IMMUNOLOGICAL CROSS-REACTIONS OF ENTEROTOXINS FROM *ESCHERICHIA COLI*

by

Norman W. Smith

A THESIS

Presented to the Department of Clinical Pathology
and the Graduate Division of the
University of Oregon Medical School
in partial fulfillment of
the requirements for the degree of
Master of Science

June 1972

APPROVED:

[redacted]

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(Professor in Charge of Thesis)

[redacted]

.....

(Chairman, Graduate Council)

ACKNOWLEDGEMENTS

Credit for the opportunity to complete this stimulating course of study must be given to many individuals.

First I would like to thank my wife, Carol, and my children for their support and understanding during my absence.

I also wish to express gratitude to Dr. T. Hutchens and Dr. M. Berroth and other members of the staff in the Clinical Pathology Department for their advice and assistance.

Special thanks must be given to my thesis advisor, Dr. R.B. Sack and to Dr. A.L. Rashad for their friendship and guidance. Their examples of the true spirit of medical research has given me an understanding of solid achievement in science.

Recognition and thanks for typing this thesis goes to Miss Stephanie Tallman. I would also like to thank Mrs. Linda Bolewicz and Miss Inez Brownlee for technical assistance.

Financial support which made this research possible was provided by a National Institute of Health Training Grant #5 A02 AH00036-05. Support for equipment and material was provided by a National Institute of Health Research Grant to Dr. Sack, #AI 100 74-01.

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INTRODUCTION

The subject of this thesis is the serological cross-neutralization of exotoxins (enterotoxins) derived from strains of Escherichia coli isolated from patients with cholera-like diarrheal diseases. It is hoped that the results of this study may demonstrate the feasibility of further research to develop a vaccine for immunization against the diarrheal-provoking enterotoxins of E. coli.

Since the disease caused by these enterotoxigenic E. coli is clinically similar to the diarrhea caused by Vibrio cholerae and the toxins mediating the diseases are similar, it will be helpful to first review what is now known about Vibrio cholerae and the disease it causes.

I. Diarrheas Caused by Vibrio cholerae

A. Epidemiology and History of Cholera

Many authentic documents have been produced which affirm that the clinical disease 'Cholera' has been endemic in and around the Ganges River systems of the Indian subcontinent for at least a thousand years (73). Felsenfield (33) notes that according to the Nei Ching Chronicle, the disease was known even in ancient China. However, there are no records of cholera epidemics involving other parts of the world prior to 1817. It appears that the pandemics of the 19th century originated in the region of the West Bengal province of India and Bangladesh (33).

The world has experienced seven cholera pandemics in the last 150 years, all having originated in the same region and subsequently spread along trade and travel routes. The first pandemic of 1817 encompassed Arabia to the west and China and Japan in the east, with

some spread into Russia. The second pandemic in 1829 involved Asia, North America, and parts of Africa and Europe. It was during this pandemic that a British physician, John Snow, showed the important role of water in the transmission of the cholera agent (43). He described a grand scale natural experiment in London wherein two competing companies supplied water to some 300,000 customers of every age, station and rank. One group, supplied with water contaminated with the sewage of London, had a mortality of 71 per 10,000, while the other group received the relatively clean water from the Thames and had a rate of 5 per 10,000. The results convinced Dr. Snow that a living contagion was contained in the water.

The third and fourth pandemics of 1852 and 1863 followed similar routes as the previous ones. The fifth pandemic of 1881-1896 was studied in Egypt by Robert Koch and others. At this time Koch described the Vibrio bacillus as the agent causing a specific gastrointestinal infection. England and the U.S. were not involved in this epidemic, as they had instituted effective public sanitation rules. Introduction of cholera into New York was averted by bacteriological recognition of the presence of the disease on an infected ship from France (33).

The sixth pandemic (1898-1923) took a great toll during and after the first world war, in India and in Central and Eastern Europe. The toll in European Russia was reported at over 300,000 alone (98).

In 1958 a seventh pandemic began on the Celebes Island of Sulavesi, Indonesia, and has spread through Southwest Asia, the Middle East and to the borders of Southeastern Europe. In 1970 the disease had spread extensively into Africa and has been reported in Korea, the Soviet

Union, Turkey and Czechoslovakia, and it appears that as of 1971 new foci pose a threat to the western world (43). This recent pandemic is caused by a newly recognized strain of Vibrio cholerae, the El Tor biotype. This strain is difficult to contain because it causes many asymptomatic and mild infections. In addition to these properties of the El Tor biotype, the population explosion, the primitive sanitation of developing countries, modern rapid transportation and lack of an effective vaccine have combined to aid in the spread of the disease over three continents in the last decade.

B. Bacteriology and Serology of V. cholerae

The Vibrio cholerae organism is a comma-shaped, gram-negative bacillus which has a single polar flagella. The organism was first described in 1854 by Pacini and isolated as the cause of cholera thirty-two years later by Robert Koch (3).

There are two biotypes: the "Classical" and the "El Tor". The 'Classical' is the more fragile and more virulent strain. The 'El Tor' biotype was isolated in 1906 at the El Tor quarantine station in Egypt, and it was noted that these vibrios had the ability to hemolyze a suspension of erythrocytes (3). It has been noted (3,32), however, that the hemolytic activity is not a constant characteristic of the El Tor strain. Ballows et al. (3) list five differential characteristics of Classical and El Tor vibrios. These include: chicken red blood cell agglutination, Polymixin B susceptibility, Mukerjee type IV phage susceptibility, Voges-Proskauer reaction, and tube sheep erythrocyte hemolysis.

Many workers have studied serological groupings of Vibrio cholerae organisms (7,53) and have recognized at least three "O" antigen

structures. The Classical and El Tor biotypes have the same antigenic structure which has been subgrouped (sub-O-groups) as follows: Ogawa = AB; Inaba = AC; Hikojima = ABC.

C. Clinical Response to Infection

During the course of an infection, large numbers of V. cholerae are found in the lumen of the small intestine (46). In experimental animal infections, the organisms are found absorbed to the mucosal surface and in the luminal fluid (73). However, they do not invade the epithelium or enter the crypts of Lieberkűhn (30). The incubation time is one to three days and occasionally five to ten days (33).

As previously mentioned, cholera may assume various clinical forms from mild to severe. Typical clinical cholera begins suddenly and painlessly with profuse diarrhea. Absence of tenesmus and cramping is characteristic (33). There is seldom blood in the stools and as the bile becomes diluted the stool gradually assumes a whitish-gray color called the "rice water" stool. Vomiting occurs in many patients but may be absent (73). The fluid loss leads to severe electrolyte depletion and may, in severe cases, cause cardiovascular collapse and death in 8-12 hours from onset (43). Patients with clinical cholera, if untreated, have a mortality rate of 60% or more. When fluids and electrolytes are replaced adequately, the mortality can be reduced to nearly zero.

The clinical features of acidosis and fluid depletion can be corrected in 2-3 hours in adults with proper treatment, but the diarrhea may continue for 2-6 more days. During this time the patient is well but may lose 1 to 60 liters of fluid which must be continually replaced (51). With a disease as dramatic and rapidly fatal as this, it is no

wonder that many theories have arisen as to its causes and many man-hours have been expended in its solution.

D. Treatment of Cholera

1. Fluid Replacement

It has been shown that the simple replacement of stool fluid volume lost with an intravenous electrolyte solution can reduce mortality in all ages to less than 1% (71). Treatment produced dramatic recovery with the patient rapidly regaining warmth and alertness. In four to seven days the diarrhea ceases and the patient is cured (51). The recent development and testing of an electrolyte solution containing glucose or glycine which can be administered orally by untrained personnel has greatly reduced the cost of treatment. This method can utilize local water sources to make the solutions, thus making treatment more readily available (51).

2. Antibiotic Therapy

Tetracycline, a vibriocidal antibiotic, has been field tested in Dacca (71) and Calcutta (12) and has been shown to reduce duration of the diarrhea (98) to about two days and the volume of fluid lost about 60% (71). Other antibacterial agents which have been tested and shown to be effective are furazolidone and chloramphenicol (11).

E. Diarrheal Animal Models of Cholera

The efforts to understand the pathophysiology of cholera by utilizing an animal model have been difficult, with many confusing experiments having been reported in the literature. One of the main reasons for this difficulty is that the disease only occurs in man, and the cholera vibrios only produce their effects naturally in the

lumen of the small intestine. Another related problem is that before the vibrios can colonize the small intestine, they must pass the host's first line defense -- the acid stomach.

Pollitzer (73) has reviewed the many animal models that were attempted up to 1959. Some of the models utilized parenteral or intravenous injections of live vibrios which lead to septecemia and death; this type of experiment is not relevent to natural cholera. Other studies, however, have relied upon oral or intra-intestinal innoculations of live vibrios which gave rise to diarrhea and dehydration, and thus did reflect the natural course of the disease. Currently there are three reliable animal models in wide use. These are: the suckling rabbit model, the adult rabbit ligated ileal segment, and the dog model. The focus of this review of current models and the earlier work will be on the rabbit model as it pertains to this thesis.

1. Infant Rabbit Gastric Intubation

Issaeff and Kolle (1894) administered V. cholerae organisms to young rabbits by stomach tube after neutralizing the gastric contents with 5% sodium bicarbonate. They noted that diarrhea occurred and the organisms remained confined to the intestine (73). Metchnikoff intraorally infected 1-4 day old rabbits which had only received mothers' milk with cholera vibrios and was able to show that these young rabbits were highly susceptible.

In 1955 this model was modified by Dutta and Habbu (28) to increase the reproducability of the results. They noted that young rabbits lose susceptibility to oral infection or to orally administered enterotoxins with increasing age. Thus, they used rabbits 8-10 days old. The

modifications involved repeated rinsing of gastric contents with tepid water until the rinsings were clear. The test material was directly injected by catheter into the stomach. Under these conditions, most animals followed the clinical course of the disease as observed in humans. Later, they reported that cell free products of V. cholerae when given intragastrically to 9-10 day old rabbits caused a diarrheal disease similar to that seen in cholera infections (18).

2. Rabbit Ligated Ileal Loop Models

(a) Isolated intact segments

In 1894, Issaeff and Kolle performed laporatomies on 11 rabbits and injected Vibrio cholerae suspensions directly into the small intestine. Four of the rabbits died in 1-9 days and showed clear signs of enteric cholera with no infection of the internal organs (73). Violle and Crendiropoulo (1915) tied off a small proximal segment of the small intestine at both ends and injected cholera organisms. They observed typical lesions and fluid-filled loops by this method, but had negative results if only one end was ligated (100).

It was 37 years before this model of Violle and Crendiropoulo was rediscovered by S.N. De and D.N. Chatterje in 1953 (22). They utilized this method to assay the intestinal fluid producing activity of peptone broth suspensions of Vibrio cholerae. They ligated several 'loops' along the small intestine and noted that segments injected with sterile peptone water were empty and collapsed while the cholera loops were swollen and necrotic (22).

Later it was found by Leitch et al. (61) that the mucosal epithelium remained intact in the toxin challenged loops for up to 12 hours, at which time there appeared histological evidence of damage due to fluid

pressure in the segment.

In 1959 De et al. (21), using the ligated intestinal loop model, was able to demonstrate that cell-free culture filtrates of V. cholerae promoted fluid accumulation in the segments. They determined that the agent causing the fluid accumulation was heat labile (56°C), non-dialyzable, and appeared in young cultures. Inasmuch as this component or exotoxin had specific activity in the small intestine, they called it an "enterotoxin".

Burrows and Musteikis (1966) (8) and Kasai and Burrows (1966) (52) extended the work of De and showed that a cell-free ultrasonic lysate of V. cholerae contained enterotoxins similar to those reported by De and his associates. Most important, they modified the ileal loop model so that reasonably reproducable, quantitative data could be obtained. Burrows and Musteikis (8) demonstrated a dose-response relationship in a Sigmoid shaped curve when the milliliters of fluid produced per centimeter of intestinal segment was plotted against the log dose of purified cholera exotoxin. The curve was asymptomatic at 2.75 ml/cm (in their variety of rabbits). From this curve they defined 'one unit' as being the amount of toxin that caused 50% of the maximal reaction. They noted large variation from animal to animal, and variation in response to the same dose injected proximally to distally along the small intestine. Consequently, they recommended using only the first 100 centimeters above the appendix.

Burrows and Musteikis (8) noted that fluid begins to noticably accumulate in 4-5 hours and was maximal in 10-12 hours. This was reconfirmed by Leitch and Burrows in 1968 (62). In this study, it was shown that the duodenum, lower ileum and upper ileum were

decreasingly responsive in that order. They also noted that the colon was unresponsive to toxin challenge.

The ligated ileal loop model has been widely used in the last decade not only in rabbits but in pigs, calves, dogs, rats and mice.

(b) Perfused ligated segments in the adult rabbit

Craig (18) has reviewed a modification of the ligated ileal loop model which attempts to overcome the problems of over-distension and subsequent necrosis and difficulty in sampling the fluid produced during the test period.

This model utilizes catheterized ileal segments which undergo constant intraluminal perfusion. The advantage is the ability to study fluid and electrolyte movements and toxin reaction time relationships. The disadvantages are the problems in maintaining an anesthetized animal, the limit of only two segments per animal, and maintaining the perfusion system for many hours. Workers have observed that the magnitude of fluid loss (approx. 0.2 ml/cm/hour) is within the range seen in natural cholera in man. Craig (18) notes that studies have shown that individual rabbits may differ 100-fold in enterotoxin susceptibility as measured by differences in the fluid flux rates. This great variability in toxin dose response makes the comparisons in potencies of two enterotoxin preparations very approximate. It appears that to obtain usable data points, approximately the same number of rabbits per dose must be used as with the ligated segment model.

3. The Canine Model

Pollitzer (73) reports that early attempts to infect dogs with V. cholerae were met with variable, but for the most part unsuccessful, results. The model was revived in 1965 by Swallow et al. (93)

who studied the effects of cholera exotoxin on the adult dog Thiry-Vella loops of jejunum, distal ileum and colon.

The dog as an experiment animal model was thoroughly established by Sack and his associates in 1966 (76), and subsequently utilized in detailed investigations of the pathophysiology of cholera (77-80). These workers were able to show that the rate of production of gut fluid during experimental cholera in dogs was independent of mesenteric blood flow and pressure over a wide range (13). Thus, it seems unlikely that filtration of fluid (transudation) from the network of capillaries in the intestinal villae is the important mechanism in fluid loss. They were also able to quantitatively study the attack, survival and reinfection rates, as well as other aspects of pathophysiology.

4. Other Animal Models

The rat has been investigated as a more economical assay model for cholera enterotoxins and has been reported to accumulate fluid in the toxin challenged ileal segments at a rate of 0.3 ml/cm for the first eight hours. So far the model seems limited to one to two segments per animal and has similar problems in reproducability as other systems (2). The guinea pig, as previously noted, has been successfully used (73), and has been more recently used in intraluminal cultivations of V. cholerae (60). However, it appears to suffer from the fact that the animals must be extensively pre-treated and the animal does not develop diarrhea, although they do accumulate fluid (77).

F. Non-diarrheal Biological Assay Systems

1. Cutaneous Vascular Permeability Factor

Craig (1965-66)(15,16,17) showed that when dilutions of V. cholerae culture filtrates or cholera rice water stools are injected

intracutaneously into rabbits and guinea pigs, the blood vessels become permeable to protein. This appears as an induration which is easily visualized and measured if a dye such as pontamine sky blue is injected intravenously shortly before the lesions are examined. This permeability factor has been shown to have all the characteristics of the cholera exotoxin that is active in the lumen of the gut. Mosely et al. (1970) (65) have introduced immunologic data from serological titrations in two systems that strongly support the view that the enterotoxin and the permeability factor are the same. This system is important not only in that it is a convenient and reproducable assay of toxin and antitoxin titers, but it may give some clues as to the mechanism of pathogenesis of cholera. It must be remembered, though, that sufficient proof has been given to show that vascular permeability per se is not the mechanism operative in cholera.

2. Rat Foot Pad Edema

When purified Vibrio cholerae enterotoxin is injected into the rat foot pad, a localized edema occurs after a delay of 2-4 hours. This reaction has been shown to be dose dependent and temporary (41). This phenomenon is probably due to the same factors that stimulate cutaneous vascular permeability.

3. Inhibition of Electrolyte Transport in Isolated Intestinal Mucosa

One of the possible mechanisms to explain the accumulation of luminal fluid in response to the cholera exotoxin is an alteration in an ion-transport system. Huber and Phillips (1962) demonstrated the presence of a sodium transport inhibition in cholera stools using the short-circuited frog skin method (18). This inhibition of sodium transport proved not to be the exotoxin of V. cholerae, but a heat stable,

dialyzable substance. Nevertheless, these studies have stimulated others to investigate the effects of the cholera exotoxin on unidirectional sodium fluxes across the intestinal mucosa stripped of its muscularis and mounted in Ussing (97) chambers. They have shown that normal transport of sodium from mucosa to serosa is reduced in the presence of cholera toxin and the normal mucosa-serosal chloride transport is reversed so that active chloride secretion occurs. These results have been verified in studies involving human ileal mucosa (72).

Similar studies have shown that enhanced sodium transport occurs when the mucosal surface is exposed to glucose and glycine, and cholera toxin does not block this effect. Clinical observations have shown that the administration of oral glucose-saline solutions result in adequate sodium absorption because of its coupling to glucose transport (48).

4. Lipase Stimulating Factor

It has been demonstrated that in the presence of cholera enterotoxin (99) isolated rat epididymal fat cells release glycerol into the surrounding medium. This lipolysis stimulation is proportional to the log of the enterotoxin concentration and is neutralized by specific cholera antitoxin. This system has been used to titrate toxin and antitoxin concentrations. The advantages of this system are that it is more reproducable than rabbit ileal loops and many samples can be assayed in 4 hours.

G. Vibrio cholerae Enterotoxin Production and Properties

Cholera exotoxin has been produced by extraction from alkaline peptone broth filtrates (21,24) from a semi-defined broth called 'syncase' (39) or by lysis of cells by ultrasonic or other means (52). Studies have shown this toxin molecule to be a true protein exotoxin; it is

non-dialyzable, precipitated by saturated ammonium sulfate, is not absorbed on membrane or glass filters, is not inactivated at 50°C, but heating at 56°C for thirty minutes completely inactivates it (18). It has also been shown to be stable at pH 6, but its fluid producing activity is maximal at pH 8 (39,48).

Recently the cholera toxin and toxoid have been crystalized and shown to contain no endotoxin (lipids) or sugar residues; they are electrophoretically and immunologically homogenous (42).

The molecular weight of the purified exotoxin has been measured by various workers to range from 10,000 to 60,000 (18,48). This variation in apparent size does not necessarily imply basic differences in toxins but is more likely related to differences in production methods which may lead to fragmentation or polymerization of toxin molecules (48).

H. Pathophysiology of Fluid Loss in Human Cholera

On July 26, 1884, Robert Koch addressed a conference in Berlin, presided over by Virchow. Koch was reporting on the identification of Vibrio cholerae as the cause of cholera in Egypt. Koch reported, contrary to the observations of Virchow, that the organisms were confined to the lumen of the intestine and did not spread to the blood or mesenteric glands. Also, on autopsy there was no visible damage to the walls of the intestine. Koch proposed that the bacilli growing in the gut caused death by producing a specific poison "that acted on the epithelium" (43). It is of interest that it was in 1884 that Loeffler suggested diptheria toxin and in 1885 Nicolaier proposed tetanus toxin. The most widely held view at that time had been proposed by Virchow, who had observed autopsies on victims that had been in prolonged shock. He proposed that the losses were due to epithelial slough and consequent

loss of plasma. Virchow's views were widely accepted and have appeared even recently in a modified form where it was suggested that the mucinase of the vibrio aids in the mucosal slough (5).

In the past ten years, the earlier views of Koch and later workers have been confirmed. Gangarosa and his associates (1960) (44) utilized the Crosby peroral intestinal biopsy instrument to obtain serial intestinal specimens from human cholera cases in Thailand. By studying acute and convalescent morphologic patterns in the intestine, they concluded that the mucosa was not damaged during the diarrhea. These studies have been extended with electron microscope studies by Elliot et al. (30) and Moon et al. (63) who also confirmed that villous epithelial cells remain intact.

With the hindsight of the last few years, the pathogenesis of cholera appears simple. The victim ingests a bacterial innoculum adequate to survive the stomach acid. The duodenum and jejunum, which normally harbor only small numbers of gram-positive bacteria, and the ileum, which may have a few gram-negative bacteria, become colonized with Vibrio cholerae cells in large numbers (10,45). The bacteria produce an exotoxin which acts on receptor sites in the epithelial cells in the lumen of the small intestines causing the cells to exsorb saline faster than it is absorbed, thus leading to rapid fluid accumulation (18,43). This fluid which enters the lumen of the intestine is derived from the blood plasma. It seems most probable that it enters as a result of ion-transport mechanisms in intact functioning cells.

I. Current Theory on the Mechanism of Fluid Production by Cholera Toxin

Field (35) noted that certain drugs such as theophylline, which

inhibits the degredation of cyclic adenosine monophosphate (cyclic-AMP) have similar short-circuit current and electrolyte effects as cholera exotoxin. It was suggested that the cholera toxin may operate via an intermediary molecule such as cyclic-AMP in stimulating chloride hypersecretion. Several lines of evidence seem to be converging to support this view. Cholera toxin increases levels of cyclic-AMP in intestinal cells (35), and appears to stimulate adenyl cyclase (58,83), the enzyme that converts adenosine-triphosphate (ATP) to cyclic-AMP. These effects of cholera toxin on levels of cyclic-AMP have been observed in non-intestinal tissues also (72,99). In summary, it is proposed that the cholera toxin activates the adenyl cyclase located in the epithelial cells of the small intestine. This enzyme functions to reduce ATP to cyclic-AMP, which then regulates sodium and chloride flux through the cells.

J. Cholera Immunity

Cholera vaccines have been used without proof of their effectiveness since the beginning of this century. It has been shown recently that the best of the currently available vaccines give an immunity to 85% of those vaccinated but the protection does not last longer than six months (70). These vaccines are prepared from whole cells and contain few extracellular products. It has been shown that when vaccine-induced immunity is overcome, such that symptomatic cholera occurs, the disease is indistinguishable in severity and length from cholera in non-protected persons (102). In contrast, workers using cholera toxoid with or without adjuvant to parenterally immunize rabbits showed significant protection to cholera exotoxin challenge (40,52). Moreover, Curlin and Carpenter (20) have recently shown that when the blood of

a hyperimmunized dog is cross circulated to the intestinal supply of a non-immunized dog, and visa versa, the cholera toxin challenge loops in the non-immunized dogs being perfused produce significantly less fluid than those in the immunized dogs receiving blood containing antitoxin. This suggests that humoral, circulating antitoxin is capable of neutralizing a luminal challenge with toxin. Currently there is no information published as to the ability of toxins or toxoids of V. cholerae to prevent cholera in man, although active research is being done.

People surviving cholera have measurable antibody titers against both the exotoxin and 'O' antigen (52,98,102); however, it is possible that the enteral exposure to the exotoxin is not long enough to stimulate effective immunity (98). This possibility is supported by the recent documentation of 14 cases of cholera reinfection. Six of the cases were within 13 months of the first infection. This implies that natural immunity may be very short lived (104). Ideally, an adequate vaccine would provide antitoxic and antibacterial immunity on a long-term basis (6).

II. Diarrheas Caused by Escherichia coli

Thus far the discussion has focused upon cholera as a disease entity and the principles of investigation that have developed to understand its pathophysiology. The knowledge and principles that have been acquired concerning Escherichia coli as the etiological agent of acute diarrhea have followed a parallel and overlapping course to the study of cholera.

A. Historical Recognition of Enteropathogenicity of Escherichia coli

The E. coli bacillus is usually thought to be a normal inhabitant of the large intestine in concentrations of 10^7 - 10^8/ml and has only recently been recognized as the cause of acute diarrhea. In the decade of 1940 to 1950, considerable epidemiological evidence was compiled that incriminated certain specific strains of Escherichia coli as causes of epidemics of neonatal diarrheal diseases. Prior to this period, E. coli isolates from infantile gastroenteritis had been studied for many years, although biochemical methods used were not adequate to differentiate the strains (29). Bray (1945) and Bray and Brovan (1948) were the first to associate a particular serotype with outbreaks of infantile diarrhea (29). Subsequently, Kauffman and his associates established definitive typing methods for E. coli and developed an antigenic scheme in which they could be classified (54).

At present, a specific strain of E. coli is defined as being enteropathogenic for infants when it is:

1. Isolated under epidemic conditions as the predominant coliform in the stools of infants.

2. Eliminated by antibiotic therapy and coincidental with recovery.

3. Associated with a specific antibody response in convalescence.

4. Relatively rare in the general community.

When a strain meets these criteria, its antigenic structure is noted and the typing antiserum is added to the reference set (47).

B. Bacteriology and Serology of Escherichia coli

E. coli are faculative gram-negative rods which give similar biochemical reactions and can be subdivided serologically into many

different subgroups (29).

The antigens that have been serologically classified are:

1. The 'O' or somatic antigens: heat stable cell wall lipopolysaccharides which are serologically divided into about 150 types.

2. The 'K' antigens: a group of related antigens which occur as an envelope or sheath (capsule) around the 'O' antigens and consequently inhibit agglutination. These antigens are inactivated by boiling at 100°C for one hour.

3. The 'H' or flagellar antigens: ones which are inactivated by heat at 100°C.

C. Clinical Response to Infections in Human Volunteer Studies

In 1950 Neter and Shumway (67) studied E. coli serotype 0111 to determine its effect when ingested orally in varying concentrations in infants. They found that approximately 10^8 organisms ingested with the milk formula resulted in diarrhea in 24 hours. Antibiotic treatment cleared the organisms and relieved the symptoms in less than 48 hours. Later, Ferguson and June (34) conducted adult feeding experiments with serotype 0111 B4. They found that oral ingestion of a large innoculum (9.0×10^9) produced symptoms ranging from nausea to acute gastroenteritis with violent diarrhea, tenesmus and vomiting. Feeding about 5×10^8 organisms caused only a mild illness. A large percentage of the volunteers developed high titers of serum agglutinins. Ingestion of non-enteropathogenic E. coli (one serotype) isolated from normal infants had no demonstrable effects.

D. Rabbit Ligated Ileal Loop Assay of Enteropathogenic E. coli

1. Investigations of Enteropathogenic E. coli from Acute Diarrhea in Infants and Adults

In 1956 De and his co-workers (23) noted that during the

annual cholera epidemics in Calcutta, a significant number of the clinical cases were negative for Vibrio cholerae by culture methods. This had been attributed to faulty bacteriologic technique. It was noted, however, that some patients seemed heavily colonized with Escherichia coli. They injected alkaline peptone water cultures of these organisms into the rabbit ileal loop model earlier developed for cholera studies, and were surprised to find these organisms gave the same fluid accumulating response as cholera vibrios. They noted that the fluid produced was clear and there was no evidence of acute inflammatory change in the cell wall of the gut. They found that three of twenty strains of E. coli from healthy patients caused fluid accumulation, fifteen of twenty from patients with acute diarrhea and nine of twenty from patients with chronic diarrhea. They also tested three strains of E. coli isolated from infantile diarrhea and found them to be positive; however, they did not test culture filtrates and did not serotype the organisms.

In 1958 Taylor (94) and her colleagues applied the rabbit loop model to further investigate the enteropathogenicity of E. coli. Although their assay technique was similar to that developed by De and co-workers, it is worth noting that they injected freshly innoculated peptone water into the loops and thus no significant amount of enterotoxin was present. The reactions in the loops were graded from very mild to very severe on a histological basis alone and with no regard to the amount of fluid produced. In subsequent studies (95), these workers found that the same serotype of E. coli gave positive or negative loop results depending upon whether or not the strain came from an infant who was sick with gastroenteritis or was healthy. By actively immunizing

rabbits with homologous live cell cultures they could demonstrate partial antibacterial protection. In experiments in which antisera and homologous E. coli serotypes were mixed prior to injection into loops, complete protection could be demonstrated while the antiserum gave no protection to heterologous E. coli serotypes.

In 1966 Taylor and Bettelheim (96) found that chloroform-killed suspensions of enteropathogenic E. coli caused ileal loop dilation in rabbits which correlated with the activity of the live organisms; however, the factor causing the activity was very labile and could not be isolated.

Sakazaki et al. (82) studied seven hundred and eighty-nine strains of enteropathogenic E. coli isolated from children and adults in Tokyo. On the basis of clinical observations they divided the enteropathogenic E. coli into two groups: one type caused gastroenteritis as does Salmonella, and one group caused a Shigella-like dysentery.

Ogawa et al. (68) in 1968 utilized the rabbit ileal loop assay to investigate the enterotoxigenicity of E. coli; they also used the "Serény test" for invasion of the conjunctival sac in guinea pig eyes, and innoculations of HeLa S_3 cell monolayers for determination of intracellular growth. They reported a definite difference in the pathogenic behavior of two types of E. coli. One type, which caused a Shigella-like dysentery disease, possessed the ability to cause keratoconjunctival infection in guinea pig eyes, and invaded and multiplied intracellularly in cell cultures. The other E. coli type caused a Salmonella-like enteritis and was non-invasive in the eye or cell cultures, but caused dilation of rabbit ileal loops.

DuPont et al. (27) in 1971 while studying isolates of E. coli from American soldiers in Vietnam, confirmed the work of Sakazaki and Ogawa and further demonstrated that on feeding the strains to adult volunteers (in appropriate concentrations), the clinical manifestations produced related to the proposed mechanisms of pathogenesis. The clinical picture seen with invasive E. coli infection included blood, mucous and pus cells in the diarrheal stool with tenesmus and urgency; the colon was the predominant site of multiplication. In contrast, the enterotoxigenic strains caused a mild cholera-like diarrhea with 5 to 10 watery stools per day, but without blood or pus in the stools and without tenesmus. These organisms were recovered in large numbers in jejunal and ileal aspirates as well as from the stool, thus implicating the small intestine as the primary target organ for the enterotoxin.

Sack et al. (81) in 1971 studied 17 adult patients in Calcutta suffering from severe diarrheal disease. None of these patients had a recognizable pathogen, but were heavily colonized with E. coli in the small intestine. They were able to demonstrate for the first time that enterotoxigenic strains of E. coli cause disease in humans and that the enterotoxin is similar in activity (in-vivo and in-vitro) to the enterotoxin of Vibrio cholerae; however, there appeared to be no immunologic cross reactivity between cholera and E. coli enterotoxins. These workers produced the E. coli enterotoxin in 'syncase' broth developed by Finkelstein (39) for production of Vibrio cholerae enterotoxin. The enterotoxin was semi-purified from one strain of E. coli and characterized as to its activity in rabbit ileal loops. The toxin was shown to be heat labile, non-dialyzable, precipitated by 40% ammonium sulfate and of rather low antigenicity in rabbits. The toxin

did not contain permeability factor when tested intradermally in rabbit skin.

2. Investigations of Neonatal Enteric Infections in Animals

Smith and Halls (84,85) in 1967, studying the veterinary aspects of neonatal diarrheas in swine, found the ligated weanling pig intestine useful for assay of enteropathogenic E. coli isolates from pigs. They demonstrated for the first time that the dilation of the ileal loops was caused by an exotoxin in the sterile culture filtrates of E. coli cultures. The activity of the sterile culture filtrate correlated with the activity of the live cell cultures. In 1968, Smith and Halls (86) found that the genetic component of E. coli that controls production of the enterotoxin (exotoxin) was transferable by conjugation from E. coli to unrelated E. coli and Salmonella organisms. This episomal factor has been labelled "Ent" and has recently been shown to occur in isolates of human enterotoxigenic E. coli as well as pig E. coli (89). This "Ent" factor has also been shown to be stable on repeated sub-cultures and by treatment with acriflavine.

Gyles and Barnum (50) described both heat stable and heat labile enterotoxins in cultures of pig E. coli isolates. Following additional work by Smith and Gyles (88) it was concluded that the heat stable enterotoxin (which was non-antigenic) and the heat labile enterotoxin (which was antigenic) were probably variants of the same toxin with their relative amounts depending upon cultural and isolation conditions.

E. Evaluation of E. coli Enterotoxins in Other Experimental Models

In addition to rabbits and pigs, the ileal segment assay has been tried in mice (74), dogs, calves, guinea pigs and rats (64). In the evaluation by Moon and Whipp (64), the rabbit ileum was found to

the most sensitive intestinal method for detection of E. coli enterotoxins. A rabbit skin test for permeability factor has been found to be more sensitive (64), but it is not known if this factor is the same as the enterotoxin. E. coli enterotoxin has also been tested for its effect on net ion transport in the Ussing chamber and found to have similar effects on ion flux across the small intestine epithelium as V. cholerae (1,97)

Currently, E. coli toxin is being evaluated in the other assay systems used for cholera enterotoxin.

F. Enterotoxins Produced by Other Organisms

Recently other investigators have begun to isolate and characterize enterotoxins from several organisms which have been causally associated with diarrheal diseases. These enterotoxins have been shown to be active in fluid production in ligated rabbit ileal loops.

Keusch et al. (57) in 1970 have reported a heat labile, non-dialyzable exotoxin of Shigella dysenterae I, which was active in the rabbit ligated loop model. This toxin had a M.W. of approximately 40,000-60,000 and appeared to be inactive in the skin permeability test.

Stark and Duncan (91,92), in a series of experiments, have shown that Clostridium perfringins produces an enterotoxin that is heat labile, non-dialyzable, inactivated by pronase, antigenic, stimulates a transient increase in guinea pig skin capillary permeability, and is active in the rabbit ileal loop.

Another enterotoxin active in the rabbit ileal loop has recently been isolated from culture filtrates of Pseudomonas aerugenosa; however, this toxin has not been characterized as well as the previously described toxins (59).

With the exception of the clostridial enterotoxin, there are no data to clarify the role, if any, of these enterotoxins in the production of disease.

STATEMENT OF THE PROBLEM

For many years certain serotypes of E. coli have been implicated as having the potential for enteropathogenicity in neonatal humans and domestic animals. It has also been recognized that these same serotypes may be present in healthy individuals in the same populations. Only in the last few years has it been known that E. coli organisms of diverse and unrecognized serotypes can be pathogenic by different mechanisms and some can cause acute cholera-like diarrhea.

Large scale field studies have shown that immunization with cholera vaccine made from whole cells of V. cholerae provides antibacterial protection for about six months to 50% of the population. On the other hand, animal studies have shown that immunization with purified cholera enterotoxin which gives antitoxic immunity to large challenge doses of enterotoxin may give even a longer period of protection.

Animal studies of E. coli enterotoxin have provided nebulous and contradictory results as far as determining if the E. coli enterotoxin is serotype specific, that is, intimately related to the cell wall antigens. There is also no clear evidence to determine if immunization will protect against E. coli enterotoxin challenge.

The research proposal of this thesis was to:

1. Isolate enterotoxins from E. coli of diverse serotypes and assay them in rabbit loops.

2. Inject rabbits parenterally with enterotoxin preparations in an attempt to elicit antitoxin production.

3. Try to demonstrate homologous and heterologous neutralizations between these E. coli enterotoxins and the antitoxins in the rabbit sera.

4. Determine by cross neutralization studies if these toxins are immunologically related to Vibrio cholerae enterotoxin.

5. Determine if immunization with E. coli enterotoxin is protective against intestinal challenge with enterotoxin preparations.

It is hoped that the results described in this thesis will yeild insight into the problems regarding possible use of E. coli enterotoxin preparations as practical immunization agents.

MATERIALS AND METHODS

A. Bacterial Cultures

Strains of Escherichia coli were isolated in Calcutta, India, by Drs. R.B. Sack, S.L. Gorbach and co-workers who utilized small bowel intubation techniques to sample various levels of intestinal flora in patients suffering from acute cholera-like diarrhea (81). Details of obtaining the specimens and culturing have been previously reported (45). These organisms were also previously tested as to their ileal loop responses by Dr. Sack (81). The serotype had previously been determined at the University of Illinois in the laboratory of Dr. Mark Lepper, and confirmed at the Center for Disease Control, Atlanta, Georgia, and the W.H.O. International Escherichia Center in Copenhagen, Denmark.

The E. coli organisms studied are shown in Table 1.

The organisms were maintained on nutrient agar slants (Difco) at room temperature. The original stock cultures had been maintained the same way since isolation in 1968-69. One strain of Vibrio cholerae, Inaba 569B, a standard potent enterotoxin-producing strain (38), was also obtained from Dr. Sack for use in these studies.

B. Enterotoxin Production

The innoculum was prepared by growing the organisms overnight in a 1% peptone broth (Difco) with 0.5% NaCl (pH 7.4). One hundredth ml of this culture was innoculated into sterile syncase, a semi-defined medium developed (40) for the production of Vibrio cholerae enterotoxin. The syncase was prepared according to the formula in Table 2, with the reagents being added in the listed sequence to avoid precipitation. These reagents were mixed on a magnetic stirrer, dispensed in volumes

TABLE 1

Escherichia coli Cultures Studied

Patient Number	Strain	Source	Serotype	Ileal Loop Response
931	408-3	stool	078H12	positive
931	408-4	stool	078H12	negative
931	411-5F	jejunum	0126H12	positive
931	411-7	jejunum	0126H12	negative
924	339	duodenum	015H11	positive
924	348	stool	09K(A)*H	negative
79	1105F	stool	06H16	positive

* K(A) means that O agglutination test was carried out with an autoclaved culture because of the presence of a thermostable K antigen of the A variety. This A antigen was not typed.

TABLE 2

Syncase Reagents

	Reagents	Mg/L	
1.	NH_4Cl	594.0	
2.	$MnCl_2$	3.6	
3.	$FeCl_3$	2.9	
4.	$MgCl_2$ $6H_2O$	41.8	
5.	Na_2SO_4	89.8	
6.	K_2HPO_4	5.0g	
7.	Na_2HPO_4	5.0g	
8.	Sucrose	5.0g	(Difco)
9.	Casamino acids	10.0g	(Difco)
10.	Distilled H_2O	1000 ml	

of either 25 ml in 500 ml Erlenmeyer flasks or 100 ml in 2.5 liter low form flasks, and then sterilized at 15 lb. for 15 minutes.

The stationary cultures were incubated at 37°C for 40-48 hours. The cells were then removed by centrifugation in a Sorvall^{R} model RC-2B at 13,000 RPB (20,200 RCF) for 45 minutes at 4°C. The supernate was sterilized by filtration through a 0.45u membrane filter with a pre-filter pad and then through a 0.22u membrane filter (Millipore Filter Corporation, Bedford, Massachusetts). The filtrate was then dialyzed in cellophane dialysis bags (pore Radius permeability of 24 Å) using an Oxford multiple dialyzer against 11 L of distilled water (1:11 ratio) for four days (three changes of water) at 4°C. The dialysate was then lyophilized on a Virtis^{R} #10-010 freeze dryer (Gardner, N.Y.) and stored at -45°C. A solution of Phosphate Buffered Saline (PBS) composed of 8.5 gm NaCl, 1.07 gm $Na_2 HPO_4$ (anhydrous), 0.39 gm $NaH_2 PO_4 \cdot 2H_2O$, in 1000 ml distilled water, was used for rehydrating all lyophilized toxin preparations making dilutions, washing the small intestine, and for negative control loops.

Vibrio cholerae enterotoxin was prepared by the same procedure as E. coli enterotoxins except that the cholera organisms were grown for 18 hours in a shaking water bath (60 shakes/minute) at 37°C.

C. Rabbit Ileal Loop Enterotoxin Assays

New Zealand strain white rabbits, 2-2.5 Kg and 8-10 weeks old, were purchased from a local source, V and R Research, Newberg, Oregon. The rabbits had been and were maintained on an antibiotic-free food, Albers Rabbit Family Ration (Albers Milling Co., Los Angeles, California).

Rabbits were starved for 24-48 hours before surgery; however, water was provided before and after surgery.

The surgical technique was essentially the same as developed by De (22) and modified by Kasai and Burrows (52). The rabbits were anesthetized with ether, shaved and a two inch mid-line incision made. Partial antiseptic precautions were observed with sterilized equipment and gloves used to avoid gross bacterial infections. The small intestine was gently manipulated onto the surgery drapes and was ligated approximately 90-100 cm proximal to the appendix.

The intestine was then washed by gravity flow from the proximal tie off distally past the appendix with an injection of 10 ml of PBS. After washing, the ileum was ligated about 5-10 cm above the appendix. The first segment was approximately 15-20 cm long and was left uninjected as a "blank". Subsequent segments were 8-12 cm long with a total of nine loops per rabbit ordinarily being used.

The intestine was handled as much as possible by the omentum to avoid undue alteration of the intestinal mucosal cells or the circulation. Likewise, ligations were placed at locations on the gut which would not occlude circulation. The segments were ligated, injected from the proximal end, and the injection site was tied off resulting in a double ligature between segments. This avoided the possibility of leakage from the injection site or from loop to loop. All segments were innoculated with 2.0 ml of controls and the preparations being tested. The surgical procedure usually took about 30-45 minutes per rabbit; the intestine was then manipulated back into the animal, the peritoneal cavity was closed and the skin was clipped shut. Each rabbit assay contained two loops injected with PBS as negative controls and a V. cholerae toxin preparation as a positive control. The cholera

control was either a 1:50 dilution of a sterile culture filtrate of a 24 hour syncase broth culture of V. cholerae Inaba 569B, or a dosage of toxin (0.015 mg) from the same strain, titrated (as discussed below) to be equal to 3 units of activity.

The animals were sacrificed at 15-18 hours by I.V. injection of Beuthanasia^{R} (Burns Pharmaceuticals, Oakland, California) and the intestinal loops removed for measurement. The volume of fluid produced in each segment and the lengths of the segments were measured. Ratios of volume to length (ml:cm) were calculated for each loop.

Data were accepted from each rabbit only if the control loops gave the appropriate response. That is, to be acceptable the negative control had to contain 2 ml or less of fluid and the positive control had to give a ratio of 0.90 or more.

D. Enterotoxin Titrations and Assays of Bacterial Strains

Two ml of a live E. coli cell suspension grown in syncase as previously discussed were injected into one loop in each of three rabbits to determine the activity of the strain.

The dose-reponse activity of each batch of E. coli enterotoxin was determined by testing serial dilutions of the preparation in PBS. The mean and 95% confidence limits of the ratios of each toxin dose were plotted on semilog graph paper. One unit of activity was defined as one half of the maximal response and was read from the graph. Potency of additional batches of toxin was compared with previous batches.

E. Immunological Studies

1. Antitoxin Preparations

Preliminary experiments were conducted to determine the most effective immunization schedules. Eight to ten week old New Zealand

rabbits were immunized in groups of 3-4 according to the following schedules with all rabbits being pre-bled to determine baseline titers.

Group 1

Freund's complete adjuvant (Difco) was mixed in equal volumes with 408-3 toxin to give a 1 mg/ml dose (3 units/ml). Mixing was performed by passage between two syringes connected by a 3-way valve.

Group 1A 0.5 ml per foot pad - total = 2 mg per rabbit (6 units)

Group 1B Subcutaneous, 2 areas on the back 0.5 ml each
Intramuscular, both rear flanks 0.5 ml each
Subcutaneous, right and left ingunal regions 0.5 ml each for a total of 5 milligrams (11.9 units)

Group 1C Subcutaneous, 2 areas on back 0.5 ml each
Intramuscular, both rear limbs 0.5 ml each
Subcutaneous, right and left ingunal regions 0.5 ml each for a total of 3 mg (9 units)

All of the Group 1 rabbits were boosted with 5 mg of enterotoxin intramuscularly in both rear legs and then immunizations were completed according to the schedule listed below for groups 3-11.

Group 2

Three rabbits in this group were immunized with E. coli 408-3 toxin by the same protocol as Group 1B; however, all died within 3 days. All subsequent groups (3 through 11) were immunized according to the following schedule suggested by Dr. Kim of the Clinical Microbiology Department at the University of Oregon Medical School (55,56).

1) Freund's incomplete adjuvant was mixed with enterotoxin 1:2

2) Axillary and ingunal node regions were injected with 0.5 ml per site

3) After 30 days a regimen of injections intravenously was begun on successive days, with 0.1, 0.2, 0.4, 0.8, 1.0 mg of toxin in a volume of 1 mg/ml

4) After one week a 1 mg booster was given intravenously

5) Rabbits were bled one week later

A list of all the rabbit groups immunized is found in Table 3.

The rabbits were bled from ear veins, about 50 ml per rabbit; the serum was preserved with 1:10,000 merthiolate and kept at 4°C.

2. Antitoxin Titrations

Toxin neutralization studies were performed according to methods described by Sack et al. (81) and Kasai and Burrows (52). Antisera were inactivated at 56°C for 30 minutes, then serial dilutions were mixed with equal volumes of 3 units of toxin (in PBS) and incubated at 37°C in a shaking water bath 70 oscilations per minute) for 60 minutes. Two ml of this mixture were injected per rabbit ileal loop for titration of the residual toxin.

The amount of antitoxin required to neutralize 1 unit of toxin was determined, as described by Kasai and Burrows (52), by calculating a neutralization coefficient (NC) which equals the mean of dose response (ml/cm) ratio, divided by the mean of the response of the toxin controls, the result being subtracted from 1.0. Thus, $NC = 1 - \frac{\text{mean experimental ratio}}{\text{mean toxin control ratio}}$. The log neutralization coefficient was plotted against the log ml of antitoxin dilution. The neutralization curve then has a range of 0 to 1.0, with 0 representing no neutralization and 1.0 complete neutralization. The point where NC = 0.5 represented the activity of 1 unit of toxin (2 units neutralized) and the neutralizing potency of a given lot of antitoxin was calculated by dividing the log dilution of antisera (read from the graph) by the number of toxin units neutralized. This was then divided into 1.0 to obtain the number of antitoxin units per ml of undiluted serum.

TABLE 3

List of Immunized Rabbit Groups

Immunized Group No.	Organism	Toxin Strain	Serotype	Loop Activity
1	*E. coli*	408-3	078H12	+
2	*E. coli*	408-3	078H12	+
3	*E. coli*	339	015H11	+
4	*E. coli*	411-7	0126H12	-
5	*E. coli*	408-4	078H12	-
6	*V. cholerae*	569B	Inaba	+
7	*E. coli*	348	09	-
8	*E. coli*	411-7	0126H12	-
9	*E. coli*	339	015H11	+
10	*E. coli*	1105F	06H16	+
11	*E. coli*	411-5F	0126H12	+

F. Hyperimmunized Rabbit Challenge Experiments

Preliminary experiments were done to determine whether immunization of rabbits with enterotoxin would protect against enterotoxin challenge of the small intestine of these rabbits.

All surviving immunized rabbits were challenged with multiple dilutions of E. coli and V. cholerae enterotoxins by the ileal loop method. Protection was determined by comparison of the response obtained (per dose given) to the previously obtained titration curve for that toxin in normal rabbits.

G. Statistical Procedures

Antiserum neutralization titers were compared by Duncan's multiple range test for correlated means (26,103) by the following method: a neutralization coefficient was calculated for each ratio in a titration, and the mean and standard error was obtained for each point on the neutralization curve. An F test was performed on the highest and lowest standard errors in a given titer to determine the validity of treating them as a homogenous population. The standard errors adjacent to the 50% neutralization point were averaged. This average standard error and the calculated unit neutralization titer were treated as the mean and variance for the calculations which were done by a program adapted to an Olivetti-Underwood programma 101 calculator.

RESULTS

A. Enterotoxin Titrations

Table 4 lists the ileal loop assays of the enterotoxigenic strains of E. coli studied in this thesis. It can be seen that the activity of the live cell cultures and their sterile culture filtrates are similar. The mg of toxin required to give a 50% ileal loop response (1 unit) was determined by titration of the culture filtrate after concentration by dialysis and lyophilization. Fifteen to twenty rabbits were usually required to satisfactorily assay a toxin.

The amount of lyophilized culture filtrate recovered per ml of syncase broth innoculated ranged from 0.17 to 0.47 mg for enterotoxigenic and non-enterotoxigenic strains alike. With these growth conditions there appeared to be no relationship between the amount of the recovered lyophilized filtrate and the enterotoxigenicity of the bacterial strain.

Figure 1 illustrates a typical dose-response curve seen in enterotoxin titrations of E. coli 339 and V. cholerae. The variability of the assay is shown by the 95% confidence limits of the mean. As observed by Sack et al. (81), the V. cholerae toxin was considerably more potent in the ileal loops than the E. coli enterotoxin.

Assay of E. coli 408-3 enterotoxin gave approximately the same results as E. coli 339, whereas E. coli strains 1105F and 411-5F required more assays to obtain comparable results since they exhibited more variability than the other strains.

Figure 2, an example of a toxin titration, shows the complete absence of fluid accumulation in the negative (PBS) control loops, and

TABLE 4

Ileal Loop Assays of Enterotoxin
Producing Strains of *E. coli*

Bacterial Strain	Serotype	Rabbit Ileal Loop Assays*		
		Live Cultures	Sterile Culture Filtrates	1 unit (mg)***
E. coli				
408-3	078H12	1.17 (2)**	2.18 (3)	0.32
411-5F	0126H12	1.79 (3)	2.00 (3)	1.6
339	015H11	1.43 (3)	1.39 (3)	0.20
1105F	06H16		1.02 (2)	0.40
V. cholerae	In. 569B		1.69 (2)	0.005

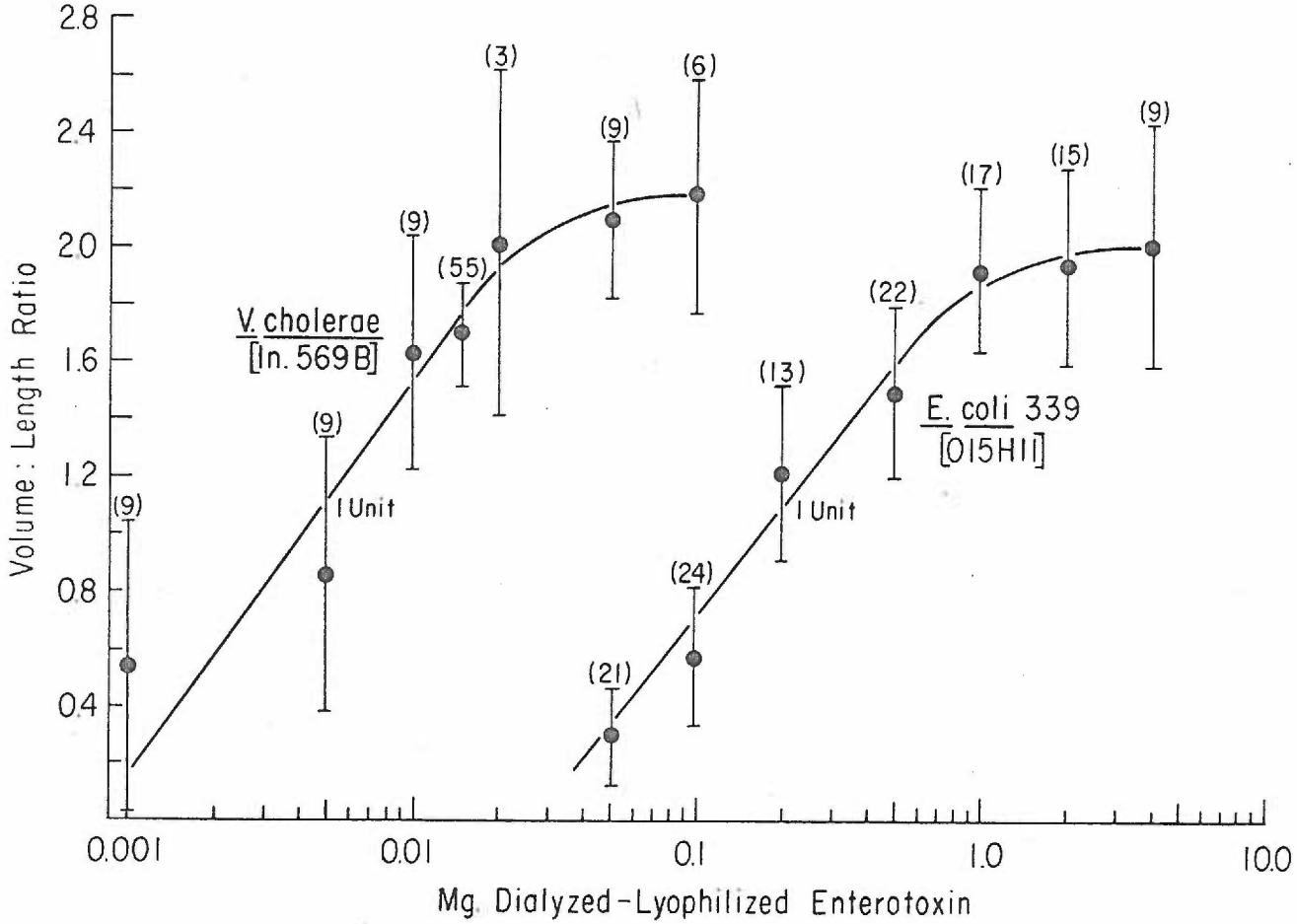
* Volume/Length Ratios

** Numbers in parentheses = number of loops tested

*** Obtained by titration of the dialyzed-lyophilized toxin

Figure I

COMPARATIVE V. CHOLERAE AND E. COLI ENTEROTOXIN TITRATION CURVES



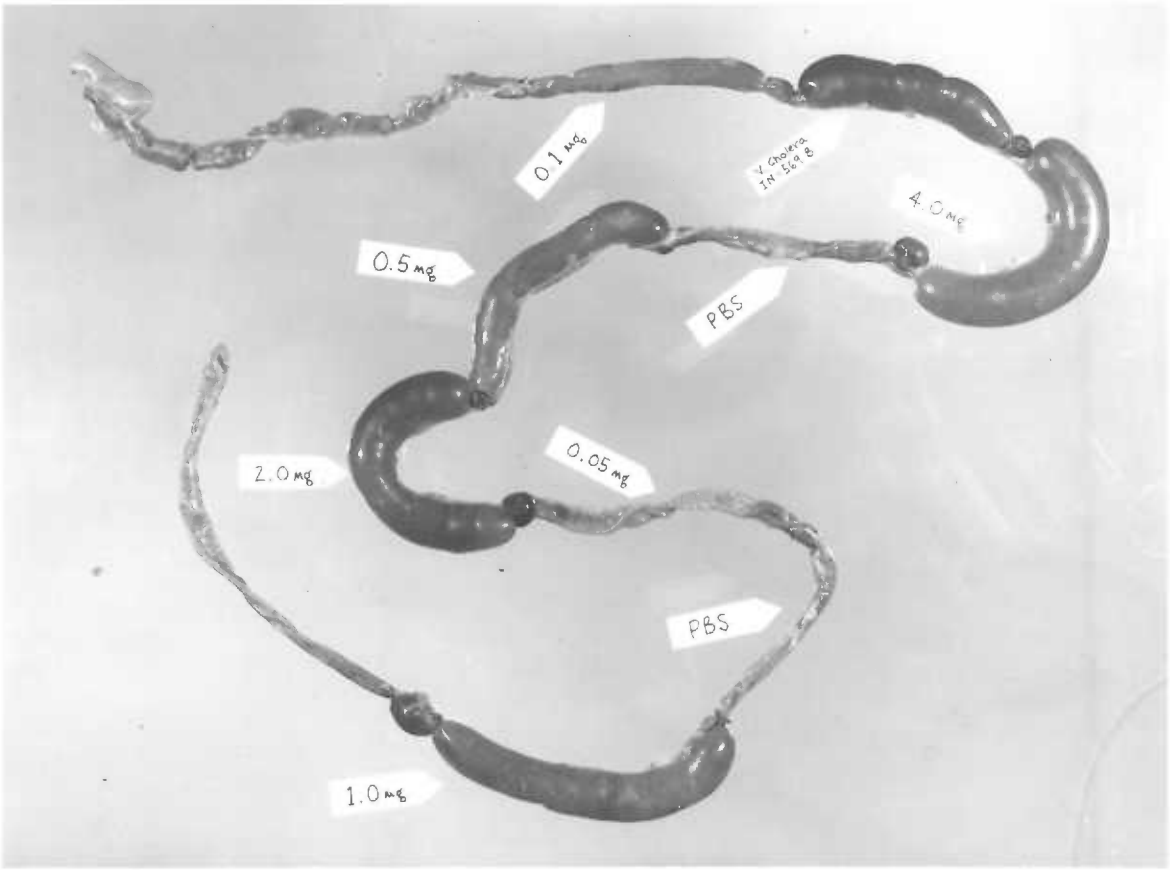
Points along the curve are the mean ratios of volume to length for increasing concentrations of enterotoxin. The numbers in parentheses indicate the number of ideal loop assays from separate rabbits for each mean value. The vertical lines represent the 95% confidence limits of the mean.

FIGURE 2

An example of an enterotoxin titration in ligated rabbit ileal loops.

V. cholerae In. 569B is the culture filtrate positive control.

P.B.S. (phosphate buffered saline) is the negative control.


0.1 mg
V. Cholera IN Sol'n
4.0 mg
0.5 mg
PBS
2.0 mg
0.05 mg
PBS
1.0 mg

the marked distension of positive V. cholerae and E. coli enterotoxin loops.

Four batches each of E. coli 408-3 and 339 enterotoxin was prepared during the course of this study. Comparisons were made to determine the variability of these different preparations. Regression lines were plotted from the 1.0 mg 'y' axis intercept to the 'x' axis, and the slopes of the lines for each batch were calculated. These slopes, compared in a two tail student T test, were found to be the same for each batch of toxin produced by a given strain. Moreover, a comparison of the mean of the slopes of E. coli 408-3 with those of E. coli 339 indidicated that there was no significant difference ($p \leq 0.01$) between the potency of the two strains.

Testing of live cells and dialyzed-lyophilized culture filtrates from non-enterotoxin producing E. coli strains gave the results shown in Table 5. No fluid was produced by the live cultures; even large amounts of lyophilized culture filtrate failed to effect significant fluid accumulation.

B. Antitoxin Production Technique

As determined in preliminary experiments, the results of immunization with E. coli 408-3 toxin by three different methods are shown in Figure 3. Three rabbits (Group 1A) which received foot pad injections, 2 mg/rabbit, gave a final titer of 129 units/ml on testing with homologous toxin. A single surviving rabbit (Group 1B) initially injected with 5 mg total in the foot pads (as well as multiple other sites) gave a titer of 333 units/ml. The three rabbits in Group 1C, which only received 2 mg intramuscularly and subcutaneously, gave a titer of 93 units/ml on testing with the homologous toxin.

TABLE 5

Ileal Loop Assays of Enterotoxin
Negative *E. coli* Strains

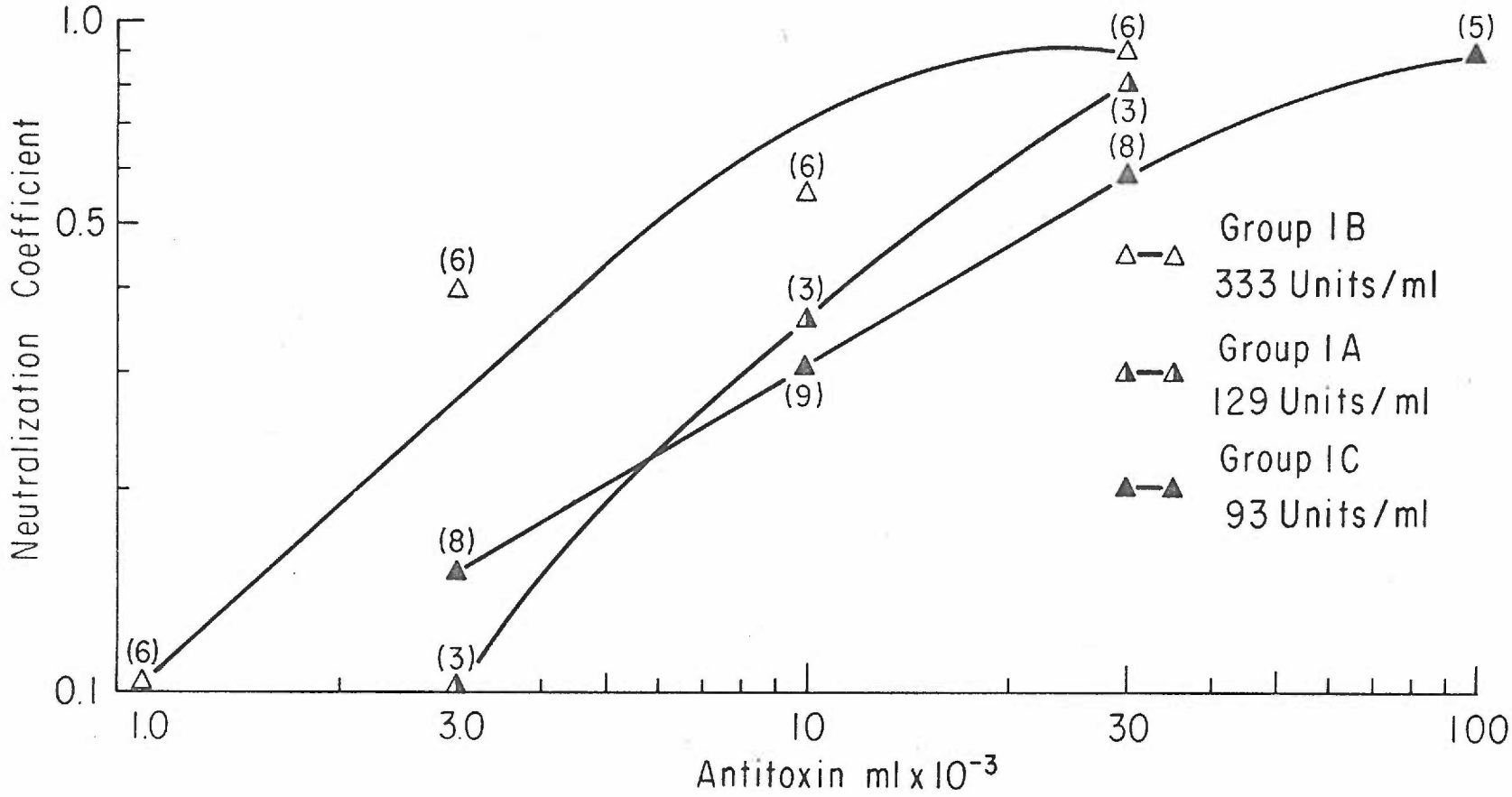
E. coli Strain	Serotype	Rabbit Loop Assay*	
		Live Cultures	Dialyzed-Lyophilized Culture Filtrates
408-4	078H12	0 (3)**	4 mg = 0 (3)
411-7	0126H12	0.13 (2)	6 mg = 0 (3)
			10 mg = 0.45 (2)
348	09H-	0.16 (2)	6 mg = 0 (3)

* Volume/Length Ratios

** Numbers in parentheses = number of loops tested

Figure 3

ANTITOXIN LEVELS ACHIEVED BY THREE METHODS OF IMMUNIZATION WITH E. COLI 408-3 [078H12] ENTEROTOXIN



The numbers in parentheses indicate the number of ideal loop assays from separate rabbits for each mean neutralization coefficient

Statistical comparisons of these three titers by the Duncan test (26) indicated that Group 1B had a significantly higher ($p < 0.01$) antitoxin titer while there was no significant difference between Groups 1A and 1C. The 1B procedure was not used, however, because of the great trauma and high mortality (50-75%) involved with the foot pad injection. Groups 3-11 were immunized by a modified method (55) which did not involve foot pad injection but was found to have satisfactory titers.

Figure 4 compares the titers of antitoxin measured at various times during the immunization of rabbit 1B. The initial bleeding was taken at 21 days because previous workers (40) had obtained high titers of V. cholerae antitoxin at this time using the same method of immunization. Duncan test comparisons showed a significantly higher titer ($p < 0.01$) at 75 days but no significant difference at 21 or 55 days.

C. Antitoxin Titrations

The results of all comparative antitoxin titrations of E. coli and V. cholerae are summarized in Table 7. The antisera used in each group (as shown in the vertical columns) is from the same pool; however, the E. coli 1105F enterotoxin indicates two different batches of toxin and their corresponding neutralization titers.

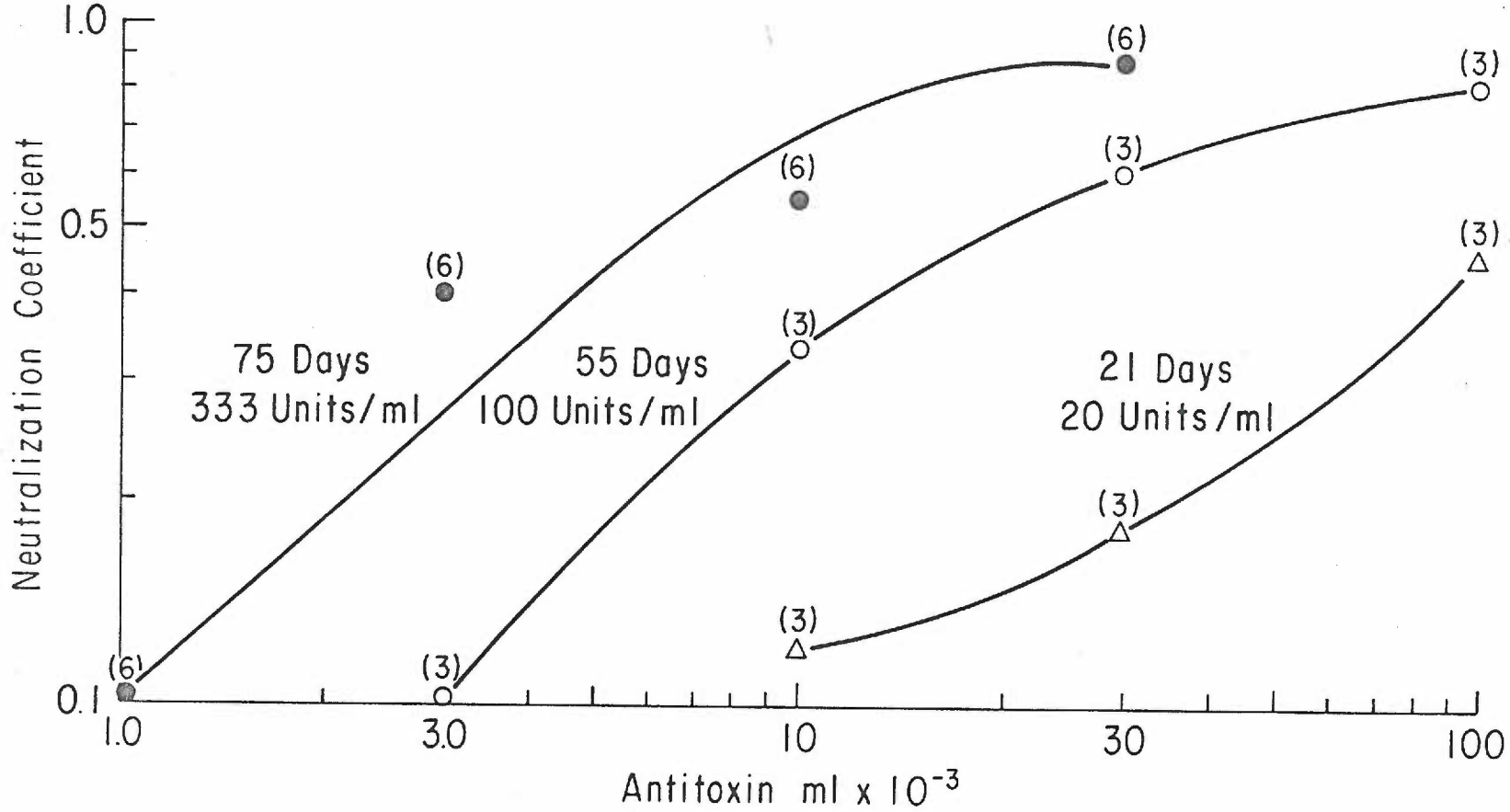
Preimmunization sera collected from the rabbits in all groups was tested with the immunization toxin and in some cases with heterologous enterotoxins to determine baseline titers. These results are summarized in Table 6. Only one case showed significant neutralization greater than 1:10; in this case (Group 9) there was approximately 20 units/ml neutralization titer.

1. E. coli 408-3 (078H12) Antitoxin Assays

This antitoxin was harvested 75 days post immunization and four

Figure 4

ANTITOXIN TITERS IN ONE RABBIT FROM GROUP IB IMMUNIZED BY E. COLI 408-3 [078H12]



The numbers in parentheses indicate the number of ideal loop assays from separate rabbits for each mean neutralization coefficient

TABLE 6

Preimmunization Serum Titrations

Preimmune Rabbit Serum Groups	Challenge Enterotoxin	Highest Serum Concentration Tested*
Group 1 (1B) (*E. coli* 408-3)	*E. coli* 339	1:10
	E. coli 408-3	1:5
Group 5 (*E. coli* 408-4)	*E. coli* 339	1:10
Group 9 (E. coli 339)	*E. coli* 339	1:10**
Group 6 (*V. cholerae*)	*E. coli* 408-3	1:10
	E. coli 339	1:5
	E. coli 1105F	1:10
	V. cholerae	1:10

* Represents the lowest antiserum dilution tested showing no significant enterotoxin neutralization

** About 20 units/ml neutralization

weeks after the last booster in rabbit 1B.

Table 7 lists the comparative titers from rabbits immunized with E. coli 408-3 toxin and Figure 5 shows the comparative neutralization titers. It can be seen that antitoxin prepared with E. coli 408-3 neutralized all three E. coli enterotoxin preparations but only weakly neutralized the cholera toxin.

Comparative analysis by the Duncan test showed significant difference at $p < 0.05$ between E. coli 339 and E. coli 408-3, and differences of $p < 0.01$ for all other comparisons in this group.

Figure 6 illustrates a rabbit loop antitoxin assay of a cross neutralization of E. coli 339 toxin with several dilutions of E. coli 408-3 antitoxin. It can be seen that as the antitoxin becomes more dilute (1:10-1:300), the loops become more distended from the effects of residual enterotoxin. Absence of neutralization is seen in the pre-immune serum dilution.

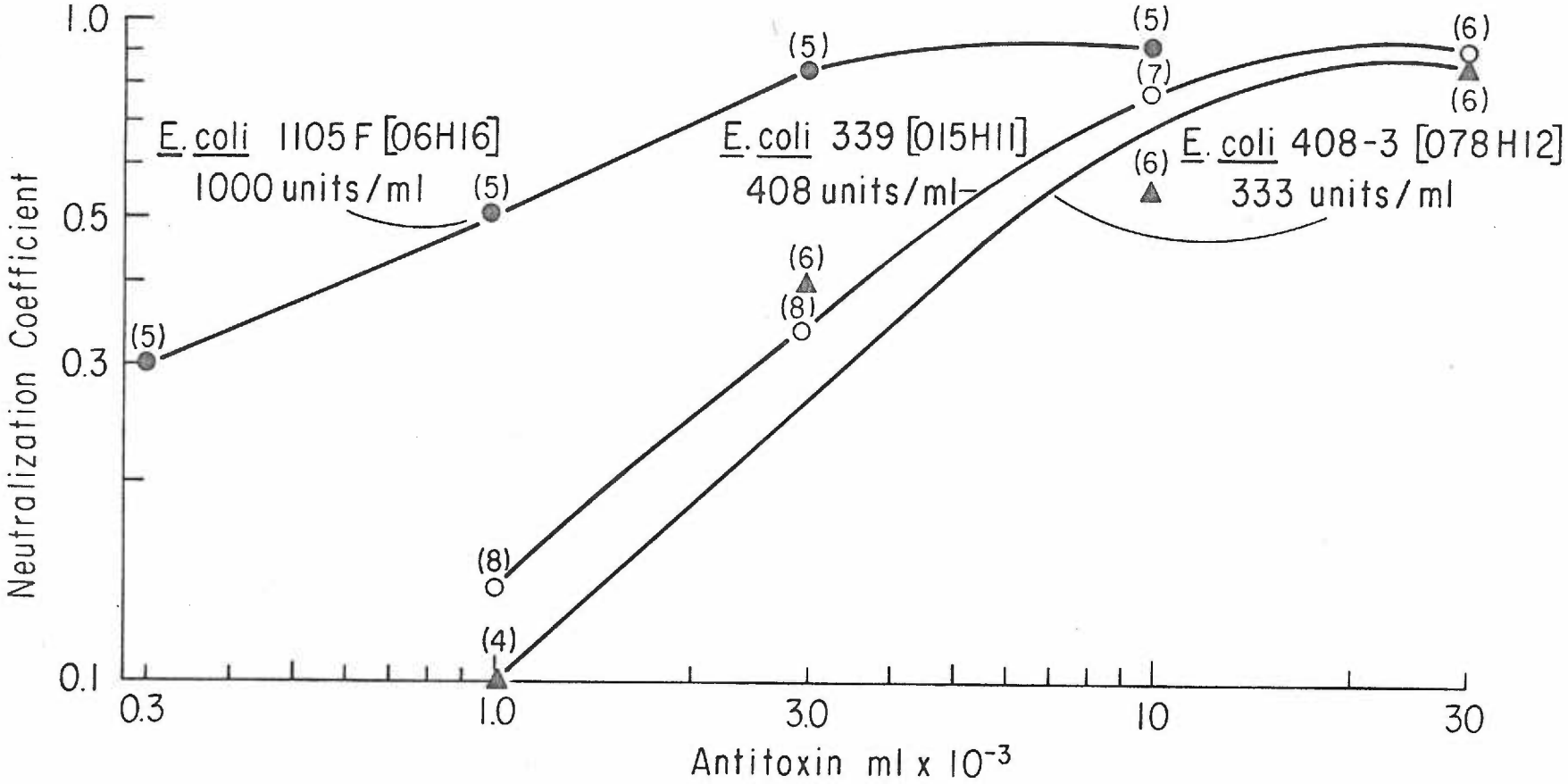
It should be mentioned that while neutralization testing routinely utilized 3 units of enterotoxin, one experiment was done using 6 units of E. coli 408-3 toxin and 408-3 antitoxin. The results were consistent with observations of Kasai and Burrows (29) that toxins and antitoxins combine in multiple proportions over a specified range.

2. E. coli 339 (O15H11) Antitoxin Assays

Two lots of antitoxin to this serotype were prepared at different times. The serum from Group 9 was selected for comparative cross neutralizations because it had a slightly higher titer on homologous neutralization than did Group 3. Table 7 lists the results of the E. coli 339 antitoxin titrations and Figure 7 shows the comparative neutralization titers. Antitoxin prepared with E. coli 339 neutralized the three E. coli toxins

Figure 5

COMPARATIVE E. COLI ENTEROTOXIN NEUTRALIZATIONS BY ANTITOXIN FROM RABBITS IMMUNIZED WITH E. COLI 408-3 [078H12] TOXIN



The numbers in parentheses indicate the number of ideal loop assays from separate rabbits for each mean neutralization coefficient

FIGURE 6

An example of a cross neutralization titration in rabbit ileal loops.

V. cholerae In. 569B is the culture filtrate positive control.

P.B.S. (phosphate buffered saline) is the negative control.

The 0.96 mg (3 unit) segment is the E. coli 339 toxin control.

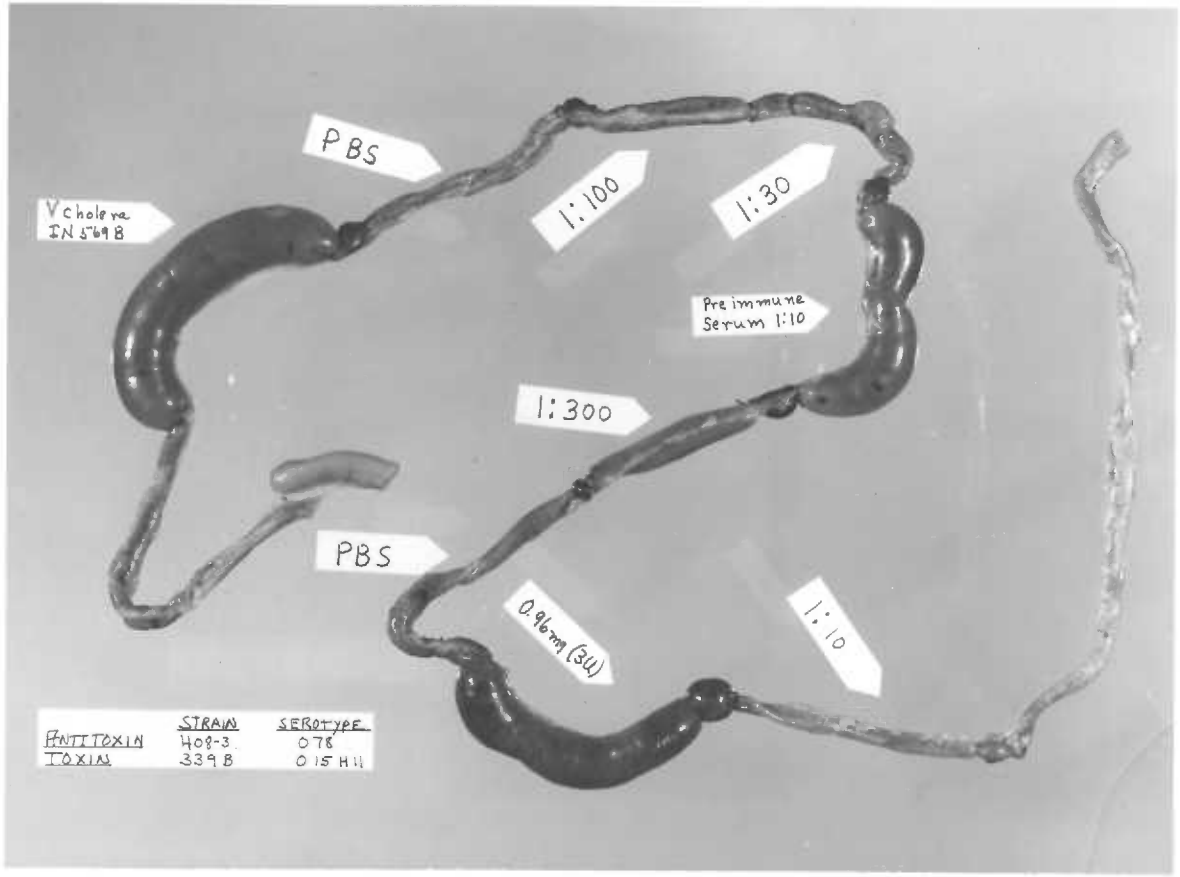
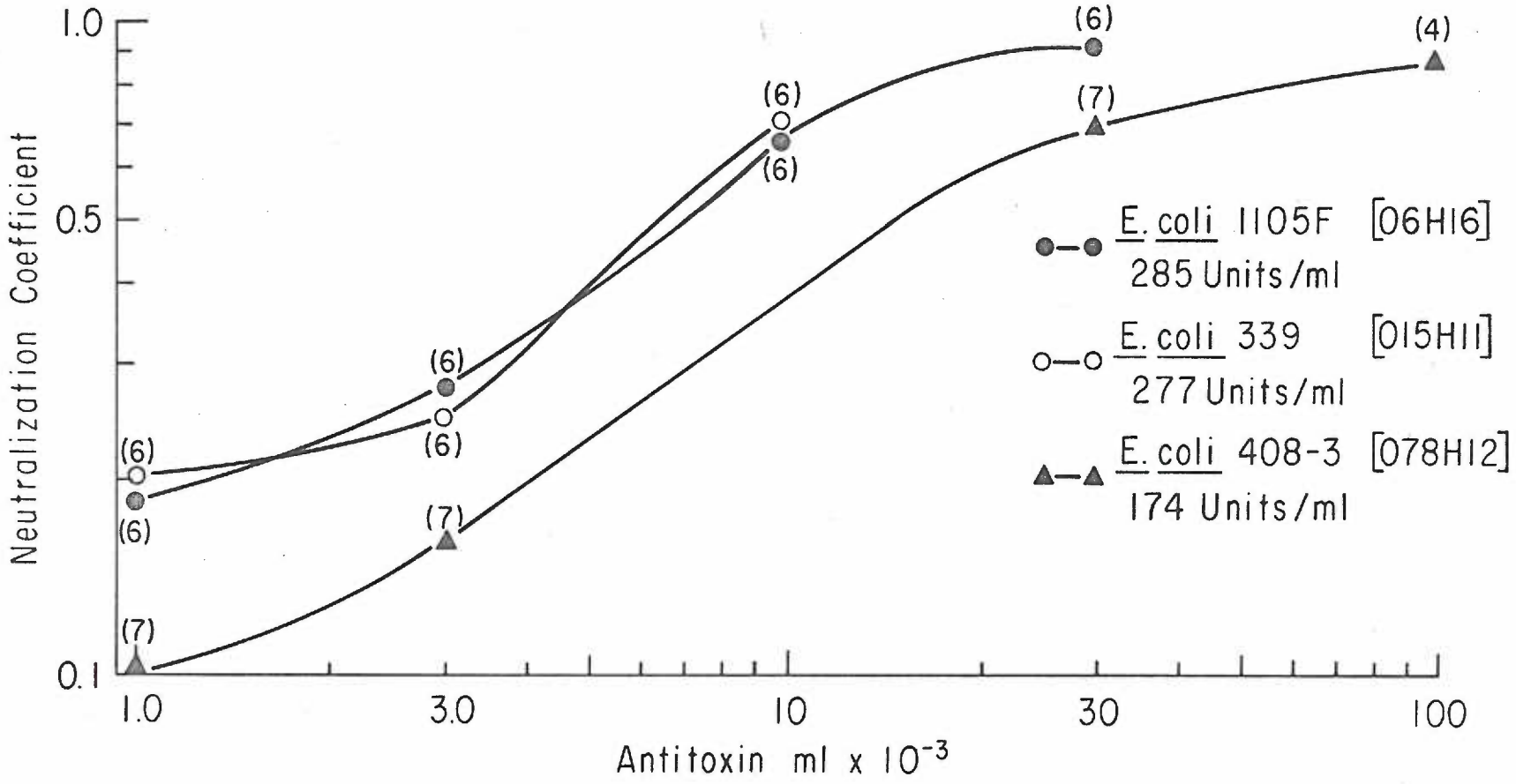

PBS
V cholerae IN 569 B
1:100
1:30
Pre immune Serum 1:10
1:300
PBS
0.96mm (34)
1:10
ANTITOXIN
TOXIN
STRAIN
408-3
339 B
SEROTYPE
078
O 15 H 11

Figure 7

COMPARATIVE E.COLI ENTEROTOXIN NEUTRALIZATIONS BY ANTITOXIN FROM RABBITS IMMUNIZED WITH E. COLI 339 [O15H11] TOXIN



The numbers in parentheses indicate the number of ideal loop assays from separate rabbits for each mean neutralization coefficient

at comparably high titers, but only weakly neutralized the V. cholerae enterotoxin. The Group 3 antitoxin gave the following titers: E. coli 339, 200 units; E. coli 408-3, 122 units; E. coli 1105F, 666 units; V. cholerae, 10 units.

Duncan test comparisons of the Group 9 neutralized enterotoxins showed no significant difference between the E. coli 339 and 1105F titers, while all other comparisons were significant at $p \leq 0.01$.

3. Assay of Antiserum from Rabbits Immunized with E. coli 1105F (O6H16) and Rabbits Immunized with E. coli 411-5F (O126H12) Enterotoxins

Rabbits were immunized and bled as previously described. Antitoxin assays indicated that no neutralizing antibodies had been produced. The rabbits were then "boosted" intravenously in an effort to stimulate a measurable antitoxin response. After one week the rabbits in both groups were bled again and tested. Assay of these sera indicated again no antitoxin activity in the 1105F rabbits and only 8 units in the 411-5 rabbits. These results are also included in Table 7.

4. Assay of Antiserum from Rabbits Immunized with non-Enterotoxin Producing Strains of E. coli

Antisera was prepared by immunizing groups of rabbits with the lyophilized culture filtrates of E. coli strains which were non-enterotoxigenic as determined by their failure to cause fluid accumulation in ileal loops either as live cells, sterile culture filtrates, or as dialyzed-lyophilized products. Table 7 also summarized the results of testing the antisera prepared with these three non-enterotoxigenic E. coli: 408-4, 348 and 411-7. It should be noted that one of these strains, E. coli 408-4 (O78H12), is the same serotype as one of the immunogenic, toxigenic strains used in the above experiments. The sera from these

immunized rabbits showed no significant neutralizing capacity for either E. coli or V. cholerae enterotoxins.

5. Vibrio cholerae In. 569B Antitoxin Assays

The sera assayed was a pool from four immunized rabbits. Figure 8 compares the neutralization titrations of enterotoxins of E. coli and V. cholerae, and Table 7 lists the comparative titers. The V. cholerae antitoxin was 10-fold more active against V. cholerae toxin than against either E. coli 339 or 408-3 toxins.

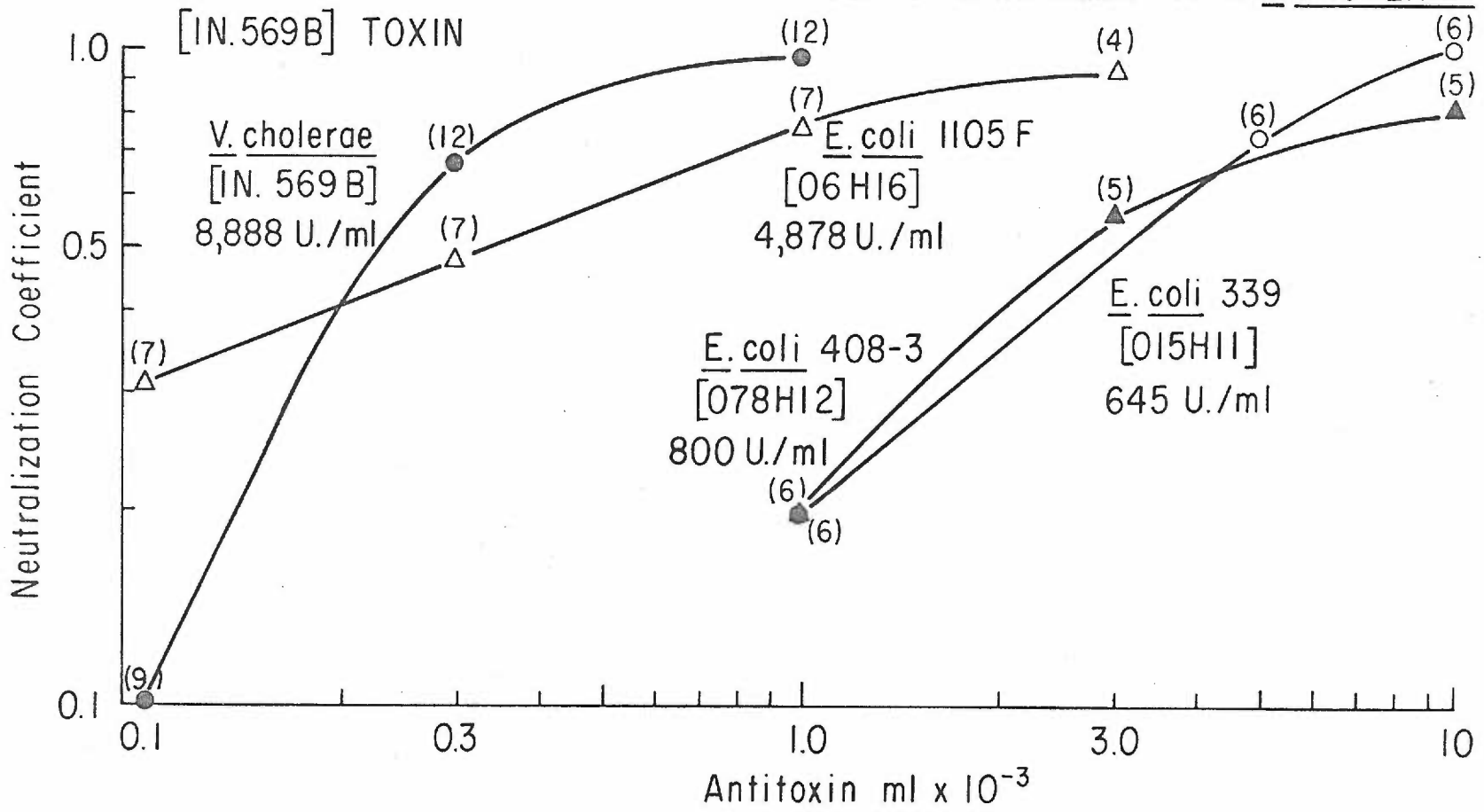
While the V. cholerae antitoxin neutralized cholera enterotoxin to a high degree (8,888 units/ml), it also significantly neutralized the E. coli enterotoxins. In fact, the titers were higher than those given by the rabbits immunized with E. coli. Of particular note is the high titer seen against E. coli 1105F; this enterotoxin was also neutralized to high titers by heterologous E. coli antisera. Duncan test analysis of the titers in this group show that there was no significant difference between the titers of E. coli 408-3 and E. coli 339; differences were significant ($p < 0.01$) between all other comparisons.

Table 7 also lists the results of assays of a commercial horse, anti-Vibrio cholerae enterotoxin antisera obtained from the Swiss Serum and Vaccine Institute at Berne, Switzerland. This standard lyophilized antitoxin, which had a stated neutralizing potency of 24,000 units/ml to V. cholerae toxin, was assayed in this lab at 25,000 units/ml.

The cross neutralization of this serum for E. coli 339 is even higher than that given by the rabbit, Inaba 569B antitoxin, but otherwise similar. The E. coli 1105F enterotoxin was neutralized at even a higher titer than the cholera enterotoxin.

Figure 8

COMPARATIVE NEUTRALIZATIONS OF V. CHOLERAE [IN.569B] AND E. COLI ENTEROTOXINS BY ANTITOXINS FROM RABBITS IMMUNIZED WITH V. CHOLERAE [IN.569B] TOXIN



The numbers in parentheses indicate the number of ideal loop assays from separate rabbits for each mean neutralization coefficient

TABLE 7

Comparative Antitoxin Titrations*

Toxin Preparations	Antitoxin Preparations: Organism / Serotype / Strain / Loop Activity	*E. coli* 078H12 408-3 Positive	*E. coli* 078H12 408-4 Negative	*E. coli* 015H11 339 Positive	*E. coli* 09H- 348 Negative	*E. coli* 06 1105F Positive	*E. coli* 0126H12 411-5F Positive	*E. coli* 0126H12 411-7 Negative	*V. cholerae* Inaba 569B Positive	*V. cholerae* Commercial Positive
TOXINS 408-3 078H12 1 unit = 0.32 mg		333	0	174					800	
339 015H11 1 unit = 0.26 mg		408	6	277	4	0	8	0	645	3,636
1105F 06H16 (Batch #2) lu=0.82 mg		(1,000)		(666)						
Batch #3 lu=0.42 mg			24	285		0	0		4,878	68,968
V. cholerae In. 569B 1 unit = 0.005 mg		20	0	20	0	0		2	8,888	25,000

*Values in the squares represent antitoxin neutralization activity in units/ml.

D. Challenge Experiments

The results of enterotoxin challenge of the surviving hyperimmune rabbits are shown in Table 8. In most cases there were not enough rabbits tested in each group to draw firm conclusions. The data do suggest, however, that there was no protection to either E. coli or V. cholerae toxin challenge in rabbits immunized with positive or negative strains of E. coli.

Table 9 shows the response ratios in Group 1 rabbits which were immunized with E. coli 408-3 (078H12) toxin. The results in this Table have been sub-grouped according to variations in immunizing methods; no significant protection was observed.

Rabbits in Groups 7 and 8 immunized with negative strains of E. coli appear to have been protected against the 0.5 and 1.0 unit E. coli 339 (015H11) challenge doses. This was not true of the E. coli 408-3 (078H12) challenges in the same rabbits.

In Group 9 (E. coli 339 (015H11)) and Group 5 (E. coli 408-4 (078H12)) there was only one surviving rabbit each. Challenges of these rabbits (not shown in the tables) also demonstrated no significant protection.

The four Group 6 rabbits which were immunized with V. cholerae (In. 569B) enterotoxin showed significant protection to the V. cholerae challenge of 9 units (0.045 mg). These results are shown in Table 8. There was no protection, however, against challenge with E. coli 339 (015H11) enterotoxin, even though a significant anti-E. coli entero-toxin had been demonstrated.

E. Enterotoxin Stability

Vials of a single lot of sterile culture filtrate of V. cholerae enterotoxin were kept frozen at -20°C. New vials were thawed and used

TABLE 8

Hyperimmune Rabbit Ileal Loop Challenges

Groups Immunized With	No. Days After Final Bleeding	Titer to Challenge Organism at Last Bleeding (units/ml)	Organism Challenged With	Enterotoxin Challenge Dose (units)	Mean Response Ratio (ml:cm)	Expected Value*
Group 3 (2 Rabbits)	13					
E. coli 339 (015H11)		200	E. coli 339 (015H11)	4	1.89	1.82
				4	1.61	1.82
				6	1.45	2.04
		114	E. coli 408-3 (078H12)	4	1.84	1.80
				4	2.04	1.80
				6	1.50	1.86
Group 7 (2 Rabbits)	4					
E. coli 348 (09)		4	E. coli 339 (015H11)	0.5	0	0.80
				1	0.11	1.14
				3	1.14	1.74
		-	E. coli 408-3 (078H12)	0.5	0.63	0.70
				1	1.77	1.14
				3	1.96	1.84
			V. cholerae (In. 569B)	3	1.74	1.70
Group 8 (2 Rabbits)	4					
E. coli 411-7 (0126H12)		0	E. coli 339 (015H11)	0.5	0.31	0.80
				1	0.57	1.14
				3	1.50	1.70
			E. coli 408-3 (078H12)	0.5	1.44	0.70
				1	2.13	1.14
				3	2.09	1.84
			V. cholerae (In. 569B)	3	1.61	1.70
Group 6 (4 Rabbits)	21					
V. cholerae (In. 569B)		8,888	V. cholerae (In. 569B)	3	0.25	1.70
				6	0.87	2.12
				9	0.82	2.18
		645	E. coli 339 (015H11)	2	1.74	1.52
				4	1.50	1.82
				6	1.92	2.04

*The expected value was obtained from the enterotoxin assays in unimmunized animals.

TABLE 9

Hyperimmune Rabbit Ileal Loop Challenges

Group I rabbits immunized with *E. coli* 408-3 (078H12) enterotoxin

Challenge Strain	Enterotoxin Challenge Dose (units)	(2)* Mean 1A	(1)* Mean 1B	(3)* Mean 1C	Total Means	Expected Ratios**
E. coli 339	0.5	0.25	0.93	0.99	0.73	0.80
	1	0.57	1.75	1.64	1.30	1.14
	3	1.74	2.50	1.70	1.85	1.70
E. coli 408-3	0.5	0.66	1.62	1.32	1.15	0.70
	1	1.50	2.00	1.51	1.59	1.14
	3	1.96	2.28	1.99	2.03	1.84
V. cholerae	0.5	0.22	1.80	0.35	0.54	0.60
	2	1.68	2.92	1.74	1.91	1.44

* Number of rabbits in each group

** The expected value was obtained from the enterotoxin assays in unimmunized rabbits

Reported mean ratios were calculated from volume/length measurements in ileal loops.

daily at a 1:50 dilution as a positive control in the ileal loops (see Figure 2). This preparation retained biological activity about two months and then rapidly became inactive. A new batch of cholera enterotoxin was prepared, dialyzed, lyophilized and stored at -45°C. This toxin was assayed and one unit determined as being 0.005 mg of enterotoxin. After about six months the potency of the toxin appeared to be the same.

Repeated assays of single lots of E. coli enterotoxin were carried out over a two to three month period with similar activity being retained. For example, two assays using a 3 unit challenge of E. coli 339 (0.6 mg) two months apart gave average response ratios of 1.40 and 1.34.

DISCUSSION

The strains of E. coli studied in this thesis were isolated in India from patients with severe cholera-like diarrhea. Of the organisms studied, only E. coli 0126 and E. coli 078 have been associated with outbreaks of infantile diarrhea. Interestingly, some of the E. coli isolates from these patients were of identical serotypes, but were non-toxigenic. This suggests that: 1. Enterotoxins are separate from the 'O' antigens of the organisms, and 2. Pathogenic E. coli occur which are unrelated to the classical enteropathogenic serotypes.

The ileal loop assay appeared to be very sensitive to low concentrations of enterotoxin, but with the considerable variability as noted by other workers. This model has been questioned as a valid reflection of the disease changes which occur in man. Craig (18) notes that a major objection is the obstruction in the intestine itself, with the accompanying distension of the test segments. The fluid pressure on the gut wall may interfere with micro-circulation and cause necrosis. Leitch et al. (61) observed that after about 12 hours ischemic damage occurs. Craig (18) concludes that the model is reasonably valid during the first 10-12 hours, but after that there was a chance of "spurious results from over-distension". Kasai and Burrows (52), who obtained results similar to those reported in this thesis, recommended using no more than six 10 cm segments, with the highest and lowest for "sham" controls and the four center segments for tests. Other workers (101), however, have used larger numbers of shorter segments and obtained satisfactory results. In this study, the number of segments used was normally 9 and the sequence of the negative controls and assay loops

were semi-randomized; this did not adversely influence the results.

The most important factor affecting the consistency of the test seemed to be the condition of the rabbits. The Animal Care Department made periodic checks for Coccidia (intestinal sporozoa) and infected animals were excluded. On occasions, the rabbits for a several week period would be either unresponsive or give many false positive loops and data from those rabbits would have to be discarded. This may have been related to the fact that the rabbits were 'batched' - that is, the local supplier gathered rabbits from neighbors and other suppliers to meet the needs.

Although the assays lacked the precision and reproducability of a chemical assay system or even assays such as the fat cell hydrolysis or the skin test of permeability factor, it was felt that this in vivo test was adequate for the purposes of this study. Moreover, there is evidence that the E. coli enterotoxin does not contain permeability factor (81); nor has the fat cell assay been developed for E. coli toxins.

The method of enterotoxin production utilized in this study was essentially the same as that reported by Sack et al. (81), except that the toxin was dialyzed against distilled water rather than PBS. This resulted in greater ileal loop activity per mg of toxin although the relative activities of E. coli to V. cholerae toxins remained similar.

Enterotoxin production has been reported in many different kinds of media and cultural conditions, and the only thing most of them have in common is an alkaline pH. Results that may reflect the varied culture methods are reports of heat stability and heat lability of enterotoxin preparations. A comparison of some of these reports and methods is

shown in Table 10. There is no agreed-upon criteria for heat lability; thus, the heat stable toxins of Smith and Gyles (88) which survive 65°C for 15 minutes may be called heat labile by another worker. Although the enterotoxins in this study were not tested for heat lability, toxin from one strain, E. coli 334 (O15H12), has been tested elsewhere (81) and found to be heat labile at 100°C for two minutes.

Various workers have attempted to identify biochemical markers to distinguish enterotoxigenic strains from non-toxin producers* (23); these attempts have been uniformly unsuccessful. Serologic studies have, however, been more promising. Bettelheim and Taylor (4) immunized rabbits with live and dead cultures of toxigenic E. coli strains. By electrophoretic methods they demonstrated a precipition band unique to the live enterotoxin producing strains of E. coli. Orskov et al. (69), in an electrophoretic study of all O and K antigen groups, found that the known enteropathogenic strains had similar migrations and could be classed in one electrophoretic group. Likewise, E. coli strains associated with 'dysentery' disease were in another group. A satisfactory immunological test has not yet been developed as a practical screening procedure, however, and the only reliable way to determine enterotoxicity is in animal models.

The antigens used to immunize the rabbits in this study were derived from 48 hour culture filtrates and consequently contained many cellular products, including enterotoxin. It was assumed that there was a significant endotoxin content in the antigen and therefore that anti-endotoxin antibodies would be formed. Moreover, the preparations from non-toxigenic strains should contain similar amounts of endotoxin. Wallace (101), using

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* Sack, R.B., unpublished observations.

TABLE 10

Comparisons of Culture Methods and Heat Stability of *E. coli* Enterotoxins

Reference	Organism	Culture Conditions	Product	Heat Stability				
				Labile	Partially Labile	Stable	Temp	Time
Etkin & Gorbach (31)	*E. coli* (078H12)	Syncase broth, pH 8 Shake flasks, 18 hours, 37°C	Lyophilized-Culture filtrate			X	60°C	30 min
						X	100°C	15 min
					X		100°C	30 min
		Syncase agar, 18 hours, 37°C	Whole cell lysate	X			60°C	15 min
Gyles & Barnum (83)	*E. coli* (08H19)	Peptone broth, pH 7.8 Shake flasks, 7 hours, 37°C	Dialyzed culture filtrate			X	100°C	30 min
		Soft agar	Whole cell lysate	X			65°C	10 min
Moon et al. (63)	*E. coli* (08H19)	T. soy broth (BBL), pH 7.4, 166 shakes/min, 20 hours, 37°C	Broth culture filtrate		X		65°C	30 min
				X			100°C	30 min
Smith & Halls (80)	*E. coli*	Soft nutrient agar, 24 hours, 37°C	Agar filtrate			X	65°C	30 min
						X	100°C	30 min
					X		121°C	30 min
				X			121°C	2 hrs
Sack et al. (81)	*E. coli* (015H11)	Syncase broth, pH 8, Stationary, 48 hours, 37°C	Lyophilized - Culture filtrate			X	56°C	30 min
					X		80°C	30 min
				X			100°C	2 min
Wallace, C.K. (101)	*E. coli* (015H11)	Syncase broth, pH 8, 200 shakes/min, 18 hours, 30°C	Lyophilized-Culture filtrate			X	56°C	30 min
						X	80°C	30 min
						X	100°C	2 min

similar cultural conditions, used the Limulus horseshoe crab assay system to test E. coli enterotoxin preparations and found an endotoxin level of greater than 1:10,000.

The presence of antibodies to other components was clearly shown in the homologous antiserum titrations. In two experiments, heavy flocculation was seen in serum dilutions up to 1:100, and yet this reaction had no neutralizing effect upon the enterotoxin activity. In the first experiment, titration of the non-enterotoxigenic E. coli 408-4 (078H12) antisera with the toxin from E. coli 408-3 (078H12) showed a large amount of flocculation in the reaction flasks but there was no neutralizing effect upon the enterotoxin. In the second experiment, this same phenomenon was seen in the reaction of E. coli 1105F antiserum with 1105F toxin. Heterologous toxin and antitoxin titrations gave no flocculation. These results indicate that the flocculating antigens were unrelated to enterotoxin. Others (50,88,90) have indirectly shown that anti-endotoxin reactions are distinct from anti-enterotoxin neutralizations because antisera prepared against live cultures or killed E. coli organisms inhibit the dilating effects of live cultures only by a type-specific bactericidal effect.

The most significant result of this study was the demonstration that antiserum from rabbits immunized with enterotoxin from a specific serotype of E. coli will cross-react and neutralize at high titers the ileal loop activity of enterotoxins from other serotypes of E. coli.

These conclusions are similar to those of Etkin and Gorbach (31), who studied essentially the same three enterotoxin producing strains of E. coli: 078, 015 and 06. In fact, the strain 1105F (06H16) was obtained

from Dr. Gorbach. Etkin and Gorbach used filtered whole cell lysates of E. coli cultures to immunize rabbits. They also used the same Kasai and Burrows (52) titration methods. However, they attained only very low antiserum titers. They showed that the 078 E. coli enterotoxin was 50% neutralized at a 1:30 dilution of homologous antisera as compared to a 1:667 dilution for the same neutralization point in this study. In heterologous neutralizations they found that E. coli 015 antisera gave 50% neutralization with E. coli 078 toxin at 1:20 and E. coli 06 antitoxin with E. coli 078 toxin at 1:19. In this study, 1:333 and less than 1:5 dilutions were necessary for comparable neutralizations (the low E. coli 06 titer is discussed below).

It was noted in this study that antiserum to E. coli 408-4 (078) (a non-enterotoxigenic strain) cross-reacted with the 1105F (06) toxin at about 1:13 (24 units/ml). Sack et al. (81), in comparing cross reactions of cholera and E. coli toxins and antitoxins, have shown that normal rabbit sera may have titers of up to 20 units/ml. This low level of partial neutralization by preimmune rabbit sera was also seen in this study. Consequently, the observations of Etkin and Gorbach might be interpreted as non-specific cross reactions, especially since no pre-immune serum titers were reported.

Other workers (31,50) have also shown that E. coli enterotoxins are cross-neutralizable; however, no one has reported anti-enterotoxin titers high enough to clearly show immunological similarities or differences of the toxins from E. coli of different serotypes.

Gyles and Barnum (50), working with procine E. coli isolates, reported heterologous cross neutralizing reactions with several serotypes of E. coli. They did not attempt to titer the antisera, however, and

the antitoxin was mixed in equal volumes with a toxin dosage of unassayed potency. Nevertheless, they did observe cross neutralizations that were distinguishable from the controls of normal unimmunized rabbit sera. Their immunizing innoculum included living cultures, formalized cultures, whole cell lysates, and peptone culture dialysate, all of which exhibited similar neutralizing ability.

In later work, Smith and Gyles (87,88), using sera against live 18-hour broth cultures of enterotoxin producing E. coli and using antiserum dilutions of 1:3 and 1:7.5, reported there was no cross neutralization of E. coli enterotoxins and no cross antibacterial activity. These results were not compatible with the earlier work of Gyles and Barnum (50).

The strong cross reactivity and the similarity in titers of antitoxin stimulated by E. coli of different serotypes seen in this study suggests that the toxins are immunologically similar. This might be expected since Smith and Linggood (89) showed that the genetic factor that controls enterotoxin production (Ent) is easily transferred by conjugation between E. coli strains and related bacterial species; it is unknown how many E. coli serotypes may produce enterotoxin.

It is apparent that there are several conflicting reports in the literature concerning the cross-neutralizability of E. coli enterotoxins. Considering the crude state of the toxins used to produce the antitoxins, the wide variations in toxin production methods, and the lack of uniformity in toxin and antitoxin assay procedures, these discrepancies may be more apparent than real.

Cross-neutralizations of E. coli enterotoxin with V. cholerae antitoxin has also had contradictory reports in the literature (50,81).

In this study of the neutralizations of E. coli enterotoxins by V. cholerae antitoxin, it appears that the homologous cholera neutralization titers are roughly related to the E. coli toxins; that it, the Swiss V. cholerae antitoxin neutralized at a proportionately higher titer than the Inaba 569B prepared antitoxin. Unfortunately, the challenge experiments involving cholera immunized rabbits do not support the in-vitro neutralization results.

A review of the literature on this subject shows there are no clear cut answers to support or refute these observations. Previous work has also suggested cross neutralization between E. coli and V. cholerae toxins. Gyles and Barnum (50) studied E. coli P307, an enterotoxin producing isolate, from swine. They reported that antisera prepared from V. cholerae In. 569B cultures, neutralized E. coli P307 enterotoxin at dilutions of 1:2. In further work, Gyles (49) found that high titered V. cholerae enterotoxin antiserum prepared in a horse, neutralized 3 ED_{50} (4.0 mg) of E. coli P307 enterotoxin at a antitoxin dilution of 1:256 in the rabbit ileal loop. In pigs the above neutralization was effected at a dilution of 1:16 with 50 mg of E. coli enterotoxin. The methods used and the results do not lend themselves to direct comparisons with our methods and results since the E. coli enterotoxin was not titrated the same, but they are suggestive of cross reations.

On the other hand, Sack and co-workers (81), using enterotoxin preparations of E. coli 334 (O15H11) found cross neutralizing activity of only 50 units/ml with anti-V. cholerae enterotoxin equine antisera, which had a homologous cholera titer of 23,255 units.

Wallace (101) has investigated the possibility of an antigenic relationship between cholera and E. coli enterotoxins by attempting to

neutralize E. coli 334A (O15H11) enterotoxin with 16 Craig units of cholera antitoxin from the Swiss Serum Institute. He found that cholera toxin was neutralized 32-fold by this dose but there was no effect on E. coli toxin. Since a "Craig unit" is defined (17) in the neutralization of cholera toxin skin permeability factor assay, a very sensitive test, it seems likely that the antitoxin was too dilute to neutralize the E. coli toxin.

From the present studies, it appears that there is definite E. coli enterotoxin neutralizing cross reactions with V. cholerae antitoxin. The nature of this reaction deserves further study. The fact that V. cholerae toxin is very antigenic and readily stimulates high antitoxin titers, suggests that similar findings might be obtained with E. coli toxin, if larger amounts free of endotoxin could be administered. One of the first steps toward these studies would be the purification of the E. coli enterotoxin.

It is noteworthy in the results presented here that the enterotoxin produced by E. coli 1105F (O6H16) had several unusual properties. It was neutralized by higher dilutions of antiserum than the other enterotoxins, and yet was non-antigenic by the method used to prepare antisera with other strains. These data do suggest the possibility that the E. coli 1105F toxin may act as a complex hapten, which is non-dialyzable, non-antigenic, and yet is biologically active and capable of being neutralized by antisera (14). It is also possible that it would be antigenic in another species of animal. Gyles (49) noted that the stable toxin he isolated was non-antigenic but was neutralized neither by antisera prepared against live cultures or labile toxin. Further studies

should be done to confirm the above work and to further characterize this unusual enterotoxin.

Although the method of immunizing rabbits in this study gave relatively high titers of E. coli antitoxin, the rabbits were not protected against toxin challenge. These findings are in accord with those reported by Etkin and Gorbach (31) who were also unable to show any protection against toxin activity in rabbits immunized to E. coli 078H12 enterotoxin. In their case this was not surprising because of the low antitoxin titers found in their rabbits. Other workers (84) using live cell cultures of enterotoxigenic E. coli to vaccinate animals have also found that circulating antibody did not afford protection against ileal loop challenges. No antitoxin titers were determined in these experiments.

These findings are different from those in the neutralization of V. cholerae toxin. Other workers have also observed in-vivo protection against V. cholerae challenge. Finkelstein and Atthasampunna (40) immunized rabbits with purified choleragen (cholera enterotoxin) in Freund's adjuvant and obtained protection against challenge with V. cholerae live cells or massive doses of cholera enterotoxin. Burrows et al. (9) reported that peak immunity to V. cholerae enterotoxin challenge in ligated loops was associated with peak titers of antibody in serum and not corproantibody. They also observed that parenteral immunization gave better protection than local intraintestinal immunization. From this they inferred that the basis of immunity to enterotoxin was a tissue contained antibody.

On the basis of reported animal studies, considerable research is being done to clarify the nature of V. cholerae toxoid induced immunity in humans. It would also appear from the animal studies reported in

this thesis that more work should be done to find better methods of immunizing with E. coli enterotoxin. If higher titers of circulating antitoxin can be obtained, then it can be determined if these are protective to intraluminal challenge. Certainly these results indicate that protective immunization against the enterotoxins of E. coli is a possibility.

SUMMARY AND CONCLUSIONS

Seven strains of Escherichia coli (comprising five serotypes, 4 toxigenic and 3 non-toxigenic) and one strain of Vibrio cholerae, were studied for enterotoxigenicity.

The enterotoxins were produced in syncase broth (39), dialyzed and lyophilized. Rabbits were immunized with these toxins and the degree of cross neutralization between them was determined. Toxins and antitoxins were assayed in rabbit ileal loops according to the method of Kasai and Burrows (52).

The results of this study indicate that enterotoxins produced by strains of Escherichia coli of different serotypes are antigenically similar, are not related to cell wall antigens and have some antigenic similarity to the enterotoxins of Vibrio cholerae.

Vibrio cholerae enterotoxin was strongly antigenic in that it stimulated high antitoxin titers in rabbits, and conferred protection against homologous enterotoxin challenge. It also stimulated the production of antibodies that neutralized E. coli enterotoxin, but did not confer protection against E. coli toxin challenge.

The E. coli enterotoxin was shown to be poorly antigenic, giving antitoxin titers less than one tenth as high as cholera antitoxin, but much higher than those previously reported in the literature. These E. coli titers, however, were not protective against homologous or heterologous intraluminal toxin challenges.

One strain, E. coli 1105F (O6H16) appeared to be immunologically related to other enterotoxins, but was different in that it was non-antigenic in rabbits, and was neutralized by V. cholerae and heterologous

E. coli antisera at unusually high titers.

Immunological similarities between *V. cholerae* and *E. coli* enterotoxins suggest that a purified, antigenic form of *E. coli* enterotoxin could be developed to provide protective immunization against acute diarrheas caused by this organism.

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