Cytotoxicity of *Fusobacterium ulcerans*

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Summary. A new species of *Fusobacterium*, *F. ulcerans*, was isolated from 46 tropical ulcers. All the isolates had shown identical soluble cell protein patterns on polyacrylamide gel electrophoresis although there were two morphological types with slightly different biochemical properties. A representative strain of each of the two groups was selected for in-vitro cytotoxicity testing on a range of tissue-culture cell lines. Both strains of *F. ulcerans* induced a marked cytotoxic effect on Vero and Int-407 cells. This effect may contribute to the pathogenesis of tropical ulcers.

Introduction

Tropical ulcers are common among young children in the tropics but the disease is not confined to those areas. The lesions start as painful papules which break down over 3–5 days to become large, necrotic, foul smelling ulcers. The rapid progress of ulceration suggests that a toxic factor may be involved in its pathogenesis. The aetiology and pathogenesis of these ulcers was investigated and 120 patients in five tropical areas (Gambia, Zambia, southern India, Fiji and Papua New Guinea) were studied. In all locations the disease followed a similar pattern. *Fusobacteria* were cultured from swabs taken from 46 of these ulcers and the same organisms could also be identified in the dermis of the ulcers by electronmicroscopy of biopsy samples (Adriaans and Drasar, 1987).

The isolates showed minor biochemical and morphological differences. However, polyacrylamide gel electrophoresis (PAGE) of their soluble cell proteins showed identical patterns (Adriaans and Drasar, 1987). The biochemical characteristics of the *fusobacteria* and their PAGE patterns differ from any other reported species of *Fusobacterium*, which suggests that a previously undescribed species may be involved in the pathogenesis of tropical ulcer. The isolates have been characterised and are now referred to as *F. ulcerans* in keeping with their source (Adriaans and Shah, 1988). Since *F. ulcerans* was isolated from patients in several geographical areas, it is suggested that these bacteria may be relevant in the pathogenesis of the disease (Adriaans and Drasar, 1987).

We report our findings on the cytotoxicity of these *fusobacteria* and discuss their role in the pathogenesis of the disease.

Materials and methods

Bacterial strains

A representative strain of each group of *Fusobacterium* isolated was used in the experiments. The strains used were those deposited with the National Collection of Type Cultures—*F. ulcerans* NCTC nos. 12111 and 12112. The isolates were grown in 50 ml of Brain Heart Infusion Broth (BHI) (Oxoid) supplemented with haemin, cysteine, and vitamin K as described by Holdeman *et al.* (1977). *Bacteroides fragilis* and *Enterobacter cloacae*, also isolated from the ulcers, were grown in the same medium.

Preparation of culture supernates

The anaerobic cultures were incubated for 48 h, at 37°C, in an anaerobic cabinet with an atmosphere of H₂ 15%, N₂ 80%, CO₂ 5% (Don Whitley Scientific, Shipley, W. Yorks). The *E. cloacae* strains were incubated aerobically for 24 h. Cultures were centrifuged at 3000 g for 10 min at room temperature and the supernates were harvested, sterilised by membrane filtration, and tested for cytotoxicity.

Cytotoxicity test

The cytotoxicity test was based on that of Giugliano *et al.* (1982). Five cell lines were used: Vero (African green monkey kidney), Chimpliver, Int-407 (human embryonic intestine), CHO-K1 (chinese hamster ovary) and MRC (human diploid lung cells). Cell monolayers were grown in either Medium 199 (Gibco) or Dulbecco's Modified Eagle's Medium (Gibco). Cell monolayers were brought into suspension with trypsin-versene treatment (Giugli-

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ano *et al.*, 1982) and the cells were resuspended in the appropriate test medium, either Medium 199 containing fetal calf serum 5%, 20mM tricine (pH 7.6) and NaHCO_3 0.22% w/v, or Dulbecco's Modified Eagle's Medium containing fetal calf serum 10%, 20mM tricine, gentamicin 25 $\mu\text{g}/\text{ml}$ and NaHCO_3 0.22% w/v, at a final concentration of 3×10^5 cells/ml. Cytotoxicity tests were performed in microwell plates (Nunc).

Two-fold dilutions of culture filtrates in test medium were prepared in 100- μl volumes to cover a range of dilutions from 1 in 2 to 1 in 256. Then 100 μl of the cell suspensions were added to each well. Plates were incubated for 24 h at 37°C and all morphological changes were observed at 18 and 24 h. These were graded as: + + +, $\geq 90\%$ of cells affected; + +, 70–80% cells affected; +, 50–60% cells affected; +/– 30–40% cells affected; and –, when < 30% cells were affected.

Cells were fixed with buffered formaldehyde for 1 h and stained with 0.001% phosphate buffered crystal violet. Excess stain was washed from the monolayer and then cell bound stain was eluted with 100 μl of acid methanol (1M HCl 1% in methanol) added to each well. The dye concentration was measured by reading the optical density at 540 nm on a Dynatec plate reader. The bound dye was used as an indicator of cell numbers (Barer *et al.*, 1986 a, b).

Each experiment was repeated on at least two occasions. Uninoculated BHI Broth served as a negative control for cytotoxicity and uninoculated growth medium as the negative control for cell survival. Known concentrations of butyrate in uninoculated BHI broth were used to challenge Vero cells.

The butyrate concentration of the culture filtrates was measured by gas liquid chromatography (GLC) (Pye-

Unicam chromatograph, series 204, Philips, Cambridge). The peak area was derived with an integrated computer (PU 4180 computing integrater, Philips). Known concentrations of butyrate were used as standards.

Results

Filtrates of *F. ulcerans* culture supernates were cytotoxic for Vero and Int-407 cell cultures (table I). Cytotoxicity was demonstrated by morphological changes in the cell monolayers. The affected cells showed long filamentous tendrils when damage was mild and rounding up of cells when the damage was more extensive. The rounded cells often became detached into the medium (figure).

Vero cells were affected most severely; Int-407 cells exhibited similar though less severe changes. The other cell lines were not affected. Cytotoxicity was not observed with the uninoculated BHI broth, nor with culture filtrates of *B. fragilis* or *E. cloacae*. The butyrate concentrations in the culture filtrates are shown in table I. Butyrate added to uninoculated BHI broth in equivalent concentrations produced a similar effect (table II).

Discussion

The role of infection in tropical ulcers has not been clearly established. Several investigators have suggested that infection as well as malnutrition and trauma may be contributory factors in the patho-

Figure. Vero cells showing cytotoxicity from *F. ulcerans*. (Crystal violet $\times 80$).

Table I. Cytotoxicity of fusobacteria on Vero cells

Strain	Butyrate concentration (M) in supernate	Cytotoxicity* of culture filtrates at dilutions of					
		2	4	8	16	32	64
<i>F. ulcerans</i> NCTC 12111	0.03	+++	+++	+++	++	+	—
<i>F. ulcerans</i> NCTC 12112	0.03	+++	+++	+++	++	+	—
<i>B. fragilis</i>	0	—	—	—	—	—	—
<i>E. cloacae</i>	0	—	—	—	—	—	—
Uninoculated control	0	—	—	—	—	—	—

* See Materials and methods.

Table II. Effect of butyrate on Vero cell cultures

Butyrate concentration (M)	Cytotoxicity
0.0025	+
0.005	++
0.01	+++

genesis of the disease (Loewenthal, 1963; Ngu, 1967). Clinical observation of patients with tropical ulcers suggests that infection is a likely factor in the aetiology of this disease because patients often improve when antibiotics are administered early in the disease (B. Adriaans, unpublished observation).

McAdam (1966) was able to transmit tropical ulcers experimentally by inoculating pus from an ulcer of an affected individual to an unaffected area of the same individual. Similar results were obtained by inoculating pus from an affected person into a healthy subject. However, by this method several different organisms from the mixed flora of these ulcers were inoculated and not all of them were necessarily involved in the aetiology of the ulcers. Our previous work has shown that many different species of gram-negative bacteria are present in these tropical ulcers (Adriaans *et al.*, 1987a). The bacteria not only colonise the surface, where they could be regarded as commensals, but they also invade the deeper levels of the ulcer (Adriaans and Drasar, 1987). Fusobacteria have been identified in the dermis by electronmicroscopy of skin biopsies from patients with tropical ulcers.

The rapidity with which the ulcers break down suggests rapid tissue destruction by a toxin or some

substance toxic to the connective tissue. Electron-microscopy showed the presence of abnormal collagen (Adriaans *et al.*, 1987b). In our present study of the effect of several culture filtrates on various cell lines *in vitro*, *Fusobacterium* culture filtrates were cytotoxic to Vero cells in culture, whereas filtrates of *B. fragilis* and *E. cloacae*, both isolated from the ulcers, did not produce the same effect. Cytotoxicity in > 50% of the cells in a well at titres ≥ 32 was produced by both test strains. This was of similar order to the 0.0025 M butyrate concentration which produced the same degree of cytotoxicity when tested separately. Previous work by Grenier and Maynard (1985) showed that butyrate from *F. nucleatum* is cytotoxic to Vero cell lines. Our species of *Fusobacterium* has not previously been isolated and has only recently been fully characterised (Adriaans and Shah, 1988). The organism is not haemolytic, does not produce extracellular DNAase and does not digest casein, characteristics which should be taken into account in the pathogenesis of destructive disease (Adriaans and Drasar, 1987). The *Fusobacterium* sp. now known as *F. ulcerans* produces large amounts of butyrate as a metabolic product and these findings are in keeping with the results of Touw *et al.* (1982) who suggested that toxic concentrations of butyrate may be of aetiological significance in periodontal disease.

We conclude that these fusobacteria may contribute to the development of tropical ulcers. Furthermore, we suggest that their cytotoxicity against Vero and Int-407 cells may be indicative of similar toxicity to the host tissues which may contribute to the rapid tissue destruction and ulcer formation.

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