Pathogenesis of Hobbs’ Heat-Sensitive Spore Forming Clostridium perfringens Type A Strain†

A.K. CHAKRABARTY* and K.G. NARAYAN2

* Department of Bacteriology and Hygiene, Haryana Agricultural University, Hissar-125 004, India

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Abstract Food poisoning in man due to heat-sensitive strains of Cl. perfringens type A appeared to be mediated through enterotoxin synthesized in vivo during sporulation. A minimum of $2.0 \times 10^5$ vegetative cells suspended in sporulation medium was sufficient to elicit gut-loop response in rabbits. The functional disturbance in the gut as well as the structural changes were progressive and proportional to the size of the inoculum up to a dose limit of $2.0 \times 10^7$ vegetative cells and beyond this the changes remained steady.

Although studies by McDonel (8), McDonel and Asano (9) and McDonel and Duncan (10) helped in the understanding of the functional disturbance brought about by enterotoxin in the intestine, the structural changes caused by the enterotoxin remained unresolved due to variable results obtained by earlier workers. Duncan et al (3) first reported epithelial damage in the ileum of rabbits in association with diarrhoea induced by vegetative cells of enteropathogenic strains of Cl. perfringens, while Hauschild et al (6) could not detect this in lambs. Niilo (12) reported partial loss of villus epithelium with the cell-free extract of sporulating cells when given intravenously and complete sloughing when given intraluminally to calves. Similar observations were obtained by Chaturvedi and Narayan (1) in the ligated ileal loop of rabbits. McDonel (8) observed desquamation at the tip of the villi in rat ileum with highly purified enterotoxin. The degree of damage was related to the doses of enterotoxin. Dose dependent structural changes were observed in rabbit ileum also by McDonel and Duncan (11). However these studies were performed with a few Hobbs’ heat-resistant strains. Knowledge concerning the mechanism of gut-loop distension vis-a-vis food poisoning in man due to heat-sensitive food poisoning strains of Cl. perfringens is lacking. Hence the present study was undertaken and the results are reported hereunder.

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1 Present address: Associate Professor Microbiology, College of Vety. Sciences, Khanapara, Gauhati-22 (Assam), India.

2 Professor and head department of Vety. Public Health, Ranchi Vety. College, Kanke, Ranchi-7, Bihar, India.


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MATERIALS AND METHODS

Strain. Stock culture of Clostridium perfringens type A strain FH/8588/66 (Hobbs' heat-sensitive serotype III) obtained from Colindale, England was used for this study.

Preparation of vegetative cells. Fresh cultures grown for 5 hr at 37 C in V.F. broth (4) were passaged twice in the same medium at a 5 hr interval before final inoculation in the same medium at the rate of a 10% inoculum (v/v). At the end of incubation the cells were harvested by centrifugation and washed three times in cold saline by centrifugation and pelleting. After final washing, cells were suspended in V.F. broth free from meat particles and stored at 4 C. The vegetative cell preparations were also checked for their freedom from spores by staining (5) and by heating at 75 C for 20 min to determine their failure to survive.

The total viable count was determined and then vegetative cell suspensions containing 2.0 × 10^8 to 2.0 × 10^8 cells were prepared. Two ml of each of the suspensions were placed in duplicate in two test tubes and centrifuged at 1,700 rpm for 45 min. The supernatant was discarded and the pellet of one tube was suspended in 0.4 ml of D.S. medium (2) and the pellet of the other in 0.4 ml of saline.

Biological test. The biological test was performed in rabbits by the method described by Duncan et al (3). Each ligated ileal loop of 6.0 cm in size was inoculated with 0.2 ml of D.S. medium or saline suspensions containing various numbers of cells. The rabbits were sacrificed 8 hr post-inoculation. The volume of fluid accumulated in the loops was measured. The pieces of ileal loop injected with various doses of vegetative cells were cut out and preserved in 10.0% formol saline for histopathological studies.

Serological, cultural and bio-assays of loop contents. The loop contents were collected along with the washings and centrifuged. The clear supernatant, supposed to contain enterotoxin produced in vivo, was collected, concentrated by dialyzing against PEG 6000 and stored at 4 C for the guinea-pig skin test (7) and immunodiffusion test. Antiserum specific to enterotoxin was obtained by absorbing the antibodies to cell extract (CE) of sporulating cells with those of the vegetative cells (13) of Hobbs' serotype III. This absorbed serum specifically neutralized the erythema effect of enterotoxin (CE) in guinea-pig skin and gave a single precipitin line with CE in the agar gel immunodiffusion test. The immunodiffusion test with the loop contents was run against antiserum specific to enterotoxin of Hobbs' serotype III keeping enterotoxin (CE) of the same strain as control. The sediments of the loop contents containing vegetative cells and spores were resuspended in physiological saline up to the original volume and divided into two portions. One portion was heated at 75 C for 20 min to kill the vegetative cells and simultaneously heat activate the spores. Serial 10-fold dilutions of the heated and unheated replicates were prepared in 0.5% peptone water (pH 7.2) and spores and total counts were determined in semisolid sulfite iron agar medium.

Histopathologic technique. Paraffin sections of 5.0 – 10 µm thickness of ileal loop
<table>
<thead>
<tr>
<th>No. of cells per loop</th>
<th>Suspending medium 0.2 ml each</th>
<th>Mean value of two loops after 8 hr</th>
<th>Immunodiffusion reaction of loop supernatant with enterotoxin specific serum to FH/8588/66</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inoculated</td>
<td>Fluid volume in ml accumulated per loop</td>
<td>Total viable cells</td>
</tr>
<tr>
<td>2.0×10³</td>
<td>DSM</td>
<td>0.50</td>
<td>9.0×10⁶</td>
</tr>
<tr>
<td>2.0×10³</td>
<td>NSS</td>
<td>−</td>
<td>3.5×10⁴</td>
</tr>
<tr>
<td>2.0×10⁴</td>
<td>DSM</td>
<td>1.00</td>
<td>2.0×10⁶</td>
</tr>
<tr>
<td>2.0×10⁴</td>
<td>NSS</td>
<td>0.40</td>
<td>1.0×10⁶</td>
</tr>
<tr>
<td>2.0×10⁴</td>
<td>DSM</td>
<td>1.75</td>
<td>1.75×10⁷</td>
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<td>0.50</td>
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<td>DSM</td>
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<td>DSM</td>
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<td>1.0×10¹⁰</td>
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<tr>
<td>2.0×10⁸</td>
<td>NSS</td>
<td>−</td>
<td>4.5×10⁸</td>
</tr>
</tbody>
</table>

DSM = Duncan Strong sporulation medium.
NSS = 0.85% per cent saline solution.
−, +, ++, and +++ = negative, positive and the intensity of erythema reactions.
were cut, processed, deparaffinized and stained with hematoxyline-eosin for histopathological studies.

RESULTS

The results of this study are summarized in Table 1. It was observed that vegetative cells suspended in saline failed to sporulate and bring about detectable ileal loop response in rabbits irrespective of the size of the inoculum. In contrast, sporulation was observed in all the loops inoculated with vegetative cells suspended in D.S. medium. Fluid accumulation was observed in loops inoculated with 2.0 × 10^4 to 2.0 × 10^8 cells. The increase in fluid accumulation in the gut cor-
responded to the inoculation of increasing numbers of cells up to a level of $2.0 \times 10^7$ cells. Any increase in the number of cells beyond this level did not lead to a corresponding increase in the accumulation of fluid in the loop. The fluid supernatant of the contents of the positive loops induced erythema in guinea-pig skin and gave a single precipitin band identical to the one given by enterotoxin of Hobbs' serotype III in immunodiffusion tests. Supernatants of the contents of loops inoculated with various doses of saline suspension of vegetative cells gave neither erythema responses nor immunodiffusion reactions with enterotoxin of Hobbs' Serotype III. The results of the immunodiffusion test are shown in Plates la and lb.

The structural appearance of both normal and challenged ligated loops in-

Plate 1b. Immunodiffusion of the contents of loops inoculated with varying doses of vegetative cells of Hobbs' serotype III. Central well contained specific antiserum to enterotoxin of Hobbs' serotype III (AS III) peripheral wells contained CE of Hobbs' serotype III (III) and contents of loops inoculated with saline (S) and DS suspension (DS) of vegetative cells. Log value indicates number of cells inoculated. Figures 4–6 demonstrate the presence of enterotoxin in loop contents challenged with only DS suspension of cells.
oculated with various doses of vegetative cells suspended in D.S. medium or saline are shown in Plate 2 (Figs. 1-4). Sections from the normal uninoculated ileal loop showed no detectable structural changes in either lacteals or in the surface epithelium. The macroscopically positive loop (showing fluid accumulation)
inoculated with various doses of vegetative cells suspended in the sporulation medium showed progressive structural damage ranging from slight separation and effacing of the villus epithelium to complete desquamation with severe edema, hemorrhage and infiltration with inflammatory cells. Increasing severity of the structural changes corresponded to the increasing doses of inoculum up to a level of $2.0 \times 10^7$ vegetative cells. Beyond this dose, the changes remained the same. For example loops receiving $2.0 \times 10^3$ vegetative cells suspended in D.S. medium revealed dilatation of lacteals, congestion of capillaries and edema in the submucosa while loops inoculated with $2.0 \times 10^4$ cells in D.S. medium revealed degenerative changes. Loop inoculated with $2.0 \times 10^5$ cells showed separation of surface epithelium from the basement membrane. Loops challenged with $2.0 \times 10^6$ vegetative cells in D.S. medium revealed complete desquamation of the villus epithelium accompanied by necrosis of the lamina propria. The same microscopic changes in epithelial structure was observed in the loops inoculated with $2.0 \times 10^7$ or $2.0 \times 10^8$ vegetative cells in D.S. medium but the degree of severity was much higher compared to the ones inoculated with $2.0 \times 10^6$ vegetative cells suspended in sporulation medium. By contrast, the loops inoculated with saline suspension of vegetative cells did not show distension irrespective of the doses. Histological examinations revealed no detectable changes in either lacteals or surface epithelium. Mild to moderate structural changes observed in sections of certain loops were not progressive and dose dependent. In general, the histological pictures in sections of these control loops resembled those of the normal intestine. Attempts to locate vegetative cells or spore of Cl. perfringens in the ileal sections failed.

DISCUSSION

The mechanism of Cl. perfringens type A food poisoning in human beings was studied using the rabbit ileal loop model with a few Hobbs' heat-resistant strains only (3). The present study was performed with Hobbs' heat-sensitive strain, the enteropathogenic mechanism of which is not known. It revealed that vegetative cells suspended in saline failed to sporulate and bring about detectable gut-loop response irrespective of the number of cells inoculated (Table 1). By contrast, a minimum of $2.0 \times 10^4$ vegetative cells suspended in sporulation (D.S.) medium when inoculated into the ileal loop showed both sporulation and fluid accumulation in the gut. The presence of enterotoxin in the contents of the positive loops was detected by erythema test in guinea-pig skin and by immunodiffusion test with specific serum to enterotoxin of Hobbs' serotype III. Contents of the control loops inoculated with saline suspension of vegetative cells did not show the presence of enterotoxin in these tests (Plates 1a and 1b). Further, the contents of the positive loops showed increased spore counts. This clearly indicated that sporulation and consequent enterotoxin production in vivo were dependent on favorable conditions provided by the sporulation medium. Under natural conditions of food poisoning a similar environment is likely to be provided by the food, especially meat eaten by consumers. The increase in fluid volume in the gut was found to
correspond to the increase in number of cells inoculated, although such conditions were not demonstrated in the ileal sections. It was, therefore, reasonable to believe that increased ileal loop response was due to \textit{Cl. perfringens} and was mediated through increased synthesis of enterotoxin \textit{in situ}. Thus a dose-dependent ileal loop response was observed in the present study, as also observed by Duncan et al (3), with the heat-resistant strains. The structural changes in the ileum were observed mainly in the villus. This ranged from slight separation and effacing of the epithelium of the villi to complete desquamation with severe edema, hemorrhage and infiltration of inflammatory cells between the lamina propria and surface epithelium. The histopathological changes were also found to be dose dependent because increased structural damage of the villi corresponded to the inoculation of increasing numbers of vegetative cells in D.S. medium as was observed with purified enterotoxin by McDonel and Duncan (11). The structures of the loop inoculated with various doses of vegetative cells in saline were similar to those of normal uninoculated loops. Considering the functional disturbance and structural changes brought about by the various doses of inoculum, it appeared that a minimum of $2.0 \times 10^8$ vegetative cells placed in the intestine along with a suitable sporulation medium like that of D.S. medium or meat would be sufficient to elicit food poisoning symptoms in man. These cells would sporulate and release enterotoxin responsible for pathological changes and the patient would excrete spores of \textit{Cl. perfringens} for further transmission of infection. Thus the heat-sensitive strain of \textit{Cl. perfringens} type A resembled the heat resistant strains in their pathogenic mechanism.

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REFERENCES


