal receptors for vibrio adhesion" (Freter and Jones, 1976). When discussing the underlying basis for the different observations made in their two systems, these workers have stressed the significance of the host's (substrate's) contribution to the binding event,

and have concluded that "the two systems most likely represent mechanisms of bacterial association with different areas of the mucosal surface" (Freter and Jones, 1976).

In contrast, our emphasis has been upon the nature of the adherence factors which mediate vibrio attachment. Initially this was a direct consequence of our interest in the virulence determinants of V. cholerae, but subsequently our approach was vindicated by experiments which suggested that these bacteria can adhere in the absence of specific receptor structures (see Section 5.2). Our studies of the vibrio adhesins enable us to explain the observations made in the different systems in terms of the nature of the factors which mediate attachment to the various substrates. This approach provides a more complete explanation of the available data, without needing to propose the existence of specific receptor structures. Since our own experiments have not supported the theory that such structures are necessary for V. cholerae adherence, we shall not consider their possible involvement at this stage; however the issue of the specificity of vibrio attachment will be discussed shortly (Section 7.5). Let us now briefly examine the nature of adherence to intestinal tissue, brush border membranes, and inert particles.

Intestinal tissue. Freter and Jones (1976) reported that vibrio attachment to rabbit ileal slices was mediated by a factor present on motile organisms but not on non-motile variants derived from them. This adhesin resisted prolonged incubation in buffer at 37C (so that bound organisms showed no tendency to spontaneously detach), and was retained by motile vibrios during growth on Trypticase soy agar (TSA). Each of these observations was confirmed in our system of adherence to everted murine small intestine; collectively they suggested that the slime agglutinin (SA) played no significant role in attachment to an intact mucosal surface.

Subsequent experiments enabled us to conclude that the adhesin responsible for binding in this system is located on the vibrio flagellum, and is probably non-LPS in nature (see Section 7.3.3). Like Freter

and Jones (1976), we found that the adherence function of this moiety could be inhibited by specific antibodies (Section 6.4.4). The fact that this effect could be demonstrated at sub-agglutinating levels of bacteria suggested that the antibodies were able to directly block the expression of the adhesin (see Section 6.4.4).

Brush border membranes. The characteristics of V. cholerae attachment to brush border membranes isolated from rabbit intestinal epithelial cells (see Jones et al, 1976) suggest that binding in this system is primarily mediated by the slime agglutinin. Like the latter, the brush-border-adhesin (BBA) is a powerful haemagglutinin, and is not retained by vibrios (of Classical biotype) during growth on Trypticase soy agar (TSA). Both factors are denatured by prolonged incubation in buffers at 37C, and as a consequence their parent vibrios spontaneously detach from their binding substrates under such conditions (see Lankford and Legsomburana, 1965; Jones et al, 1976).

Freter's group subsequently concluded that their BBA was associated with the vibrio flagellum (Jones and Freter, 1976) but, as discussed previously, this is probably an erroneous impression arising from their routine growth of vibrios in TSB at 37C (see Section 5.4.2). Lankford's SA could be extracted from the vibrios without affecting their motility, and seemed to be normally associated with a slime envelope which surrounded the cells (Lankford and Legsomburana, 1965; Chulasamaya and Lankford, 1970). Accordingly we can readily explain the finding that specific antibodies could only inhibit the expression of the BBA under conditions where bacterial agglutination could occur (Freter and Jones, 1976). Since the SA is distributed over the entire vibrio surface, a direct antibody-blocking mechanism would not be demonstrable (unless, perhaps, with Fab fragments); rather, the antibodies would agglutinate the bacteria, and in this way achieve an indirect inhibition of attachment.

Inert particles. Our studies with inert substrates suggested that the attachment of V. cholerae 569B to Ballotini beads was mediated primarily by the SA; in contrast, both the flagellar adhesin and the SA effectively promoted binding to Pevikon beads (see Section 5.4.3(ii)). In view of the significance of the SA in both systems, it was puzzling why our earlier experiments with these substrates had not demonstrated the lability of the adhesin involved (Section 5.3.1).

The likely explanation for this seeming paradox became apparent later, during an attempt to isolate the SA from V. cholerae 569B. It was found that, although incubation in borate buffered saline at high pH (after Lankford and Legsomburana, 1965) effectively removed the haemagglutinin from the surfaces of the vibrios, similar treatment with phosphate buffered saline at pH 7.4 was unsuccessful (Attridge and Rowley, unpublished observations). The latter had been used for our previous studies with inert particles (Section 5.3.1), and the stability of the SA in this buffer was presumably responsible for the apparent stability of the adhesins involved in these systems. Since at that stage we did not appreciate the significance of the SA as a mediator of vibrio attachment, we were similarly unaware of the need to ensure the denaturing potential of the incubation conditions.

7.4.3 The significance of the flagellar adhesin, the slime agglutinin and motility per se to the in vivo colonization of V. cholerae.

Our investigation of the adherence phenomenon in V. cholerae has suggested the existence of at least two discrete factors which can mediate attachment in vitro. In this section we shall briefly consider the possible pathogenic significance of these adhesins, and also discuss the likelihood that the property of motility might be important for colonization in vivo. It should be emphasized that, although both in vitro and in vivo studies attest to the likely involvement of the flagellar adhesin, the relevance of the slime agglutinin (SA) to the colonization process remains uncertain.

Following ingestion, those vibrios which survive passage through the stomach (Section 1.1.2) enter the small intestine, where they need to colonize the mucosal surface in order to establish an infection (Section 2.2). Although motility per se seems of little relevance to the in vitro attachment of V. cholerae (Jones and Freter, 1976; Sections 5.3.2 and 7.3.3), there is some evidence to suggest that this property might significantly assist colonization in vivo, by promoting the widespread distribution of the organisms prior to the actual binding event. Thus, when Berry's group compared the distribution of related motility variants of V. cholerae in the intestines of infant mice, they observed that the motile bacteria were able to penetrate the intervillous spaces and enter the intestinal crypts, whereas the non-motile organisms were mainly confined to the lumen (Guentzel et al, 1977; also see Schrank and Verwey, 1976).

In view of the dual motility and binding functions of the vibrio flagellum, and the demonstration that immobilized vibrios can still attach in vitro (Section 5.3.2), it is difficult to assess the in vivo significance of motility per se. Nevertheless it seems reasonable to propose that the property of motility is responsible for the effective distribution of the vibrios independently of, and prior to, the expression of the adherence function of the flagellar structure. There are at least two ways in which motility per se might thus assist the colonization process. Firstly, it would provide the organisms with the capacity to respond positively to chemotactic stimulants diffusing away from the intestinal surface (see Allweiss et al, 1977). Secondly, and perhaps as a consequence of such chemotactic responsiveness, the beating of the flagellum might enable the vibrios to "burrow" through the mucous layer which protects the intestinal villi from the contents of the lumen (see Schrank and Verwey, 1976), or at least to migrate along "tracks" within this mucous zone (see Jones et al, 1976).

Although the property of motility might facilitate the initial interaction between vibrios and intestinal epithelium, it seems likely that

enduring colonization would be dependent upon the expression of an adherence capacity. Thus our in vitro studies have suggested that the prime determinant of the protective potential of specific antibodies is their capacity to directly block vibrio attachment; a capacity to inhibit motility is not necessary (see Section 6.6).

Of the two recognized vibrio adhesins, the factor located on the vibrio flagellum would seem the more likely mediator of in vivo adherence. The binding capacity provided by this factor was found to underlie the significance of the flagellar structure to the process of attachment to intestinal tissue in vitro (Sections 5.3.2 and 7.3.3); in contrast the slime agglutinin (SA) was unable to promote adherence to this substrate (Section 5.4.3(i)). Furthermore the motile variants of V. cholerae 111 and V. cholerae SC198 were found to colonize the infant mouse small intestine almost as effectively as our most virulent strain, V. cholerae 017 (see Section 4.4.3), despite the fact that they are unable to produce the SA in vitro (Section 5.4.2). Finally, although the SA was able to facilitate the attachment of non-motile V. cholerae 569B to Ballotini beads (Section 5.4.3(ii)), it did not increase the virulence of this strain (Section 5.4.4).

However, in a recent electron microscopic study of the nature of V. cholerae adherence in vivo, it was concluded that "the bacteria adhered via their surface coats" to the microvilli of the epithelial cells (Nelson et al, 1976). In each of two models of experimental cholera, "the majority of bacteria were aligned horizontally with the epithelial surface, but some were attached in an end-on manner, with their flagella extending into the lumen" (Nelson et al, 1976). These observations suggest the involvement of a different adherence factor, one whose location is not restricted to the flagellar structure. The obvious candidate for such a surface adhesin would be the slime agglutinin, particularly in view of the fact that the vibrios used in the above study were of El Tor biotype (and would therefore probably express the SA - see Section 5.4.4) and were grown under conditions which favour the development of this moiety (TSB at 37C - see Nelson et al 1976,

and Section 5.4.2). It is thus conceivable that the SA promotes the initial binding of the vibrio to the host epithelium, and that this association is subsequently stabilized by the action of the flagellar adhesin.

At present, however, there is no direct evidence to support the thesis that the SA can facilitate attachment to an intact intestinal surface (see Section 7.4.1), and so its role in the colonization process remains purely speculative. Indeed, there seems just as much reason to suppose that the main function of the SA is to assist the survival of the vibrios in the external environment. The three distinguishing characteristics of organisms of El Tor biotype are their capacity to agglutinate (chicken)erythrocytes, and their resistance to polymyxin B and Mukerjee's Type IV cholera phage (see Section 1.1.1). The first of these is almost certainly a reflection of the constitutive expression of the SA (see Section 5.4.4); if the other traits can similarly be attributed to the presence of the slime layer around the periphery of the cell, they may be just two examples of the way in which this moiety can protect the vibrios in the wild. A similar function has been proposed for the "glycocalyx" which surrounds many other bacteria (Costerton, Geesey and Cheng, 1978).

In addition, the SA might favour the replication of V. cholerae outside the human body. We have observed that, when grown in standing broth cultures, vibrios of El Tor biotype form thick surface pellicles (Attridge and Rowley, unpublished observations), which seem analogous to those produced by other bacteria under similar cultural conditions (see Duguid et al, 1966; Duguid and Campbell, 1969). Duguid's group has shown that pellicle formation in static cultures is a characteristic of fimbriated organisms; the same bacteria do not form pellicles when in the non-fimbriated phase. Furthermore, the fimbriated organisms are able to multiply much more rapidly in standing cultures than their non-fimbriated counterparts, and are also able to agglutinate vertebrate erythrocytes (Duguid et al, 1966; Duguid and Campbell, 1969). Since the SA is also a haemagglutinating, possibly - fimbriate

(see Chulasamaya and Lankford, 1970) factor, it seems likely that it might similarly be responsible for in vitro pellicle formation. If so, the same capacity might favour the multiplication of vibrios in static surface waters in the field.

7.5 The Specificity of *V. cholerae* Adherence.

It is now apparent that the adhesins of *V. cholerae* can collectively mediate attachment to a variety of physiological substrates, including mammalian erythrocytes (Bales and Lankford, 1961; Jones et al, 1976; Section 5.4.2), leucocytes and HeLa cells (Lankford and Legsomburana, 1965), the intestinal epithelium of mice (Guentzel and Berry, 1975; Section 4.3) and rabbits (Freter, 1969; Freter and Jones, 1976; Nelson et al, 1976), the surfaces of isolated intestinal epithelial cells (Bellamy et al, 1975; Jones et al, 1976; Section 4.A), and the serosal surface of adult mouse small intestine (Section 4.A). However, from the viewpoint of maintaining the species in the wild, all of these substrates may be considered "irrelevant", in the sense that they either originate from resistant animals or, where of human origin, represent surfaces which would not be encountered during the infective process.

Obviously selective adherence does not underlie the completely restricted natural host range of *V. cholerae* (see Section 2.3), but in the present context the relevance of the above studies lies in their implications for the specificity of vibrio attachment. To rigidly apply the dogma of selective bacterial adherence, it would be necessary to propose that each of the surfaces mentioned above displays the specific receptor structures to which vibrios are able to bind. Similar assumptions would be necessary to explain the binding of Shigellae (see Savage, 1972) and gonococci (Tebbutt, Veale, Hutchison and Smith, 1976) to the cells of irrelevant tissues.

However the finding that vibrios can also attach to inert substrates - including glass, polyvinyl and polystyrene beads (Section 5.2.1; also see

Lankford and Legsomburana, 1965) - suggests an alternative explanation for the studies mentioned above. Since these substrates would be unlikely to display any surface structures which remotely resemble specific host receptors, the concept of a non-specific adherence phenomenon would seem to merit consideration. It should be noted that bacteria of other genera can also bind to a wide variety of inert substrates, including discs of epoxy resin (Jones, Roth and Sanders, 1969) or plastic (Costerton et al, 1978), the enamel surfaces of teeth (Gibbons and van Houte, 1975), cellulose fibres, and charcoal, kaolin and sand particles (see Daniels, 1972).

Before attempting to reconcile the concept of non-specific adherence with the many documented examples of the selectivity of bacterial attachment, it seems necessary to briefly discuss two important aspects of the former. Firstly, what is the likely in vivo significance of adherence factors which mediate non-specific attachment in vitro? Secondly, how might non-specific adherence take place?

With respect to the first point, it might be argued that the slime agglutinin (SA) is likely to be one important mediator of non-specific attachment in vitro (Sections 7.4.1 and 7.4.2), and yet this factor may have no relevance to the establishment of the infective process (Section 7.4.3). However systems which monitor the adherence capacity of the SA have also provided data which have been interpreted as supporting the theory that V. cholerae attach specifically to special receptor structures (see Jones and Freter, 1976, and Section 7.4.2), and it is clear that the same criticism would apply in this instance. Nevertheless there remain two reasons to suppose that mechanisms of non-specific adherence in vitro may be of relevance to the process of colonization in vivo. Firstly, the functional expression of the flagellar adhesin - which seems likely to play a significant role in vivo (see Sections 7.3.1 and 7.4.3) - is not dependent upon the presence of specific receptor structures. Thus, this factor can promote attachment to Pevikon beads (Section 5.4.3(ii)), as well as intestinal tissue (Section 5.3.2).

Secondly, it should be emphasized that, following in vitro passaging on either Ballotini or Pevikon beads, non-motile V. cholerae 569B not only acquired the capacity to attach to intestinal strips in vitro, but also displayed greater virulence in vivo (Section 5.2.2).

Turning to the second of the questions posed above, it is possible that vibrios attach to inert and physiological substrates in a manner analogous to that proposed by Edwards to explain binding between eukaryotic cells (Edwards, 1978). He suggested that many instances of differential cell adhesion may result from non-specific interactions between the glycoprotein "coats" present on the surfaces of the various cell types. For example, it was postulated that tissue formation during embryogenesis might occur simply as a result of the aggregation of cells bearing the same array of surface glycoproteins, in a manner analogous to the incompatible phase separation which occurs in mixed solutions of large organic polymers (see Edwards, 1978).

It seems quite possible that the adhesins of V. cholerae might similarly facilitate attachment by increasing the number or strength of the various non-specific attractive forces (see Edwards, 1978) tending to bind the organisms to a given substrate. Thus, because of its widespread distribution over the surface of the cell, the presence of the slime agglutinin (SA) might significantly alter the charge characteristics of the vibrio surface, and hence its affinity for a given substrate. For example, if the SA increased the net surface charge of the organisms, it would automatically increase the likelihood of binding to substrates of opposite charge. Such an effect might even facilitate attachment to surfaces with the same overall charge, provided that multivalent ions of opposite charge are available to act as "ionic bridges" (see Costerton et al, 1978). It is interesting to note in this latter regard that the SA-mediated (see Section 7.4.2) attachment of V. cholerae to brush border membranes or human erythrocytes was found to be dependent upon the presence of calcium ions (Jones et al, 1976).

Since attachment to these latter substrates could be partially

inhibited by the presence of free L-fucose - and to a lesser extent D-mannose - in the suspension medium, Freter's group proposed that this monosaccharide might constitute part of the natural host (rabbit) receptor for V. cholerae (Jones and Freter, 1976). However, we have found that both of these sugars also cause a slight inhibition of V. cholerae 569B attachment to Ballotini beads (see Table 5.3 and Section 5.4.4), and in this system the mimicking of receptor structures is not a feasible explanation. It therefore seems possible that the inhibitory capacities of these sugars are a reflection of their abilities to neutralize some of the non-specific attractive forces between bacterium and substrate.

Freter's group has also reported that the adherence of V. cholerae to intact ileal slices can be significantly reduced by a soluble factor prepared from scrapings of rabbit intestinal mucosa (Freter and Jones, 1976). The details of the preparation procedure suggest that this factor might normally be exposed on the surfaces of the epithelial cells, and that its inhibitory capacity may indeed be attributable to the fact that it can function as a substrate for vibrio attachment. However, we would emphasize the point that this moiety might not be essential for attachment, but merely be one of a number of substrates for which vibrios can express a binding affinity. When discussing his suggestion for the nature of differential adhesion in general, Edwards (1978) commented that his "explanation of apparently specific cell interactions in terms of large numbers of weak non-specific forces conflicts particularly with the widely held view that cells interact through specific binding by recognition proteins with lock-and-key active sites. Such proteins probably do play a part in some mechanisms; the point is that they may not be necessary." Exactly the same comment applies to our suggestion that the adherence phenomenon in V. cholerae may lack substrate specificity.

To conclude this section, it should be acknowledged that in several situations bacterial attachment would indeed appear to be dependent upon the presence of specific receptor structures on host epithelial cells (see Section 2.3). It is interesting to note, however, that nearly all of the strong evidence in favour of selective adherence has been derived from studies which have examined the binding of commensal organisms to cells from their respective hosts. Bacteria which are now indigenous to a given host may well have evolved in concert with that host; in view of the non-specific mechanisms acting to inhibit colonization¹, it may have been necessary for the organisms to evolve a recognition system in order to be able to occupy a particular habitat within the host. In those instances where the advantages which accrue to the host² outweigh the disadvantage of supporting an indigenous flora, there would be no evolutionary selection pressure to counter such bacterial adaptation.

In contrast, any similar specialization by pathogenic organisms might be expected to elicit a prompt evolutionary response from the host species. For example, Rutter and co-workers found that some piglets of a given herd were resistant to challenge with enteropathogenic E. coli, apparently because their intestinal epithelial cells lacked the receptor structures for which the latter had evidently become adapted (Sellwood et al, 1975; Rutter, 1976 - cited by Smith, 1977). The enormous survival value of such a mutation would ensure its rapid spread throughout the population, with obvious and serious implications for the persistence of the specialized pathogen. It is tempting to speculate that other pathogenic bacteria might have evolved mechanisms of non-specific adherence in an attempt to avoid such rapid and effective host responses. This might well explain the capacity of V. cholerae, Shigellae and gonococci to bind to "irrelevant" cells (see above).

¹ These would include the microbicidal activities of gastric acid (Section 1.1.2) and intestinal fluid (Table 6.7), the cleansing activity of the flow of mucosal secretions, peristalsis and epithelial cell desquamation (see Section 2.2).

² A commensal bacterial flora might prevent the colonization of potentially more damaging organisms (see Section 2.3.1, and footnote 3 in Chapter 4), or provide the host with additional sources of nutrients (eg. the breakdown of cellulose by the organisms indigenous to the ruminant gut).

SECTION B

IMMUNITY TO *V. CHOLERAE*

THE PROPHYLACTIC SIGNIFICANCE OF THE NON-LPS ANTIGENS OF *V. CHOLERAE*

In this section we shall discuss the results of our evaluation of the collective prophylactic potential of the battery of non-LPS antigens of *V. cholerae*. We shall consider the implications of our findings for the future development of cholera vaccines, and also discuss relevant studies from other laboratories. Many of the points to be considered below have already been discussed in detail in Chapter 6, and so will be dealt with more briefly here.

7.6 The Existence of non-LPS Protective Antigens in *V. cholerae* 569B.

7.6.1 Initial studies.

As discussed previously (Section 6.1) there seemed several reasons to be optimistic about the possible existence of non-LPS protective antigens in *V. cholerae*. In particular, previous studies in this department had suggested the presence of such determinants in the 569B strain, and we began our present investigation by attempting to confirm these findings. Based on the work of our previous colleagues, we employed two different experimental approaches, and each successfully demonstrated the existence of non-LPS protective antigens in *V. cholerae* 569B.

The first approach involved the repeated absorption of a mouse anti-live 569B serum with highly purified 569B-LPS (after Neoh and Rowley, 1972). Comparison of the antibacterial capacities of the original and residual sera indicated that this treatment had completely removed the haemagglutinating capacity of the former, but that the latter retained significant levels of bactericidal and protective antibodies (see Section 6.2). Indeed, despite the potent immunogenicity of LPS, approximately 20% of the protective activity of the original serum was evidently mediated by antibodies directed against non-LPS determinants (Table 6.1a).

Another interesting point to emerge from this line of investigation was the finding that, at least in the mouse, antisera prepared against live vibrios contain higher levels of IgG antibodies than those raised against isolated LPS. Thus the average mercaptoethanol-resistance of the bactericidal capacities of two sera prepared against highly purified V. cholerae LPS was only 0.27%; in contrast, immunization with live organisms generated antisera in which 1.8 - 6.7% of the bactericidal activity was mediated by ME-resistant antibodies (see Table 6.2). It was calculated that, by comparison with the two anti-LPS sera, a hyperimmune anti-live 569B serum possessed a 25-fold higher IgG : IgM antibody weight ratio (see Section 6.2).

If this finding could be repeated in humans, it would have great relevance for the practice of parenteral immunization. Thus, of the IgM and IgG antibodies elicited by vaccination, the latter are selectively transmitted to the site of the potential infection (Section 1.3.4(ii)); moreover, IgG antibody responses are more enduring and demonstrate anamnesis when recalled. It therefore seems likely that the most effective parenteral vaccines will be those which induce the greatest proportions of IgG antibodies. In this regard the non-LPS protective antigens of V. cholerae should be superior to the killed cellular suspensions being used at present (see Section 1.3.7).

The second approach adopted for our attempted demonstration of non-LPS protective antigens in V. cholerae 569B was prompted by the work of Bellamy et al (1975), and involved the use of a specially constructed hybrid vibrio, 569B/165. The preparation of this strain has been described elsewhere (Section 6.3); the important feature is that it carries the non-LPS antigens of the 569B parent strain, but none of the LPS determinants of this strain. Accordingly the 569B/165 hybrid offered an excellent opportunity to assess the prophylactic significance of the non-LPS antigens in the absence of the highly immunogenic LPS determinants.

Our studies indicated that live 569B/165 organisms were able to generate mouse antisera which were extremely efficient in the protection of infant mice

from challenge with the parental 569B strain. Since the reported antigenic relationship between the hybrid and its V. cholerae parent has been repeatedly confirmed (see Sections 6.3 and 6.5), and since the shared protective antigens were shown to be heat-labile (Sections 6.3 and 6.5), it seems certain that these determinants are non-LPS in nature. The finding that the protective capacity of a hyperimmune anti-live 569B/165 serum was almost equivalent to that of a similar serum raised against organisms of the challenge strain itself - despite the fact that the former lacked anti-LPS antibodies which are known to be protective in the infant mouse model of cholera (Neoh and Rowley, 1972; Bellamy et al, 1975; Horsfall and Rowley, 1979; also see Table 6.11b) - strongly suggested that further investigation of these non-LPS antigens was warranted.

7.6.2 The stability of the non-LPS protective antigens of V. cholerae 569B.

Although our initial studies had demonstrated the prophylactic significance of the non-LPS antigens of V. cholerae 569B, it was felt that the relative lability of these determinants might limit their effectiveness in cholera vaccines. In particular, it was of interest to determine the likely consequences of oral administration and vaccine inactivation upon the immunogenicity of such potentially protective non-LPS antigens.

As discussed previously (see Section 1.3.6), the optimal route of vaccine administration has yet to be defined; however recent studies with combined oral-parenteral immunization schedules suggest that future vaccines may be presented orally, at least in part (see Section 1.3.6). Since one of the disadvantages of oral immunization is the denaturation of the vaccine in the hostile environment of the gut (see Section 1.3.6), we used the 569B/165 strain to attempt to assess the suitability of the relatively labile non-LPS protective antigens as oral immunogens (see Section 6.5.1). Our experiments revealed that, although capable of eliciting protective antibodies when presented systemically, live 569B/165 vibrios could generate no protective

response when given by the oral route (Section 6.5.2). Subsequent investigation demonstrated that this was indeed a result of the denaturation of the protective antigens in the intestinal environment (Section 6.5.2). This work suggests that the relatively labile non-LPS antigens may only constitute effective prophylactic immunogens when given systemically. It is interesting to note in this regard that such antigens apparently generate a high proportion of IgG antibodies (see Table 6.11a, and footnote 14 in Chapter 6), making them ideally suited for parenteral vaccines (see Section 7.6.1).

Although it has received much less attention to date, the question of the optimal method of vaccine inactivation is likely to assume greater importance with our increasing appreciation of the possible significance of the non-LPS components of cholera vaccines. Our ultimate goal will be to identify, isolate and immunize with these antigens, but as this will require considerable time and effort it seems essential in the meantime to inactivate the conventional cellular vaccines in such a way as to cause the minimal level of antigenic denaturation. Once again the 569B/165 strain provided a means of assessing the stability of the non-LPS protective antigens to the various methods of inactivation and preservation, in the absence of the highly resistant LPS determinants (see Section 6.5.1). Our examination showed that some treatments were definitely more deleterious than others (see Section 6.5.3), and suggested that this issue should receive more consideration in the future production of cholera vaccines.

The identities of the non-LPS protective antigens of V. cholerae 569B remain undefined. Since the anti-live 569B/165 serum was able to inhibit the attachment of the 569B parental strain to intestinal tissue in vitro (see Section 5.3.2), it seems likely that at least some of the antibodies present in this serum are directed against the 569B adhesin which mediates attachment, i.e. the flagellar adhesin (see Sections 5.3.2 and 7.3.3). Berry's group has recently reported the presence of a highly protective factor which is present in crude preparations of vibrio flagella; its exact nature remains uncertain,

although it would appear to be discrete from both LPS and the flagellar core material (see Eubanks et al, 1977).

7.6.3 The relative prophylactic significance of the LPS and non-LPS antigens of *V. cholerae* 569B.

Before embarking upon the detailed identification and careful isolation of the non-LPS protective antigens of *V. cholerae*, it is essential to determine whether this is likely to be a sufficiently rewarding line of research. Thus it seemed important to attempt an evaluation of the relative prophylactic significance of the LPS and the non-LPS antigens in the one strain known to possess the latter. The fact that pure preparations of non-LPS antigens were not available limited the experimental approach that could be employed for this study (see Section 6.7.1); it was eventually decided to compare the protective efficiencies of antibodies of the same class which were specific for either type of determinant (see Section 6.7.1).

Accordingly, IgG and IgM fractions were prepared from our sources of anti-LPS and anti-non-LPS antibodies; when the "class purity" (see Section 6.7.2) of each fraction had been confirmed, its protective efficiency - expressed as the protective activity per RIA binding unit (see Section 6.7.1) - was determined. The resulting data were extremely encouraging, for they suggested that antibodies directed against non-LPS antigens were at least 15 times more protective than those specific for the LPS determinants. Since conventional vaccines rely heavily on their LPS components to generate protective responses in their recipients (see Sections 1.3.7 and 6.1), this study suggests that it might be possible to dramatically improve the significance of immunization as a cholera control measure (see Section 1.1.4).

7.6.4 Restricted distribution of the non-LPS protective antigens.

The studies discussed above indicate that, in at least one strain of *V. cholerae*, the non-LPS antigens are potentially of much greater

significance than the LPS determinants. Obviously the impact of such a finding on the development of cholera vaccines will be critically dependent upon the distribution of these non-LPS protective antigens among the strains of the V. cholerae species. We have been unable to detect such determinants in either the 017 or T50 strains; the underlying basis for this restricted distribution remains undefined. As discussed previously (Section 6.4.3), it is possible that the presence of non-LPS protective antigens is a constant feature of the Classical biotype or the Inaba serotype, and we are currently investigating this possibility. In conjunction with our findings, the studies of Watanabe and co-workers suggest that the latter might be the case (see below).

7.7 The Protective Antigens of V. cholerae : studies in other laboratories.

Several groups have attempted to define the nature of the protective antigens of V. cholerae, and in this section we shall briefly discuss some of the findings which have been made. Since the capacity of the LPS determinants to elicit protective antibodies has been well demonstrated in a number of experimental systems (Watanabe and Verwey, 1965; Neoh and Rowley, 1972; Svennerholm, 1975; Holmgren et al, 1977; also see Table 6.11b), and in view of the present emphasis upon the possible prophylactic significance of the non-LPS antigens, we shall concentrate upon those studies of relevance to the latter.

Watanabe and co-workers have investigated the nature of the antigens which generate antibodies capable of mediating protection in the active mouse protection test (Watanabe and Verwey, 1965; Watanabe, Verwey, Guckian, Williams, Phillips and Rocha, 1969). They reported that, whereas the protective antigen of an Ogawa strain was LPS in nature, that isolated from an Inaba strain was a complex of LPS and protein (Watanabe et al, 1969). This complex contained a heat-labile phenol-sensitive protective immunogen. Our own experiments have similarly demonstrated the presence of non-LPS protective antigens in an

Inaba strain, and their absence in two Ogawa strains. Together these studies suggest that such determinants might be generally associated with strains of Inaba serotype. If this can be confirmed, then the non-LPS antigens of V. cholerae might indeed be of great significance in the development of future cholera vaccines.

At least two other groups have suggested the involvement of the non-LPS determinants in the generation of protective antibodies to V. cholerae. Actor's group reported the purification of a protein-carbohydrate-lipid colloid which functioned as a protective immunogen in the active mouse protection system (Jensen, Gregory, Naylor and Actor, 1972). Although this material was isolated from an Ogawa strain, it was stated that the protein component was essential for its capacity to induce protective antibodies (Jensen et al, 1972), in contrast to the results discussed above. Finally, Berry's group recently isolated a non-LPS component which, on a weight basis, was 50 - 100 times more effective than a commercial cholera vaccine in the stimulation of protective antibodies in mice (Eubanks et al, 1977). This factor was prepared from an Inaba strain of V. cholerae, and seemed to be associated with the flagellar structure (Eubanks et al, 1977). It is conceivable, in this latter regard, that the correlation between high levels of anti-flagellar antibodies and resistance to typhoid (Benenson, 1964; Wahdan, Sippel, Mikhail, Rahka, Anderson, Sparks and Cvjetanovic, 1975) might similarly be a reflection of a flagellar-associated protective antigen (see Wahdan et al, 1975).

Although two studies in other laboratories failed to detect the presence of non-LPS protective antigens in V. cholerae, there would appear to be quite valid explanations for these reports. Thus, Sinha and Bhaskaran (1973) were working with the T50 strain and our own studies have confirmed that this strain, does indeed lack non-LPS protective determinants (see Section 6.4.2). Svennerholm (1975) reported that all of the ileal loop protective activity could be removed from an antiserum prepared against formalin-fixed Inaba vibrios by absorption onto purified homologous LPS.

Firstly however, the formalin-treatment of the immunizing bacteria may well have reduced the subsequent prophylactic potential of the non-LPS antigens (see Table 6.8). Secondly, the LPS used to absorb the resulting antiserum contained 7% protein (see Svennerholm, 1975), and may therefore have removed any protective anti-protein antibodies (especially in view of the findings of Watanabe and co-workers - see above).

7.8 The Protective Antibacterial Function of Specific Antibodies.

Our previous discussions of the possible means by which specific antibodies might mediate protection against V. cholerae led to the conclusion that the inhibition of bacterial adherence was the most likely mechanism (see Sections 1.3.5 and 6.6.1). Such a capacity had been demonstrated both in vitro (Freter, 1969; Bellamy et al, 1975; Freter and Jones, 1976) and in vivo (Schrang and Verwey, 1976; Guentzel et al, 1977). However, since several antibacterial functions might contribute to the capacity of specific antibodies to inhibit attachment (see Section 1.3.5), we attempted to evaluate the relative protective significance of each.

Although bacterial agglutination might indirectly inhibit bacterial adherence by facilitating the peristaltic removal of the challenge organisms, this mechanism was found to be insufficient for protection (see Section 6.6.2). Subsequent experiments further suggested that the capacity to immobilize V. cholerae was not necessary for the expression of the protective capacity of specific antibodies (see Section 6.6.3). The only antibacterial function which provided a consistent in vitro correlate of in vivo protective activity was the ability to directly block attachment at the single - cell level (see Section 6.6.4). We therefore reasoned that the capacity to inhibit the expression of the vibrio adhesins might be the essential antibacterial function of specific antibodies. Such a capacity might explain the weak but significant protective activity of antibody Fab fragments in the infant mouse model of cholera (Steele et al, 1975)..

SECTION C

CONCLUSIONS

The major findings which have resulted from the studies described in this thesis may be conveniently summarized by drawing the following conclusions :

(i) There is a correlation between the capacities of V. cholerae strains to attach to murine intestinal tissue in vitro and their virulence in the infant mouse model of cholera.

(ii) Motile vibrios are able to adhere to intestinal tissue in vitro, and colonize the small intestine in vivo, much more efficiently than their non-motile counterparts. The superior binding capacity of the former is not a reflection of their motility per se, but rather is due to their possession of an adherence factor located on the flagellar structure.

(iii) Specific receptor structures are not essential for the binding of V. cholerae, since these organisms can also bind to inert substrates. The slime agglutinin is an important mediator of such non-specific adherence.

(iv) The existence of non-LPS protective antigens in V. cholerae 569B has been confirmed. These determinants are relatively labile, and consequently seem unsuitable for use as oral immunogens. Similar antigens could not be detected in the 017 and T50 strains; the basis for this restricted distribution remains undefined.

(v) Antibodies directed against the non-LPS protective antigens of V. cholerae 569B mediated protection much more efficiently than those specific for the LPS determinants.

(vi) Regardless of antibody specificity, the essential antibacterial function of protective antibodies is their capacity to directly block the expression of the vibrio adhesins.

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