Between 1951 and 1959, Sambhu Nath De made crucial discoveries on the pathogenesis of cholera that changed the course of our understanding of the disease. The discovery that cholera is caused by a potent exotoxin (cholera enterotoxin) affecting intestinal permeability, the demonstration that bacteria-free culture filtrates of *Vibrio cholerae* were enterotoxic, and the development of a reproducible animal model for the disease are considered milestones in the history of the fight against cholera. In this commentary, a classic article by De & Chatterje published in 1953 and its public health and research impact are highlighted.

From endotoxin to exotoxin: De's rich legacy to cholera

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Vibrio cholerae, the causative agent of the disease known as cholera, which causes watery diarrhoea, was first described by the Italian anatomist Filippo Pacini in 1854. That same year British physician John Snow demonstrated that the disease is water-borne. Thirty years later, Robert Koch found the characteristic comma-shaped bacterium in the intestinal tissue of Egyptian patients who died after developing the typical clinical symptoms of cholera. Later that year, Koch cultured the bacterium in Calcutta (now known as Kolkata), India, and is credited with the discovery of *V. cholerae*, which became known as "the comma bacillus".

Having isolated the organism from cholera patients and grown it in culture, Koch had fulfilled two of his famous postulates for proving causality, but he had yet to fulfil the third, i.e. to show that pure cultures of the comma bacillus obtained from cholera victims could cause the disease in an animal model. This third postulate remained undemonstrated for the next 75 years, until the toxin that caused cholera was discovered by Sambhu Nath De in Kolkata in 1959.[1] De, in effect, also proved Koch's third postulate by reproducing the disease in an animal model. The full significance of De's discovery is highlighted by the fact that it took Koch just under 8 months to discover the more elusive and fastidious etiologic agent of tuberculosis, which he did in March 1882, including replicating the disease in a guinea pig model. It was the availability of an animal model for tuberculosis that enabled Koch to discover the pathogen.[2] However, in the case of cholera success eluded him because there was no animal model to provide proof that the comma bacillus could cause the disease. In 1959, when De reported the discovery of the cholera toxin,[1] another group in Bombay led by NK Dutta reported the development of an infant rabbit model for cholera and demonstrated that the symptoms of the disease were caused by a toxin.

Between 1951 and 1959, Sambhu Nath De, born in 1915 in Garibati near Calcutta, made critical discoveries on the pathogenesis of cholera that radically changed our understanding of the disease. The pioneering 1953 article of De & Chatterjee,[3] reproduced in the original with this commentary, is a classic. It was the first in a series of papers that examined the action of *V. cholerae* on the intestinal mucous membrane and that culminated in the discovery of cholera toxin.[1] Prior to the above work, almost all research had consisted of administering the stools of cholera patients or various toxic preparations derived from *V. cholerae* to different animals by various routes using a multiplicity of techniques to check for potential systemic or lethal effects, and conflicting results had been obtained. De, however, contended that the primary site of activity of *V. cholerae* and/or its toxin was the intestinal mucosa.[4] Few of the earlier studies had examined the effect of the toxic material on the intestinal mucosa because of the entrenched belief that an endotoxin was the main toxic principle in cholera. Thus, the 1953 article of De & Chatterje[3] displayed a paradigm shift in thinking.

In the simple experiments that led to the article, living *V. cholerae* cultures were first introduced into the intraperitoneal cavity of a rabbit and later into the lumen of the rabbit's ligated intestine. In this way, De & Chatterje demonstrated that *V. cholerae* alters the permeability of the intestinal mucosa and thereby causes fluid secretion. The intravenous injection of Evans blue dye, which combines firmly with plasma albumin, was an ingenious way to prove that the leakage of fluids in the intestinal lumen was from intestinal capillaries. De also had a rational explanation, based on experimental evidence, for why the intraperitoneal fluid was rich in protein, unlike cholera stools, and why the fluid that accumulated in the ligated intestine of rabbits was low in protein, like cholera stools.

The prodigious work of De & Chatterje[3] was followed by the demonstration that the pathogenicity of some strains of *Escherichia coli* was very similar to that of *V. cholerae*, and such strains were what we know today as enterotoxigenic *E. coli*.[5] The discovery of the cholera enterotoxin and its effect on intestinal permeability,[3] the demonstration that bacteria-free culture filtrates of *V. cholerae* are enterotoxic[1] and the development of a reproducible animal model for cholera[1,3,4] are milestones in the history of the fight against the disease.

The work of De & Chatterje had a profound impact on public health. The realization that the cholera toxin impairs intestinal permeability without disrupting the intestinal mucosa, altering intestinal motility, or producing an inflammatory response set the stage

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for the impressive discovery in the late 1960s of oral rehydration therapy, a simple, cheap and effective treatment for the severe, rapid dehydration produced by cholera. Oral rehydration therapy dramatically brought down the cholera case fatality rate from 30% in 1980 to around 3.6% in 2000. The effectiveness of oral rehydration therapy became fully evident during a cholera epidemic that broke out during the Bangladesh Liberation war in 1971.[6] Oral rehydration therapy was introduced globally by the World Health Organization in 1979 and rapidly became the cornerstone of programmes for the control of diarrhoeal diseases. Its use brought the annual number of deaths attributable to dehydration from diarrhoea among children aged less than 5 years from an estimated 4.6 million in 1980 to about 1.5 million in 2000.[7] Recent trends suggest that diarrhoeal deaths among children continue to decline as a result of its use.

De's work has made a mark in the history of efforts to understand cholera[8] and in the history of cellular physiology and biochemistry[9] because it marked the beginning of a new way of examining the complex process manifested as diarrhoea. The work of De also paved the way for the discovery of entire families of labile toxins from enterotoxigenic *E. coli*, and Shiga and Shiga-like toxins from *Shigella* spp. and diarrhoeagenic *E. coli*. To the immunologists, De's work opened new vistas, particularly from the perspective of exploring the immune responses to the toxin and developing a vaccine containing antitoxin. A search done on 19 November 2009 in the PubMed database using the keyword "cholera toxin" yielded a phenomenal 11 168 publications that the work of De spawned.

The year 2009 heralded the 50th anniversary of the discovery of cholera toxin by De, and 128 years have elapsed since the first isolation of pure cultures of the comma bacillus by Koch. Despite the great wealth of knowledge accrued on *V. cholerae* over the past 128 years, including the sequencing of the entire genome of 24 isolates of *V. cholerae*, the problem of cholera continues unabated in many parts of the world. It has worsened since the 1990s, and Zimbabwe offers a striking recent example of how cholera can ravage a country. Good hygiene, sanitation and the provision of safe water can effectively reduce the burden of cholera, but implementing these measures realistically in low-resource settings is a complex matter with which we continue to grapple. Population growth and rising poverty, global climate change and rapid, unplanned urbanization are perfect ingredients in the recipe for cholera. The burden of this dangerous disease will continue to rise, for ultimately it is a question of "hygiene versus hunger" in the most impoverished areas, where the priorities are different from those in more prosperous parts of the world. We would need De's pragmatic wisdom to solve the problem of cholera. Is there a simple solution? ■

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AN EXPERIMENTAL STUDY OF THE MECHANISM OF ACTION OF *VIBRIO CHOLERÆ* ON THE INTESTINAL MUCOUS MEMBRANE

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It is difficult to regard the massive outpouring of fluid in cholera as an inflammatory phenomenon in the generally accepted sense. Neither the cholera stool nor the wall of the choleraic intestine shows the presence of inflammatory cells. On the other hand, several workers suggest that the toxin of the cholera vibrio may alter the permeability of local capillaries. Manwaring *et al.* (1923) found that 2-7 days' culture filtrate of *V. choleræ* perfused through an isolated mammalian heart caused œdema of the myocardium. Burrows *et al.* (1944) demonstrated an increased outflow of fluid from isolated strips of rabbit small intestine immersed in cholera endotoxin. De *et al.* (1951) showed that intraperitoneal injection into rabbits of a suspension of killed and washed cholera vibrios causes an accumulation of fluid within the peritoneal cavity; this fluid is rich in protein and poor in cells. Evans blue solution injected intravenously leaks into the fluid that collects in the peritoneum. The present investigation is concerned with permeability changes in the capillaries after the introduction of living *V. choleræ* into loops of small intestine isolated by ligatures.

Materials and methods

Rabbits weighing 1200-1500 g. were not allowed food or water for twenty-four hours. With aseptic precautions and local procaine anæsthesia, a midline incision about two inches long was then made just below the middle of the abdomen, which was opened by cutting through the muscles and peritoneum. A segment of small intestine taken midway between its upper and lower ends was isolated with two silk ligatures; blood vessels were carefully avoided. One ml. of Dunham's peptone-water medium inoculated freshly with one loopful of a twenty-four-hours liquid culture of an Ogawa strain of *V. choleræ* was injected slowly into the lumen of the isolated loop. Previous experiments had shown that this was the most suitable dose. The abdomen was closed in two layers with thread. The animal was not allowed food or water and was killed after a further twenty-four hours by the rapid intravenous injection of 5 ml. of air. A careful examination was made of the isolated loop and of parts of the small intestine above and below it. The fluid contained in the distended parts of the small intestine was aspirated with a sterile syringe and measured, cultured on MacConkey plates and in Dunham's peptone-water medium for the detection of *V. choleræ*, and centrifuged. The deposit was examined microscopically as a wet preparation, both unstained and after staining with Loeffler's alkaline methylene blue. A smear was also stained for cells with Ehrlich's acid hæmatoxylin and eosin. The albumin of the supernatant fluid was estimated after precipitating the mucus and globulin

with a saturated solution of sodium sulphate. Equal lengths (5-6 in.) of the ligated and of the adjacent proximal and distal parts of the small intestine were slit open, washed, wiped dry and weighed. Small pieces were fixed in 10 per cent. formol-saline and embedded in paraffin; sections were stained with Ehrlich's hæmatoxylin and eosin. This experiment was performed on ten rabbits and fifteen rats. Six additional rabbits and five rats served as controls in which the sterile, uninoculated medium was injected into an isolated segment prepared in the same way.

Three test animals and two controls were operated on and injected as above; four hours later 4 ml. of a 2 per cent. solution of Evans blue (T. 1824) in normal saline were injected into the ear vein. Evidence of leakage of the blue dye into the contents and walls of the different parts of the small intestine was looked for next day.

Results

Injection of V. choleræ *into isolated segments of rabbit small intestine*

The small intestine proximal to the isolated loop was distended with fluid both in the test animals and in the controls. The fluid was yellowish in colour and its albumin content ranged from 0·42 to 0·50 per cent. The part distal to the ligated segment was collapsed in both groups of animals and no fluid could be expressed. The isolated segment in the control animal was also collapsed and empty in contrast to that of the test animal, which was distended with fluid and swollen to the diameter of the thumb. The blood vessels in the wall of this part were markedly injected and the peritoneal surface of the isolated loop, which appeared dull, was loosely adherent to neighbouring loops. As much as 14-20 ml. of fluid could be aspirated from this segment. The fluid was sometimes frankly blood-stained and often rice-watery with a pinkish hue, but it never showed any trace of yellow colour. Culture from the contents of this segment alone was positive for *V. choleræ*, the other parts of the small intestine giving negative results. Microscopical examination of the fluid revealed the presence of flecks of mucus, with numerous epithelial cells and vibrios. A pus cell was encountered occasionally, but never any macrophages. Red cells, though sometimes numerous, were usually few in number. The albumin content was invariably high, ranging from 1·0 to 3·8 per cent.; the highest figures were obtained from frankly blood-stained specimens. The mean weight of the wall of the ligated segment in control animals was almost equal to that of equal lengths of the proximal and distal parts, but was about 12·7 per cent. heavier than the latter in the experimental animals.

Histological changes. The outstanding microscopic change in the experimental animal was marked œdema and widening of the submucosa of the wall of the isolated loop. The tissue spaces as well as the lymphatic channels appeared to be dilated. The larger blood vessels were much engorged although the minute ones seemed to escape, perhaps due to the pressure exerted by the œdema fluid in the tissues around. The summits of the villi mostly appeared to be necrotic,

with evidence of nuclear pyknosis. Many of the stroma cells of the mucosa showed hydropic change, which, however, was absent in the lining epithelium. The muscle layers appeared normal but there was evidence of deposition of fibrin in the subserosa. Nowhere in the wall was there any evidence of cellular infiltration.

Experiments on rats

Closely similar changes were seen in these animals. The quantity of fluid in the isolated loop of the experimental animals amounted, however, to no more than 0·5 ml. *V. choleræ* could not be isolated from the contents except in one instance. More detailed examination and further experiments were therefore not continued in these animals. Histologically the wall showed much evidence of vascular engorgement and of hæmorrhage and necrosis in the mucosa, but less œdema than in rabbits.

Observations on rabbits injected intravenously with Evans blue

The fluid formed in the isolated segment of small intestine in the cholera animals was coloured blue. Fluid from the small intestine proximal to the isolated loop, both in the test animals and in the controls, showed little or no trace of blue. What little fluid soaked into a piece of clean blotting paper from the collapsed distal parts of both groups of animals and from the isolated segment in the control animals did not exhibit any blue tinge. The wall of the isolated loop in the test animals was a deeper blue than the rest of the intestine.

Discussion

The fluid that accumulated in the isolated loop of small intestine after the introduction of *V. choleræ* resembled the peritoneal fluid in the experiments with suspension of killed vibrios reported by De *et al.* The albumin fraction ranged from 1·0 to 3·8 per cent., and the total protein was in all probability high. This suggests that proteins had leaked out from the plasma through the intestinal capillaries and intestinal tissues into the lumen. Support for this view is provided by the observation that the contents of the experimental loop were coloured by Evans blue injected intravenously. This dye is known to be firmly bound to the plasma proteins (Courtice, 1943-44) and to behave like plasma albumin with regard to the permeability of membranes (Rawson, 1942-43). Hence it may be concluded that *V. choleræ* or its toxic products have increased the permeability of the intestinal capillaries, as a result of which plasma proteins have escaped into the tissue, raised the osmotic pressure and held back the tissue fluid with consequent œdema of the submucosa. A comparable observation was made by Seneviratne (1948) in his experiments with Shiga toxin, although the œdema and increase in weight of the cæcal wall which he encountered were much more marked. This difference is possibly due to the free escape of the larger part

of the fluid in our experiments through the necrosed superficial portion of the mucosa into the lumen where the main collection of the fluid had taken place.

The cholera stool is well known to be poor in protein (Schmidt, 1850, cited by Peters and Van Slyke, 1946) and this has been advanced as evidence against the conception of increased permeability in cholera (Saha and Das, 1952). However, Evans (1949) notes that if an animal's own serum be introduced into a loop of its intestine the whole of that serum is absorbed. Homologous protein is thus specifically absorbed from the small intestine, so much so that when protein is found in the stool its source is invariably the large gut (Harrison, 1947). A low protein content of the cholera stool does not, therefore, necessarily disprove increased permeability of capillaries of the small intestine. In an investigation which one of us has been carrying out with Sengupta, it has been found that the content of the small intestine in cholera shows a high percentage of albumin while that from cases of non-choleraic diarrhœa and from some other conditions contains a very small amount of albumin.

Summary

Injection of living *Vibrio choleræ* into the lumen of a loop of rabbit small intestine isolated by ligature is followed after twenty-four hours by accumulation within this loop of a large amount of fluid having gross, microscopic and cultural similarity with the cholera stool. The albumin content of the fluid is high and Evans blue solution injected intravenously leaks into this fluid. These results suggest that *Vibrio choleræ* alters the permeability of intestinal capillaries to proteins.

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