GROWTH AND CHARACTERISTIC FEATURES OF SHIGELLA

The characteristic features of *S. flexneri* in the present study i.e. growth in Mac Conkey agar, nutrient agar, triple sugar iron agar, simple glucose ammonium salt media are consistent with those of the *Shigella* species in general (Sleigh and Duguid, 1989).

The species *S. dysenteriae* did not grow in the simple glucose ammonium salt media. This finding is similar to the report of Ahmed et al. (1988).

PRODUCTION OF TOXINS BY SHIGELLA SPECIES

It has been hypothesized that *Shigella* strains produce an enterotoxin and that the secretion of fluids in the gut is a consequence of toxin production by *Shigella* species (O’Brien and Holmes, 1987). Also, except for the cytotoxin/enterotoxin elaborated by *S. dysenteriae*, little convincing proof has been generated that the other species of *Shigella* in fact produce enterotoxins (Fasano et al., 1995).

In the present study, the extracts of all the *Shigella* strains i.e., *Shigella dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* were found to be cytotoxic in the HeLa cell assay. The order of cytotoxicity of the species was as follows:

*S. flexneri > S. dysenteriae > S. boydii > S. sonnei*. A comparison of the profile of the lysates of the four species on native and SDS-PAGE reveals a characteristic pattern which is unique to the species and can help to distinguish each species from the other members of the genus.

PRODUCTION OF THE TOXIN BY *S. flexneri*

According to Fasano et al. (1995) earlier attempts by other investigators to detect enterotoxic activity by *S. flexneri* yielded negative
results with the exception of two groups namely O'Brien et al. (1977) and Bartlett et al. (1986). O'Brien and Holmes (1987) had observed that the toxin from S. flexneri has not been obtained in pure form and had questioned whether or not it is similar to closely related to Shiga toxin. The present study is an attempt to purify and characterize the toxin from S. flexneri.

**TOXICITY OF S. flexneri**

The supernatant and lysate were used to check cytotoxicity in prokaryotic test system involving rec assay. The negative results confirm the fact that Shiga and Shiga-like toxins are active on eukoryotic cells (Keusch et al., 1972).

The crude preparation and the purified protein yielded negative results on rabbit skin test, an observation in agreement with Keusch et al. (1972), who reported no increase in vascular permeability or signs of redness in rabbit skins when treated with toxin from S. dysenteriae.

When checked on HeLa cells, the crude samples, the supernatant and purified samples from S. flexneri were found to be toxic. Similar results were obtained in case of the crude extracts of the species S. dysenteriae, S. sonnei and S. boydii. These results are in agreement with those of Bartlett et al. (1987). The Shiga and Shiga-like toxins have been known to be cytotoxic on HeLa cells (Keusch et al. 1972, Gentry and Dalrymple, 1980).

**EXTRACTION OF PROTEINS FROM S. flexneri**

Different methods have been reported for the extraction of the toxin. For this French press lysate was used by O'Brien and LaVeck
(1983). Osmotic shock was used to obtain the lysate by Donohue-Rolfe and Keusch (1983). Sonication was performed by Ramotar et al. (1990).

To extract the toxin from the bacteria, lysis buffer was used by Brown et al. (1982). Extraction with polymyxin B was reported to be used by Griffin and Gemski (1983) and Petric et al. (1987).

Most of the above methods involve lysis of the whole cell. Shiga toxin and other toxins are, however, cell associated and may be located in the periplasmic space. To avoid unnecessary contamination with other cellular proteins and to prevent a cruder starting toxin preparation lysis buffer was used in this study to extract the periplasmic proteins of Shigella flexneri.

**PURIFICATION**

Different protocols for toxin purification have been reported in literature. van Heyingen and Gladstone (1953) reported the neurotoxin of Shigella shigae (now Shigella dysenteriae) to be one of the most toxic substances occurring in extremely low concentrations in cultures of the organisms and consequently its purification presented some problems. Brown et al. (1982) used ultracentrifugation, ammonium sulphate precipitation, ammonium sulphate fractionation, chromatography followed by gel filtration and preparative isoelectric focusing for purification of the toxin.

Kongmuang et al. (1987) used immunoaffinity column chromatography to purify the protein. Other workers have used overproducing clones (Calderwood et al., 1990) for this purpose.

In the present study gel permeation chromatography was used to purify the protein and fractions 11-18 had cytotoxic effect on HeLa cells.
MOLECULAR WEIGHT

In the strain *S. dysenteriae* it was reported that the protein responsible for cytotoxicity has a $A+5B$ configuration (O'Brien and Holmes, 1987). In *S. flexneri*, the data obtained and shown in Fig. 4.14 can be interpreted and postulated using the same configuration. The bands of 19,050 and 9,120 Daltons are parts of the $A$ subunit and 7,586, 15,140 and 22,190 are monomers, dimers and trimers formed by subunit $B$ (Table 5.1).

ISOELECTRIC POINT

Andrews and Aserjo (1984) have noted that many bacterial proteins have pIs in the acidic range. This is similar to the observation made in the present study which estimates the pI to be 3.6 as it corresponds to the position of the molecular weight marker amyloglucosidase (pI 3.6) when the proteins were run on an ultra-thin polyacrylamide gel.

The observed isoelectric point is in contrast to that of the toxins of *E.coli* H30 and Shiga 60R which were estimated to have a pI of 7.03 ± 0.02 (O'Brien and LaVeck, 1983), 6.72 for *E.coli* verocytotoxin (Petric *et al.*, 1987) 6.7 for VT-2 (Head *et al.*, 1988), 9.0 for VT-2e (MacLeod *et al.*, 1991) and 5.2 for the unique verocell cytotoxin from *E.coli* 0157:H7 (Padhye *et al.* 1987).

TREATMENT WITH PROTEOLYTIC ENZYMES

It has been reported that proteolytic enzymes enhanced the toxicity of bacterial proteins (MacLeod *et al.*, 1991). However, in the present study a loss of cytotoxic activity on treatment with proteolytic enzymes was observed. Treatment with trypsin however allowed the
<table>
<thead>
<tr>
<th>TREATMENT WITH REDUCING AGENTS</th>
<th>EXPERIMENTALLY ESTIMATED MOL. WT. (DALTONS)</th>
<th>POSSIBLE SUBUNIT COMPLEXES</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td>66,070</td>
<td>A+5B</td>
</tr>
<tr>
<td>YES</td>
<td>22,910</td>
<td>B₃</td>
</tr>
<tr>
<td></td>
<td>19,050</td>
<td>PART OF A SUBUNIT</td>
</tr>
<tr>
<td></td>
<td>15,140</td>
<td>B₂</td>
</tr>
<tr>
<td></td>
<td>9,120</td>
<td>PART OF A SUBUNIT</td>
</tr>
<tr>
<td></td>
<td>7,856</td>
<td>B</td>
</tr>
</tbody>
</table>

IF, A = 19.050 + 9.120 kDa  
= 28.170 kDa  
AND B = 7.586 kDa  
THEREFORE 5B = 37.930 kDa  
THEN A + 5B = 66.100 kDa
toxin from *S. flexneri* to retain its activity. In contrast, treatment with proteinase K resulted in a loss of toxic activity which could be the result of its broad and non-specific proteolytic effect.

**HEAT TREATMENT**

The toxin from *Shigella flexneri* was observed to be more heat-labile than the Shiga toxin which retained its toxic activity after incubation at 65°C for 30 min (O'Brien and LaVeck, 1983). The results of the present study are however similar to that of SLT-IIv which lost 75% of its activity by heat treatment at 50°C and completely lost it at 65°C (MacLeod *et al.*, 1991).

**EFFECT OF REDUCING AGENTS**

The cytotoxic activity of the toxin from *S. flexneri* was completely destroyed by incubation with 2-mercaptoethanol and dithiotheitol. This observation is similar to that reported for SLT-IIv (MacLeod *et al.*, 1991). They attributed this property to the role of disulphide linkages which were reported to be essential for biological activity.

**DIFFERENCES WITH OTHER TOXINS**

Keusch and Jacewicz (1973) first suggested that the species *S. flexneri* and *S. sonnei* may produce toxic substance that are antigenically related to Shiga toxin. Thompson *et al.* (1976) established that *S. flexneri* 2a does produce a Shiga-like toxin although at very low levels compared to those of Shiga toxin.

Earlier attempts by other workers had failed to detect enterotoxicity in supernatants of *S. flexneri* with the exception of Thompson *et al.* (1976) and the work of Keyti *et al* (1978) who reported the production of a heat-stable enterotoxin by *S. flexneri* 2a. The protein
isolated in the present study is different from the heat stable toxin which is reported to be having a low molecular weight, is optimally produced in the absence of glucose and is resistant to proteotytic enzymes (Tsuji, 1995).

Askenazi et al. (1990) reported a 100-125 kDa protein in Shigella species which was distinct from the known Shiga and Shiga-like toxins 1 and 2. It was not neutralizable with antiserum to purified Shiga toxin and the strains lacked the structural genes for Shiga toxin production. The toxin was obtained under conditions of iron- limitation and showed multiple bands in each preparation. The protein reported in the present study is apparently different from that reported by Askenazi et al. (1990) on the basis of its molecular weight. In contrast to their observation that a few bands of >100 kDa were noted in SDS-PAGE, no such bands have been observed in the present study.

Fasano et al. (1995) have reported two distinct enterotoxins SLET-1 and SLET-2 produced by S. flexneri 2a. Iron limitation was a factor necessary for the production of these toxins. SLET-1 was reported by Noriega et al. (1995) to be a 55kD protein prevalent in all S.flexneri 2a strains studied by DNA colony blot assay and PCR amplification, but its prevalence was rare (only 3.3%) in isolates of other serogroups and was not found in EIEC. Iron limitation, its rarity and reported molecular weight make it a protein different from the protein isolates in the present study.

ShET-2, a plasmid encoded protein present in the 80% of the Shigella isolates has been described by Nataro et al. (1995). It was reported to be single moiety protein. The sen gene product was found to have a molecular mass of 63 kDa. The protein isolate reported in the
present study however shows multiple protein bands in the lower molecular weight range which makes it different from ShET-2.

The result of the present study are consistent with the observations of Bartlett et al. (1986) and Ashkenazi et al. (1990). On this, the former group reported that among Shigella strains other than S. dysenteriae, production of one or more cytotoxins is far more common than the production of Shiga toxin itself. The latter authors endorsed this view point and observed that toxic products other than Shiga toxin may play a role in the manifestations of the disease.

Shiga toxin and Shiga-like toxins have different molecular characteristics i.e., isoelectric point, heat stability and different behaviour with proteolytic enzymes. The molecular weights of the holotoxin are however, of similar range (60-70 kDa). The toxin from S. flexneri may have the same 1A: 5B configuration as the toxin of the Shiga family. It is similarly cytotoxic to HeLa cells (Gentry and Dalrymple, 1980) but not to prokaryotic cells or to rabbit skin (Keusch et al., 1972). Its response to reducing agents is also similar to that of the toxins of Shiga family (MacLeod et al., 1991).