

**The Aetiology and Pathogenesis**

**of**

**Tropical Ulcer**

**Beverley Adriaans**

**Tropical Dermatology Unit**

**Department of Medical Microbiology**

**London School of Hygiene and Tropical Medicine**

**Keppel Street**

**London WC1E 7HT**

**England**

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## ABSTRACT

Tropical ulcer is a very specific form of cutaneous ulceration. It occurs worldwide in most tropical and a number of subtropical areas.

The disease occurs mainly in older children and young adults with children under the age of 5 and adults over 45 years rarely being affected. Ulcers occur most commonly on the lower leg but may occur on the upper limb. Although most ulcers normally heal slowly over many weeks or months, some ulcers may recur. Recognised complications include squamous cell carcinoma, gangrene and osteitis, although these are rare.

A number of authors have reported on the disease and suggested diet, trauma and infection as aetiological factors for this condition.

This survey was thus conducted to assess as many of these factors as possible. The study took place in 5 tropical areas, namely Zambia, Gambia, southern India, Fiji and Papua New Guinea. Consultations took place at hospitals, rural clinics, health centres and villages.

Although many authors have suggested that the disease is related to malnutrition, few have objectively assessed the nutritional status of the patients and compared it with controls. Those studies which included objective assessments were limited to small areas and only investigated specific parameters.

In order to investigate the immune response of the host to an anaerobic infection, the antibody levels to the organisms isolated from the ulcers were measured by an ELISA test. The local host response to an infection with a Fusobacterium species was assessed by the number of antibody secreting B-lymphocytes at the site of the ulcers. These parameters may play a role in the localisation of the ulcers and account for recurrent infections.

In this survey the nutritional status of the patients was objectively assessed and compared to a control population in each area. A questionnaire was used to obtain personal details and information about the present and/or previous ulcers.

Several authors have also reported fusiform bacilli and spirochaetes on smears from the ulcers. These organisms have not been fully characterized as they are anaerobic bacteria and require a strict anaerobic environment to survive. A suitable transport system was therefore developed to transport the specimens from the tropical study areas to London. This kept the samples anaerobic and ensured that the organisms remained viable. During this study, swabs were taken from the base of the ulcers and biopsies from the edge. Both types of samples were examined bacteriologically and all were cultured aerobically and anaerobically.

The biopsies were also examined histologically. Electron microscopy was performed on some biopsies.

The isolates were tested for in-vitro cytotoxicity as a possible mechanism for the development of ulcers. The pathogenicity of all the bacterial species recovered from the ulcers was further assessed by their ability either singly, or in combination, to induce an experimental abscess in a guinea pig. In-vivo synergy between the various organisms was also investigated. The guinea pig proved to be the most suitable animal model.

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## CONTENTS

	Page
<u>SUMMARY</u>	1
<u>INTRODUCTION</u>	
Study plan	3
<u>REVIEW OF THE LITERATURE</u>	7
Epidemiology	8
Clinical manifestations	16
Nutritional status	25
Role of infection	32
Pathology	41
Management	43
<u>CURRENT STATE OF THE PROBLEM</u>	48
<u>MATERIALS AND METHODS</u>	
Epidemiology	50
Clinical manifestations	53
Nutritional status	59
Role of infection	61
Pathology	90
Mechanisms of infection	102
<u>RESULTS</u>	
Epidemiology	116
Clinical manifestations	121
Nutritional status	136
Role of infection	145
Pathology	172
Mechanisms of infection	191
<u>DISCUSSION</u>	208
<u>CONCLUSION</u>	218
<u>APPENDIX 1</u>	
Tropical ulcer questionnaire	220
Transport fluid	226
Medium for spirochaete isolation	227
Ingredients for PAGE	228
Cell growth medium	229
<u>APPENDIX 2</u>	
Suppliers	230
<u>BIBLIOGRAPHY</u>	235

## LIST OF FIGURES

- Fig. 1. Distribution of tropical ulcer.
- Fig. 2. Schematic representation of ulcer progression.
- Fig. 3. Parts of a Hungate tube.
- Fig. 4. Cannulae for delivery of gas.
- Fig. 5. Method for preparation of transport fluid.
- Fig. 6. Hungate tube with anaerobic transport fluid.
- Fig. 7. Hungate tube showing loss of anaerobiasis.
- Fig. 8. Acute tropical ulcer.
- Fig. 9. Chronic ulcer.
- Fig. 10. Multiple tropical ulcers well separated.
- Fig. 11. Multiple adjacent tropical ulcers.
- Fig. 12. Squamous cell carcinoma.
- Fig. 13. Histology of squamous cell carcinoma.
- Fig. 14. Tropical ulcer scar.
- Fig. 15. Tropical ulcer and early leishmaniasis.
- Fig. 16. Histology of leishmaniasis.
- Fig. 17. Leishmaniasis in a child.
- Fig. 18. Fusobacterium type 1 colony.
- Fig. 19. Fusobacterium type 1 morphology.
- Fig. 20. Fusobacterium type 2 colony.
- Fig. 21. Fusobacterium type 2 morphology.
- Fig. 22. Electron microscopy of Fusobacterium type 1. (BHI)
- Fig. 23. Electron microscopy of Fusobacterium type 1. (FABA)
- Fig. 24. Electron microscopy of Fusobacterium type 1. (WC)
- Fig. 25. Electron microscopy of Fusobacterium type 2. (BHI)
- Fig. 26. PAGE of Fusobacterium types 1 and 2.
- Fig. 27. PAGE of Fusobacterium types 1 and 2, and other species of Fusobacterium.
- Fig. 28. Carbol fuchsin stain of cultured spirochaetes.
- Fig. 29. Histology of tropical ulcer showing spongiosis.
- Fig. 30. Histology showing vascular changes.
- Fig. 31. Dieterle stain showing bacteria.
- Fig. 32. Dieterle stain showing spirochaetes.
- Fig. 33. Electron microscopy of fusobacteria in the dermis
- Fig. 34. Electron microscopy of spirochaete in the dermis
- Fig. 35. Immunohistochemistry (T-cells) of infiltrate.
- Fig. 36. Immunohistochemistry (B-cells) of infiltrate.
- Fig. 37. Vero cells in culture with medium.
- Fig. 38. Vero cells exposed to Fusobacterium supernatant.
- Fig. 39. Mouse model showing cutaneous necrosis.
- Fig. 40. Guinea pig model showing abscess formation.
- Fig. 41. Histology of experimental ulcer.

## LIST OF TABLES

1. Nutrition and tropical ulcer.
2. The role of infection in tropical ulcer.
3. Bacterial culture media.
4. Selective media for bacterial culture.
5. In-vitro cytotoxicity cell lines.
6. Bacterial strains for in-vitro cytotoxicity.
7. Bacterial strains for animal inoculations.
8. Mouse strains for animal inoculations.
9. Frequency of tropical ulcer in Papua New Guinea.
10. Number of tropical ulcers per location.
11. Number of ulcers per patient.
12. Anatomical sites of tropical ulcers.
13. Age distribution of patients with tropical ulcers.
14. Sex of patients with tropical ulcers.
15. Relationship of tropical ulcers and trauma.
16. Body mass index of patients with tropical ulcers.
17. Body mass index of ulcer patients and controls.
18. Dermatophyte infections in Fiji.
19. Number of swabs analysed per location.
20. Bacterial isolates from each location.
21. Number of isolates per swab.
22. Analysis of single isolates.
23. Frequency of multiple anaerobic isolates.
24. Bacterial species recovered from swabs.
25. Duration of ulcer and recovery of anaerobes.
26. Number of isolates from mud samples.
27. Biochemical profile of fusobacteria isolated.
28. Different characteristics of Fusobacterium species.
29. Characteristics of Bacteroides species isolated.
30. Pathology of tropical ulcer.
31. Patient serology - antibody levels to fusobacteria.
32. ELISA - antibody to bacteria from the ulcers.
33. ELISA - Rabbit IgG levels.
34. ELISA - Rabbit IgM levels.
35. Percentage of Vero cells affected.
36. Cell lines and cytotoxicity.
37. Cytotoxicity of supernatant fractions.
38. Butyrate concentrations causing cytotoxicity.
39. Inoculation of single bacteria.
40. Inoculation of multiple organisms.

## SUMMARY

Tropical ulcer is an acute or chronic skin disease seen in the tropics and subtropics that is characterized by necrosis of the epidermis and underlying subcutaneous tissue. The disease has been reported from China to the West Indies. Although it occurs mainly in children adults may also be affected. The disease often has a protracted course as the ulcers may continue for several months.

Previous reports on the disease, over the past 90 years, have included diet, trauma and infection as aetiological agents.

This study investigated one hundred and seventy-nine patients with tropical ulcers in five tropical areas. The nutritional status of the patients was assessed objectively. This study shows that there was no consistent relationship to overt malnutrition.

The clinical, bacteriological and histological features of the disease are also presented. The clinical findings were similar in all the areas studied. The acute ulcers (less than six weeks duration) were painful and oedematous whereas the chronic ulcers were relatively painless with a fibrous rim around the ulcers.

Histology was available on 20 patients and electron microscopy on 7. The main findings were loss of epidermis, dermal oedema, a polymorphonuclear inflammatory cell infiltrate and blood vessel proliferation. There was no evidence of vasculitis in any of the sections. Bacteria were seen at the site of tissue damage.

As previous reports have implicated anaerobic bacteria such as fusobacteria in the pathogenesis of tropical ulcers, a new method was developed for transporting samples anaerobically. Swabs were taken from patients in remote areas and transported to London for culture.

A number of bacterial species were isolated which included a new species of Fusobacterium. This new organism, F. ulcerans, could also be identified by ultrastructure of the histological sections. This species of Fusobacterium is cytotoxic to Vero cells in-vitro and may account for some of the tissue destruction which occurs at the onset of the ulcers. This species of Fusobacterium itself does not produce an experimental ulcer, but when used together with coliform bacteria, also isolated from these tropical ulcers, an abscess resulted. This has been demonstrated in the guinea pig model which confirms that this is a synergistic rather than a simple polymicrobial infection.

## INTRODUCTION

### Study Plan

The aim of this study was to determine the cause of tropical ulcer. The study was carried out by myself with assistance from Dr. D.C. Robinson, a paediatrician, who was particularly involved in the epidemiology of tropical ulcers. The study took place in five tropical areas where tropical ulcer was thought to be endemic. A questionnaire was prepared and adhered to throughout the investigation to maintain uniformity. The questionnaire provided details of the patients personal, family and dietary history.

The survey was conducted over two years, and each area investigated for a month. Information about the living conditions and social attitudes of the patients in the areas studied, was obtained from local health visitors and nursing, medical or para-medical staff.

The clinical aspects of the disease were investigated and all the ulcers were photographed. The bacterial flora of the ulcers was studied and particular emphasis placed on the identification of the anaerobic flora. Fusiform bacilli have often been reported in smears, and have been implicated in the aetiology of tropical ulcers. However, these organisms have not been fully identified or characterised. As these organisms are anaerobes, detailed anaerobic bacteriology and the best method of sampling ulcers were undertaken. Swabs were taken from the most recent ulcer for bacteriological culture. The bacterial samples had to be transported anaerobically from the study areas to London. A transport system was devised to transport samples anaerobically and to ensure viability of the organisms.

As the ulcers develop rapidly, a toxic effect of the bacteria seemed a possible pathogenic mechanism for the infection. In-vitro cell culture systems were used to assess the aetiological role of the organisms isolated from the ulcers. A variety of cell lines were used to detect cytotoxicity. In-vivo synergy of bacteria to produce ulcers in guinea pigs was also studied.

The nutritional status of patients with tropical ulcer was assessed objectively to see whether the patients affected were malnourished. Patients were also examined for other superficial skin infections, such as ecthyma, impetigo, scabies or fungal infections to see whether these conditions predisposed to, or were associated with the development of tropical ulcer. The role of person to person spread in communities was also investigated.

Skin biopsies were performed whenever possible. The sections were processed and stained with haematoxylin and eosin as well as special bacterial and fungal stains. The inflammatory cellular infiltrate was further characterised by means of a selection of monoclonal antibodies and immunoperoxidase techniques.

The host response to the ulcers was assessed by an enzyme linked immunosorbant assay, which used whole cell preparations of all the organisms isolated frequently from the ulcers.

The hypothesis which this study hoped to fulfill, was that tropical ulcer is a defined clinical entity due to a specific infection with fusobacteria and spirochaetes.

## Definition

Tropical ulcer has been defined in several ways. For instance it has been described as an acute sloughing ulcer which occurs on the leg below the knee. The lesion may be superimposed upon a wound or may arise apparently spontaneously (Clements 1936). In 1932, Smith emphasised the description of "tropical sloughing phagedaena" as the foul necrotic slough left a raw granulating base which was surrounded by a raised, rolled sometimes indurated edge. Tropical ulcer has also been defined as a specific acute, ulcerative skin disease with a characteristic slough containing numerous fusiform bacilli and spirochaetes in the early stages (Blank, 1947). Hare (1948) also described tropical ulcer as a specific disease associated with infection with the fusiform bacillus. Marsh (1945) quoted the definition used by Roy (1938) in the British Encyclopaedia of Medical Practice which defined the disease as "a rapidly spreading ulcer, occurring on the lower extremities of the body, which quickly assumes a phagedenic character and is accompanied by considerable pain, local oedema, sloughing and a sero-sanguinous discharge." Kariks (1957) defined tropical ulcer as "an acute, specific, inflammatory ulcerative process, involving the skin and subcutaneous tissues, with characteristic adherent, foul-smelling, slough overlying a soft, very tender and easily bleeding granulomatous base with surrounding oedema, caused by spirochaetes and fusiform bacilli." This definition, like several of those mentioned above, is broad and includes the clinical sites affected as well as the aetiological agents.

All the definitions therefore include the common characteristics of these ulcers, namely that they develop rapidly and produce profuse amounts of foul-smelling slough which covers the friable base.

In this survey all patients who presented with an ulcer of rapid onset, which had a friable base covered with an exudate of slough, or those ulcers which had evolved through that phase and become chronic i.e. static in size and shape with a thick fibrous rim around the margins, were included. For the purpose of this study, tropical ulcer was defined as an acute or chronic skin disease characterized by dermal and epidermal necrosis associated with the rapid development of slough. This definition is based on the medical definition of "phagedenic" which refers to obstinate ulceration of the skin and subcutaneous tissues associated with sloughing and quick extension to the surrounding tissues (Butterworth).

## TROPICAL ULCER-REVIEW OF THE LITERATURE

The first cases of tropical ulcer were recorded in 1857 by Vinson in Mozambique and those, together with all the other cases which were reported around that time were reviewed by Le Denteg (1899). Tropical ulcer was described under several synonyms in the past. These include Naga sore (Fox 1920) tropical septic ulcer (Apostolides 1922), *ulcus tropicum* (Panja 1945) and tropical phagedenic ulcer (Yesudian 1979). Despite the many synonyms, most writers believe that tropical ulcer is a definite entity. In the past the disease has been confused with several other ulcerative conditions which may occur on the leg and have a similar clinical appearance (Blank 1947). These include streptococcal and diphtheritic ulcers and yaws. Burnie (1931) reviewed the other conditions included the literature as tropical ulcers. These include leishmaniasis, tuberculosis, syphilis and other "sores". In his review he stated that several authors previously included different types of sores under the heading of tropical ulcer. Some patients with leishmaniasis for instance, were reported to present after their lesions had developed into tropical ulcers. He thought that most "sores" were caused through secondary contamination of minor injuries, and were not due to specific infective organisms. Similarly Adams (1923) regarded *ulcus tropicum* as a comprehensive and uncertain term, to designate ulcers that terminate several diseases such as syphilis, cutaneous tuberculosis, framboesia (yaws) and leishmaniasis.

## Epidemiology of tropical ulcer

### Geographical distribution

Tropical ulcer occurs widely throughout the world. The disease is found predominantly in warm countries and is particularly frequent in the tropics and subtropics (Costa 1944, Loewenthal 1963, Ngu 1967, Golden and Padilla 1946). Cases have been described from the Caribbean (Clements 1936, Earle 1942) and from Asia (Yesudian 1979, Fox 1920). Many reports from Africa confirm the existence of the disease on that continent. For instance cases were reported from Uganda (McAdam 1966), Nigeria (Ngu 1967), South Africa (Ferguson et al 1959), the Gold Coast (Ampofo and Findlay 1950) and Kenya (Jarvis 1945). In Africa, the disease is seen most commonly in central Africa. Few cases have been reported from North Africa including Libya (Castellani 1948).

The disease has also been reported from South America including Bolivia, Brazil and Argentina (Costa 1944), and much less commonly from Europe (Clements 1936, Castellani 1957). A map (figure 1) outlines the distribution of the reported cases of tropical ulcer. The hot, humid conditions were thought to predispose to the development of these ulcers, (Clements 1936, Kariks 1957) although it is known that tropical ulcers also occurred in the cold highland regions in Somalia (Adamson 1949).

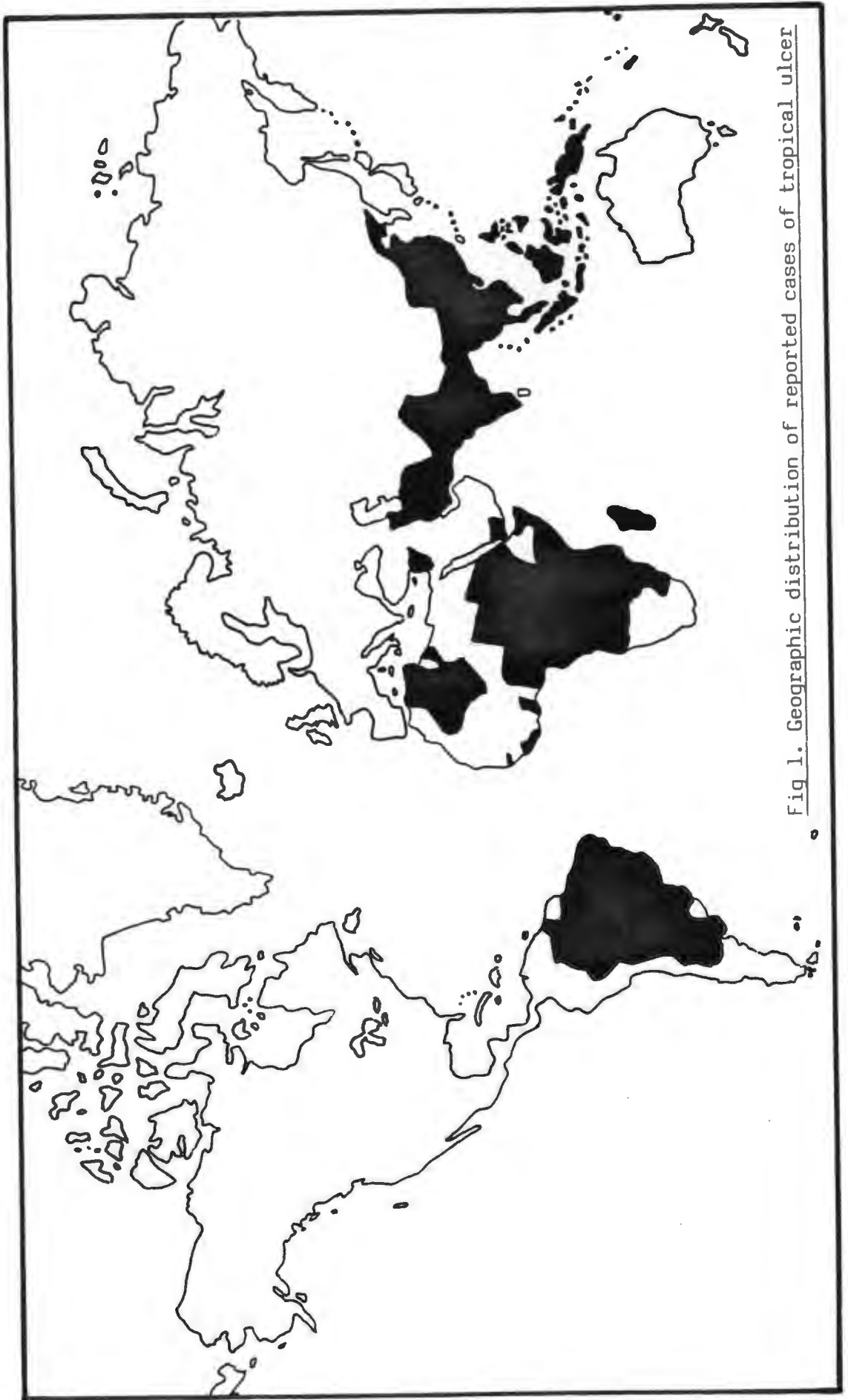


Fig 1. Geographic distribution of reported cases of tropical ulcer

Castellani (1957) however stated that tropical ulcer did not occur in the temperate zone. Other reports on the distribution of the disease, showed that the disease could occur in both dry and swampy areas (Burnie 1931). These reports confirm that the disease may occur in many areas irrespective of the climate or the degree of humidity.

### Prevalence

The prevalence of tropical ulcers is unknown because the figures quoted by some authors refer to hospital admissions or patients who attend out-patient clinics. In many areas where the disease occurs, patients do not seek medical attention at all. Also in many countries the hospitals are often situated in the major towns. Those individuals with tropical ulcers who live in the rural areas never attend these centres because of the great distance. A report of the Sudan Medical Service for 1936 stated that 3% of all hospital admissions for the whole country were patients with tropical ulcer (Corkhill 1939). The figure implied that the government figures reflected the incidence of the disease in the country. However the statement provides no information on the prevalence of the disease in the country, only the percentage of those patients who were able to attend hospital and were admitted for their tropical ulcers. Several authors who have lived and worked in endemic areas recorded their personal impressions. Such impressions are notoriously inaccurate. Where figures are available tropical ulcer is a very common condition in many countries. For instance, Burnie (1931) estimated that ninety-five percent of ulcers of the skin seen in Kano, Nigeria were tropical ulcers.

Koerber (1950) in the former French West Africa reported an incidence of 2.6% in out-patients and 7% among in-patients (Loewenthal 1963). Thomson (1956) in northern Nigeria described tropical ulcers as the commonest disease among the local inhabitants. Nelson and Semambo (1956) examined 1,945 people family by family in an area in Uganda where tropical ulcers were common. They found an incidence of 1% of the population affected with ulcers. Similarly Ngu (1967) published figures from Nigeria and reported an incidence of tropical ulcer in up to 33% of registered hospital patients. Loewenthal (1963) reported that over half the patients admitted to hospital in an area in Uganda between 1932 and 1933 had tropical ulcers. The same author also found that 2.5% of the Iteso tribe were affected with tropical ulcers and 13.6% of the same tribe showed the typical scars of tropical ulcer. Until population studies are carried out throughout the year on all the persons in the areas where tropical ulcers are common only rough estimates can be made of the prevalence of the disease.

There are well documented epidemics of tropical ulcer. For instance Apostolides (1922) reported an outbreak of these ulcers in Palestine in 1919. Up until that time ulcers were not seen in that area. In Assam only isolated cases occurred up to 1942. Thereafter clusters of cases developed and the disease became endemic in the area (Hare 1948). The author attributed the influx of cases to a gradual build-up of infection among the population in the area as all other conditions such as nutritional status, occupation and climate were constant.

## Seasonal variation of tropical ulcers

Reports on the seasonal variation of the disease are conflicting. For instance Steinhauser in Aden, Smits in Sumatra and Chisholm in the West Indies found that the prevalence of ulcers was greatest in the wet, rainy seasons (Burnie 1931). Similarly Corpus (1924) reported that ulcers occurred more frequently in damp weather. On the other hand Onarato (1927) attributed the high incidence in Tripolitania to the drought and heat (Burnie 1931) whereas Burnie (1931) himself reported an increase in the number of tropical ulcers after the rainy season. Although Charters (1947) found an increase in the incidence of tropical ulcers just after the rainy season he attributed this to the lack of milk and fresh vegetables rather than a seasonal variation in the prevalence of the disease. Similarly Corkill (1939) and Dixon (1951) also noted an increase in the number of cases just after the beginning of the rainy season but he also ascribed this finding to the lack of vitamins available at the time.

Marsh and Wilson (1945) found that cold weather and rain favoured the development of tropical ulcers. On the other hand Hare (1948) in India found that tropical ulcers did not occur at all during the cold, dry months. After the cold season no cases persisted. New cases which developed thereafter were sporadic and thus unlikely to be related to carry over of infection from the previous season. He favoured an insect vector as an explanation for the seasonal prevalence throughout the year.

Other authors have found that the prevalence of tropical ulcer is constant throughout the year. Thus the report from Trinidad by Earle (1942), suggested that tropical ulcer occurred with almost equal

frequency throughout the year. He found the number of cases were only slightly increased during February to August when atmospheric temperature, rainfall and relative humidity were low.

The overall impression is that tropical ulcers can occur in any climate although the evidence presented seems to suggest that the disease is more frequent during the rainy season.

#### Age specific incidence

Tropical ulcer occurs more frequently in children than adults and those in their second decade have been reported to be most commonly affected (Loewenthal 1963). However other authors have suggested that the condition is most frequent during the third decade (Hughes 1931, Earle 1942). Kariks (1957) stated that all ages can be affected and that age has little influence on the onset of the disease. In his experience the disease was seen most commonly in patients aged 15-45 years. Most of those cases occurred in labourers who were usually adults. Hare (1948) reported that patients in the third decade were affected most often followed by those in their second and fourth decades. Most authors agree that the disease rarely affects children under the age of 5 years. This was possibly due to the fact that children were carried by their mothers and were less exposed to trauma. However James (1938) stated that 2% of his patients were under the age of 2 years. Most authors also agree that tropical ulcers rarely appear for the first time in patients older than 45 years although patients may develop recurrent ulcers over for many years.

### Sex specific incidence

Earle (1942) reported that 67% of patients were male. He suggested that the difference reflected work practice. Men worked in oil fields and were thus more liable to trauma. Similarly Mohanty (1945) and Pattanayak (1944) described a male predominance of cases. Hare (1948) also observed more male cases and stated that this was related to the recruitment of males to the fields in the area where he worked. Hughes (1931) went as far as saying that he had never seen a case of tropical ulcer in a female. Kariks (1957), on the other hand, felt that in the cities and on plantations sex may play a role. Males rather than females worked in these areas and they were more likely to sustain trauma which facilitated the development of a tropical ulcer. However in the villages because women were more actively involved in the agricultural work, and worked with men, the incidence of ulcers was distributed equally between the sexes. In contrast Loewenthal (1963) found a female predominance of cases. Thus males and females may be affected and the number of cases is most likely related to trauma sustained in employment.

### Racial incidence

In the report by Earle (1942) negroes were affected more than any other racial group. He attributed this increased incidence among adult negroes to their employment in the oil fields where they were constantly exposed to trauma. Children of negroid and Indian extraction provided most of the juvenile cases. Individuals of European, Chinese or American descent were relatively free of ulcers. Hughes (1931) in Malaya stated that the Chinese were particularly prone to the disease.

Burnie (1931) found the disease almost confined to the natives of tropical and subtropical countries and that only malnourished Europeans developed tropical ulcers. Hare (1948) reported that he had seen ulcers in civilian Europeans although this was rare. He felt that in most cases their mode of dress or their work may have predisposed them to attack by insects which he thought were vectors of some infective agent. Although racial differences were observed tribal differences were also recorded. Loewenthal (1963) in Uganda noticed a difference in the incidence of the disease in people of the same tribe. Those who were chiefs, policemen or house servants were not affected even though they lived in close proximity to the rest of their tribe.

## Clinical Manifestations of Tropical Ulcers

Tropical ulcers may occur as acute or chronic ulcers. The clinical manifestations which separate the two forms of ulceration are described.

### The acute ulcer

Ulcers start with a tiny papule a few millimetres in diameter (Marsh & Wilson 1945, Loewenthal 1963, Kariks 1957). The initial lesion is very painful. Although this stage is rarely seen by the attending physician it may be observed when the patient develops a new lesion while another ulcer is treated. The papule may become a vesicle which contains sero-sanguinous fluid. Within a few days the surrounding area becomes black due to tissue necrosis. The papular lesion may become haemorrhagic or even bullous. Most commonly though it becomes frankly pustular. A few days later the central area of necrosis sloughs to leave an ulcer. Over the next few days the ulcer rapidly enlarges in all directions, becomes very painful and a thick purulent slough covers the base of the ulcer. At this stage the ulcers are foul smelling and produce copious amounts of exudate which attracts flies. The base of the ulcer is often friable and bleeds easily on contact. The ulcer is usually round to oval, 2-6 centimetres in diameter and has a slightly raised edge and surrounding hyperpigmentation. There may be some swelling around the ulcer but the ulcers seem to provoke very little reaction in the surrounding tissues.

Most of these acute ulcers remain unchanged without treatment and heal slowly but spontaneously over a period of up to eighteen months although on average they heal within in six months (Loewenthal 1963, Earle 1942).

### The chronic ulcer

Except at the onset of the ulcers the lesions are rarely painful (Nelson and Semambo 1956, Loewenthal 1963). After the acute ulceration and rapid enlargement the ulcers cease to extend. The surrounding oedema settles and the edge becomes prominent. Often the surrounding area forms a firm lip of fibrous tissue around the ulcer. The base of the ulcer becomes less friable and the slough diminishes (Roberts and Highet 1986). On average chronic ulcers develop six weeks after the initial onset of ulceration. These ulcers may take months or even years to heal. When healing finally begins the edge flattens and granulation tissue gradually fills the defect. The subsequent scar takes up the shape of the original ulcer. The scars are often atrophic and liable to break down. Recurrent ulceration is not uncommon. The schematic representation of ulcer progression is shown in fig. 2. At present there are no obvious features that predict which ulcers will become chronic.

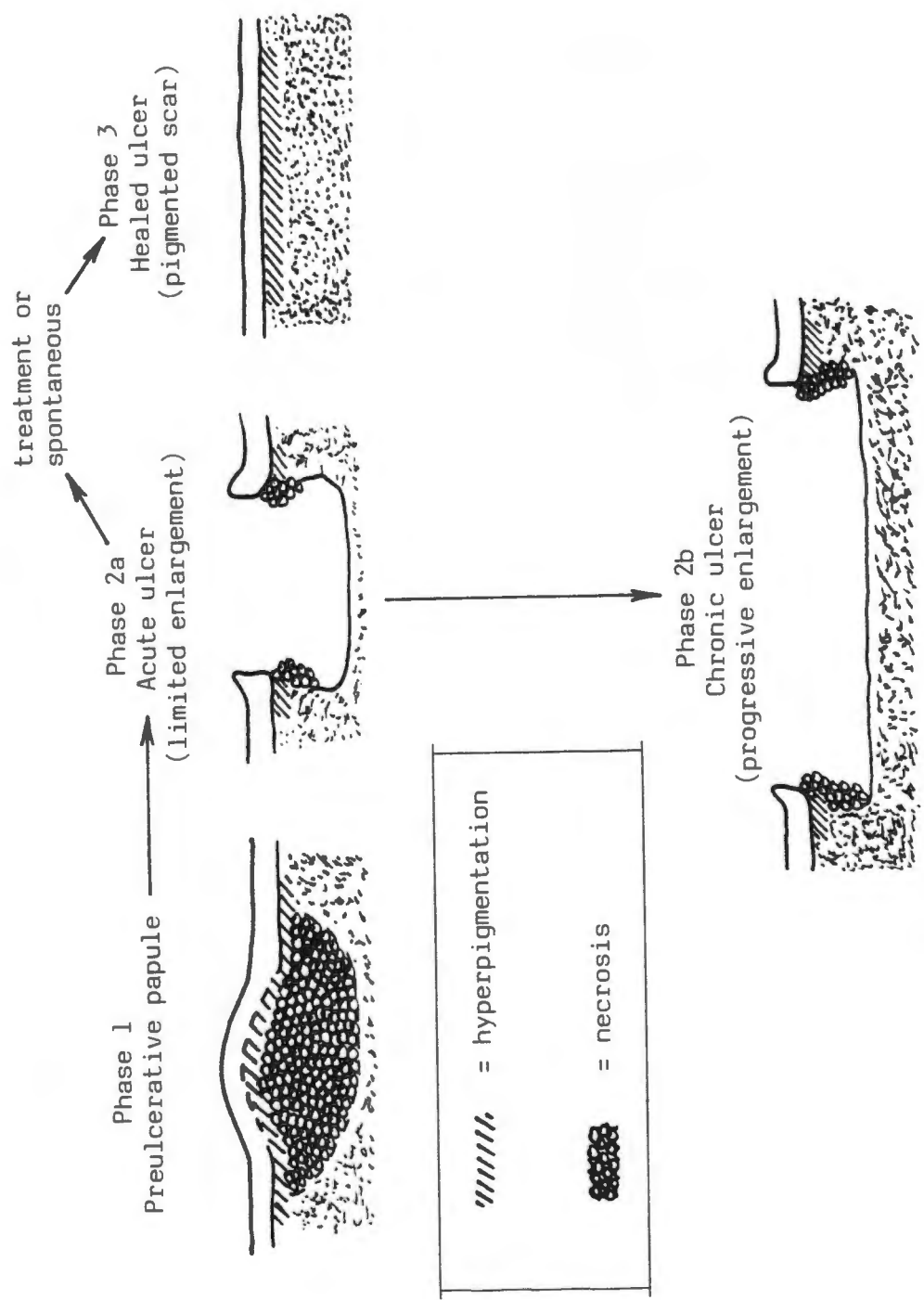


Fig 2. A scheme illustrating the possible development of tropical ulcers

### Sites affected

Most ulcers occur on the lower leg, foot or toes. Less commonly the hand or arm is involved (Hare 1948). Lasbrey (1952) observed tropical ulcers on the nail bed of the toes. Any of the toes were affected. Ulcers may be single or multiple. Where they are multiple and exist side by side they have been referred to as "kissing ulcers" (McAdam, 1966). Ulcers may be widely separated on the same limb or may develop on the upper and lower limbs. The trunk is rarely affected.

### Systemic manifestations

Systemic signs are uncommon in patients with tropical ulcers. Despite the extent of these ulcers there are few instances where systemic features have been reported. When secondary infection supervenes, regional lymphadenopathy occurs and there may be considerable erythema and swelling at the site of the ulcer (Apostolides 1922). Fever may be associated with the secondary infection. In most cases though the disease remains localised to the skin.

The frequency of extensive disease or systemic symptoms varies among different populations. For instance O'Brien (1951) regularly found bone and tendon involvement as a complication of these ulcers, whereas Loewenthal (1963) rarely found these features in his patients. O'Brien (1951) also observed fever and lymphadenopathy as associated features in some cases.

## Role of Trauma

Burnie (1931) was convinced that trauma played a large part in the aetiology of these ulcers. He stated that "there appears no doubt that the great majority of the ulcers originate at the site of superficial injuries such as slight skin abrasions produced by scratching, contused wounds, injuries caused by stones, thorns, guinea-worm infections and the like." He reviewed 250 patients with tropical ulcers and all except six ascribed the ulcer to trauma. Over 32% of the new patients who sought treatment attended for tropical ulcers which had followed trauma. Most of these patients were adults who worked as railway construction workers.

Earle (1942) reported that any form of trauma which damaged the epidermis could initiate a tropical ulcer. The injury could vary from a crushing blow followed by a haematoma to a laceration. He reported two cases of tropical ulcers which followed a dog bite. He felt that scratches, abrasions, or punctures that followed accidental injury by plants or nails could initiate an ulcer. A nurse developed a tropical ulcer at the site where she accidentally injured herself with a scalpel used to debride a wound in a patient with a tropical ulcer (Apostolides 1922). Earle (1942) also reported that tropical ulcer could follow scabies, impetigo or mosquito bites. Jarvis (1945) suggested that tropical ulcers could develop after jigger sores especially those which affected the toes.

Many authors have stressed the correlation between trauma and occupation. However, Hare (1948) observed that many of his patients were heavy manual labourers but never experienced any form of trauma at the site of the ulcer.

Moreover when ulcers and abrasions developed on the same leg the abrasions remained clean and did not necessarily develop into tropical ulcers. He postulated that when an insect vector bit the soft granulation tissue of an abrasion, the infection remained close to the surface and healed rapidly. Whereas when the insect vector bit unbroken skin the infection remained deep. A vesicle formed and when it broke down a larger ulcer was visible. Thus some form of trauma (even minor) was necessary for an ulcer to develop. For example an insect bite.

Kariks (1957) also thought trauma was important in the aetiology of tropical ulcers. He reported that 94% of his patients recalled some form of trauma prior to the onset of the ulcer. In his patients there was a close correlation between trauma and occupation. O'Brien (1951) emphasised how difficult it was to obtain a history of trauma from the patients. In his study, 181 out of 209 patients could recall a history of trauma.

The evidence presented suggests that trauma does play a role in the aetiology of tropical ulcers. Often trauma and occupation are linked and the contribution of each is difficult to define.

## Complications of tropical ulcer

### 1. Osteitis

Tropical ulcers may extend to bone although fortunately this is rare. Bone involvement was reported by Golden and Padilla (1946) and Brown and Middlemiss (1956). Golden and Padilla reported marked radiological cortical opacity and thickening which encroached on the medullary canal, periosteal thickening and fine radial lines which were interpreted as calcification. Exostoses which limit patients' mobility, were reported by Nelson and Semambo (1956). However, no X-rays were included in the report so periosteal proliferation cannot be excluded. Despite many reports on the complications of tropical ulcer osteitis is rarely reported which suggests that it is rare.

### 2. Tendon necrosis

Ulcers which extend deeply may involve and damage tendons. Once the tendons slough, healing of the lesion is delayed and the result is severe deformity and a painful scar (Jarvis 1945).

### 3. Gangrene of the limb

Gangrene of a limb may follow tropical ulcers and has been observed by Carayon (1957) and Apostolides (1922). Healing may subsequently occur with extensive scarring but many patients will require surgery. Other authors rarely reported this complication and it is possible that tropical ulcers were confused by some investigators with other conditions as the descriptions given were vague and did not appear to fit the description of tropical ulcers.

#### 4. Recurrent ulceration

Ulcers may recur at the same or a different site. The recurrent lesions may develop shortly after one lesion heals or a year or two later. James (1938) suggested that recurrent ulcers in scars were not uncommon because the previously damaged skin had a poor blood supply and this predisposed to further ulcers. Another reason for a recurrence was the continued presence of organisms dormant after the lesion healed (Smith 1933).

#### 5. Squamous cell carcinoma

The malignant transformation of a tropical ulcer to a squamous cell carcinoma is rare and may occur in up to 2% of ulcers (Vint 1935). The lesions become progressively heaped up, with vegetating growths. Only one report describes the development of metastases to the groin (Nelson and Semambo, 1956). The authors observed 9 patients with squamous cell carcinomas among 350 patients treated. The diagnoses were confirmed histologically.

#### 6. Tetanus

There is a theoretical risk of tetanus following contamination of tropical ulcers although reports of this complication are rare (Ngu 1967).

## 7. Hepatitis B infection

Recently Tibbs (1987) reported an incidence of 26% for hepatitis B infection in patients with tropical ulcers in Kiribati (formerly the Gilbert Islands). Of 47 patients with tropical ulcers tested for hepatitis B infection, 10 carried hepatitis B surface antigen and two hepatitis B e antigen. Hepatitis B surface antigen was detected in all the ulcer exudates from subjects with hepatitis B surface antigen in their blood. The same was observed with hepatitis B e antigen. These patients had open wounds and transmission from older to younger siblings could have accounted for the high incidence of hepatitis B infection among siblings. Boys had a higher rate of infectivity than girls. This was thought to be related to greater contact during play between boys who were encouraged to play with their peers outside the family whereas girls were often confined to close contact with their mothers for several years.

## Role of nutritional status in tropical ulcer

Many authors have emphasised the role of malnutrition in the development of a tropical ulcer. Burnie (1931) reported that as far back as 1867, in the Abyssinian war, British soldiers who were well supplied with food remained free from ulcers whereas the Hindus who for religious reasons were not allowed to eat fish developed ulcers. McCulloch (1928) described malnutrition as a major cause of tropical ulcers. He analysed the diet of those who developed tropical ulcers and observed that their diet contained large amounts of glucose but lacked protein and salt. He even stated that tropical ulcer was a "dietetic ulcer" and felt that the role of infection was minimal. Similarly Dalrymple (1928) felt that consumption of certain foods, such as fresh meats, reduced the frequency of ulcers. In Kenya two tribes were compared with regard to the frequency of tropical ulcers and the inclusion of meat in the diet. According to Orr and Gilks (1931) those who ate meat rarely developed ulcers. Loewenthal (1963) observed that animal protein and calcium were required to prevent the development of a tropical ulcer.

Loewenthal (1932) also reported that the ulcers were related to the availability of food. During episodes of drought usually associated with famine, ulcers were much more frequent. He reported that two tribes who lived under very similar socio-economic conditions had a different incidence of tropical ulcers. The only difference in behaviour which he could detect was that one tribe ate fish regularly whereas the other omitted fish from their diets because of tradition. He also observed that even though tribes lived in close proximity to each other the incidence of tropical ulcers differed among the different classes.

For instance chiefs, native troops and policemen rarely developed ulcers whereas the rest of the tribe often did. The implication was that those living under poorer socio-economic conditions developed ulcers.

Similarly Charters (1943) reported the prevalence of tropical ulcers among troops in East Africa. Of the East Africans admitted to the troops 0.2% (n=1) had ulcers and that patient may have had necrotic bone on the shin rather than a tropical ulcer. However 49% of Somalis admitted to the troops had tropical ulcers. He thought that the only difference between these two groups of people was their diet. In particular the East Africans ate more dried beans, ghee and maize meal whereas Somalis refused to eat dried beans. He felt that there was perhaps an essential substance in dried beans or ghee which protected people from tropical ulcers. Later (1947) the same author reported that milk prevented tropical ulcers. When milk was in short supply the number of ulcers increased considerably whereas when milk became available again they decreased. However none of these studies objectively assessed a control population.

Other authors observed that malnutrition was not an essential factor for the development of tropical ulcers. Marsh and Wilson (1945) observed that all their patients with tropical ulcers were malnourished, with dietary deficiencies similar to those reported by other workers. However although they were unable to correct the diets, most ulcers healed albeit slowly. Similarly Buchanan and Sanderson (1935) found that dietary therapy did not necessarily heal ulcers. Patients failed to respond to a diet rich in vitamins. Corkill (1939) treated his patients with oral vitamin supplementation and used cod liver oil dressings on the ulcers.

The results showed that ulcers treated with cod liver oil dressings healed more rapidly. Oral vitamin A provided no further benefit and proteins, rather than vitamins, were thought to be more important for healing.

To quantitate nutritional deficiency Corkill (1939) used clinical signs such as the presence of black patches on the tongue and blueness of the gums as indicators of malnutrition. He scored all patients with ulcers and tried to correlate nutritional deficiency with both the number of ulcers and their recurrence rate. The assumption was that those with recurrent or chronic ulcers would exhibit greater degrees of malnutrition than those with acute or recent ulcers. He found that patients with recurrent ulcers were more likely to be malnourished and that ulcers healed more rapidly in those less malnourished. However there were patients with ulcers who had no signs of severe malnutrition. He concluded that although malnutrition was an important factor it was not the only factor which predisposed to the development of tropical ulcers.

Further evidence against malnutrition as a major factor in the aetiology of tropical ulcers was recorded by Hare (1948). He examined several patients on the tea estates in India for evidence of malnutrition and found no correlation between malnutrition and the development of a tropical ulcer. He stated that "there is no valid evidence that food deficiencies play any part in the aetiology of tropical ulcer, and any apparent relationship is merely due to the fact that the diets of the poorer classes in most countries is imperfect and that it is the poorer classes who by their habits are liable to contract infection". Finally, McAdam (1966) found that the majority of patients in Uganda showed no evidence of malnutrition.

He was able to produce experimental ulcers by inoculating ulcer material in healthy subjects just as easily as in ulcer patients.

#### Role of Calcium and Vitamins

Several authors suggested that ulcers were related to a deficiency of calcium. Earle (1942) found that patients with tropical ulcers had low serum calcium levels. Loewenthal (1932) also found a low serum calcium in patients with tropical ulcers and reported an improvement after intravenous administration of calcium. In contrast Pawan and Camps-Campins (1931) reported the results of calcium levels in patients in Trinidad with a variety of conditions which included tropical ulcers. They could identify two groups of ulcer patients. The first appeared well except for the presence of an ulcer. The second were clinically malnourished and their dietary histories confirmed that they were chronically malnourished. Blood calcium levels were always normal or even elevated in the first group whereas they were always low in the second. Charters (1943) experimented with dietary supplementation to see which nutritional supplements would be most beneficial. Patients were given calcium at a daily dose of 0.5 g intramuscularly or intravenously. Other patients were given vitamin A in the form of cod liver oil. Some patients were prescribed nicotinic acid (or coramine, the diethyl amide of nicotinic acid), others vitamin C or riboflavin in the form of condensed milk. All the patients treated were compared with controls. Sixty percent of controls improved. The author found that vitamin C and nicotinic acid had no effect on the rate of healing. Those patients treated with calcium and cod liver oil healed rapidly. He observed that East Africans had a low calcium intake in their diet yet they rarely developed tropical ulcers.

From these studies he concluded that the ulcers resulted from a deficiency of vitamin A. Yet despite the dietary shortage of this vitamin there were no other clinical signs of vitamin A deficiency such as xerophthalmia or phrynoderma. Similarly Scott (1941) reported a deficiency of vitamin A in Tanzania in areas where tropical ulcer was frequent. On the other hand Jarvis (1945) also in Tanzania found that patients in that area who developed bony fractures healed very well which suggested that they are not in fact calcium deficient. He suggested instead that the soil in the area may be calcium deficient.

Corkhill (1939) reported improved healing rates with topical cod liver oil. However this therapy made no difference to acute ulcers although chronic ulcers improved. This did not therefore prove that vitamin A was beneficial to all tropical ulcers. Blank (1947) found that ulcers responded to therapy with vitamin B complex. By contrast Hare (1948) found that the disease was patchy in distribution which could not be related to dietary deficiencies but to variations in the terrain and of the vegetation.

#### Role of zinc

The role of malnutrition in the development of tropical ulcer was investigated by Watkinson et al (1985). Patients with tropical ulcers were compared with an age and sex matched control population in the Gambia. Although most of the patients had low serum levels of zinc many of the controls also had low values. Despite adequate replacement therapy with restoration of the serum levels of zinc to normal there was no improvement in the healing of these ulcers. The study was well conducted and based on quantitative zinc levels.

The evidence that malnutrition predisposes to tropical ulcer is contradictory. Many of the studies were anecdotal and few assessed objective data. A summary of the reports on the role of malnutrition in the development of tropical ulcers is shown in table 1.

Table 1. Nutrition and tropical ulcer (TU)

Author	Dietary Deficiency	Incidence of TU	Evidence
McCulloch 1928	Protein and "salt"	increased	Quantitative
Loewenthal 1932	Calcium	increased	Calcium supplementation & clinical observation
Corkhill 1939	Vitamin A	no effect	Vitamin A supplementation & clinical observation
Charters 1945	Ingredients in dried beans	increased	clinical observation
Panja 1945	none	no effect	clinical observation
Loewenthal 1963	Fish	increased	clinical observation
Watkinson 1985	Zinc	no effect	Quantitative with Zinc supplementation

## Role of infection

The role of infection in the aetiology of tropical ulcer has been emphasised by some authors (Earle 1942, Burnie 1931, Blank 1947) and discarded by others (McCullouch 1928). The first report which describes the bacilli associated with these ulcers was by le Dantec, Plaut and Vincent (1899). He observed swarms of bacilli on the smears taken from the surface of the sores. Subsequently several authors confirmed the presence of the fusiform bacilli in the smears and later (1909) the spirochaetes in the ulcers were recognised (Clements 1936). Many other authors subsequently suggested that there may be more than one fusiform bacillus involved in the disease process (Brienl 1915, Fox 1920, Hughes 1931). Initially several workers thought that the two morphological types were different stages of the same organism (Burnie 1931). The organisms were later identified as Spirochaeta schaudinni (spirochaete) and Bacillus fusiformis (fusiform bacillus). The presence of these organisms was thought to be indicative of acute ulceration. When organisms were scarce, the ulcers were usually of longer duration or the patients had some form of treatment (Earle 1942).

Webb (1946) noted clusters of ulcer cases as if in an epidemic however there was no definite spread from one person to another. Thus although cross infection from person to person was thought likely this was not proven. Similarly Jarvis (1945) also reported epidemic outbreaks.

Kotrajaras (1982) reported an outbreak of endemic leg ulcers which resembled tropical ulcers. However they never isolated fusiform bacilli from any of the ulcers.

### Relation to oral sepsis

The source of the presumed infective agent was investigated by several authors. Apostolides (1922) and Clements (1936) both suggested that tropical ulcer might be related to infection from the mouth. Gordon Thomson (1956) suggested that eating with the hands allowed transfer of the organisms from the mouth to the legs. The organisms could then be scratched into the skin or introduced by insects. Clements (1936) investigated patients in New Guinea and found that 25% of tropical ulcer patients had chronic periodontitis. Contrary to this Earle (1945) reported that whereas infection with Vincent's organisms was common in northern Peru tropical ulcers were uncommon. Similarly Panja (1945) recorded that in his experience patients almost always had healthy gums. Apart from these clinical impressions Hare (1948) objectively studied seven tropical ulcer patients and seven controls who lived in intimate contact with the affected patients. He examined swabs from the ulcers, the skin around the ulcers and the gums of all the patients and controls. He found that swabs from all seven ulcers but from only one patient's skin surface showed fusiform bacilli. Only one ulcer patient and one control had fusiform bacilli in the oral swabs. No controls had fusiform bacilli in skin surface smears. He concluded that although the mouth might be the source of infection in a small number of cases it was by no means the only or even the major source of infection.

### Fusiform bacilli

These bacteria were frequently reported from swabs of tropical ulcers (Clements 1936, Forbes-Brown 1935, Charters 1943). They have been regarded as normal commensals of the mouth and are found in normal healthy people (Finegold 1977). Marsh (1945) reported that the organisms were found on "dirty" skin of affected patients. The fusiform bacilli have also been considered to be of secondary importance in these ulcers, particularly as they were often associated with other organisms in smears (Ferguson et al 1959). A report of an epidemic in South Africa documented the presence of streptococci, staphylococci and fusiform bacilli in ulcers. The authors suggested that the primary lesion was a pyoderma caused by streptococci or staphylococci. Lesions were secondarily infected with fusiform bacilli which caused extensive spread.

Hare (1948) performed smears on early lesions in the vesicular stage and noted fusiform bacilli. Yet they were rarely seen on histology. He thought that the bacilli were not seen on the histology sections because they were present in small numbers. After 12-24 hours the number of fusiform bacilli increased considerably. In the 18 patients investigated either staphylococcal or streptococcal infections were common.

## Spirochaetes

Several reports have documented the presence of Vincent's organisms in smears of ulcers (Clements 1936, Forbes-Brown 1935, Eggers 1915, Jarvis 1945). Eggers (1915) reported tropical ulcers from China. He observed six types of spirochaetes in the ulcers he examined although the cases investigated included ulcers other than tropical ulcers. His descriptions include straight forms of spirochaetes which he regarded as variations of the spirochaetes. These may have been bacteria. Associated with these spirochaetes he described fusiform bacilli and bacilli with clubbed ends. Similar spirochaetes were also observed in patients with chancroid and gonococcal urethritis. Thus the relationship of the spirochaetal infection to tropical ulcer is unclear.

## Combination of fusiform bacilli and spirochaetes

Most of the previous authors concentrated on a combination of micro-organisms as aetiological agents in tropical ulcer. Apostolides (1922) produced transmission tropical ulcers using "parcels of pseudomembrane" from cases of ulcero-membranous angina. He postulated that the fusiform bacilli and spirochaetes seen on the pus smears might be the causative agents. He was cautious when he interpreted his findings and stated that other conditions such as debility or malnutrition were prerequisites for the development of a tropical ulcer. He was adamant though that the only causative agents in this condition were fusiform bacilli and spirochaetes.

A list of the authors and the results of smears and cultures from ulcers is set out in table 2.

Table 2. Organisms recovered from tropical ulcers

Author	Year	Bacteria implicated	Source of bacteria	Culture
Eggers	1915	Spirochaetes	Smears from ulcers	Aerobic
Fox	1920	Fusiform bacilli	Smears and cultures	Anaerobic
Apostolides	1922	Spirochaetes & fusiform bacilli	Smears from ulcers	None
Burnie	1931	Spirochaetes, fusiform bacilli Cocci and Pseudomonas	Smears from ulcers	Aerobic
Forbes-Brown	1935	B. fusiformis, Gram-positive cocci and bacilli	Smears from ulcers	Aerobic
McAdam	1966	Fusiform bacilli & spirochaetes	Smears from ulcers	None

## Transmission experiments of tropical ulcers

Many workers have tried to produce an animal model of tropical ulcer. For instance Burnie (1931) reported that Lloyd Patterson (1908) tried to inoculate material from ulcers intradermally, kept the inoculated sites bandaged and typical ulcers developed. Balliano (1916) repeated the experiment but only fusiform bacilli were recovered in smears from the experimental ulcers. Subsequent experiments by Fox (1920), Smith (1932), Panja (1945) and McAdam (1966) all showed an alleged aetiological role for the fusiform bacilli. However these organisms had not been characterised and the observations from experimentally produced ulcers were based on Gram stains of smears of pus. The aim of these experiments was to show that tropical ulcer was an infective condition.

Fox (1920) performed several experiments after his attempts to culture the organisms. He used an emulsion of the discharge from the ulcers and also mixed cultures of cocci grown from the discharge of the ulcers. A pure culture of fusiform bacilli inoculated intramuscularly into a guinea pig failed to produce an abscess or an ulcer. Burnie (1931) tried to reproduce ulcers and used flies which were commonly found near ulcers. He was unable to transmit the infection through these insects.

## Fusiform bacilli: identification and cultivation

Fusobacteria are Gram-negative, obligately anaerobic rods which are pleomorphic in shape and size. Some bacteria may be almost coccal in shape whereas others are long and filamentous. Yet others may show a central swelling. Although many have tapered ends others appear rounded. They are nutritionally demanding in laboratory culture but with adequate techniques they may be recovered with relative ease from clinical specimens. However many will not survive repeated subculture when exposed to air. The fusobacteria are predominantly found in human and animal intestinal tracts, but may be commensals of the respiratory tract (Finegold 1977).

The term Fusobacterium (Latin, fuso : a spindle) was probably first used by Knorr (1922) to define the long thin spindle shaped Gram-negative bacilli. According to Duerden (1984), the bacteria originally described by Knorr as Fusobacterium plauti-vincentii (as the organisms resembled those of Vincent's bacillus) should probably be classified today as Leptotrichia buccalis. The term Fusobacterium therefore now refers to Gram-negative spindle shaped rods.

Attempts to culture the fusiform bacilli date back to the early part of this century when Krumwiede and Pratt (1913) tried several culture methods. These included shake and surface cultures. Initially these were unsatisfactory. They then used agar contained between two halves of the petri dish with the bottom of the dish placed in the inverted cover. Plates were covered with paper to prevent air entering at the sides. The rim was covered with paraffin to prevent dessication. These methods proved partially successful.

Contamination with aerobes still occurred but was limited to the surface areas. The addition of horse serum was essential for growth. This was probably the first successful culture of the fusiform bacilli. Subsequently Fox (1920) had only limited success when he cultured fusiform bacilli from ulcers. He used the technique described by Krumwiede and Pratt (1913).

As culture techniques have improved many more species of the genus Fusobacterium have been recognised and characterized (Holdeman, Moore and Cato 1977). There are now 15 species recorded in the Virginia Polytechnic Institute manual (1977). The main characteristic common to all species of Fusobacterium is the production of butyric acid as the major end product of metabolism. Although they may sometimes resemble the Bacteroides species the production of butyric acid differentiates them from the latter. Most species are sensitive to a wide range of antibiotics although there may be interspecies variation. In general most are sensitive to penicillin and neomycin and resistant to rifampicin.

The role of different species of Fusobacterium in disease has been reported by several authors (Moore-Gillon et al 1984, Ydenfeld et al 1982). Of all the recognised species, F. necrophorum and F. nucleatum are most frequently reported in clinical infections. The former causes necrobacillosis in man and animals, a disease associated with necrotic tonsillitis and post-anginal septicaemia (Mitre and Rotheram 1982). In man, oral and dental infections, as well as cerebral, hepatic and abdominal abscesses may follow infection with F. necrophorum (Finegold, 1977). Ovine foot rot is an example of a fusobacterial infection in sheep.

The sheep develop ulcerative lesions on the feet after being in wet conditions for a prolonged period. The disease is due to a synergistic infection with F. necrophorum, a diptheroid and Bacteroides nodosus (Hine 1983).

On the other hand F. nucleatum has often been isolated from the mouth where it is considered part of the normal flora (Willis 1977). It is often recovered from this site and may play a role in periodontal disease. F. necrophorum produces haemolysin and leukocidin. Both may contribute to the pathogenicity of the organism. F. nucleatum is not haemolytic. Other species of Fusobacterium are only rarely isolated from clinical infections.

Fusobacteria are difficult to recognise without special expertise as the morphology is not always spindle shaped as expected. Only F. nucleatum is regularly spindle shaped. This has to be taken into account when these bacteria are identified. Bennett and Duerden (1985) have provided a good practical method for distinguishing the various Gram-negative anaerobic rods. These techniques were used in this study to facilitate identification of the fusobacteria.

## Pathology of tropical ulcer

Harvey Blank (1947) reported that the characteristic histopathology is only seen in sections taken from an actively phagedenic part of the ulcer. The central slough which he described as a "pseudomembrane" was a structureless mass of tissue which had undergone coagulation necrosis and the granulation tissue extended to the fascia. On the other hand Burnie (1931) examined 20 ulcers histologically and found no distinctive findings. Golden and Padilla (1946) reported the pathology of 24 ulcers seen in Guatemala. They described epidermal hyperplasia, pseudoepitheliomatous hyperplasia but none showed evidence of malignancy. Polymorphonuclear cells infiltrated the epidermis and in some instances formed microabscesses. Loss of pigment was thought to be more common in older lesions and late lesions showed no pigment at all.

The dermal changes described included oedema, loose connective tissue, dilated capillaries and haemorrhagic foci. Lymphocytes and polymorphonuclear leukocytes were scattered in the dermis. In the older lesions the dermis was devoid of inflammatory cells and showed considerable fibrosis. Nerves were not affected. The subcutis showed a moderate number of inflammatory cells, mostly lymphocytes. There were foci of necrosis associated with foreign body giant cells. The authors (Golden and Padilla) also observed the arteries and veins were thickened although the latter less so. Larger arteries showed either marked muscular hyperplasia with narrowing of the lumen, or a mixture of hyperplasia and interstitial fibrosis. They felt that the vascular changes were primary and not a result of the ulcer as the changes were noticeable in early lesions, and also outside the areas of heavy inflammation.

They examined a control population who had post mortems for other reasons. No similar vascular changes were identified in the sites where tropical ulcers usually occurred. They also compared the changes in leishmaniasis, which was common in Guatemala, with those of tropical ulcers. No similar vascular abnormality was observed. They concluded that the vascular changes could be used to differentiate tropical ulcers from other ulcers which occurred in Guatemala.

Anderson and Roberts (1932) also reported success with grafting and emphasised the advantage that a large ulcer or a small ulcer took about the same time to heal. When the graft took after surgery the average hospital stay was 14.9 days. Of 154 cases grafted, 103 grafts were completely successful. In 25 out of the 154 cases, more than half the graft took and only in 7 cases the graft failed.

In Uganda where tropical ulcers occurred commonly, Nelson and Semambo (1956) described a form of surgery which could be performed by an itinerant team of health workers. They excised the ulcer under spinal anaesthesia then grafted the area with a split skin graft. Patients received penicillin for a week before surgery and a week after. They emphasised that extended trials of other topical therapies were unnecessary and whenever possible, excision and grafting should be carried out. Their results were impressive (72% cure rate). Surgery was mainly indicated for patients with chronic ulcers. Surgery cut down the number of visits to the health centres. Patients who had been house bound for up to ten years with crippling ulcers, which extended to bone and caused gross deformity, were given a new lease of life after surgery. They also found that patients could be grafted at the health clinics and did not have to be admitted to the central hospital.

## Management of tropical ulcer

Over the years various treatments have been recommended for tropical ulcers. Prevention of these ulcers was regarded as the most important aspect of therapy. Charters (1943) recommended protection of the feet and legs with boots and trousers. This suggestion has to be taken in context with the prevalence of tropical ulcers in the area studied. For instance a report by Capt. Fyvie, Medical officer to the East African unit, stated that his troops wore only sandals with no socks for six weeks yet none of the men developed an ulcer despite several episodes of trauma (Charters 1947). Therefore it seems clear that trauma does not necessarily lead to tropical ulcers. An additional factor such as infection is possibly necessary.

### Topical therapy

Before the development of penicillin a topical treatment with tar was common (Anderson and Roberts 1932). Topical therapy with dilute solutions of hydrochloric acid, iodine or potassium permanganate was often recommended (Apostolides, 1922). The same author also used topical antiseptics before removal of the slough from the ulcers daily. Other forms of topical therapy included cod liver oil dressings (Corkill 1939), magnesium sulphate and glycerine paste or copper sulphate (Earle 1942). Jarvis (1945) reported the beneficial effect of eusol for the initial stages of an acute ulcer which contained a large amount of slough. As the lesions cleared he found acriflavine more suitable.

Marsh and Wilson (1945) described the "lock-up " treatment for tropical ulcers. They used a variety of topical applications such as bismuth, whale oil, vaseline and zinc oxide. The ulcers were measured, washed or powdered and a layer of ointment applied to the ulcer. After a layer of vaseline was applied the leg was bandaged in a plaster cast. Ulcers healed in 7 weeks. They found that the rate of healing was proportional to the size of the ulcer, the site and, in their opinion, the dressing applied. A topical application of BIPP (bismuth, iodoform and paraffin) or ZIPP which contained zinc oxide instead of bismuth seemed the most effective for healing the ulcers. However Earle (1942) reported no benefit from systemic bismuth unless syphilis coexisted. Jarvis (1945) confirmed the plaster of Paris cast method of treatment in Kenya. He suggested that if patients walked on the cast they could be restrained from doing so by incorporating the bottom bar of the bed into the plaster!

Lasbrey (1952) reported the beneficial effect of topical aureomycin to tropical ulcers. The ointment was placed on the ulcer with a piece of gauze. Dressings were changed every five days. He observed a good response to this form of therapy which was cheap and easy to apply. Local treatments used by populations where ulcers are frequent include banana leaves, extract of the manchineel tree and saliva (Earle 1942).

Earle (1942) suggested that rest was invaluable for healing ulcers and observed that absolute bed rest decreased the healing time for tropical ulcers by half.

## Systemic therapy

Intravenous neosalvarsan was recommended for severe ulcers (Apostolides 1922). Earle (1942) reported success with sulphonamides. His results were subsequently confirmed by Dostrovsky and Sagher (1943). In 1946 Webb reported that penicillin was the treatment of choice for tropical ulcers. Up until then no systemic agent was uniformly successful for their treatment but ulcers healed rapidly with penicillin.

Later similar reports on the response of ulcers to penicillin were documented. Penicillin was used "experimentally" in a patient in Iran in 1947 (Pinkerton). After 100,000 units of penicillin administered intramuscularly daily for only three days there was significant healing. Subsequently the author used penicillin on further cases for up to five days with a beneficial effect. Not all patients responded though. One of the patients had a temporary recovery but the infection then spread up the leg and the patient required surgery. Smears showed fusiform bacilli and spirochaetes so the ulcers may well have been tropical ulcers which extended rather more than is usual.

In 1950 Ampofo and Findlay reported the successful use of oral aureomycin for tropical ulcers at a dose of 250 mg 4-6 times a day. Treatment continued for 4 to 7 days. Within 72 hours there was improvement and organisms were no longer visible on smears from these ulcers. In some cases no organisms were visible after just 24 hours. They claimed that penicillin only worked if administered in a large oral dose and found that aureomycin in a dose of 750 mg daily for three days was as effective as penicillin in large doses.

Lindner and Adeniyi-Jones (1968) reported successful results with oral metronidazole. Yesudian et al (1979) also administered metronidazole orally. The patients received 400 mg three times a day. Within 48 hours patients reported relief from pain. All ulcers healed in two weeks. They concluded that metronidazole was very effective for tropical ulcers.

### Surgery

Surgery has often been advocated for tropical ulcers. Indications included:

- ulcers which failed to respond satisfactorily to topical therapy
- necrotic tissue which required surgical removal (James 1932)
- progressive disease where local surgery failed to halt the disease process. Then amputation was performed (Apostolides 1922).

Braithwaite (1930) treated the ulcers with curettage under general anaesthetic. He curetted the area and cauterised it with pure carbolic acid. Healthy granulation tissue formed in fourteen days. If a large area was curetted he grafted the defect. Jarvis (1945) also operated on some patients with tropical ulcers and his indications for surgery were similar to those described above. He observed that if he scraped the ulcer base it was sufficient to promote healing. Where bone or tendon necrosis developed, surgery allowed more rapid healing of the ulcer.

Anderson and Roberts (1932) also reported success with grafting and emphasised the advantage that a large ulcer or a small ulcer took about the same time to heal. When the graft took after surgery the average hospital stay was 14.9 days. Of 154 cases grafted, 103 grafts were completely successful. In 25 out of the 154 cases, more than half the graft took and only in 7 cases the graft failed.

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## CURRENT STATE OF THE PROBLEM

Tropical ulcer has been reported worldwide. Though much of the data is anecdotal it is clear that children and young adults are often affected and the sequelae may lead to long term incapacity due to contractures, bony exostoses and squamous cell carcinoma.

Tropical ulcer still affects many children in the tropics. The morbidity from these lesions is considerable as patients may have recurrent ulcers over many years. Many people are incapacitated by crippling ulceration of the legs and are unable to work. In many instances the patients previously worked as subsistence farmers which involved heavy manual labour. The presence of leg ulcers limited their normal activities. Verbal reports from health workers in various tropical areas suggest the disease is still endemic in certain areas. This prompted the study into this disease.

In the Gambia for instance ulcers occur frequently among the rural population. Therapy is not easily available to all because of the lack of treatment facilities in the rural areas. In some clinics run by voluntary organisations, penicillin may be available but, needles and syringes for administration are in short supply. Most of the reports on the role of malnutrition and infection in the aetiology of tropical ulcer are anecdotal, and few compared the results in the patient population with those of a control population in the same area. For this reason these factors were investigated to obtain objective data on the nutritional status of patients.

Although several reports allude to the fuso-spirochaetal nature of the disease the fusiform bacilli have not been identified or characterized. This is probably due to the lack of good anaerobic techniques which have only recently become available. These bacteria are fastidious anaerobes which require detailed anaerobic microbiology for their isolation. Also well-equipped laboratories are rarely found in the areas where tropical ulcers are common. For this reason samples for bacteriological investigation need to be transported over large distances. Anaerobic bacteria do not survive unless maintained under anaerobic conditions. The role of these bacteria in the pathogenesis of the disease has not yet been assessed. Experimental infections were largely based on Gram-stain appearances. An in-vivo model to demonstrate synergy between the bacteria isolated from these ulcers was required to determine the pathogenicity of the bacteria.

There are no data on the host response to the mixed infection with fusiform bacilli and spirochaetes. The reason recurrent ulcers occur has not been previously investigated. This study was therefore performed to objectively assess as many of these parameters as possible.

## MATERIALS AND METHODS

### Epidemiology

Patients with tropical ulcers were seen in five countries where tropical ulcers were said to be frequent namely Zambia, the Gambia, southern India, Fiji and Papua New Guinea. (Patients were seen by B.A. in Gambia and Fiji). The study took place between April 1984 and April 1986. Consultations took place at hospitals, rural clinics, health centres and villages. Details of the personal and family history were obtained through the consultations as well as details of the socio-economic conditions prevalent in the area. Dr. Robinson and I designed a questionnaire to use throughout the study. The questionnaire is included in Appendix 1a.

The following information was obtained:

- age of patient
- occupation or parent's occupation
- duration of ulcer
- history of previous ulcers
- relation of ulcer to trauma
- number of persons affected in the household

In the Gambia patients were seen at the out-patient clinics of two large hospitals. These were at the Medical Research Council centre in Fajara and the local government hospital situated in the capital Banjul. In the rural areas patients were seen at the mission hospital clinics at Sabayinda and Marakissa as well as the Medical Research Council stations based at Farafenny and Kenaba. Village visits were arranged at Sabayinda and Bassey which are inland villages situated along the main river.

In Zambia patients were seen at the hospital in the Eastern Province at Katete and at rural clinics in a further six provinces served by the Zambian Flying Doctor service. On a few occasions home visits were carried out in the villages.

In Papua New Guinea patients were seen at the mission hospital clinics at Fatima and Sissano as well as the refugee camps at Kamporotoro on the Western border of West Sepik.

In Fiji patients were sought in the hospital out-patient clinics in Suva, Lotaka, Nadi, Savusavu, and at local schools and villages throughout the main island and Viti Levu which is one of the smaller islands. Many health worker clinics were visited in the rural areas.

In southern India (Madurai) patients were seen at hospitals on both sides of the city. Patients were also sought in the tea plantations and at the Karagiri leprosy centre.

The age of the patient with a tropical ulcer was recorded. When patients were uncertain of their age, every effort was made to try and obtain the details of the age. For example by recollection of past events or other circumstantial evidence of the age was sought so that the data (particularly the anthropometric data) could be interpreted. Details of any previous ulcers were recorded. This included the age at which a previous ulcer developed and the duration of any previous ulcers.

A household was defined as those who normally eat together. i.e. two wives in separate huts count as two households. Children away at school were included. The main activity of the patient was described in detail. e.g. desk worker, outdoor worker, farm labourer etc.

Most patients attended the clinics specifically for treatment of their ulcers although some were referred by health visitors because of the research project. Most patients were English speaking in the Gambia, Papua New Guinea and Fiji but whenever necessary information was obtained via an interpreter who was well versed in both English and the local language.

### Clinical manifestations of tropical ulcer

The number of ulcers present at the time of examination were documented. All ulcers were represented on the questionnaire by a drawing which indicated the site, size and presence of associated features such as cellulitis, gangrene or lymphadenopathy. The skin was also examined for evidence of previous ulceration which could be recognised by scars. Other concurrent superficial skin infections such as impetigo, ecthyma, scabies and ringworm were recorded on the questionnaire. All tropical ulcer patients had their teeth examined for dental caries. An age and sex matched control population was evaluated in a similar manner in each location. The controls were persons seen in the same clinics as the patients but who attended for other unrelated conditions, or they were inhabitants of the same village. All ulcers investigated in the survey were photographed. Where multiple ulcers occurred the most recent ulcer was included for bacteriological and histological assessment in the study. The older ulcers were documented and included in the diagram.

### Differential diagnosis of tropical ulcer

Tropical ulcer may resemble ecthyma, leishmaniasis, sickle cell ulcers, Buruli ulcers, cutaneous diphtheritic ulcers and yaws, where the latter still occurs. Prior to the breakdown of the ulcer the swelling may resemble an area of folliculitis or furunculosis.

## Ecthyma

Ecthyma is a pyogenic infection caused by streptococci or staphylococci. The infection usually follows an insect bite, an abrasion or a laceration. In the early stages of the infection the ulcer is superficial and may resemble the early lesion of a tropical ulcer. Ecthyma may occur at identical sites to those of tropical ulcer. i.e the leg, foot or thigh. The lesions differ clinically in that crust formation is the hallmark of ecthyma whereas crusts rarely occur in tropical ulcer. Ecthyma lesions are usually not as deep nor as large as tropical ulcers. The former are usually 0.5 - 2 centimetres in diameter whereas the latter are often at least a few centimetres in diameter. The natural history of the two diseases is also different. Ecthyma usually settles in a few days or at the outside a few weeks. Once the crusts are removed the lesions heal well although scars are common. Tropical ulcers persist longer. Usually several weeks or even months. The histology of ecthyma shows that the infection extends to the upper dermis and rarely affects the reticular dermis. Gram stains show gram-positive cocci on the surface of the lesion. Bacteriological culture of lesions almost always yields group A streptococci or less frequently Staphylococcus aureus .

## Leishmaniasis

A range of lesions may occur in leishmaniasis. The surface of the lesions may crust, but often indurated, granulomatous lesions are present together with ulcers. In the early stages the clinical appearance of the papular lesion of leishmaniasis may appear almost identical to the early lesion of a tropical ulcer. The early papular lesions of both leishmaniasis and tropical ulcer may progress to become pustular. Lesions of leishmaniasis tend to occur on the trunk, arms, upper legs or face and are less frequent on the lower legs. The subsequent course of the two diseases usually distinguishes the two conditions. The granulomatous lesions of leishmaniasis develop gradually whereas the papule of the early tropical ulcer extends very rapidly over a few days to become an ulcer.

The two lesions differ histologically. Leishmaniasis shows the Leishman-Donovan bodies, with a chronic inflammatory infiltrate in the dermis. The infiltrate consists of histiocytes and lymphocytes. The overlying epidermis is acanthotic. The infiltrate may contain histiocytic granulomas and giant cells. A Giemsa stain readily shows the organisms in early lesions whereas they are rarely observed in older lesions. On the other hand tropical ulcer shows true ulceration with a mixed dermal inflammatory infiltrate.

### Sickle cell ulcers

Leg ulcers in sickle cell disease cause considerable morbidity to the affected population. The ulcers usually start after the age of 10 years with peak age of between 10 and 20 years. Sickle cell ulcers rarely occur for the first time after this age. The age range for patients affected with tropical or sickle cell ulcers is very similar. Clinically there are both similarities and differences between the two types of ulcers. Sickle cell ulcers which may be of two types (spontaneous or traumatic in origin) almost always affect the lower third of the leg around the malleoli. They are virtually never seen above the middle third of the lower leg. Tropical ulcers may be found on any part of the lower leg, the foot or the thigh, or less commonly on the upper limbs. Both lesions are painful initially. Epidermal involvement results in necrosis and ulceration and follows dermal necrosis a few days after the onset of clinical symptoms. On the other hand tropical ulcers start with a very superficial papule and clearly involve the epidermis at the onset. The two conditions differ in their geographical distribution. Whereas sickle cell ulcers are the commonest form of leg ulcers in children in the West Indies, tropical ulcers are rarely seen in that area. In West Africa on the other hand tropical ulcers are more common than sickle cell ulcers. The clinical course of the ulcers differs in the two diseases. Sickle cell ulcers are more persistent and respond poorly to systemic antibiotics whereas tropical ulcers respond rapidly to the early administration of antibiotics. This is probably due to the different pathogenic mechanisms involved in the two ulcers. Sickle cell ulcers follow infarction of the skin due to sludging of red blood cells in the vessels.

Histologically sickle cell ulcers shows endothelial cell proliferation in the small arterioles whereas tropical ulcers do not show any histological vascular abnormality. The bacteriology of sickle cell and tropical ulcers differ. MacFarlane (1986) reported that in sickle cell ulcers, cultures yielded predominantly aerobic bacteria although anaerobic cultures were also performed. They found that S. aureus , Pseudomonas aeruginosa , and b-haemolytic streptococci were most commonly isolated from swabs. In only 8 out of 80 ulcers Corynebacterium diphtheriae was isolated and 4 of the strains were toxigenic. Fusiform bacilli and spirochaetes are usually seen on the smears of tropical ulcers.

#### Buruli ulcers

These ulcers are caused by Mycobacterium ulcerans and usually occur in young children. The population of patients affected is similar to those with tropical ulcer. The lesions differ clinically and histologically from tropical ulcer. In Buruli ulcers, the subcutaneous tissues are primarily affected. Epidermal and dermal involvement follow secondary to this. Initially the lesions are painful as in tropical ulcer but there is no papular or pustular lesion to start with. Buruli ulcers extend rapidly, may become circumferential and cause massive tissue necrosis as they extend. The ulcers are large and may reach several centimetres in diameter. Buruli ulcers are deeply undermined and one may probe many centimetres under the lip of the ulcer. They are also irregular.

Although tropical ulcers also extend rapidly they are very rarely more than 3 or 4 centimetres in diameter. The ulceration extends from the epidermis to the dermis and rarely affects the subcutaneous tissues. The histology of Buruli ulcers shows extensive subcutaneous tissue necrosis with extension to the overlying dermis and epidermis. A Ziehl-Neelsen stain shows the causative organisms in those areas.

Other conditions which may be confused with tropical ulcer include;

- a) varicose ulcer in the older patient
- b) syphilitic gumma
- c) primary or tertiary yaws

## Nutritional status of tropical ulcer patients

The nutritional status of the patients was assessed objectively to:

- a) identify whether or not the patients with tropical ulcers were malnourished and
- b) assess whether malnutrition contributed to the aetiology of tropical ulcer.

The study did not aim to eradicate malnutrition, nor to put into effect measures to maintain good nutrition subsequently. To this end a detailed dietary history was obtained. Particular emphasis was placed on the consumption of first class proteins at the time of onset of the ulcer. The availability of food, particularly proteins, throughout the year and the type of food normally consumed was documented. The overall clinical appearance of the patients was recorded as well as any specific signs of overt malnutrition such as follicular keratoses, glossitis and angular stomatitis.

Information about the socio-economic and cultural background of the groups was recorded. Details were obtained on the food habits, the cooking practises and the beliefs and taboos of the population in the area studied. The information was obtained from the patients, their parents or the local health workers. The information provided an overall idea of the agricultural methods, predominance of cash crops, food imports or exports and the purchasing power of the local population. During the consultations information was obtained regarding distribution and storage of food, especially during the lean months.

The most appropriate techniques were mandatory for a survey on clinical nutrition so that flaws in the design or interpretation of the results were minimised. Standardization of survey methods have been emphasised and with this in mind the patients were evaluated using the criteria recommended (WHO tech. report 1977).

All patients were examined and their weight (kg) and height (metres) recorded. The nutritional status of the patients was assessed with the formula  $\text{Weight} / \text{Height}^2$ . This is often referred to as the "Quetelet's index" after an early proponent but is now referred to as the "body mass index". This correlates well with skin fold thickness (Thomas et al 1976). The method provided a continuous quantitative scale for relative weight which facilitated comparisons of individuals with population norms. Measurements of nutrition which give an indication of acute or chronic malnutrition were applied to the same patients to see whether the results showed any discrepancy. These included weight for height and height for age (Rao and Singh 1970, Waterlow 1972).

### Treatment

Patients were questioned about the treatment of their ulcers, particularly whether or not traditional medication was used and whether saliva was placed on the ulcer. Patients were also asked at what stage of the ulceration they sought medical attention, whether medical therapy was sought for previous ulcers and how often they could attend clinics for treatment.

## Role of Infection

### Bacteriology

#### Preparation of equipment prior to field trips

Before each field trip, 0.25% Ringers solution was mixed together with 0.25% peptone water (Oxoid, Basingstoke, U.K.) (peptone-Ringers) and dispensed into 5ml glass Bijoux. They were then autoclaved at 121<sup>0</sup>C for 15 minutes. This was used to disperse the exudate/pus collected from the ulcers.

Cotton wool swabs were prepared using non absorbent cotton wool on an orange stick. Groups of four swabs were wrapped in foil and then autoclaved at 121<sup>0</sup>C for 15 min.

Transport medium to convey the bacteriological specimens was prepared at the London School of Hygiene and Tropical Medicine. Pre-reduced anaerobically sterilised (PRAS) peptone yeast extract (PYG) agar and broth were prepared by a method modified from the Anaerobe Laboratory Manual (Holdeman, Moore and Cato 1977) and dispensed into Hungate tubes (Macey 1972). Hungate tubes were supplied by Bellco Glass, Vinelands, New Jersey, U.S.A. The tubes consisted of a glass tube (8" in length and 1/2" in diameter), a butyl rubber stopper and a screw cap (figure 3). Once the tubes were filled with medium, they were closed with the butyl rubber stopper, and the system was then held in place by means of the screw cap. The advantage of this system was that the medium could be pre-reduced to provide the necessary anaerobic conditions.

After the tubes were closed, anaerobic conditions were maintained until the tubes were opened. The self-sealing rubber stopper allowed materials to be introduced or removed with a needle and syringe without causing disruption to the anaerobic conditions within the tube. The tubes also served as individual anaerobic culture systems which could be used in place of anaerobic jars.

The formula used for the transport fluid is listed in Appendix 1b. Sodium sulphoxalate (0.03%) was added to the fluid to increase the anaerobic conditions.

#### Method for preparation of transport fluid

The salt solution was prepared and stored at 4°C until use. A suitable flask (just slightly larger than the volume of the ingredients) was chosen to allow only a small amount of space between the top of the fluid and the top of the flask. An open rubber stopper and a plastic bottle with a hole cut into the top to allow steam to escape, served as a chimney. This ensured minimal loss of fluid during the boiling stage.

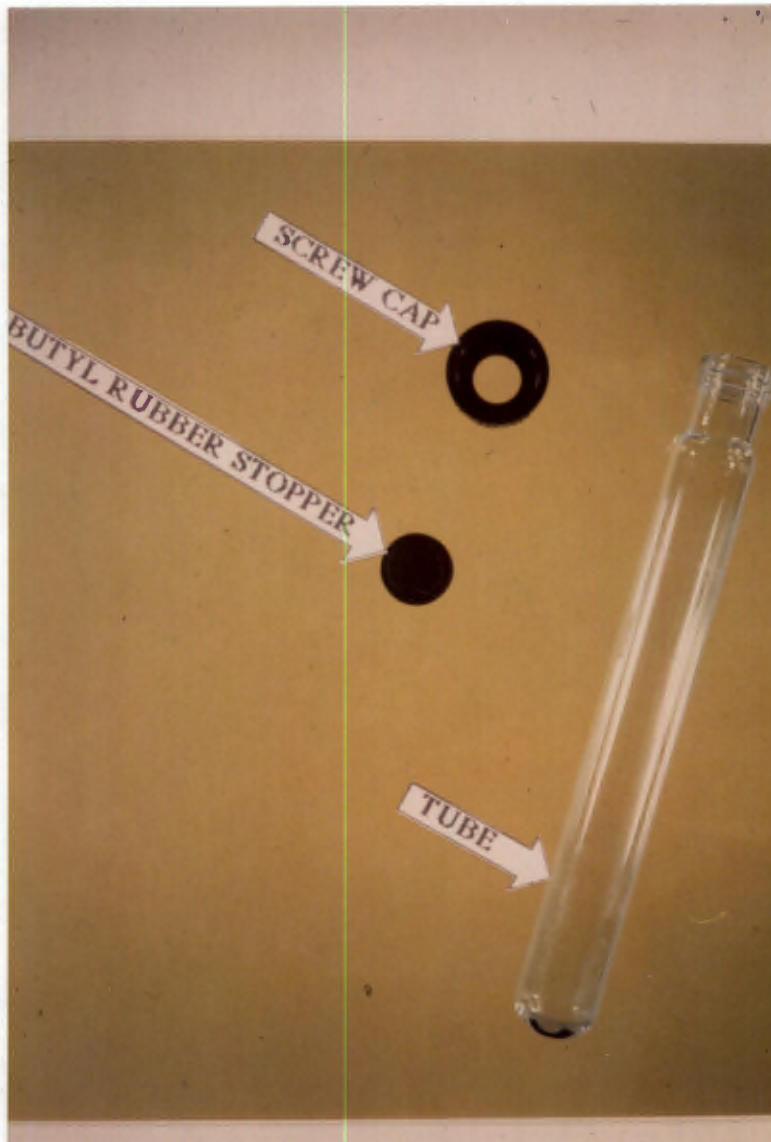


Fig. 3. Parts of a Hungate tube

The peptone, rezazurin, trypticase, yeast extract, sodium sulphoxalate and distilled water were weighed or measured and boiled under oxygen free carbon dioxide (British Oxygen Special Gases Division, U.K.). The gas was delivered through one of the cannulae from a multi-pronged delivery system (Don Whitley, Shipley, U. K.) attached to the cylinder of oxygen free carbon dioxide. The other cannulae supplied gas to four Hungate tubes (figure 4). The medium was boiled until the rezazurin Eh indicator changed the colour of the fluid from pink to colourless. After reaching boiling point, the medium was cooled while oxygen free carbon dioxide continuously bubbled through the medium. Cysteine was added to the cooled medium to provide a further reduction of the Eh. Thereafter haemin and vitamin K were added. The medium was allowed to cool further under a constant stream of oxygen free gas.

While the medium was cooling the individual Hungate tubes were flushed with oxygen free gas (fig. 5). When the medium cooled sufficiently, 10 ml aliquots of the medium were transferred with a glass pipette from the flask to each individual tube. (Adequate cooling of the medium prevented the formation of a vacuum. If the medium was transferred to the tubes while still warm a vacuum could have occurred and air drawn into the tubes when they were opened). The tubes were then sealed with the butyl rubber stopper and screw cap, autoclaved at 121°C for 15 minutes and were then ready for use (fig. 6) The agar tubes contained the same ingredients as listed above together with 2% Davis agar which was added during the boiling stage.

### Collection of specimens: Sampling procedure

Swabs were taken from all the ulcers investigated. Where multiple ulcers occurred, the most recent ulcer was included for bacteriological assessment. Sterile cotton wool swabs were used to take the swabs taken from the base of the ulcers, beneath the advancing edge. The early pre-ulcerative papules were deroofed and the swabs were taken from the base of the centre of the papule. The swabs were then placed in the sterile peptone - Ringers solution and vigorously stirred. An aliquot of the inoculated solution was aspirated with a 26 gauge needle and a 2 ml syringe. All the air in the syringe was expelled. The sample was then inoculated into a Hungate tube with Peptone yeast glucose broth. A further 2 ml aliquot of the inoculated transport fluid was then aspirated and inoculated into a Hungate tube with molten Peptone yeast glucose agar at 45°C. All the inoculations were done through the self-sealing butyl rubber stopper of the tubes after the top was swabbed with 70% alcohol. The broth tubes were then stored at room temperature due to lack of storage facilities. The agar tubes were rapidly rotated to simulate the formation of roll tubes, as described previously in the Anaerobe Laboratory Manual (1977), and stored at room temperature. The tubes contained rezazurin, as the Eh indicator, which caused the straw coloured medium to change to a pink colour as soon as anaerobic conditions were lost. This alerted one to the loss of anaerobiasis (figure 7).

Mud samples were collected from areas where patients with tropical ulcer lived. The same technique was used to introduce the mud into the Hungate tubes. Only broth tubes were inoculated with mud. The samples were stored in the same way as the clinical specimens.

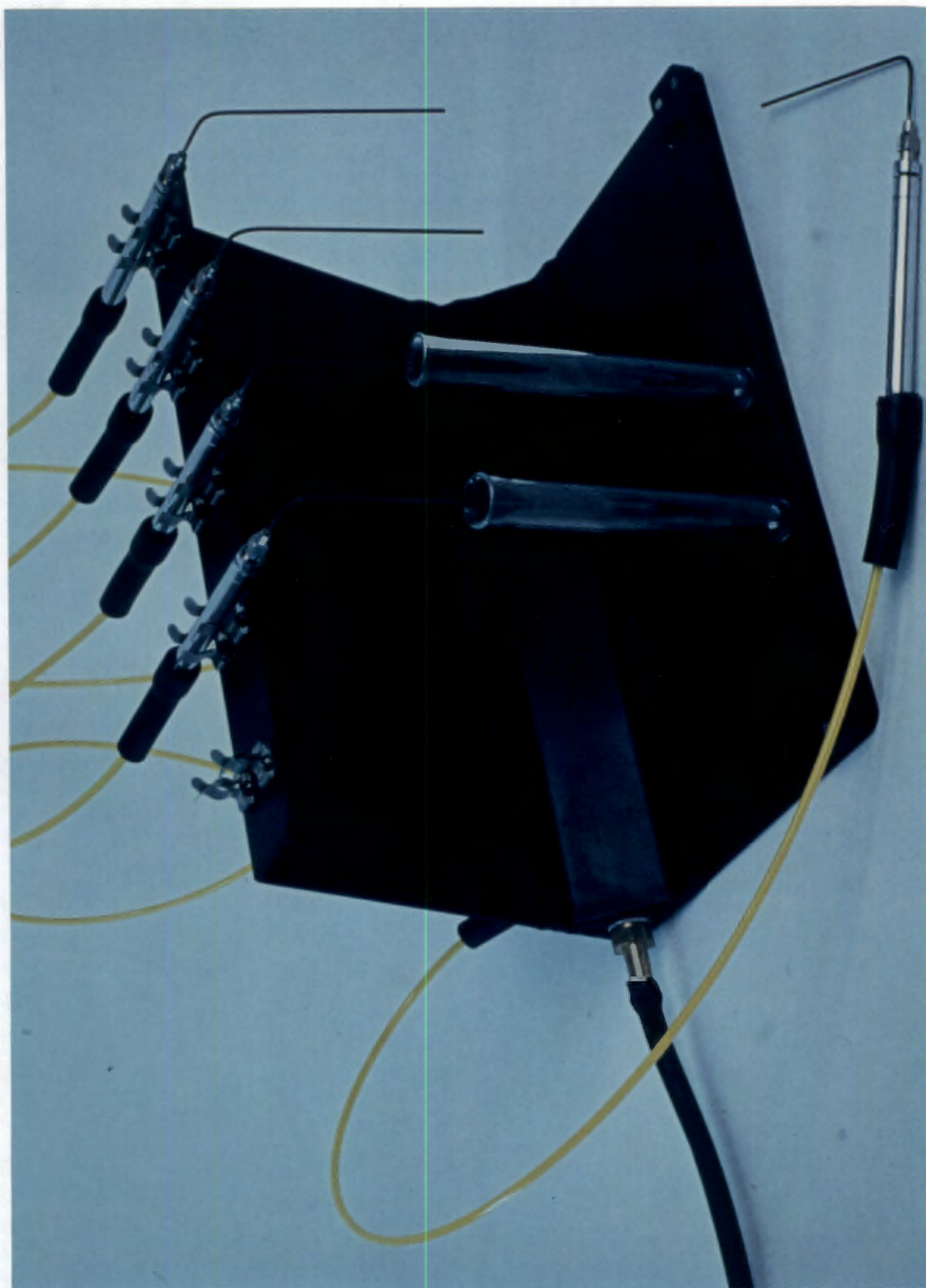


Fig. 4. Method for preparation of transport medium

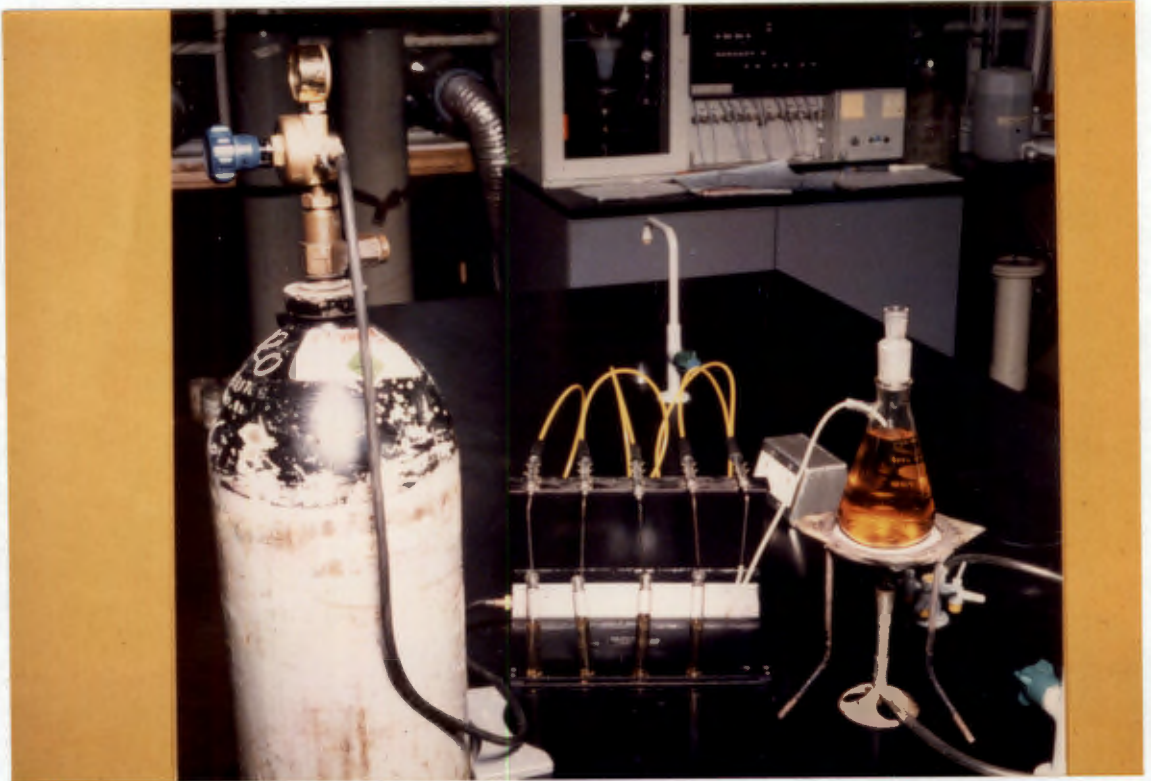


Fig. 5. Cannulae delivering oxygen free gas to Hungate tube

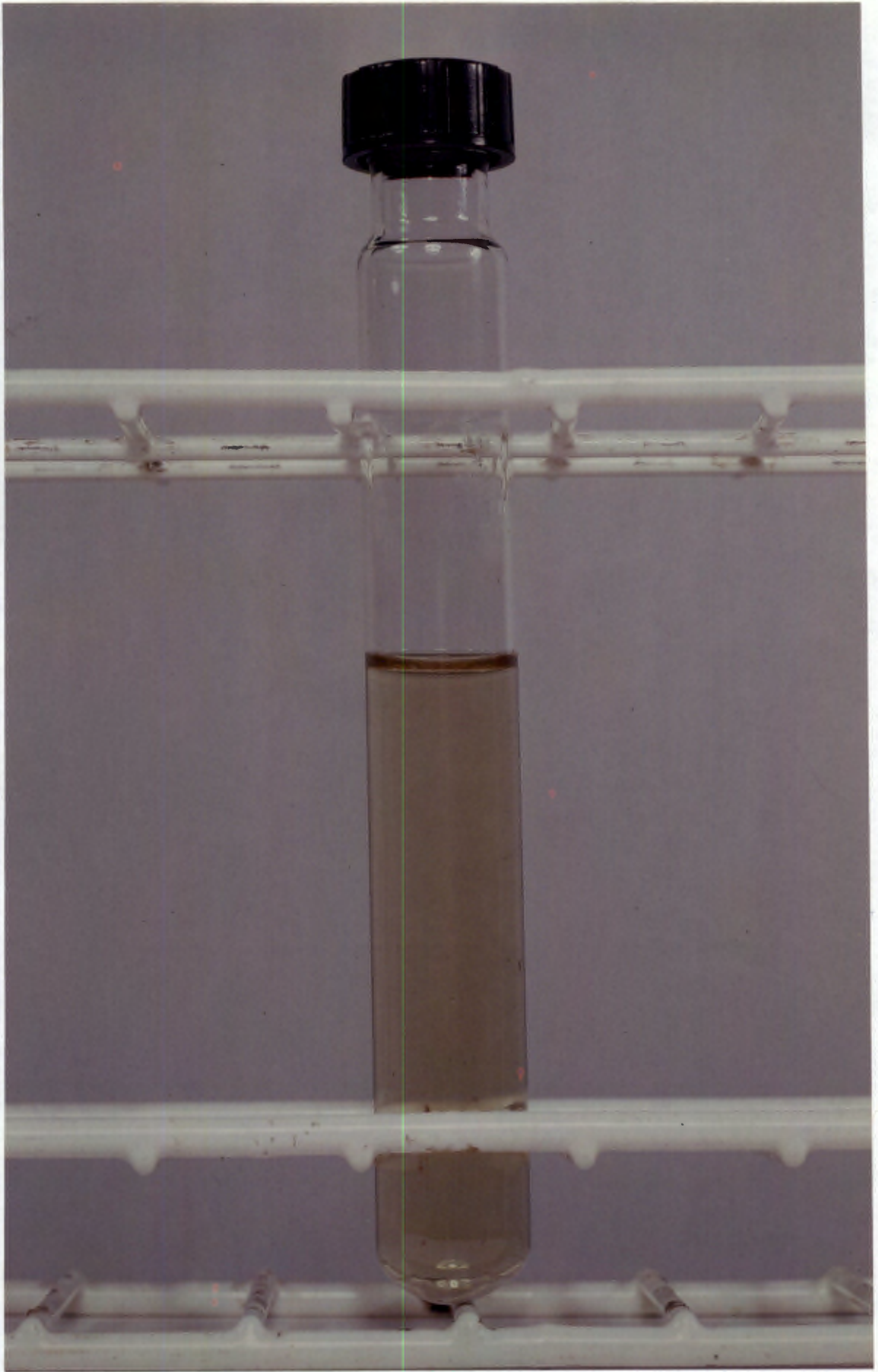


Fig. 6. Completed Hungate tube with anaerobic transport medium

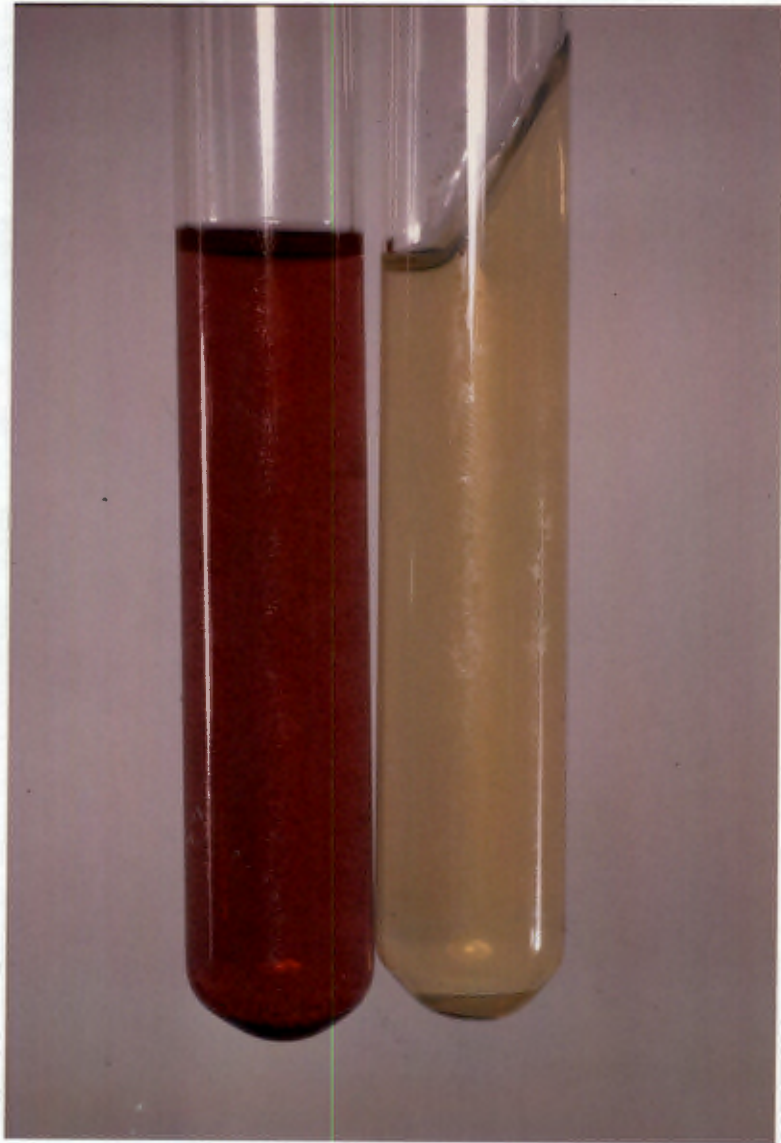


Fig. 7. Hungate tube showing loss of anaerobiosis

### Skin biopsies for culture

Skin biopsies were obtained from as many patients as possible. Verbal consent for the biopsy procedure was obtained from the patients or their relatives if they were under the age of consent. Biopsies were taken from the outer edge of the ulcer. After the area for biopsy was cleaned with an aqueous solution of Savlon (I.C.I., Macclesfield, U.K.) the area was infiltrated with 2% lignocaine with adrenalin (Astra, King's Langley, U.K.) which was administered with a 26 - gauge needle and 2ml syringe. The biopsy specimen incorporated a few millimetres of normal skin. A 5 mm disposable punch (Stiefel, High Wycombe, U.K.) was used in most cases. A few elliptical biopsies were performed using a no. 15 blade and scalpel (Gillete, Isleworth, U.K.). The wounds were closed with 3.0 silk suture material (Ethicon, U.K.) and the sutures removed a week later. Half the biopsy material was homogenised with a glass homogeniser (Gallenkamp, Loughborough, U.K.) and dispersed in the peptone-Ringers solution. The inoculum was aspirated with a 2ml syringe and 18 gauge needle. All the air was expelled from the syringe and the contents inoculated into a Hungate tube with Peptone yeast broth as described previously. The samples were left at room temperature.

### Processing of specimens

Bacteriological specimens were transported to London from the field and cultured under aerobic and anaerobic conditions. All the Hungate tubes were opened and the plates inoculated in an anaerobic cabinet (Don Whitley, Shipley, U.K.). Each sample of transport medium was inoculated onto a set of plates as shown in table 3.

The plates were inoculated mechanically with transport medium by means of a spiral plater (spiral systems Inc 6740 Clough Pike, Cincinnati). This method facilitated the recovery of individual colonies as a very small aliquot of medium was dispensed.

Two sterile plastic disposable cups were filled with 70% alcohol and sterile water respectively. These were used to clean the stylus of the spiral plater. An aliquot of transport medium was aspirated from each of the Hungate tubes and placed in a sterile plastic disposable cup. A volume of fifty microlitres of the inoculated transport broth was aspirated by vacuum. After the dispensing stylus was lowered onto the surface of the agar plates, the plates were mechanically inoculated. The stylus was cleaned with alcohol and water between inoculations. After the plates were inoculated, transport fluid was streaked onto a non-selective plate with a plastic disposable loop (Don Whitley, Shipley, U.K.). A 5 ug metronidazole disc (May and Baker, Dagenham, U.K.) was placed on each of the non-selective plates. The plates for anaerobic incubation were incubated at 37°C for up to five days in an anaerobic cabinet filled with 85% nitrogen, 5% carbon dioxide and 10% hydrogen.

Those plates which were incubated under CO<sub>2</sub> were transferred from the cabinet to an incubator set at 37°C and supplied with 5-7% CO<sub>2</sub>. Plates were examined at 24 and 48 hours.

All those colonies which grew in an anaerobic environment, were sensitive to metronidazole or which produced no comparable growth on Columbia blood agar under aerobic conditions were regarded as obligate anaerobes. These colonies were picked out and streaked for single colonies onto a BHI non-selective plate.

Their colonial morphology and staining characteristics were examined after 48 hours and 5 days growth on the non-selective plate. All the anaerobic Gram-negative non-sporing rods were further tested with various antibiotics described below.

The Hungate tubes which contained agar transport medium were also opened in the anaerobic cabinet. The individual colonies which had grown in the agar by then were "picked out" with a sterile pasteur pipette and plated onto non-selective plates for aerobic and anaerobic incubation. The plates which were incubated anaerobically included a 5 ug metronidazole disc.

Table 3. Bacterial culture media

- 1) Brain Heart Infusion Agar (BHI) plates (Difco, East Molesey, U.K.) supplemented with cysteine, hemin, vitamin K (Holdeman, Moore and Cato 1977) and 10% horse blood. This served as a non-selective general purpose medium for comparison of growth on antibiotic - containing media. This plate will be referred to as supplemented BHI plate.
- 2) Supplemented BHI agar with 10% horse blood and crystal violet 1:1000 for the isolation of fusobacteria (Ninomiya, 1972).
- 3) Supplemented BHI agar with 10% horse blood and rifampicin 7.5 ugr/ml for the isolation of fusobacteria.
- 4) Supplemented BHI agar with 10% horse blood and kanamycin 1000 ugr/ml and vancomycin 7.5 ugr/ml for the isolation of Bacteroides spp.
- 5) Supplemented BHI with 100 ugr/ml nalidixic acid for Gram-positive rods.
- 6) Columbia agar with 10% horse blood (Oxoid, Basingstoke, U.K.) for the isolation of aerobic organisms.
- 7) Mac Conkey agar (Oxoid) to differentiate aerobic Gram-positive cocci and Gram-negative rods.
- 8) Sabouraud's medium (Oxoid) for isolating yeasts.

## Preparation of media

The following media were prepared for the isolation and identification of bacteria:

For aerobic incubation:

- 1) Columbia blood agar with 10% horse blood
- 2) Mac Conkey agar
- 3) Sabourauds medium

Anaerobic incubation:

- 1) Brain heart infusion agar supplemented with 10% horse blood  
cysteine  
vitamin K  
hemin
- 2) Fastidious anaerobe agar supplemented with 10% horse blood
- 3) Supplemented BHI with vancomycin 7.5 ug/ml and kanamycin  
1000 ug / ml
- 4) Supplemented BHI with rifampicin 7.5ug/ml
- 5) Supplemented BHI with crystal violet 1:1000
- 6) Supplemented BHI with 2% oxgall (Oxoid).

For all the above media, BHI and Fastidious anaerobe agar (FABA)(Lab M, Salford, U.K.) were prepared according to the manufacturers instructions. All antibiotics were filter sterilised. The medium was autoclaved and cooled and blood and antibiotics where indicated, were added. The crystal violet and oxgall were added prior to autoclaving the media. Media were poured into standard disposable plastic petri dishes (Sterilin, U.K.).

Several selective media were used to facilitate isolation of the various bacteria from the swabs. These are included in table 4.

Table 4. Selective media for the isolation of organisms

Media	Isolates
Air with 5% CO <sub>2</sub>	
1. Columbia blood agar (CBA)	Staphylococci, Streptococci, Proteus & Pseudomonas spp.
2. CBA with netilmicin	All of above except Proteus
3. Sabouraud's medium	Yeasts
Anaerobic incubation	
1. Supp. BHI	All anaerobic cocci & bacilli Facultative anaerobes
2. Supp. BHI + rifampicin	Fusobacteria
3. Supp. BHI with kanamycin and vancomycin	Bacteroides
4. Supp. BHI with crystal violet	Fusobacteria
5. NOS media with rifampicin	Spirochaetes
6. BHI with pectin and starch	Spirochaetes
7. M10 medium	Spirochaetes
8. Kelly's medium	Spirochaetes

## Spirochaete isolation

Several methods were used to isolate spirochaetes. The ingredients of the various media are included in Appendix 1c. The first method used was described by Leschine et al in 1980. The medium was prepared as described. All the ingredients were used except the volatile free fatty acids, rabbit serum and sodium carbonate. The medium was prepared in bulk under oxygen free nitrogen, dispensed into individual pre-reduced Hungate tubes and then autoclaved. The last three ingredients were filter sterilized. Tubes were cooled and when the temperature reached about 45<sup>0</sup>C the last three ingredients were added. The ingredients were mixed, aspirated with a needle and syringe and the appropriate aliquots were dispensed carefully into each tube through the butyl rubber stoppers to prevent the introduction of air into the system. A filter sterilized solution of rifampicin was added to each tube to give a final concentration of 7.5 mg/ml. When required, the tubes were heated to just above 42<sup>0</sup>C which allowed the agar to melt. Transport fluid (0.5 ml) from each specimen was inoculated and the tubes rotated and incubated at 37<sup>0</sup>C for a week.

The second method used Brain heart infusion agar (BHIA) plates. The agar was prepared according to the manufacturers instructions and 10% horse blood was added. After the medium was boiled it was allowed to cool before the plates were poured. Once the BHIA set in the petri dishes, filter papers were placed on the surface of the plate and aliquots of transport fluid were poured onto the filter paper. The papers were sealed around the edges with autoclaved vaseline to prevent organisms from entering on the sides.

The third method used M10 agar as described in the VPI manual (1977). The medium was again prepared in bulk and dispensed into individual Hungate tubes after they were flushed with oxygen free nitrogen. Each individual tube was inoculated as described above.

Kelly's medium was prepared as described (Kelly 1971, Stoenner 1974). Individual Hungate tubes were prepared as described above.

Aliquots were taken from the transport fluid and examined under a dark ground microscope (Leitz, U.K.) for motile spirochaetes.

## Identification of bacteria

### Staphylococci

S. aureus: This organism was recognised by the presence of haemolysis around the colony and by its ability to produce coagulase as described by Cowan and Steel (1974). Those staphylococci which did not produce coagulase were regarded as coagulase negative staphylococci. Further identification was not done.

### Streptococci

All streptococci were identified by gram stain, as described by Cowan and Steel (1974), and then tested with a STREPTEX kit (Wellcome Diagnostics, Dagenham, Essex, UK). Their ability to grow anaerobically was documented as well as the presence of haemolysis around colonies. Anaerobic cocci were tested with the API 20A kit.

### Coliforms

Gram-negative facultative anaerobes were identified using the criteria described by Cowan and Steel (1974). All isolates were then tested with the API 20A kit for coliform bacteria (API Laboratories, Paris, France).

Those samples which contained Proteus species, were then grown in the presence of varying concentrations of gentamicin, tobramycin or netilmycin to inhibit the growth of Proteus. The minimal inhibitory concentration for each of these antibiotics was calculated using the method of Miles and Misra (Cruikshank, 1973).

Detailed identification of the Proteus and Pseudomonas species was not carried out.

### Biochemical tests

The following biochemical tests were performed on all Gram-negative rods:

#### 1) Catalase production:

A 10% solution of  $H_2O_2$  was added to a colony taken from the 48 hour culture of the organism. The presence of bubbles arising from the colonies indicated the presence of catalase production.

#### 2) Oxidase production:

Blotting paper strips were soaked in a freshly prepared 1% solution of tetramethyl-p-phenylene-diamine hydrochloride in distilled water. A colony of the organism to be tested was streaked onto the paper with a glass rod and the production of a purple colour was regarded as a positive result. Pseudomonas aeruginosa served as a positive control.

#### 3) Carbohydrate fermentation:

The API 20A kit (API Laboratories, Monralieu, Vercieu, France) was used for all the anaerobic gram-negative bacteria isolated. A heavy inoculum of the organism on a was collected on a cotton wool swab and dispersed in the API 20A medium supplied with the kit. Each of the tubes was filled to the required depth using a sterile pasteur pipette. Mineral oil sealed the tubes to test for indole to prevent evaporation of the indole. The completed strip was placed in the plastic holder which contained some water to prevent dessication.

The strip was closed with the plastic cover and incubated anaerobically for 48 hours in the anaerobic cabinet. A BHI purity plate was streaked at the same time. Carbohydrate fermentation caused a colour change on the medium from purple to yellow.

The API 20E system (API Laboratories, Monralieu, France) was used for the Enterobacteriaceae. The instructions of the manufacturer were adhered to and the strip was incubated aerobically for 24 hours.

### Anaerobic bacteria

Those bacteria which only grew under anaerobic conditions were regarded as obligate anaerobes. All the anaerobic Gram-negative rods were further tested for their:

- 1) antibiotic susceptibilities using the discs recommended by Duerden et al (1976);
- 2) products of metabolism by gas liquid chromatography.

The antibiotics discs contained penicillin (2µg), kanamycin (1000 µg), vancomycin (7.5 µg) and phosphamycin (300 µg).

### Chromatographic procedures for analysis of acid and alcohol products of Gram-negative anaerobic rods

One of the characteristics of anaerobic bacteria is their ability to produce typical fatty acids which can be detected by gas liquid chromatography (GLC). To detect these acids, cultures have to be acidified and extracted with ether. These ether extracts can be chromatographed. This technique was used to identify the

Gram-negative anaerobic rods isolated from the ulcers. The methods used were described in the Anaerobe Laboratory Manual (p 134). A Pye-Unicam chromatograph, series 204 (Philips, Cambridge, U.K.) was fitted with a 1.5 x 4mm glass column and packed with Chromosorb W (80-100 mesh). The chromatograph was equipped with a flame ioniser and was attached to a computer (PU 4180 computing intergrater, Phillips, U.K.). The flow rates for hydrogen, nitrogen and air were 44ml/min, 40ml/min and 400 ml/min respectively. The temperature settings for the injection port, the detector and column were 250<sup>0</sup>, 250<sup>0</sup>, and 150<sup>0</sup>C respectively. The chromatograph was cleaned with methanol initially and in between samples to clear any residual gases. Known volatile and non-volatile acids were used as standards. These were tested every day before any test samples. The retention times of these standard fatty acids were recorded daily.

#### Preparation of samples for GLC

All the anaerobic organisms isolated were grown in Brain heart infusion broth for 24-48 hours and the media analysed for the end products of metabolism by gas liquid chromatography. The samples were centrifuged at 3000 rpm for 10 minutes at 4<sup>0</sup>C. The supernatant was treated in various ways before chromatography.

1) with DUOLITE (BDH chemicals, Poole, U.K.) and centrifuged again.

A fixed amount (3ul) of the supernatant was injected into the injection port.

2) acidified and extracted with ether. A fixed amount (3ul) of supernatant was injected into the injection port.

### Calculation of fatty acids produced

The retention times of standard fatty acids were compared to those of the test samples. The amount of fatty acid produced was measured by the peak in the graph which recorded the amounts of volatile fatty acids produced. The area under the curve was quantitated by an intergrated computer and the results printed.

### Conversion of threonine to propionate

A further characteristic of fusobacteria is the conversion of threonine to propionate. All isolates of fusobacteria were grown in broth culture with and without threonine. The ether extracts were of both cultures were chromatographed and the amounts of propionate compared as described in the Anaerobe Laboratory manual (1977 p128.)

The Gram-negative anaerobic rods identified after these tests included fusobacteria and bacteroides.

### Fusobacteria

All the fusobacteria isolates were investigated in detail to determine their species. The following additional tests were done on all fusobacteria:

#### 1) Aesculin hydrolysis

A freshly prepared 0.5% w/v solution of aesculin and ferric citrate 0.05% w/v were added to Fastidious Anaerobe Agar (FABA). Blackening of the agar around the colonies after 48 hours incubation indicated hydrolysis of aesculin.

## 2) Gas production

Stab cultures were performed into freshly prepared FABA with 1% glucose in glass tubes. Immediately after a heavy suspension of the organisms was inoculated into the molten agar, the caps were replaced and the tubes incubated aerobically for 48 hours. Gas production was shown by bubbles disrupting the agar.

## 3) Hydrogen Sulphide production

This test was included in the API 20A kit and was performed according to the manufacturers instructions. A precipitate near the base of the cupule indicated H<sub>2</sub>S production.

## 4) Extracellular DNAase production

DNA ase agar (Oxoid) with 3% yeast extract was heavily seeded with the test organism. Plates were incubated anaerobically for 48 h or until adequate growth was observed. The plates were flooded with a 10% solution of hydrochloric acid. A clear zone around the colonies indicated DNAase production. Staph. aureus NCTC 6571 was used with each test as a positive control.

## 5) Casein digestion

Casein methylene blue agar was prepared to the following formula:

Brain Heart Infusion Broth (Gibco, Uxbridge, U.K.) 3.7%

Casein (BDH, Poole, U.K.) 3.0%

Yeast extract (Oxoid) 0.5%

Agar (Davis) 2.0%

Aqueous methylene blue 1% w/v.

The agar was seeded with a heavy inoculum of the test organism and incubated anaerobically for 48 hours. The plates were left on the bench until the blue colour returned and then observed for opacity around the colonies. Casein digestion occurred when the plate was flooded with a 10% solution of HCL and a clear zone appeared around the growth.

#### 6) Nitrate reduction

A filter sterilised solution of potassium nitrate was added to FABA to give a final concentration of 1%. The solid agar was streaked with the fusobacteria isolates. Nitrate A (0.8% sulphanic acid in 5N acetic acid) and Nitrate B (0.5% a-naphthylamine in 5N acetic acid) solutions were added to 48 hour cultures. A pink colour indicated nitrite ions.

#### 7) Nitrite reduction

A filter sterilised solution of sodium nitrite was added to FABA to give a final concentration of 0.001%. The presence of a pink colour as above indicated the presence of nitrite ions.

#### 8) Indole production

This test was included on the API 20A kit. The cupule was sealed with mineral oil to prevent evaporation of the indole.

#### Guanine and cytosine ratio

Dr. H. Shah (London Hospital, U.K.) measured the G+C ratios of the fusobacteria by the methods described by Owen et al (1979) and Marmur (1961).

## Soluble cellular protein electrophoresis

Different species of Fusobacterium may be identified by their biochemical reactions and also by their soluble cellular membrane proteins. All the isolates of the genus Fusobacterium which were recovered from the ulcers, were characterised by analysis of their cell membrane proteins with polyacrylamide gel electrophoresis. A strain of Streptococcus faecalis, isolated from a tropical ulcer served as an internal reference standard for all the experiments as it produced a thick band half way down the column. This allowed easy comparison of bands produced by other organisms. Bacteroides species isolated from the samples were examined in the same way.

### Method

The method used was a modification of those described by Moore et al (1980) and Laemmli (1970). The electrophoresis tank apparatus was supplied by Biorad (Watford, U.K.) The cleaned glass plates were wiped with ethanol to remove any grease and then dried thoroughly. While one of the plates lay flat on a table, a perspex spacer strip was placed at each edge. The spacer strips touched the edges at the top and bottom and abutted on the sides of the glass plate. A second plate was clamped to the first with a screw type clamp at each side which ensured that the glass plates reached as far as possible into the clamp. A rubber sealer in the grooves at the base of the plate holder provided a water tight seal.

The samples (fusobacteria, bacteroides and faecal streptococci) used were prepared as follows:

1) Isolates were cultured for 48 hours in Brain heart infusion broth supplemented with 0.1% calcium carbonate (BHI-C) (for gram negative bacteria) and BHI-C plus 0.025% tween-80 for gram positive bacteria after a gram stain of the colony confirmed a pure growth.

2) Samples were transferred to a conical centrifuge tube and centrifuged at 8000 rpm for 10 minutes at room temperature. The supernatant was discarded.

3) 0.15 gr glass beads (0.1mm diameter) (Ballotini, Stanmore, U.K.) together with 0.10 ml of 0.15 M Tris buffer at ph 7.0 + EDTA (1.02gr/L) were added to the samples.

4) Tubes were vortexed for 10 sec and placed on a shaker for 2 intervals of 2 minutes each.

5) Samples were heated in a water bath at 55<sup>0</sup>C for 5 minutes to precipitate the cell wall structures which cause smudging of the protein patterns.

6) Tubes were then centrifuged at 8000 rpm for 10 minutes.

7) Powdered sucrose equal to 1/3 volume of the supernatant was added to each tube.

### Preparation of gels

All gel solutions were prepared with autoclaved or degassed water. The ingredients used to obtain an 8.5% gel are listed in Appendix 1d.

With a pasteur pipette, the glass plates were filled with resolving gel up to a pre-marked line approximately 4 cm from the top of the plates. The acrylamide solution was layered with about 3 cm of water delivered from a Pasteur pipette and the gel was left to set for about 1 hour. When the gel had set, the layer of water was removed.

The stacking gel was added to the resolving gel with a pasteur pipette. The gel was layered up to about one inch from the top of the plates. A comb was washed with water and alcohol then dried and carefully inserted into the stacking gel to prevent entrapment of air bubbles under the comb. The stacking gel was covered with electrode buffer allowed to set (about 30min). After the stacking gel set the comb was removed and excess fluid removed. The wells were flushed with electrophoresis buffer to prevent wavy bands in the gel. The samples were added to the wells and carefully covered with electrode buffer to the top of the glass plates.

The upper buffer reservoir was placed on top of the glass plate assembly with the rubber sealers fitted in the grooves. The locks were removed from the bottom of the plates attaching them to the plate holder and the locks were placed in the holes at the top to clamp the upper reservoir to the glass plate assembly.

The above apparatus was placed into the buffer tank fitted with a water cooling system and a magnetic stirrer. The tank was filled with approximately 3.5-4 litres of electrode buffer.

Air bubbles underneath the glass plates were expelled with a modified pasteur pipette which forced buffer between the plates. The remaining electrode buffer was carefully poured into the upper reservoir tank. The safety lid was fitted and the apparatus connected to the power supply. The water cooler and magnetic stirrer were started and the apparatus set at 150 volts and 33 mA. current. The gels were allowed to run for about 5 hours. When the dye front reached the bottom of the gel, the power pack was turned off and the glass plate unit removed and dismantled. The gel was carefully removed from between the glass plates. The gel was fixed in 12% trichloroacetic acid for 30 minutes and stained in 0.08% Coomassie blue stain (Moore et al 1980). The gel was destained with 10% glacial acetic acid in water (vol/vol) until a suitable colour was obtained. Gels were transferred to "ziploc" bags for photography and storage.

## Pathology

### Preparation of fixative prior to field trips

The fixatives for the skin biopsies included 10% formal saline and periodate-lysine paraformaldehyde (PLP) (McLean and Nakane 1974) which were prepared and transported in aliquots in separate containers. Aliquots of 3% gluteraldehyde and 1 M cacodylate buffer were prepared and used as fixatives for skin biopsies obtained for electron microscopy.

### Skin biopsy technique

Skin biopsies were obtained as described previously. After the specimens were divided for bacteriologic culture the remaining half was fixed in either 10% formal saline or PLP and submitted for histology. Eight biopsies were fixed in 3% gluteraldehyde for electron microscopy.

### Processing of biopsies

#### Light microscopy

Specimens for light microscopy were embedded in paraffin. Sections (5µm) were cut and stained with haematoxylin and eosin (H&E) for routine histology. Special stains included the Giemsa, Gram, Ziehl-Neelsen, and Periodic acid Schiff (PAS) for bacteria and fungi, and the Dieterle stain for spirochaetes, as described by Pinkus and Mehregan (1981). The sections were processed at St. John's Hospital for Diseases of the Skin, London.

### Immunohistochemical stains

Skin biopsies were dewaxed, and the inflammatory infiltrate assessed with the aid of T- and B-cell markers (Dako, High Wycombe, U.K.) using the method described by Taylor (1978). Skin sections were also tested for fusobacteria as identified with an antifusobacterium antibody. Mangan and Lopatin (1983) reported that fusobacteria may stimulate B-cell formation in-vitro. This was thus investigated.

### Indirect Immunoperoxidase staining

An explanation of the method used is described below.

1) Tissue fixation may block immunoreactive sites. Enzyme pretreatment of sections "unmasked" the immunoreactive sites. This was carried out as follows:

Paraffin embedded slides were dewaxed and placed in running tap water for five minutes. Protease VII was diluted in PBS to a final concentration of 0.001% and placed on the slide for 15 minutes in a water bath at 37°C. The enzyme reaction was stopped by washing the slides under running tap water.

2) Endogenous tissue peroxidase activity was blocked by the addition of 1% hydrogen peroxide in methanol to the slide for 20 minutes. The slides were then washed in running water.

3) After the sections were wiped to remove excess PBS, 100 ul of the first antibody (antifusobacterium antiserum) was applied to the sections. The sections were incubated at room temperature for 45 min. The slides were then rinsed in PBS - tween and then in PBS.

4) The second antibody labelled with peroxidase (100ul) was applied to the sections and the slides were incubated at room temperature for 30 minutes. The slides were again rinsed in PBS, followed by distilled water.

5) Sections were washed in water.

6) Solutions A and B were prepared for Peroxidase development with;

Solution A: 2.9 mls distilled water added to  
0.1 ml Hydrogen peroxide

Solution B: 9 mls Tris buffer added to 1 ml stock DAB.

The developer contained 0.1 ml solution A which was added to solution B immediately before use.

The slides were flooded with developer and left for 5 minutes in a dark room. The reaction was stopped by placing the slides under running tap water.

7) Sections were counterstained with haematoxylin after washing with tap water.

8) A cover slip was fixed onto the slide with glycerol.

### Electron microscopy

Samples for electron microscopy were fixed in 3% glutaraldehyde in 0.1M cacodylate buffer overnight. The following day, the biopsies were transferred into 0.1M cacodylate buffer until they were processed in the department of Electron Microscopy at the London School of Hygiene and Tropical Medicine. The samples were post fixed in 1% osmium tetroxide ph 7.2, dehydrated through graded alcohols and embedded in epoxy resin Epon 812. Semithin sections of 0.5  $\mu$ m were cut on an ultramicrotome and treated with lead acetate. The sections were examined with an AE1 801 transmission electron microscope at 60 and 80 kv acceleration voltages and 50  $\mu$ m aperture (Wetherhed et al 1986). For comparison, fusobacteria, Enterobacter cloacae and Bacteroides spp. cultured in-vitro were examined in the same way.

### Haematology and Serology

#### Venous blood samples

Whenever possible venous blood samples were obtained from patients with tropical ulcers and from a control population in the same area. The blood was taken from the antecubital fossa with a 10 ml syringe and an 18 gauge needle. Haemoglobin estimation and electrophoresis were performed whenever possible. Samples were collected in an EDTA coated tube and gently rotated to prevent clotting.

A serum sample was collected from each venesection and kept in glass containers until the specimens were centrifuged (usually 4 to 6 hours later). Samples were centrifuged at 3000 rpm for five minutes.

The supernatant was aspirated with a pasteur pipette and stored at 4°C until departure. Serum samples were used to screen for anti-cardiolipin antibodies with the disposable SYFACARD system (Wellcome diagnostics, Dagenham, U.K.). The sera were diluted to a titre of 1:8 and used according to the directions of the manufacturer. More specific tests to detect antibodies to Treponemes were not used. Serum samples were also used to measure the antibody levels to the organisms commonly isolated from tropical ulcers.

#### Patient serology

Most human sera contain antibodies to Fusobacterium as these organisms are thought to be present in the alimentary tract (Hofstad 1974). The specificity of these antibodies has not yet been clearly established. Kristoffersen (1969) detected antibodies to a purified protein antigen from an oral strain of Fusobacterium. Hofstad (1974) examined human sera for antibodies which reacted with sheep erythrocytes sensitised with lipopolysaccharide purified from F. nucleatum. He found that all sera tested reacted with the polysaccharide, although when he divided the sera into those obtained from adults and children, the sera from the children contained lower titres of antibody. The antibodies were of the IgM class. This suggested that from birth, children exposed to the continuous stimulation from antigens derived from indigenous non-sporeforming, anaerobic bacteria produced antibodies to these antigens.

The bacterial antigens could have reached the tissues through the intestinal mucosa or the non keratinized epithelium of the gingival crevices.

As patients were regularly exposed to these fusobacteria, antibody production in the case of an infection would probably of the IgG class (Hofstad 1974). Patients with tropical ulcers seem to develop recurrent ulcers for several years. This suggests that they do not mount a significant antibody response to overcome the infection. IgG antibody levels were measured by immunofluorescence in patients with tropical ulcers and controls. A whole cell preparation of the species of Fusobacterium isolated from tropical ulcers served as the antigen.

#### Immunofluorescence method to detect antibodies to fusobacteria

The antigen was prepared from a 48 hour brain heart infusion broth culture of Fusobacterium. The culture was centrifuged and the supernatant discarded. The deposit was washed three times with phosphate buffered saline (PBS) and centrifuged at 10000 rpm. for 10 minutes at 4<sup>0</sup>C. A gram stain of the deposit showed there was >90% cell disruption. Glass slides were cleaned with alcohol and imprints made with a fixed prong inoculator dipped in glycerol. The glass slides were sprayed with teflon spray and allowed to dry. The glycerol was washed off with hot water and the slides dried at 37<sup>0</sup>C for 1 hour. The whole cell preparation was diluted in PBS to serial dilutions of 1:10, 1:20 and 1:40. Gram stains were performed on all these dilutions to assess the concentration of organisms present.

The most appropriate dilution was chosen for the experiments i.e. where the amount of cells was neither too small, nor too large. The slides were inoculated with the antigen using the same fixed prong inoculator. After the antigen dried on the slides in a dessicator (Polysciences Ltd., Northampton, U.K.) the slides were fixed with acetone. The slides were prepared in bulk from the same preparation and stored at  $-70^{\circ}\text{C}$  until required. This provided a standardised inoculum.

Four-fold dilutions of the patients' sera were prepared from an initial dilution of 1:16 (750 ul PBS and 50 ul serum). Six further fourfold dilutions were made by adding 250 ul of the first dilution to 750 ul PBS. This covered a range of dilutions from 1:16-1:16384. The prepared antigen slides were fixed with acetone and allowed to dry. Initially slides were also fixed with 1% hydrochloric acid to assess the effect of different fixatives.

Aliquots of 25 ul serum from each dilution were added to each well in duplicate. One well was left with only PBS as a control. Slides were incubated for 30 min at room temperature then washed in PBS for 1 hour on a shaker. The slides were then flooded with a fluorescein-conjugate mixture which contained:

2.6 ml PBS

0.3 ml Evans Blue

0.1 ml fluorescein labelled anti-human IgG conjugate (1:4 dilution)  
(Dako).

The final concentration of the conjugate was 1:120.

Slides were kept at room temperature for 30 min. and then washed in PBS on a shaker for 1-1.5 hours at room temperature. The slides were rinsed quickly in acetone and then PBS. The slides were mounted with glycerol and a cover slip. All slides were viewed with a fluorescence microscope (Nikon, U.K.) and graded accordingly (+ to ++++).

#### Animal serology

Several biopsies from the patients with tropical ulcers showed Gram-negative rods. The gross morphology was insufficient to separate the organisms especially since many cultures yielded facultative as well as anaerobic bacilli. An antibody to the Fusobacterium was raised in a rabbit. With an immunoperoxidase technique this anti-fusobacterium antibody was used as the primary antibody to locate the fusobacteria and anti-rabbit IgG antibody was used as the secondary antibody.

#### Preparation of antigen and rabbit inoculation

The method used was described by Falkner and Hawley (1977). Forty-eight hour broth cultures of fusobacteria were used for the experiments. Each culture was centrifuged at 10000 rpm for 15 min and washed three times with PBS. The deposit was suspended in PBS to a dilution of 1:10. The amount of protein in the suspension was measured by the method described below. For each injection, 325 ug of protein was administered intramuscularly to the rabbit. The first two injections, given a week apart were suspended in Freund's complete adjuvant in a ratio of 1 part antigen to 2 parts adjuvant.

The solution was mixed well and the injections given on the outer aspect of the thigh. A total of 6 injections were given the last 4 without Freund's adjuvant. The rabbit was bled from the ear two weeks after the last injection. An hour after the blood was collected it was centrifuged at 3000 rpm for 5 min and the serum kept at  $-70^{\circ}\text{C}$  until required. Antibody concentration was quantitated by an enzyme - linked immunosorbant (ELISA) technique.

#### Method for Protein Estimation

1) The dye stock solution was prepared by mixing:

2 vol 85%  $\text{H}_3\text{PO}_4$

1 vol absolute alcohol

A 0.33% weight dye/vol solution was prepared with 0.33 g Coomassie brilliant blue dye added to 100 ml  $\text{H}_3\text{PO}_4$  and ethanol mixture.

The dye solution was kept in a dark bottle. The method used was described by Bradford (1976).

2) Standard protein (bovine serum albumin) was prepared by dissolving 0.5 gram bovine serum albumin in 50 ul buffer solution (PBS). The concentration of protein in the stock solution was therefore 10 ug protein per 1 ul buffer. The stock solution was then divided into aliquots and stored at  $-20^{\circ}\text{C}$  until further use.

#### Dilutions of BSA

Protein (BSA) samples were prepared with 0.1 ml 1% BSA and 0.9 ml water. Then 100 ul of diluted BSA were added to 400 ul water to give a final concentration of 100 ug BSA/500 ul water

### Preparation of test solutions

The fusobacteria antibody was detected with

- 1) 50 ul test serum
- 2) a 1:10 dilution of the protein to be tested.
- 3) 50 ul control medium (BHI)

### Preparation of dye reagent

The following ingredients were added:

Dye stock solution 3.0 ml

85%  $H_3PO_4$  8.0 ml

Abs. Alcohol 3.8 ml

$H_2O$  to 85.2 ml

### Method

The mixture was filtered through a number one Whatman's filter paper into a brown bottle and covered with foil to prevent any light from affecting the solution. The filtrate was brown in colour as compared to the blue colour of the original mixture. The spectrophotometer was standardised with buffer/water (950 ul water with 50 ul buffer). A representative sample of the dye was placed in a spectrophotometer set at an optical density of 550 nm, to test the optical density of the dye solution. The dye solution (950 ul) was added to 50 ul buffer in a plastic cuvette and allowed to stand for up to four minutes before the optical density (O.D.) was read. An O.D. of between 0.5 and 0.7 meant the solution was acceptable. If the value was less than 0.5 a fresh solution was prepared.

The following concentrations of BSA were tested:

tube	vol BSA (ul)	vol buffer (ul)
1	0	50
2	5	45
3	10	40
4	20	30
5	40	10
6	50	0

#### Rabbit antibody production

The amount of antibody was measured by the enzyme linked immunosorbent assay (ELISA). The following were prepared prior to the experiments:

1) The test serum was diluted with PBS to cover a range of concentrations from 1:10 -1:160.

2) Substrate OPD was prepared by adding 1 ml ethanol to OPD. The mixture was vortexed well. Citric acid buffer (50 ml) was then added. The Citric acid buffer (pH 9.6, 0.06M) was prepared from;

NaHCO <sub>3</sub>	0.38%
Na <sub>2</sub> CO <sub>3</sub>	0.192%

3) PBS-Tween was prepared from;

Sterile distilled water	60 ml
6% Bovine serum albumin	10 ml
PBS-Tween x 10	6 ml

### Method for Enzyme linked immunosorbent assay

A whole cell preparation of Fusobacterium antigen was used. The amount of protein in the antigen sample was calculated as described previously. The antigen was diluted with buffer (PBS) to a final concentration of 2 ug protein/100 ul. Each well of a microtiter plate was coated with 100 ul antigen/buffer and left overnight at room temperature. The following day the plates were washed three times with PBS-Tween.

Serum dilutions (1:10-1:160) were added (100 ul) to the wells. All tests were carried out in duplicate with normal rabbit serum as a negative control and a known positive sample as a positive control. After 2 hours at room temperature the plates were washed three times with PBS-Tween. Antirabbit conjugate IgG (horse radish peroxidase) (Dako) was diluted in PBS to a final concentration of 1:10,000, and 100 ul added to each well. The plates were incubated at room temperature for 2 hours.

The microtitre plates were thoroughly washed three times with PBS-Tween and substrate-OPD added. The plates were incubated for 30 minutes in the dark. After incubation 10 ul hydrogen peroxide was added and the plates were fixed with 100 ul 2N  $H_2SO_4$ . The results were read with a Dynatek plate reader and integrated computer.

## Mechanisms of infection

### Cytotoxicity

Tropical ulcers start as tiny papules and break down to ulcers very rapidly. This suggests that a toxic factor may be involved in the pathogenesis of the disease. This aspect of the pathogenesis was thus investigated with an in-vitro cell culture system. Representative strains of four groups of isolates recovered from the ulcers were tested for cytotoxicity in-vitro. The test was based on the method described by Giugliano et al 1982.

Table 5 shows the cell lines used which were obtained from the Tissue Culture laboratory of the London School of Hygiene and Tropical Medicine, London, U.K. The solutions required during the test were prepared and are listed in Appendix 1e. These included two types of growth support medium, medium 199 and Dulbecco's modified Eagles Medium.

### Preparation of test samples

Table 6 shows the strains used for the experiments. Two strains of Fusobacterium isolated from the tropical ulcers, a strain of Bacteroides fragilis , Enterobacter cloacae and Streptococcus faecalis were included.

Table 5. Cell lines for in-vitro cytotoxicity

- 1) Vero (African green monkey kidney cells)
- 2) MRC 5 (human diploid lung cells)
- 3) Intestinal 407 (human embryonic intestine)
- 4) Chimpanzee liver
- 5) Chinese hamster ovary

Table 6. Bacterial strains for in-vitro cytotoxicity

- 1) F. ulcerans NCTC 12111
- 2) F. ulcerans NCTC 12112
- 3) B. thetaiotomicron
- 4) E. cloacae
- 5) S. faecalis

## Bacteriological media and culture conditions

The bacterial strains used in the experiments were obtained from patients with tropical ulcers. The isolates were subcultured for purity and grown on BHI agar plates. The bacteria were harvested from the purity plates, suspended in aliquots of peptone glycerol and stored at  $-70^{\circ}\text{C}$  until required. The bacteria tested for cytotoxicity were recovered from the peptone glycerol and cultured in 100 ml BHI broth at  $37^{\circ}\text{C}$  for 24 h aerobically or 48 h anaerobically (as appropriate). A BHI purity plate was always streak inoculated simultaneously. Cells were harvested from the broth culture by centrifugation at 3000 rpm at  $4^{\circ}\text{C}$  for 15 minutes. The supernatant was filtered through a membrane filter (Millipore, 0.45u, Millipore, Middlesex, U.K.), and kept in aliquots at  $-70^{\circ}\text{C}$  until required. Uninoculated brain heart infusion broth was the negative control for cytotoxicity and uninoculated growth medium the negative control for cell survival.

The supernatants of all the fusobacteria isolates were tested for cytotoxicity after:

- 1) The culture filtrates were dialysed against PBS for 18-24 hours across a permeable dialysis membrane which was boiled in distilled water for 15 minutes.
- 2) dialysed against PBS-with added cysteine (0.05%) for 18-24 hours.
- 3) dialysed against polyethylene glycol for 18-24 hours.
- 4) heated at  $50^{\circ}\text{C}$  for one hour.
- 5) heated at  $80^{\circ}\text{C}$  for one hour.

The treated supernatants were stored at  $-70^{\circ}\text{C}$  in aliquots of 10 ml until the experiments.

### Method to demonstrate cytotoxicity

Cell monolayers were grown in either medium 199 or Dulbecco's modified Eagle's medium. After 24 hours when the cells had grown to confluence, the growth medium was decanted. Cell monolayers were stripped from the bottle with a mixture of trypsin and versene. After the addition of trypsin and versene the bottle was gently rotated for 15 - 20 sec. The solution was decanted and the cells were incubated for 15 minutes to separate from the container. The cells were suspended in 10 ml medium and the cell concentration calculated with a haemocytometer. A sample of the medium which contained the suspended cells was added to the chamber with a capillary tube, and covered with a cover slip. All the cells were counted in the 16 squares in each corner. The cell count was repeated from the diluted cell mixture. The cells were resuspended in medium to give a final concentration of 300,000 cells/ml.

Cytotoxicity tests were carried out in microwell plates (Flow Laboratories, Rickmansworth, U.K.). Two-fold dilutions of culture filtrates in test medium were prepared in 50 ul volumes to cover dilutions from 1:2 to 1:256. These were transferred to the appropriate wells. Then fifty ul of the cell suspensions were added to each well using a multichannel pipette with disposable tips (Titertek, Leighton Buzzard, U.K.). Plates were incubated for 24 hours at 37°C. All tests were carried out in duplicate. Cytotoxicity was assessed morphologically at 18 and 24 hours. The changes visualised were graded as +++ if 90% or more of cells were affected; ++ if 70-80 % cells were affected; + if 50-60% cells were affected; +/- if 30-40% cells were affected, and - when < 20% cells were affected.

Cells were fixed with buffered formaldehyde for one hour and stained with phosphate buffered crystal violet (0.0001%). Excess stain was eluted with 100 ul acid methanol (1% of 1 M HCL in methanol). 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 number (Barer et al 1986a, Barer et al 1986b).

All experiments were repeated on at least two separate occasions. The five cell lines were tested individually with each of the test samples. Only Vero cells were used for further fusobacteria supernatant experiments.

#### Gas Liquid Chromatography (GLC)

Volatile fatty acids in the supernatants were analysed by GLC. The technique and operating conditions were described previously. The butyrate content in the Fusobacterium culture filtrates was quantitated from the peak area under the curve. This value was derived with an integrated computer (PU 4180 computing integrater, Philips, U.K.). Known concentrations of butyrate were used as standards.

#### The role of protein and cytotoxicity

The role of protein in the supernatant, on the cytotoxicity to the cell lines was investigated. The protein in the supernatant was subjected to two forms of treatment. Firstly the protein was precipitated with ammonium sulphate and secondly the supernatant was passed through a column of Sephadex to see whether or not a specific protein with a defined molecular weight caused the cytotoxicity.

A saturated solution of ammonium sulphate was added to an equal part of the fusobacteria supernatant to give a ratio of one to one. The mixture was shaken well and centrifuged at  $10,000 \times g$  for 10 minutes. After standing for one hour the supernatant was collected into a glass container and stored at  $4^{\circ}\text{C}$  until required. After the preliminary experiments showed that the cytotoxicity was lost after dialysis through a membrane which allowed passage of molecules of around 5000 MW, Sephadex G-25 beads (Pharmacia Chemicals, Hounslow, U.K.) were used in the column. The column, 100 cm long and with a diameter of 2.6 cm, was fixed in a suitable place which avoided draughts or direct sunlight.

The volume ( $v$ ) of Sephadex required was calculated from the formula

$$v = 11 r^2 h.$$

One gram of Sephadex was equivalent to 5 ml volume. The Sephadex beads were weighed and soaked in degassed PBS for three hours until the beads were sufficiently swollen. The column was packed with beads, and no air entered the column. The top of the column was closed and the fine particles which had collected at the top were decanted.

The column outlet was opened and the column was flushed with one bed volume of PBS during which time the flow rate was monitored. A pump adjusted the flow rate to between 10 and 20 ml/hour and the buffer (PBS) continuously flowed until the volume selected (2 bed volumes) had been completed. The tubing was then connected to the supernatant. A further bed volume of buffer passed through the column after the supernatant.

The outlet of the column was connected to a fractionator, which mechanically collected and divided the eluent into a predetermined number of fractions, with a set volume per fraction.

The column was connected to a graph. The collection times of each fraction and changes in the molecular weights of substances eluted were recorded and expressed in an elution diagram. The variation of solute concentration in the eluent was detected by an ultra-violet monitor.

#### Experimental design for the repeat cytotoxicity studies

After the supernatant fluid was treated with ammonium sulphate and gel filtration, the individual fractions were assessed for cytotoxicity. For the ammonium sulphate experiments, ammonium sulphate (one part) and one part of PBS served as a control for cytotoxicity and growth medium the control for cell survival. For the gel filtration experiments, each fraction which showed an increase in the solute concentration was used individually to test for cytotoxicity. The original supernatant fluid served as a positive control and growth medium as a negative control. All other conditions remained the same.

### In-vivo synergy

Several authors have attempted to assess the pathogenicity of bacteria isolated from tropical ulcers (Burnie 1931, McAdam 1966, Smith 1933). Usually a mixture of organisms were inoculated but none of the studies were quantitative. In most cases pus from ulcers was inoculated into human subjects, or guinea pigs. The assumption had always been that organisms present in the pus could initiate an ulcer at a fresh site. These were therefore transmission experiments. Only a few experiments used pure cultures of organisms. For instance, Fox (1920) used pus which showed only Gram-negative rods and spirochaetes on the Gram stain. However a Gram stain did not exclude small numbers of other organisms which were not seen on the Gram stain, and which if unintentionally inoculated, could have proliferated in the tissues.

### Background to experimental work

The role of obligate anaerobes in serious infections has received much attention in the last few years. Previously the anaerobic component of an infection was rarely thought to be important in its pathogenesis. Anaerobes were often regarded as innocent bystanders. However since anaerobic bacteria are frequently isolated from clinical specimens, recent work has highlighted their importance in clinical infections (Finegold 1977). Anaerobic and aerobic bacteria are often isolated together from specimens and frequently interact to produce infections-yet each separately is not pathogenic. Various animal models have been developed to evaluate this pathogenic mechanism referred to as synergy.

The intra-abdominal sepsis models (Onderdonk et al 1974, Kelly 1978) clearly demonstrated synergy between E. coli and B. fragilis causing abscesses in rats and guinea pigs. Synergy has also been demonstrated in post operative wound infections and post partum infections (Gorbach and Bartlett 1974). Most studies of bacterial synergy have involved the inoculation of small numbers of bacterial combinations into a wound or the peritoneal cavity of mice. Other reports also confirm the potential for synergy between aerobic, anaerobic and facultative anaerobic bacteria (Weinstein 1975, Ingham et al 1981). These experiments used a variety of animal models such as mice, hamsters, rats and guinea pigs. The success of the experiments depended on the organisms used, the animal model and the method of inoculation.

In this study animal experiments were performed to assess abscess formation by each organism or a combination of bacteria isolated from the tropical ulcers (Hampp 1962). The experiments aimed to identify which bacteria could produce an abscess in an animal model after subcutaneous inoculations. All the bacteria used were isolated from the tropical ulcers. Those bacteria isolated most commonly were included in the experiments (table 7).

Table 7. Bacteria used in animal experiments

Streptococcus faecalis

Anaerobic cocci

Bacteroides thetaiotomicron

Fusobacterium ulcerans NCTC 12111

Enterobacter cloacae

Citrobacter freundii

Table 8. Mouse strains used in animal experiments

1. Balb/c HEA2 (Dutch)
2. C57 black 10-BH2 haplotype
3. B10R (from C 57)
4. CBA/Ca
5. STS/A
6. CXS Balb/c x STS

## Animals

All experiments were first performed with six to eight week old mice in the London School of Hygiene and Tropical Medicine animal unit. The strains used are listed in table 8. The mice were housed and fed under conventional conditions. Two outbreak strains of guinea pig, weighing 500-1000 g, were later used for the animal experiments. The studies were designed to develop an animal model simulating cutaneous ulceration.

## Preparation of the inoculum

The bacteria were stored in peptone glycerol at  $-70^{\circ}\text{C}$ . Strains were identified by Gram stain, biochemical tests and gas liquid chromatography (anaerobes). Bacterial suspensions were thawed to room temperature, subcultured for purity on BHI blood agar and incubated at  $37^{\circ}\text{C}$  for 48 hours in an anaerobic cabinet, or 18 hours at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  (aerobes). Broth cultures (100 ml BHI broth) were prepared for each of the test organisms, divided into 0.5 ml aliquots and stored at  $-70^{\circ}\text{C}$  until required. These were the stock cultures. Quantitative determinations of viable cell density were obtained for all bacterial strains. Aliquots of each species were used for 10 fold dilutions of aerobes and anaerobes in BHI broth. Samples (0.1 ml) of each dilution were plated onto prereduced BHI agar plates, and incubated at  $37^{\circ}\text{C}$  for 24 hours (aerobes) or 48 hours (anaerobes). Colonies were counted and the viable cell density was expressed as cfu/ml. Bacterial stock cultures were diluted to appropriate concentrations in BHI broth (Cruikshank 1973).

## Experimental design

### Mouse model

For the initial experiments the mice were shaved over the dorsal skin before subcutaneous injections were performed. All the isolates were inoculated singly into the skin of the back of the mice. The injections were repeated on at least two separate occasions and the reactions recorded.

### Guinea pig model

The guinea pigs were shaved, the skin cleaned with 70% alcohol solution and the inocula injected into the back using a 18 gauge needle. Inoculations were done into the dermis of the skin. In some experiments, a silk suture (Mersilk, U.K.) was placed in the skin prior to the inoculation. The behaviour of the guinea pigs was assessed daily. The wounds were inspected daily for induration, erythema and abscess formation. A similar amount of brain heart infusion broth without bacteria was inoculated as a control.

The guinea pigs were inoculated intradermally with a constant volume of either a single pure organism or a combination of 2 or more organisms. The size and type of lesion which resulted were recorded and observed over one week, or less if the lesion was biopsied. There were three groups of organisms isolated from the ulcers and these were used in the experiments. They were coliform bacteria, cocci and anaerobic gram-negative bacilli (A,B and C). There were thus seven combinations of organisms inoculated (A+B, A+C, B+C and one of all three isolates A+B+C plus three of each organism alone).

Every experiment was repeated twice. The importance of each of the organisms was determined by its ability to cause an abscess or survive, or both when injected by itself, or by its ability to survive in a mixed infection which produced an abscess with other organisms.

#### Preparation of the sonicated fragment of fusobacteria

A 48-hour broth culture of fusobacteria was centrifuged at 10,000xg for 10 minutes. The cells were washed three times with phosphate buffered saline. The supernatant was collected and stored. The cells were resuspended in PBS and sonicated in a sonicator (MSE homogeniser, MSE, Crawley, Sussex, U.K.) with 8 bursts of 30 seconds each, with a probe tip at 6 A. This produced more than 95% lysis of the cells. The sonicated preparations were centrifuged at 2000 rpm x g for 10 minutes and the supernatant fluid was discarded. The pellet formed the sonicated whole cell preparation. The inoculum consisted of 0.1 ml of the sonicated fragment (Falkner and Hawley 1977).

#### Cultivation of abscess contents

Animals were anaesthetised with intramuscular fentanyl (Janssen, Wantage, U.K.) once an abscess developed. The abscess was excised and confirmed with haematoxylin and eosin stained histological sections. The pus from the abscess was diluted in one ml of BHI broth and swabbed onto supplemented BHI blood agar with a 5 ug disc of metronidazole and Columbia blood agar. Plates were incubated at 37<sup>o</sup> for 24 and 48 hours aerobically and anaerobically respectively. Bacterial growth was documented and colonies of all organisms isolated were identified by Gram stain and biochemical tests.

### Histopathology of experimental abscess

The skin biopsies were stretched to avoid deformation and fixed in 10% formal saline for at least 24 hours at room temperature. After fixation, sections were cut through the length of the skin biopsy and embedded in paraffin. Thereafter 5 um sections were cut and stained with haematoxylin and eosin and Gram's stain. These sections were processed at St. John's Hospital for Diseases of the Skin, London.

## RESULTS

### Epidemiology

#### Areas surveyed

A total of 179 patients with 253 ulcers were interviewed and examined in five areas, namely Zambia, Gambia, Fiji, southern India and Papua New Guinea. The first survey was carried out in Zambia by Dr. D.C. Robinson. Patients were seen at St. Francis Hospital, Katete (Eastern province) and in rural clinics in six other provinces visited by the Zambian Flying Doctor Service. The second survey was carried out in Gambia where patients were seen at the major government hospital in Banjul and at the hospitals run by the Medical Research Council in Fajara and Farafenny. Patients were seen also in health care centres and rural clinics run by missionaries in Sabayinda and Marakissa, and those run by the MRC in Bassey. In southern India patients were seen at urban clinics at two hospitals in Madras city and Madurai. In addition patients were examined in two rural areas:

- 1) The Schiefflin Leprosy Research and Training Centre at Karigiri near Vellore, Tamil Nadu.
- 2) The Tata Tea Estate at Munnar, Kerala State.

In Papua New Guinea, four main areas were surveyed. These included the Fatima mission (West Sepik province), Sissano and Warapu in West Sepik, the Kamporotoro refugee camp and the area around the towns of Aitape and Wewak.

In Fiji patients were seen at the government hospitals in Nadi, Suva and Lautoka and health centres at Nausori and Vunindawa on the main island of Viti Levu. Patients were also sought on the smaller island Vanua Levu at the coastal towns of Labasa and Savusavu. Village visits were made to the inland towns of Bua and Nabouwalu.

#### Social and environmental conditions

The patients examined in Gambia and Fiji mostly lived in rural villages of 10-100 houses. The huts were well separated and not more than 6 persons shared a hut at any one time. The size of the villages varied considerably with between 10 and 40 huts per village. On average each hut contained 2 rooms which were used as bedrooms. The huts consisted of thatch, leaves and dried mud. A few cement houses were scattered in a few villages but the majority of houses were of the thatched variety. In Zambia huts were similarly made of leaves and dried mud with corrugated iron as the roofing but many more were constructed out of mud based walls.

Water for drinking and washing was normally obtained from wells or in some areas from taps located at varying intervals. The taps (standpipes) each served a population of between 20 and 100 people. In Madurai the patients who developed tropical ulcer came from the underprivileged areas where overcrowding was common. Water for washing and drinking was supplied by street taps but washing was also carried out in the river which could be reached only by crossing wide bands of mud. The second hospital where patients were sought in India, served the more prosperous part of the city. There were no patients with tropical ulcers.

In the rural clinics of the Tata Tea Estate in the mountainous country around Munar in southern India, no cases of tropical ulcers were found even though the tea pickers walked barefoot and were exposed to frequent trauma. Tropical ulcer has rarely been seen at Karigiri.

In Papua New Guinea, (Fatima, Sissano, Warapu) individuals lived in villages of up to 300 households with an average of five to six persons per household. By contrast the individuals in Kamporototo refugee camp were living in very crowded conditions, often with several families in one house or tent.

In most areas, women washed inside the hut whereas men washed outside. Overcrowding was seen in some areas but was not a constant feature. Cooking was done outside in most instances. In most areas individuals ate with their hands. Food was stored in the coolest parts of the hut or occasionally in a paraffin-fuelled refrigerator.

Of the patients examined 95% were barefoot and all went barefoot for most of the year. In most areas young girls wore long wrap round skirts but also, for the most part, went barefoot. A small percentage (12%) said they sometimes wore shoes. Most (109/179, 61%) of the patients attended school. The presence of a tropical ulcer did not interfere with their attendance at school and in many areas dressings were done at the village health centres after school.

Person to person transmission was not a frequent finding. All patients were questioned about simultaneous involvement of other family members. This was only seen in 9 patients in all.

Of 80 families surveyed in the refugee camp there were only two with more than one member who had an active ulcer. This suggests that person-to-person infectivity is uncommon.

In the Gambia most of the patients were muslim by religion so pigs were never seen in the villages. However cats, dogs, horses and chickens were commonly seen in and around the huts. Similarly, in the other areas surveyed, animals roamed freely around the huts.

#### Frequency of tropical ulcer

Currently there are no published figures available for the frequency of the disease in affected areas. In Papua New Guinea the number of patients seen during 1987 is listed in table 9. (Dr. G. Morris, personal communication). The figures were compiled from the health centres where treatments are carried out. In all other areas surveyed there were no recorded figures available.

Table 9. Frequency of tropical ulcers in Papua New Guinea

District Aid Post	Angoram	Wewak	Maprik	Ambunt	Total
Jan	30	14	21	23	88
Feb	30	17	25	29	166
March	14	3	8	11	36
April	9	19	5	0	33
May	2	34	4	12	52
June	8	28	29	18	83
July	1	34	10	14	59
August	2	45	5	0	52
September	4	30	0	9	52
October	7	36	0	25	68
November	6	30	7	6	49
December	32	18	10	6	66
Total					730

## Clinical manifestations

The ulcers observed could be divided into acute or chronic ulcers on the basis of the duration of the ulcers. Those less than six weeks old were referred to as acute ulcers.

### Acute ulcers

The pre-ulcerative lesions described by Hare (1948) were only seen in 6 cases. These lesions were small pustules no greater than 2-3 mm in diameter. They were characterized by a raised, loose epithelium which was easily removed with a needle. This procedure was painless. Below the epithelium an ulcer, less than a centimeter in diameter, could be seen. However the underlying ulcer had a greater diameter than the overlying papule. These lesions were usually referred to as "boils" or "pimples" by the patients. The pre-ulcerative lesion was usually recognized by patients attending the clinic for treatment of an existing ulcer. The pre-ulcerative lesions developed at any time from 2 days to three weeks after the alleged episode of trauma. In 46 (26%) of patients the pre-ulcerative lesions occurred spontaneously. The number of acute ulcers observed is shown in table 10.

The ulcers less than six weeks old were all less than 5 cm in diameter and showed the features of acute ulcers. They were round to ovoid with a protuberant lip and a deep crater with a dense foul smelling slough. The ulcers bled frequently so that dressings had to be changed regularly. Almost all patients complained of pain at the onset of the ulcers but this symptom only lasted from a few days to about a week.

In 25% of cases the ulcer developed at a site of a previously recorded ulcer. In 65% of cases there was evidence of previous ulceration at a different site. The overall features of the acute ulcers were similar in patients from all locations (figure 8).

### Chronic ulcers

The chronic ulcers were less tender to touch. This was especially noticeable when the bacteriological swab was taken. These ulcers had a thick rim of fibrous tissue round the outside of the lesion (fig 9). The ulcers ranged in size from 3-6 cm in diameter, except in one patient where the ulcer was virtually circumferential (Robinson et al 1987). The base was less friable with less slough. The ulcers lacked the foul smelling purulent exudate characteristic of acute ulcers. The chronic ulcers gradually healed from the edges. The ulceration extended only to the mid-dermis in the majority of cases.

Chronic ulcers were less common. Only 19 patients could confirm that they had an ulcer for more than six months (table 10). Of these five had had ulcers for more than a year. In two patients the lesions started three years previously, healed after a year and then broke down spontaneously over 6 months previously. However many patients (22,(12%)) were uncertain of the duration of the ulcer. Direct questioning of the group uncertain of the duration of their ulcers (as to whether the ulcer was present during the previous "wet season") suggested that most ulcers were of several month's but less than one years duration.

### Number of ulcers

There were 121 patients with solitary ulcers but up to four were seen in a single individual. When multiple ulcers occurred they developed with equal frequency on the same or contralateral limb. Multiple ulcers were for the most part well separated (fig. 10) although in a few patients (n=4) ulcers occurred adjacent to one another (kissing ulcers, fig.11). Table 11 shows the number of ulcers for each patient.

### Sites affected

The majority of ulcers occurred on the lower leg (81%).The sites of involvement are shown in table 12. Only the limbs were affected but the nail beds were never involved. In no patients was the trunk involved.



Fig. 8. Acute tropical ulcer showing surrounding oedema



Fig. 9. Chronic tropical ulcer showing thick fibrous rim



Fig. 10. Multiple ulcers on the hand and leg  
(Courtesy of Dr. D. Robinson)

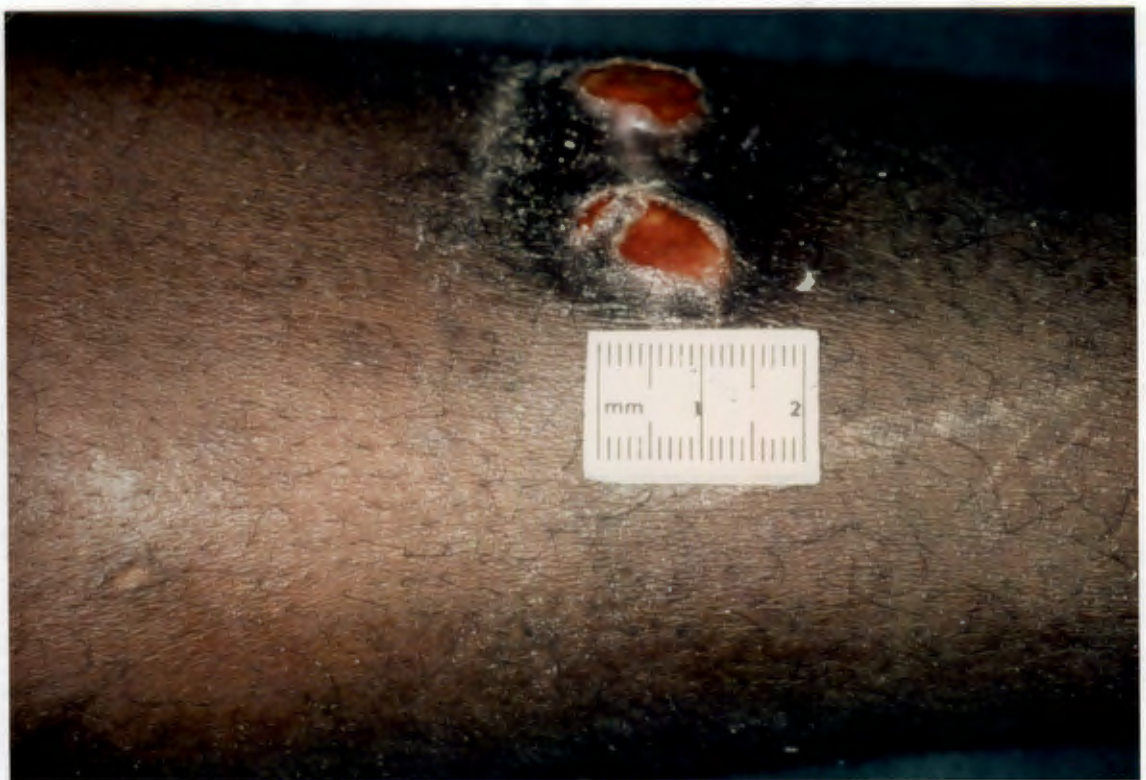


Fig. 11. Multiple adjacent ulcers on the leg

Table 10. Duration of ulcers at time of examination

<u>Duration</u> <u>(weeks)</u>	<u>Zambia</u>	<u>Gambia</u>	<u>India</u>	<u>Fiji</u>	<u>PNG</u>
0-6	42	15	9	3	35
6-12	13	7	5	1	8
>24	3	8	1	0	7
Uncertain	6	11	0	0	5

Table 11. Number of ulcers

<u>Number</u>	<u>Zambia</u>	<u>Gambia</u>	<u>India</u>	<u>Fiji</u>	<u>PNG</u>
1	32	31	11	4	43
2	18	5	1	0	10
>2	14	5	3	0	2

Table 12. Sites of Tropical ulcers

	Zambia	Gambia	India	Fiji	PNG
	No.(%)	No.(%)	No.(%)	No.(%)	No.(%)
Lower leg					
Upper third	6 (7)	5 (7)	0	0	1 (1)
Middle third	15 (17)	21 (32)	12 (55)	2 (50)	26 (34)
Lower third	53 (62)	24 (36)	9 (41)	2 (50)	29 (38)
Foot	11 (13)	13 (19)	0	0	15 (20)
Upper leg	1	2	0	0	2 (3)
Upper limb	0	0	1	0	3 (4)
	-----				
	86	65	22	4	76

Total number of ulcers: 253

Frequency at ulcer sites

Lower leg : 81%

Foot : 15.4%

Upper limb: 1.6%

Upper leg : 2.0%

### Age of the patients

Table 13 shows the age distribution of the patients. Most of the patients were aged between 5 and 15 years (96/179, 54%). In Zambia and India, more than 2/3 of the patients fell into that age range. However in Gambia and Papua New Guinea a number of patients were older than 15 years (47). This difference can be accounted for by the predominance of adult women working as farmers in Gambia, or in fishing in PNG. There were only 3 patients older than 45 years (2 in Gambia, 1 in PNG). All three had several episodes of ulceration previously and in all three the lesions had occurred in a site of previous ulceration.

### Sex of patients

Table 14 shows the sex distribution of the patients. Males outnumbered females only slightly except in India where there was a significant male predominance. In Zambia and Gambia there was an equal sex incidence. In Papua New Guinea females outnumbered males.

Table 13. Age distribution of patients with tropical ulcer

Age group (years)	Zambia			Gambia			India			Fiji			PNG		
	M	F	Total	M	F	Total	M	F	Total	M	F	Total	M	F	Total
0-4	0	0	0	1	3	4	0	0	0	0	0	0	0	1	1
5-15	20	21	41(64%)	9	9	18(44%)	9	1	10(67%)	1	0	1(25%)	12	14	26(47%)
> 15	13	10	23(36%)	10	9	19(46%)	5	0	5(33%)	2	1	3(75%)	11	17	28(51%)
	64			41			15			4			55		

Total number of patients seen = 179.

Table 14. Sex distribution of tropical ulcer patients

Sex of patients:	Male (%)	Female (%)
India	14 (93)	1 (7)
Papua New Guinea	23 (42)	32 (58)
The Gambia	20 (49)	21 (51)
Fiji	3 (75)	1 (25)
Zambia	33 (51.5)	31 (48.5)
	93 (52)	86 (48)

### Complications of tropical ulcer

In only one patient (seen in Gambia) an area of ulceration had undergone malignant change (fig.12) to a squamous cell carcinoma. This was confirmed histologically (fig 13). In four cases, however, there was clinical evidence of spread to bone. These patients were exquisitely tender to touch in the area of ulceration and adjacent tissues.

One third of all the patients investigated could remember a previous ulcer and 25% of patients thought that the present ulcer had recurred in the same site. In a further 40% of cases, a history of a previous leg ulcer was confirmed by a large atrophic scar, which took up the shape of the previous ulcer (fig.14).

### Relationship to trauma

Patients frequently reported a history of trauma albeit minor. A definite history of trauma was obtained from 56 (31%) patients. The types of trauma sustained are tabulated in table 15. The most frequent injuries were associated with farming, walking in the bush or playing outside. Other injuries resulted from falling off a bicycle, playing soccer or patients were scratched by bushes. When relatives accompanied the children, they confirmed the initial injury. Only 5 patients thought that their ulcer was preceded by an insect bite. The interval between trauma and the onset of the ulcer varied from 2 days to three weeks.



Fig. 12. Squamous cell carcinoma developing  
after a tropical ulcer

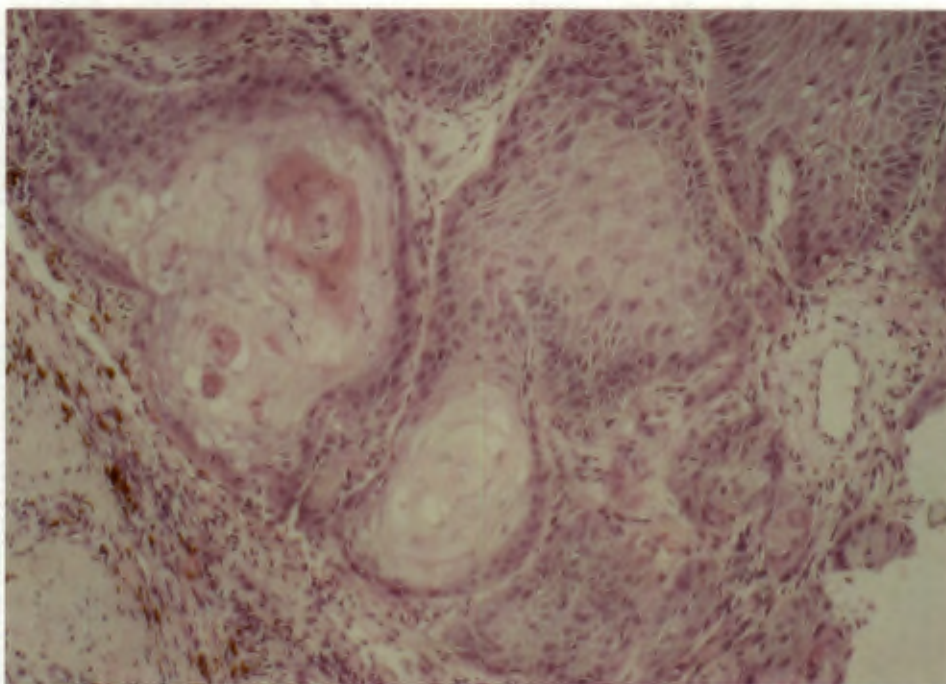


Fig. 13. Histology of Squamous cell carcinoma



Fig. 14. Scar of previous ulcer

Table 15. Injuries sustained prior to the onset of a tropical ulcer

Injury	No. of patients
Playing soccer	9
Riding a bicycle	8
Wading in the river	6
Fall	18
Farming	6
Walking outside	4
Insect bites	5

## Nutritional status of tropical ulcer patients

### Dietary habits of patients with tropical ulcer

Fish was freely available during the rainy seasons and in the Gambia and Fiji fish were regularly caught in the nearby rivers. Fish was frequently air-dried and the dried fish consumed during the dry and lean seasons. Fruit and vegetables were grown in most areas in Fiji but in Gambia only tomatoes and root vegetables were frequently obtained. Ground nuts was the main produce of the Gambian farmers. In PNG most of the patients were employed in fishing. Goat's milk was more readily obtainable than cow's milk but condensed milk was often used instead of fresh milk (Gambia).

### Assessment of nutritional status

The results for the body mass index for 139 tropical ulcer patients are shown in the table 16. While the majority had values less than 19, that is below the quoted desirable range for an American adult, no significant differences were found when tropical ulcer patients were compared to controls in each area as shown in table 17.

Haemoglobin values recorded in Zambia and Gambia showed levels above 10 gr% in all cases. Only one patient in Gambia showed a sickle cell trait.

Table 16. Body mass index of patients

$W/H^2$			No of ulcer pts
>19	normal	(group A)	31
15-19	slightly malnourished (group B)		67
<19	grossly malnourished (group C)		41
<hr/>			139

Table 17. Nutritional status of patients with available controls

	Zambia		Gambia		India		Fiji		PNG	
	P	C	P	C	P	C	P	C	P	C
Group A	4 (25)	6 (37)	9 (34)	12 (46)	0	0	4 (100)	3 (75)	7 (32)	7 (32)
Group B	10 (63)	9 (56)	11 (42)	9 (35)	4 (40)	5 (50)	0	0	4 (18)	6 (27)
Group C	2 (6)	1 (6)	6 (23)	5 (19)	6 (60)	5 (50)	0	1 (25)	11 (50)	9 (41)
	16	16	26	26	10	10	4	4	22	22

No patients or controls were less than 75% of the predicted weight for height as calculated from the nomograms adapted from the Society of Actuaries (1959) and published in the report of the WHO Expert Committee on Nutrition (1971). Of those studied 80% were above the 90% value for the desired ratio. There was thus no evidence of recent or current malnutrition.

All those patients less than 18 years old were assessed for evidence of chronic malnutrition. The results showed no patients or controls had values less than 85% of the predicted values as calculated from the charts produced by Rao and Singh (1970). There was thus no evidence of chronic malnutrition in the patients or in the controls.

#### Vitamin deficiencies

There were no clinical signs of vitamin deficiencies, particularly no angular stomatitis (riboflavin deficiency) follicular keratosis (vitamin A deficiency) or follicular haemorrhages (vitamin C deficiency) although subclinical deficiencies could not be ruled out.

None of the patients interviewed received concurrent treatment for either tuberculosis or leprosy. None of the patients showed overt signs of acute infections but chronic infections, such as malaria or parasitic disease, could not be excluded.

### Association with other medical conditions

Although all patients were examined for other medical conditions or skin conditions there were no patients with evidence of either cutaneous tuberculosis or leprosy. One patient had clinical and histologic evidence of concurrent leishmaniasis and a tropical ulcer. Other skin diseases such as dermatophyte infections, impetigo and leishmaniasis were common.

Impetigo was present in 28 patients (16%). No patients received specific treatment for this condition. In Fiji, *tinea imbricata* was endemic. Large sections of the population were affected and the disease affected several members of the family. The diagnosis was confirmed by culture in 37 cases. None of the patients with positive fungal cultures had tropical ulcers. *Tinea capitis* and *corporis* were common in Fiji and Gambia. Children were affected more often than adults. In Fiji the causative organisms were *T. rubrum* or *T. tonsurans* (table 18).

Leishmaniasis was seen only in Gambia. The early lesions were identical to those of early tropical ulcers. One patient had a tropical ulcer on the lower leg and an early papular lesion adjacent to the ulcer showed histological features of leishmaniasis (see figs 15 & 16). The patients with Leishmaniasis were older (average age 26 years) although there was one patient only 4 years of age who showed the characteristic clinical features of leishmaniasis (fig 17).



Fig. 15. Tropical ulcer adjacent to leishmaniasis

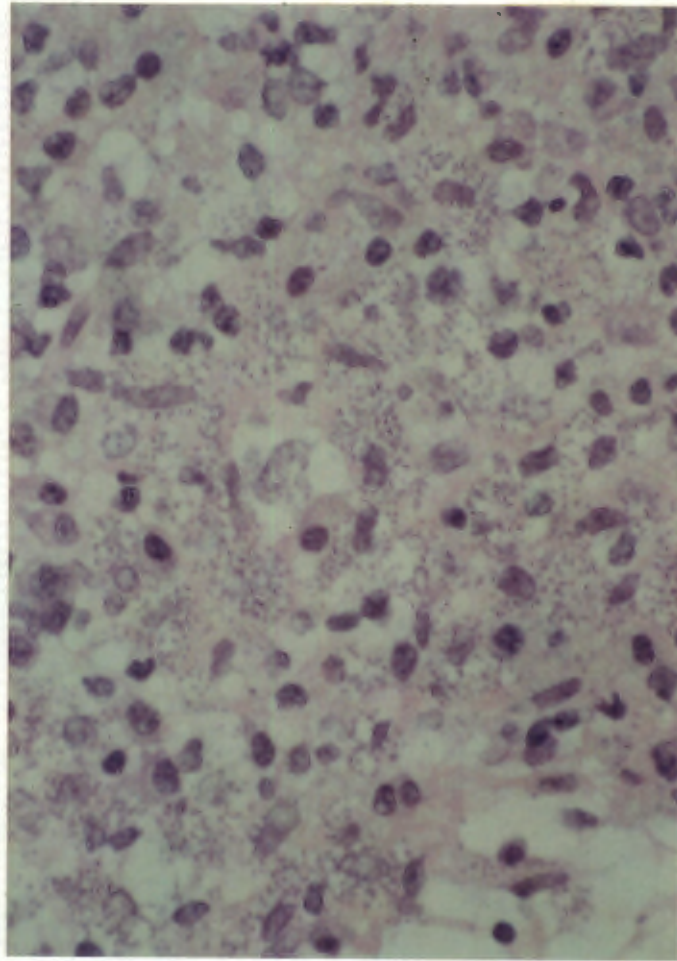


Fig. 16. Histology of Leishmaniasis papule



Fig. 17. Leishmaniasis in a child

Table 18. Dermatophyte infections in Fiji

<u>Source of sample</u>	<u>Fungus cultured</u>
Scalp hairs (1)	T. tonsurans
Trunk (17)	T. rubrum (6) T. tonsurans (3)
Palm (1)	T. rubrum

## Role of infection

### Bacteriology

Table 19 shows the number of swabs analysed from each location.

The results of the bacterial cultures from the swabs of the patients are summarized in table 20. From the aerobic cultures, E. cloacae, coagulase negative staphylococci and faecal streptococci were isolated most frequently. Of the anaerobic bacteria, fusobacteria and anaerobic cocci were isolated most often.

There were a total of 376 isolates (240 aerobes and 136 anaerobes), which represented an average of 2.52 isolates per specimen (1.6 aerobes and 0.9 anaerobes). Several swabs had more than one organism present. The detailed analysis of the number of isolates is shown in table 21.

Where only one isolate was obtained, the organism present varied as shown in table 22.

Table 19. Number of swabs analysed from each location

Country	No. of Patients swabbed	No. of Controls swabbed
Zambia	34	0
Gambia	41	3
India	15	2
Fiji	4	0
PNG	55	0
Total	149	5

Table 20. Bacteriology of cultures (from ulcers)

Aerobic culture

Gram-positive

Organism	Gambia	Zambia	India	Fiji	PNG	Total
-----						
<u>Staph. aureus</u>	1	1	2	-	2	6
Coagulase negative staphs (CNS)	17	9	8	3	14	51
Streptococci	23	8	2	3	11	47
group A	0	0	0	0	0	
D	23	8	2	3	11	
-----						

104

Table 20 continued

## Gram-negative

Organism	Gambia	Zambia	India	Fiji	PNG	Total
<u>Enterobacter</u> <u>cloacae</u>	25	12	3	1	13	54
<u>Eschericia</u> <u>coli</u>	2	1	1	0	2	6
<u>Citrobacter</u> <u>freundii</u>	15	5	6	2	8	36
<u>Proteus</u> spp	11	2	0	0	0	13
<u>Pseudomonas</u> spp	14	3	4	1	5	27
Total						136

Total number of aerobic isolates: 240.

Anaerobic culture

Organism	Gambia	Zambia	India	Fiji	PNG	Total
<u>Fusobacterium</u> spp	18	3	7	1	17	46
<u>Bacteroides</u> spp	6	4	5	0	7	22
Anaerobic cocci	12	13	5	1	9	40
<u>Propionobacterium</u> sp	6	10	5	2	5	28
Total number of anaerobic isolates						136

Table 21. No. of isolates per swab

	Gambia	Zambia	India	Fiji	PNG	Total
No growth	7	3	4	0	4	18
Single organism	10	1	5	2	0	18
2 organisms	15	7	0	1	9	32
3	16	7	6	0	15	44
>3	4	5	0	1	27	37

Table 22. Analysis of single isolates

Single isolate	No. of specimens
Non-lactose fermenting coliform bacilli	9
Anaerobic cocci	4
Coagulase negative staphylococci	3
<u>Pseudomonas</u> spp.	2
Total	18

Gram-negative aerobes such as E. cloacae , C. freundii and Proteus or Pseudomonas spp. were frequently isolated as shown in table 20. E. coli was isolated less frequently (6/149).

All the streptococci isolated were faecal streptococci, and were facultative anaerobes. Specimens from the Gambia were heavily contaminated with Proteus spp. The organisms were difficult to inhibit on the aerobic plates as they were resistant to nalidixic acid, gentamicin, tobramycin and were only sensitive to netilmicin at a minimum inhibitory concentration of 64 ug/ml. Pseudomonas spp. were often isolated from samples obtained in the Gambia. Detailed speciation was not performed on these isolates.

#### Results of anaerobic culture

Fusobacteria were isolated most commonly from the swabs (table 20). Anaerobic cocci were also isolated frequently. Two or more anaerobes were recovered from the swabs on several occasions as shown in table 23. The fusobacteria were frequently isolated in association with facultative anaerobes (42 samples, 91%). The frequency of isolation of all bacteria from the ulcers is listed in table 24.

Table 23. Frequency of isolation of 2 or more anaerobes from the same sample

Anaerobes	Number of samples
<u>Fusobacterium</u> and <u>Bacteroides</u> spp.	8
<u>Fusobacterium</u> and anaerobic cocci	18
<u>Bacteroides</u> and anaerobic cocci	7
Bact., Fusobac., and an. cocci	4

Table 24. Bacteria isolated from swabs

Organism	% total isolates
<u>E. cloacae</u>	14
<u>C. freundii</u>	10
<u>Pseudomonas</u> spp	7
<u>Proteus</u> spp	3
<u>E. coli</u>	2
<u>S. aureus</u>	1.6
CNS	14
Feacal streptococci	12
<u>Fusobacterium</u> spp.	12
Anaerobic cocci	11
<u>Bacteroides</u> spp.	6
<u>Propionobacterium</u> spp.	8

### Duration of ulcer and the presence of anaerobes

Anaerobes were only present in samples of less than six weeks duration. Table 25 compares the duration of ulcers with recovery of anaerobes.

### Bacteriology of mud samples

The mud samples were cultured aerobically and anaerobically and the results are shown in table 26. All samples had more than one isolate present. All the samples contained anaerobic cocci and Bacteroides spp. 3 contained fusobacteria, and 2 contained Clostridia spp, whereas Clostridia spp. were never isolated from ulcers.

All the isolates recovered from the ulcers were stored at  $-70^{\circ}\text{C}$  until required.

Table 25. Duration of ulcers and recovery of anaerobes

Duration of ulcer with anaerobes (weeks)	No of ulcers (%)
0- 6	54 (36)
6-12	2 (1)
>12	0

Table 26. Number of isolates per sample of mud

No of isolates per sample	Number	Location
Single isolate	0	
2 isolates	0	
3 isolates	0	
>3 isolates	4	PNG, India

## Description of fusobacteria

Those Gram-negative, non-sporing rods which produced large amounts of n-butyric acid and no iso-butyric acid as end products of metabolism were regarded as members of the genus Fusobacterium. All these isolates also converted threonine to propionic acid and thus fulfilled the requirement for membership of this genus. All the Fusobacterium species were isolated from ulcers of less than six weeks duration.

The fusobacteria were recovered from the BHI plates, but were easily recognised on the BHI plates supplemented with rifampicin. The isolates were sensitive to metronidazole, penicillin and phosphamycin. They did not grow on Columbia blood agar plates incubated in air with 5% CO<sub>2</sub>, nor on the MacConkey plates and were thus obligate anaerobes. The two distinct morphological types isolated from 46 ulcers will be referred to as type 1 & type 2 fusobacteria.

### Type 1 Fusobacteria

#### Colonial morphology

The 40 isolates that belonged to this group were identical. The organisms grew relatively quickly and colonies were visible at 24 hours but reached their maximal size by 36-48 hours. The optimal temperature for growth was 37°C. The colonies were 3-4mm in diameter, dull white in colour initially but became yellow with age. They produced no haemolysis on blood agar nor any pigment. The edge of the colony was smooth and entire.

Colonies were dome shaped in appearance (fig. 18). Growth on different media e.g. BHI, FABA or Wilkins-Chalgren agar produced no change in the rate of growth although the organisms showed considerable morphological pleomorphism. In broth culture the organisms grew readily and dispersed well throughout the medium. They grew easily in BHI broth without blood supplementation. Broth cultures were malodourous due to the butyrate.

#### Cellular morphology

A Gram-stain showed long thin rods which displayed pleomorphism of size and shape with some organisms short and almost coccal. The ends were pointed. Others were filamentous and fusiform. The cells stained evenly as Gram-negative, non-sporing rods (figure 19).

#### Type 2 fusobacteria

##### Colonial morphology

These colonies were smaller than type 1 colonies, about 2mm in diameter. They were translucent, butyrous in consistency and grew much slower than type 1 colonies. Type 2 colonies were not visible at 24 but at 36 hours and attained their maximal size by 48-72 hours. They produced neither pigment nor haemolysis on blood agar. The colonies were round with an entire edge and low convex shape (Fig. 20)

### Cellular morphology

Gram stain showed marked pleomorphism of size and shape. The rods were larger than type 1 organisms and showed a central bulbous swelling. The edges were also pointed but the bacteria were very irregular in length. They stained irregularly but on the whole the organisms were Gram-negative (figure 21).

### Guanine plus cytosine ratios

The guanine+ cytosine ratio was 29.2 mol% and 29.5 mol% for type 1 and 2 respectively.

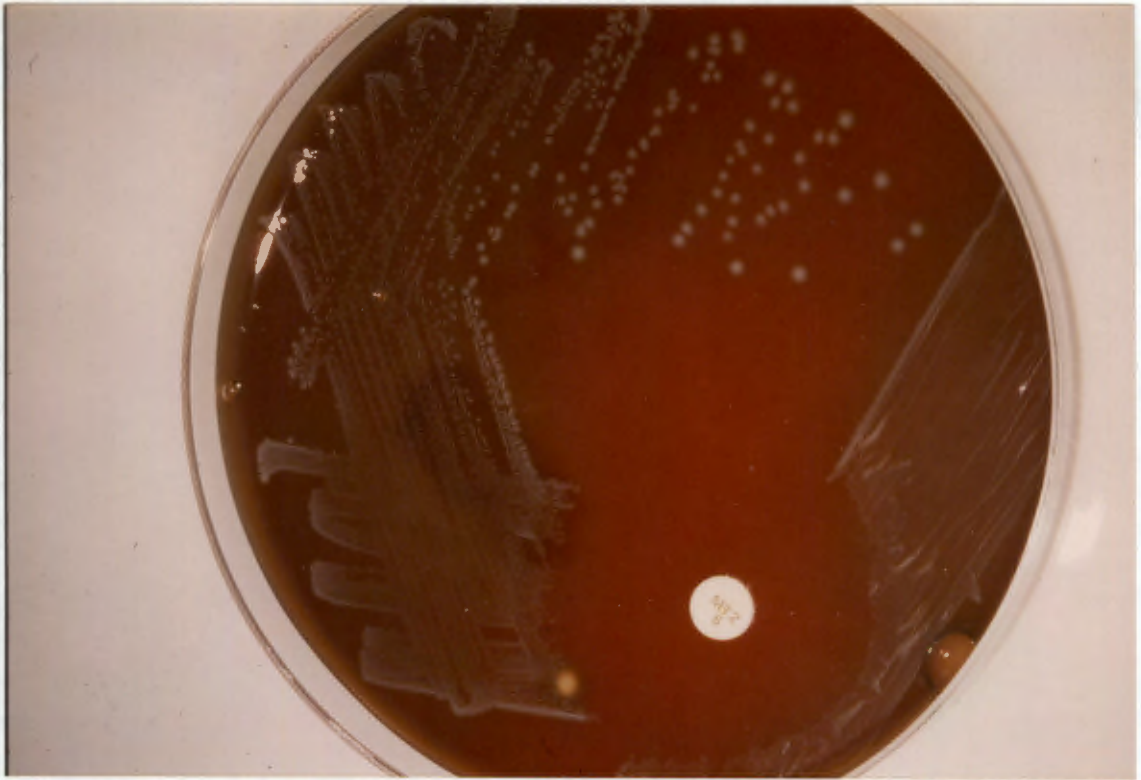


Fig. 18. Type 1 fusobacteria colony

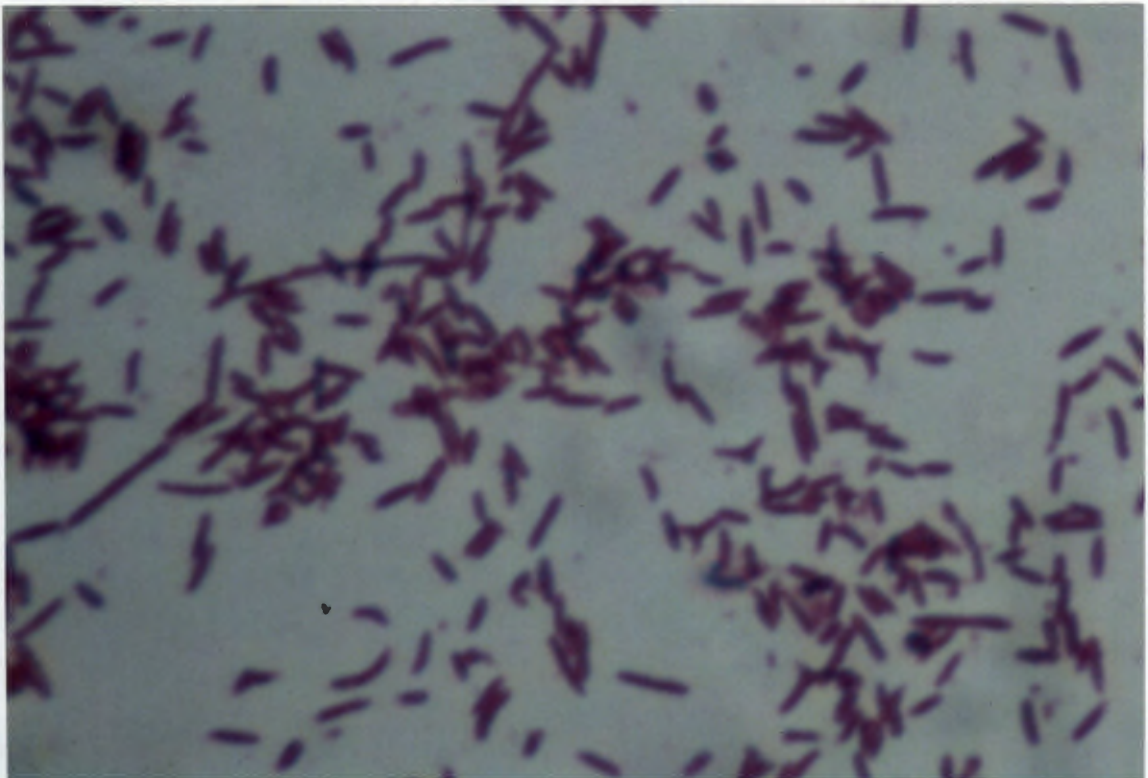


Fig. 19. Type 1 fusobacteria morphology (x1000)

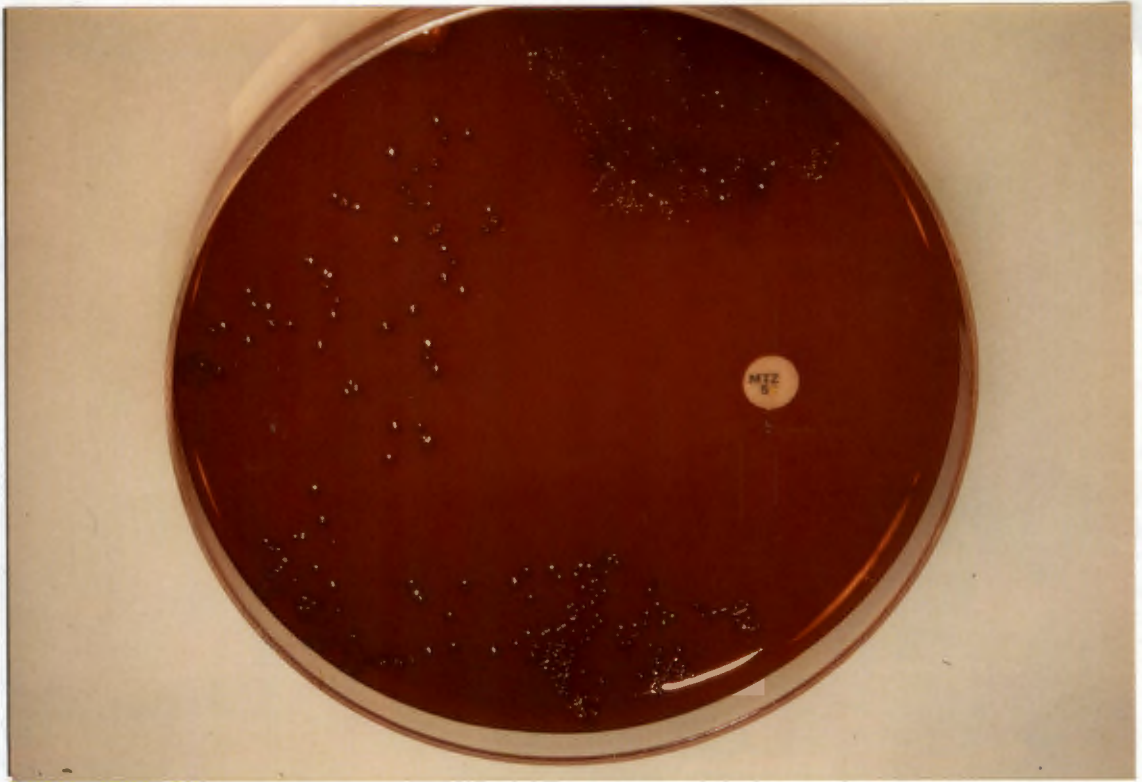


Fig. 20. Type 2 fusobacteria colony

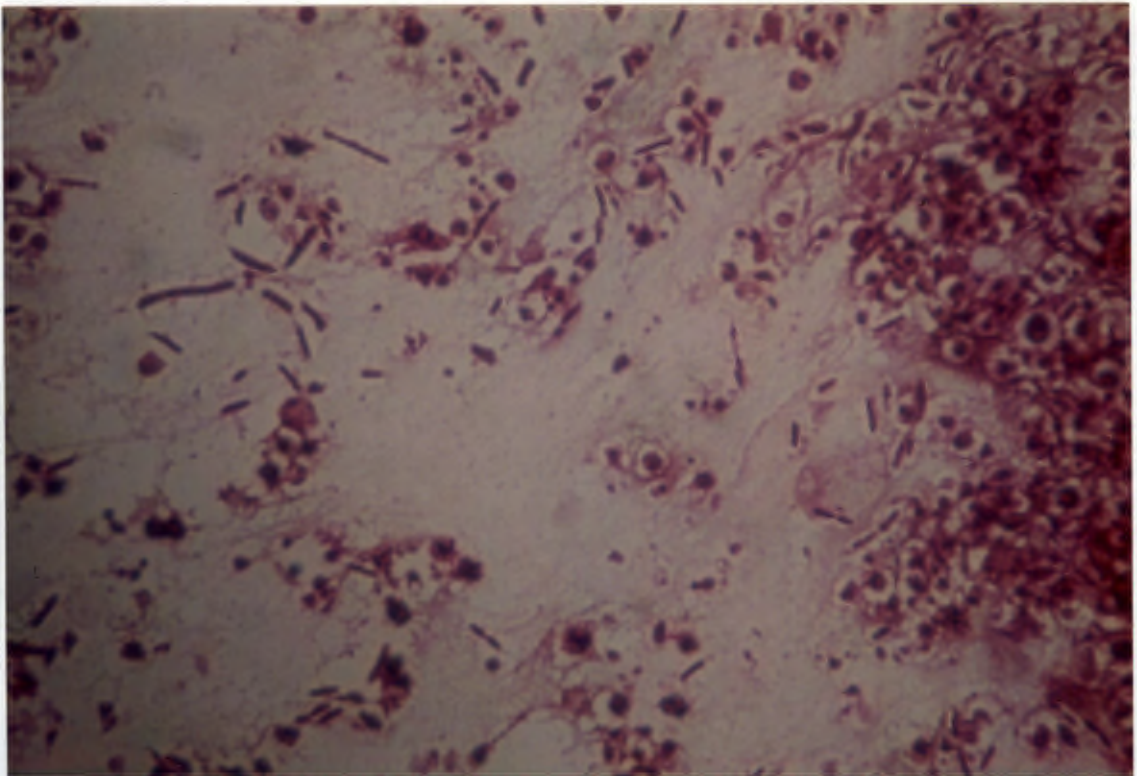


Fig. 21. Type 2 fusobacteria morphology (x1000)

## Electron Microscopy of fusobacteria

### Type 1 fusobacteria

The organisms showed significant morphological variation when grown on different media. Figure 22 shows these organisms harvested from supplemented BHI with 10% horse blood. The cells were 0.5-3 $\mu$  in diameter and 0.5-4.5 $\mu$  in length. The pleomorphism in shape was easily recognised. Some organisms were long and straight whereas others were curved. Figure 23 shows the same organism on Fastidious Anaerobe agar with 10% horse blood. The pleomorphism is again evident, more so than on BHI. Figure 24 shows the organisms on Wilkins - Chalgren agar supplemented with 10% horse blood. The picture shows again the considerable difference in morphology compared to the organism on BHI.

### Type 2 fusobacteria

On supplemented BHI agar the pleomorphism was apparent. The bulbous swelling previously recognised on direct Gram stain was confirmed. These organisms were larger than the Type 1 strains. The cells were 0.8-4.5 $\mu$  in length and 0.2 $\mu$  in width with a central width of 0.5 $\mu$  at the bulbous swelling (figure 25).

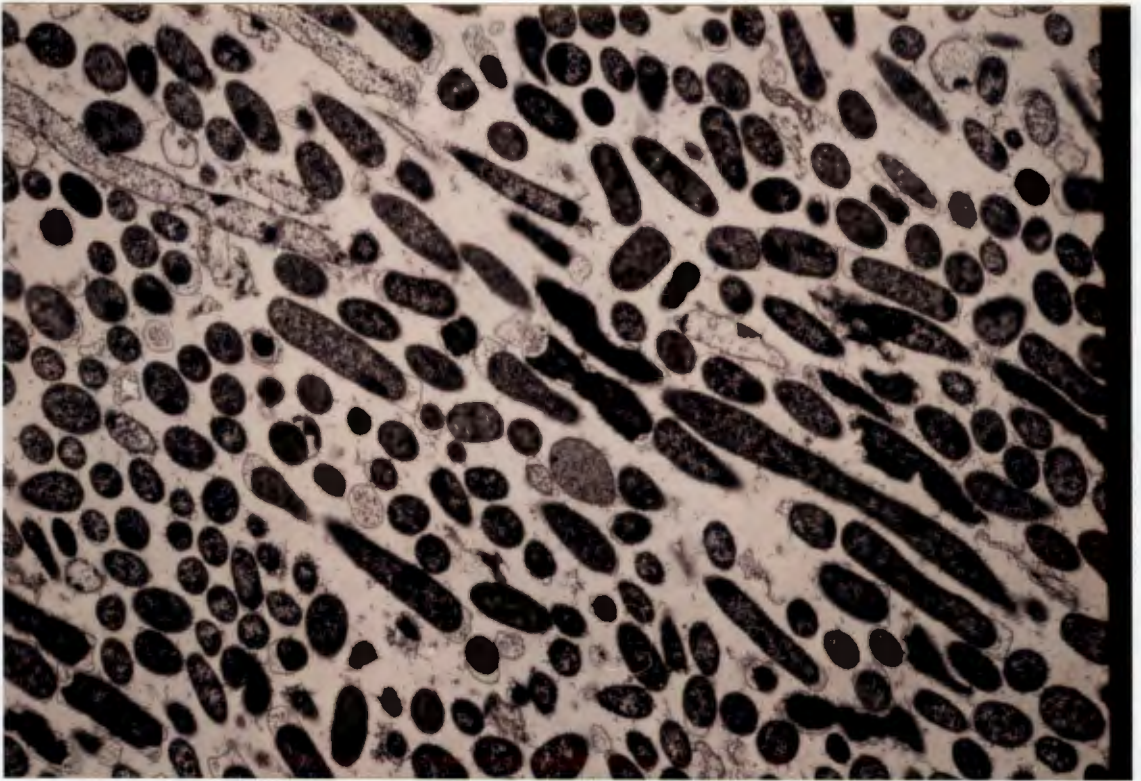


Fig. 22. Electron microscopy of type 1 fusobacteria (BHI) (x5000)



Fig. 23. Electron microscopy of type 1 fusobacteria (FABA) (x5000)

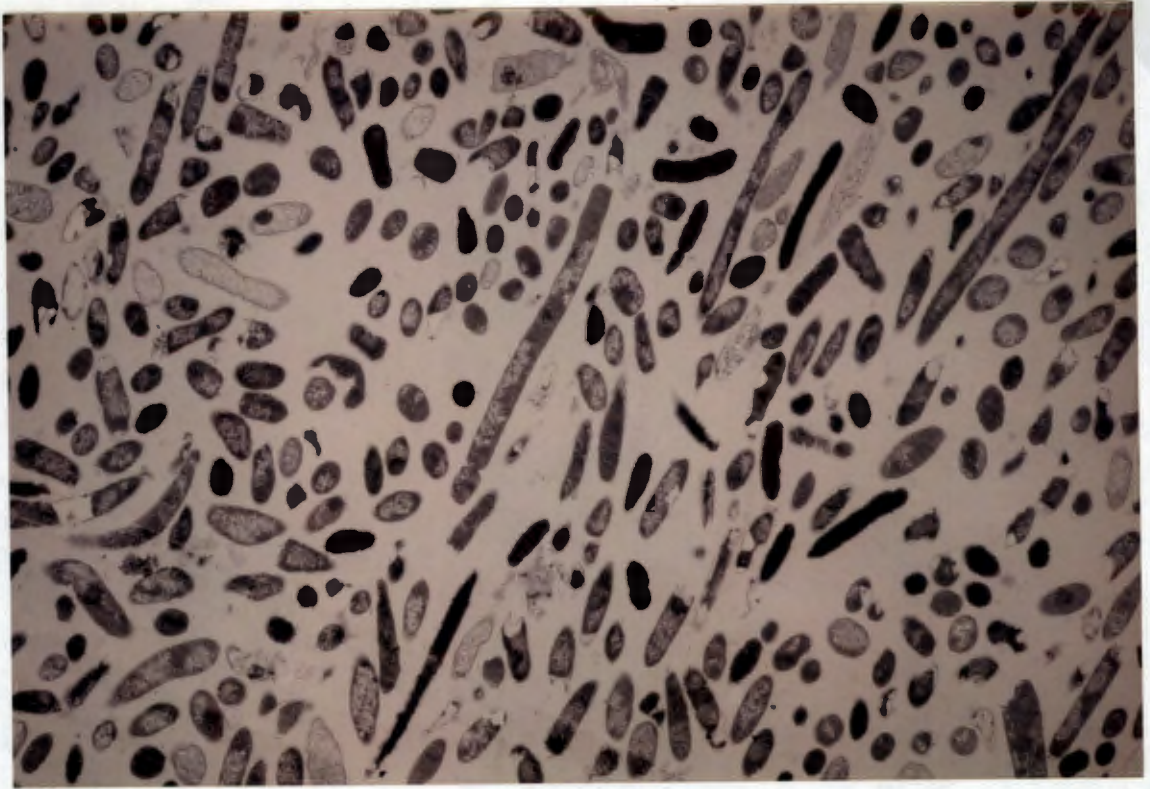


Fig. 24. Electron microscopy of type 1 fusobacteria (WC) (x5000)

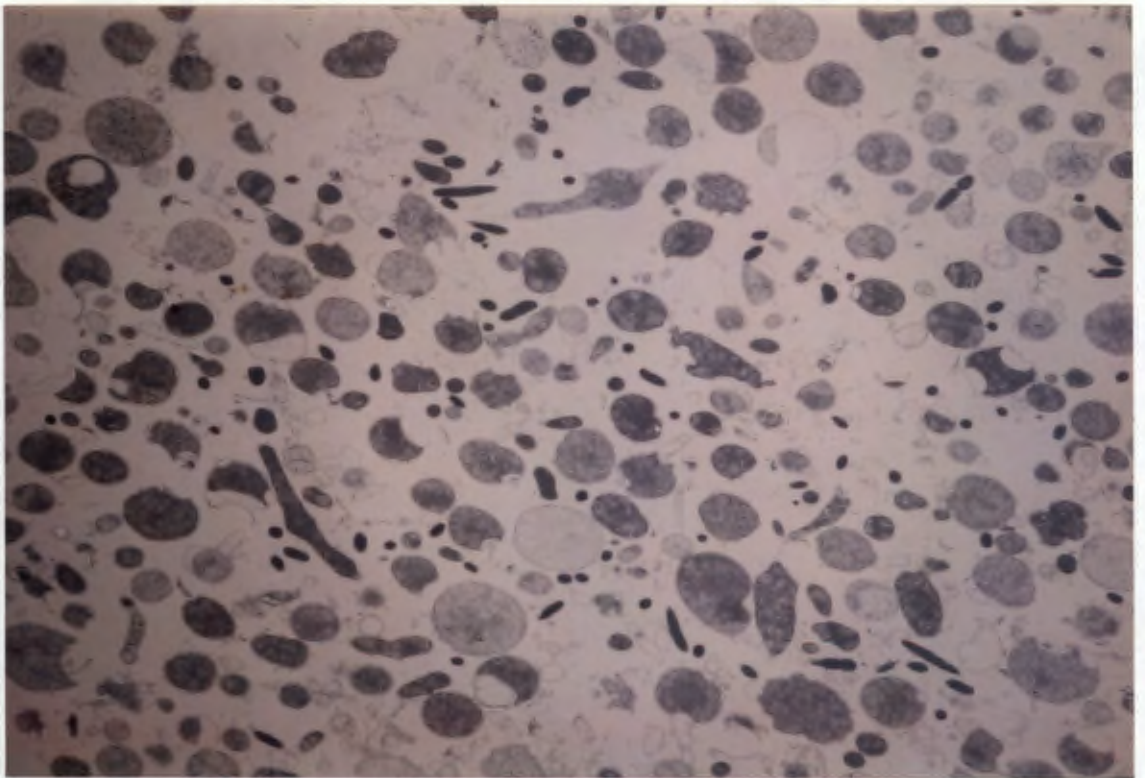


Fig. 25. Electron microscopy of type 2 fusobacteria (BHI) (x5000)

## Biochemistry of fusobacteria

The biochemistry profiles of the fusobacteria isolated are shown in table 27. All strains convert threonine to propionic acid which confirms their membership of the genus Fusobacterium. Both colonial types produced large amounts of gas in deep FABA after 24-36 hours incubation in air. No H<sub>2</sub>S was detected. All strains produced acid from glucose and type 2 strain fermented glucose and mannose. No acid was produced from fructose, lactose, sucrose, maltose, arabinose, raffinose or rhamnose. Indole, catalase, lecithinase, urease, lipase and oxidase were not produced. Aesculin and starch were not hydrolysed. Nitrate was reduced by all the strains. No digestion of gelatin or chopped meat occurred. These biochemical reactions differ from those species which the two morphological types of fusobacteria resemble namely, F.varium and F. mortiferum. The differences are shown in table 28.

Table 27. Biochemistry profiles of the fusobacteria

Test	Type 1	Type 2
Catalase	-	-
Oxidase	-	-
Aesculin hydrolysis	-	-
Casein digestion	-	-
Hydrogen sulphide production	-	-
End products of metabolism	acetic, butyric & propionic acid	acetic, butyric propionic acid
Bile tolerance	+	-
Carbohydrate fermentation	Glucose	Glucose & mannose
Nitrate reduction	+	+
Starch hydrolysis	-	-
DNAase production	-	-
Lipase	-	-
Conversion of threonine to propionic acid	+	+
Indole	-	-
Gas production	+	+

Table 28. Differentiating characteristics of Fusobacterium species

Test	F.mortiferum	F.varium	F.necrogenes	F.ulcerans
Indole	-	+	-	-
Aesculin hydrlyolysis	+	-	+	-
Nitrate reduction	+	-	-	+
Gas producion	+	+	+	+

### Antibiotic disc susceptibility tests

The fusobacteria isolated were sensitive to phosphamycin (300 ug/disc), kanamycin (1000 ugr/disc) and penicillin (2 u/ml). All were resistant to rifampicin (7.5 ug/disc).

### Soluble cellular protein pattern of fusobacteria

The soluble cellular proteins were identical for all the isolates. This is shown in figure 26. The reference standard ( Strep. faecalis ) is shown on the inner lane of the gels. The protein bands showed identical patterns for the two different morphological types of fusobacteria isolated. Compared with other Fusobacterium species, these protein patterns were completely different (W.E.C.Moore, personal communication). That the Bacteroides and other Fusobacterium species differ is shown in figure 27.

In view of the differences in the morphology, biochemical profiles and soluble cellular proteins, these fusobacteria have been identified as a new species and are referred to as Fusobacterium ulcerans . This nomenclature has been accepted by the International Journal of Systematic Bacteriology. The type strain deposited with the National Collection of Type Cultures is NCTC 12111 and NCTC 12112 is a further reference strain.

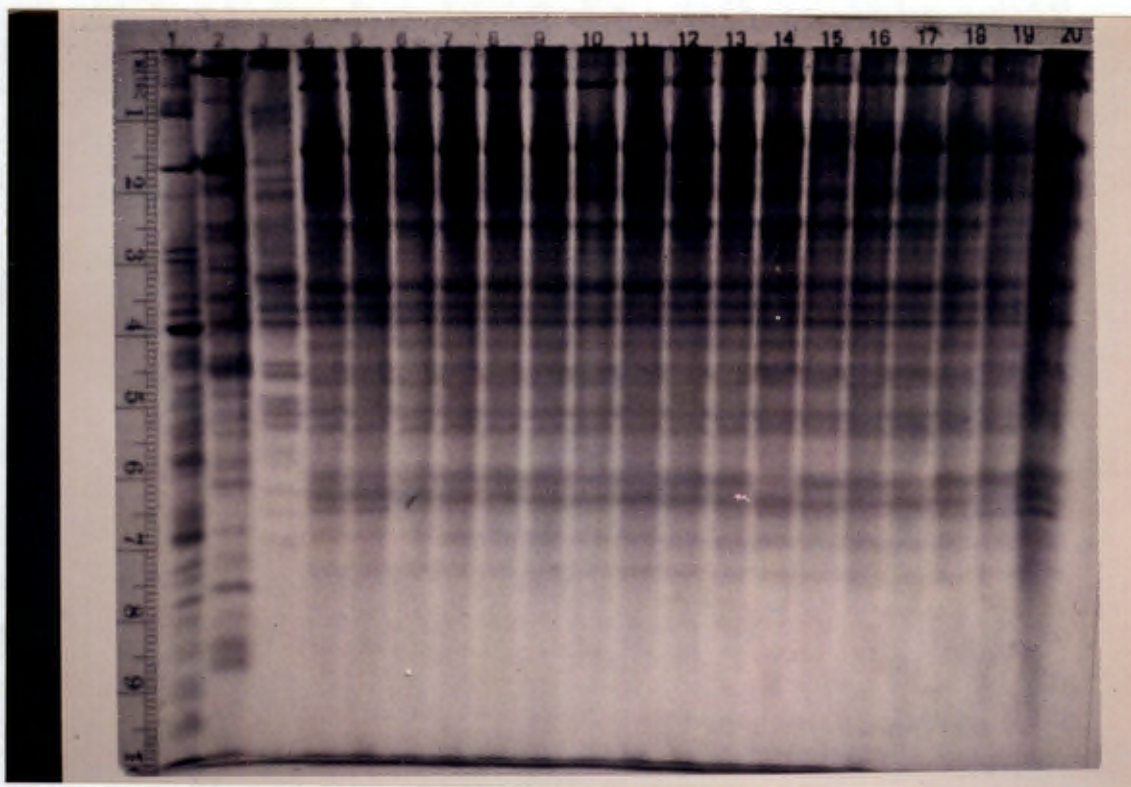


Fig. 26. PAGE of fusobacteria isolates

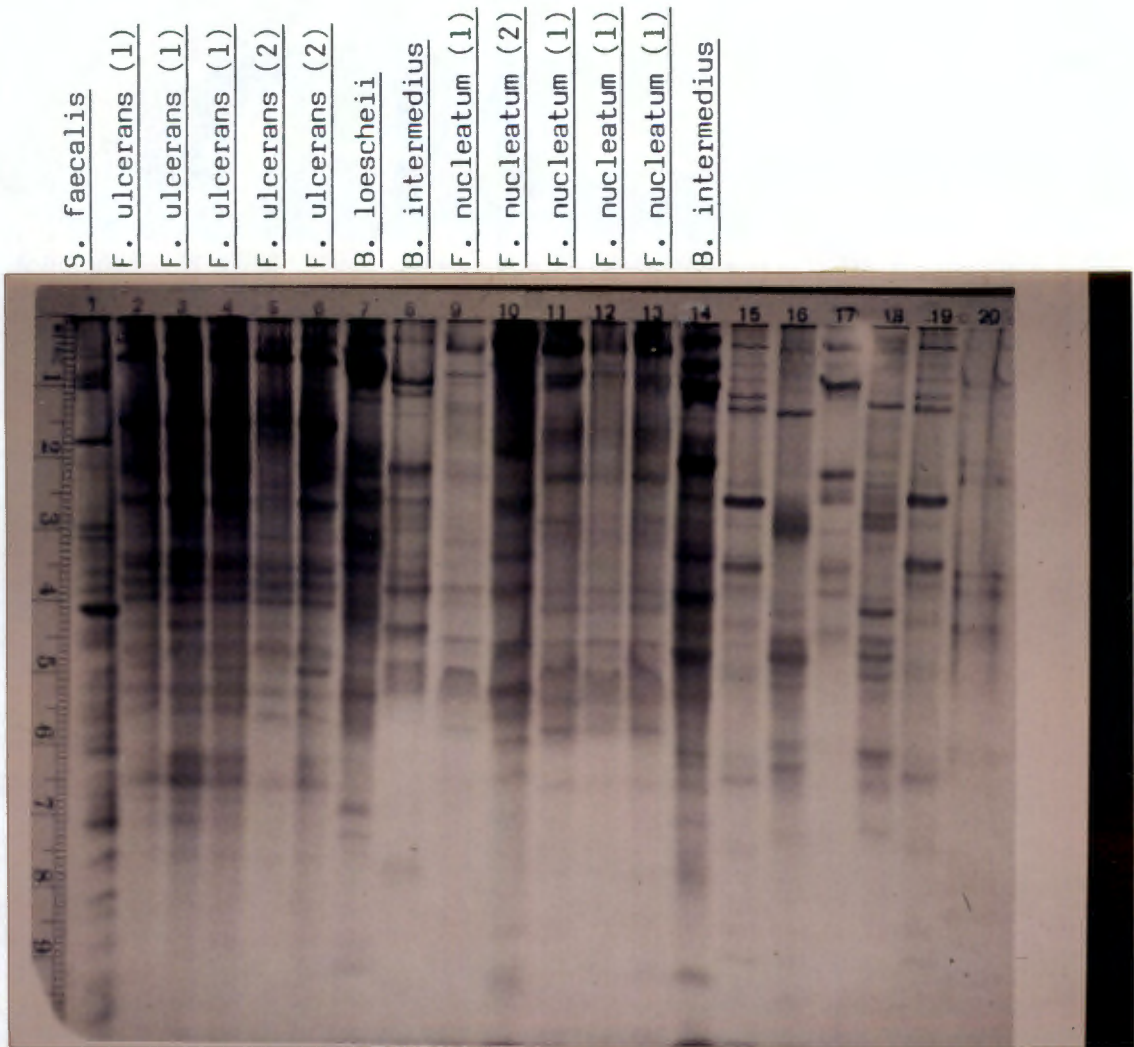


Fig. 27. PAGE of fusobacteria isolates  
with other species of Fusobacterium

### Bacteroides spp.

Those gram negative rods which were non-sporing, anaerobic and which produced iso-valeric or valeric acids on gas liquid chromatography were tentatively regarded as belonging to the Bacteroides genus. Their antibiotic sensitivities and biochemical profiles were recorded and are shown in table 29. The most frequently isolated species were B. thetaiotomicron and B. distasonis. Both organisms have been well described previously and the strains isolated showed no difference from the recognised patterns. These Bacteroides species were always isolated in association with other organisms.

### Anaerobic cocci .

Peptostreptococcus and Peptococcus spp. were isolated most commonly. One isolate of Veillonella was recovered.

### Propionobacterium spp.

These bacteria were short Gram-positive rods which grew only anaerobically and were sensitive to metronidazole. The organisms produced large amounts of propionic acid.

### Clostridium spp.

These thick Gram-positive spore-forming rods were only isolated from mud samples and not from ulcers. Further identification of these bacteria was not performed.

Table 29. Characteristics of Bacteroides spp isolated

Test	Result
Oxidase	-
Catalase	-
Bile tolerance	+
Antibiotic susceptibilities	Sensitive to rifampicin Resistant to kanamycin and vancomycin.
End products of metabolism	acetic, isovaleric and valeric acids

## Spirochaetes

Spirochaetes were visible on dark ground microscopy in 20% of the samples. Attempts to culture the spirochaetes were only partially successful. Of all the media used only NOS agar yielded growth of spirochaetes from three samples. The colonies were 4-5 mm in diameter. They were white, fluffy and appeared round in the tubes. The organisms were visible after one week's growth. However the culture was mixed and further characterisation of the organism was not possible. A carbol fuchsin stain shows the organism in mixed culture (fig 28).

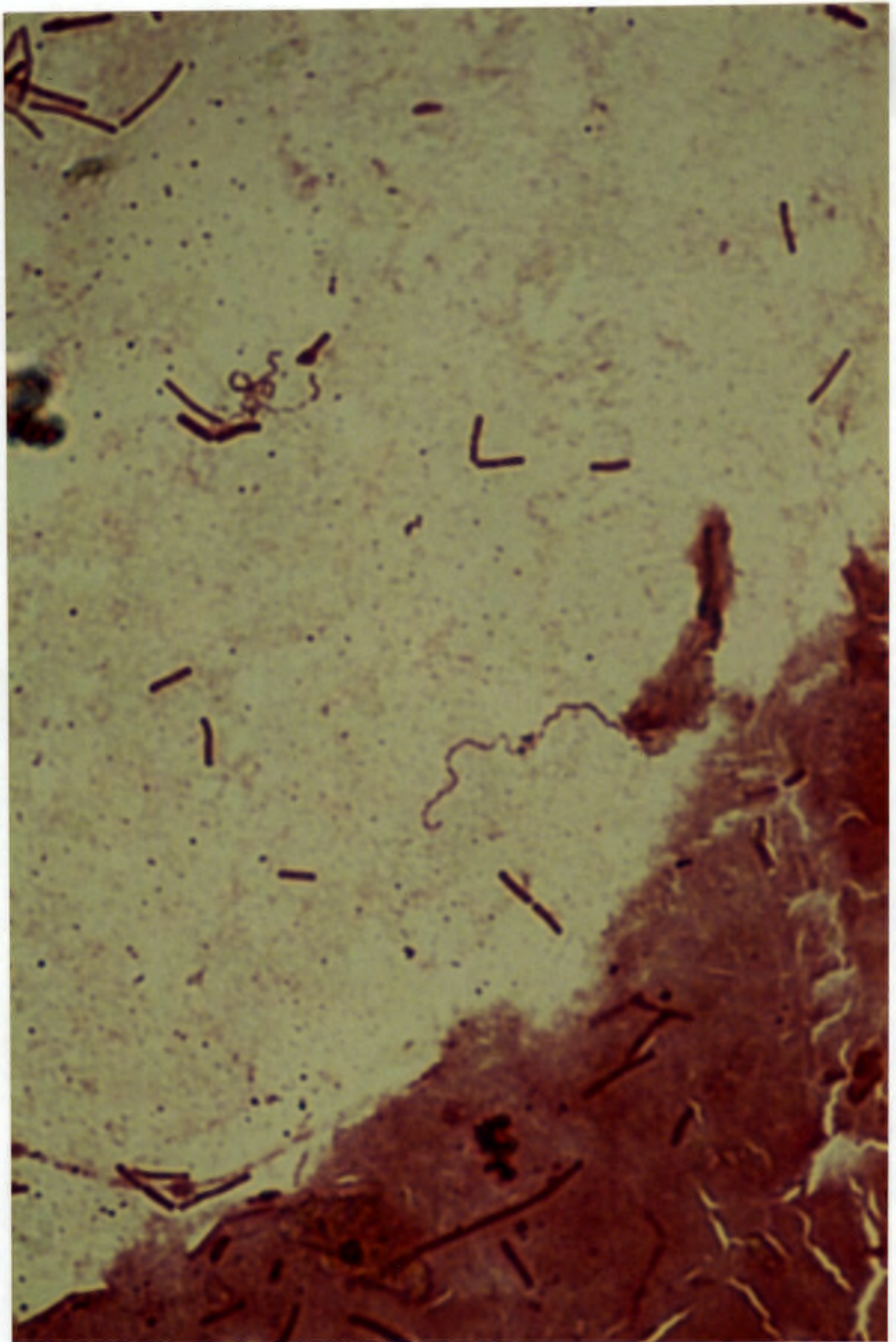


Fig. 28. Spirochaete morphology (x1000)

## Pathology

Biopsies were available on 20 patients from four endemic areas namely Gambia, Zambia, Papua New Guinea and India. The duration and treatment details were noted and are listed in table 30. In 17 patients the duration of the ulcer was known and of these, nine had been present for less than one month. The rest were between four and twelve weeks old. Seven patients had been treated with antibiotics (penicillin = 6, metronidazole = 1).

### Light microscopic findings

#### 1) Epidermal changes

##### A) Haematoxylin and Eosin stain

The main abnormality seen in all patients was acanthosis around the ulcer rim accompanied by a variable degree of hyperkeratosis. The ulceration extended through the epidermis to the dermis. Around the ulceration the epidermis showed pseudoepitheliomatous hyperplasia. Spongiosis was seen in the overlying epidermis of five biopsies all of which were less than six weeks in duration (fig. 29). A diffuse infiltrate of polymorphonuclear leukocytes in all layers of the epidermis accompanied the spongiosis. At the edge of the ulcer the epidermis appeared orderly and intact. In older lesions elongation of the rete ridges accompanied marked epidermal thickening. Remnants of stratum corneum were occasionally seen in the slough covering the ulcer.

Table 30. Duration of ulcers before biopsies

Duration (weeks)	Number of biopsies
0-4	9
4-8	4
8-12	4
unknown	3

## B) Dieterle stain

Bacteria were seen in the epidermis in six patients, only one of whom had received antibiotics. All of these were ulcers less than one month old. In four cases the organisms were seen in the outer stratum corneum together with polymorphonuclear leukocytes. In two patients bacteria were seen scattered throughout all the epidermal layers. For the most part these were pleomorphic Gram-negative rods or Gram-positive cocci. The Dieterle stain proved very useful for demonstrating all bacteria clearly. No fungi were identified in any of these sections.

## 2) Subepidermal changes

### A) Haematoxylin and Eosin stain

The pattern depended on the zone examined and the duration of the ulcers and the treatment received. The ulcer beds were unremarkable and showed granulation tissue with necrotic slough, polymorphonuclear leukocytes and nuclear debris. At the base of the ulcer vascular proliferation was common. None of the ulcers showed extension to the subcutaneous tissues. Under the intact epidermis there was marked oedema of the dermis particularly in ulcers less than one month old. This was absent in patients with ulcers of short duration who had received antibiotics (penicillin). The cellular infiltrate was dense and maximal around the epithelial defect. Most of the cells were polymorphonuclear leukocytes although in two patients large numbers of eosinophils were seen. Lymphocytes were also present in moderate numbers. Collagen bundles in the area of the infiltrate were widely separated suggestive of oedema.

Next to the ulcers there was a dense inflammatory infiltrate of chronic inflammatory cells around the blood vessels and adnexal structures. Involvement of nerves was not apparent. Pigmentary incontinence was seen in all biopsies and usually extended well beyond the borders of the ulcer.

While the dermal blood vessels were dilated and increased in number (fig. 30) there was marked thickening of the vessel wall with endothelial proliferation in several chronic ulcers. There was no evidence of vasculitis at any stage but one chronic ulcer showed endarteritis obliterans.

#### B) Special stains

In acute lesions the sides and base of the ulcer were occupied by inflammatory slough containing bacteria. In untreated lesions these appeared to consist of a plug of necrotic material and organisms. The predominant bacteria were rod-like or pleomorphic and were Gram-negative. Long and arcuate forms were also seen (fig. 31). These differed from those described by Kuberski and Kotega (1980). In four untreated cases spiral bacteria were also found in the slough or at the ulcer rim (fig.32). These were associated with other organisms. Gram-positive bacteria were rarely seen. The PAS stain failed to show any fungi and the Giemsa stain was useful for detecting the Leishman-Donovan bodies in those patients with leishmaniasis.

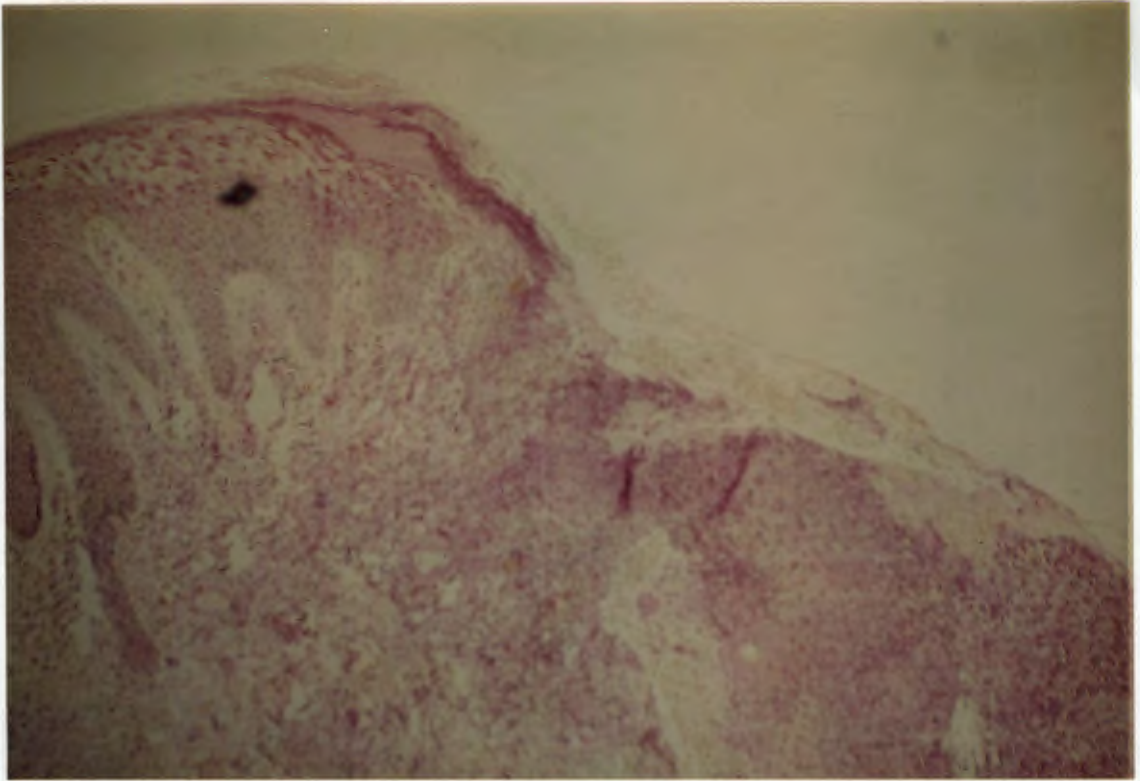


Fig. 29. Pathology of tropical ulcer showing spongiosis  
(haematoxylin and eosin) (x400)

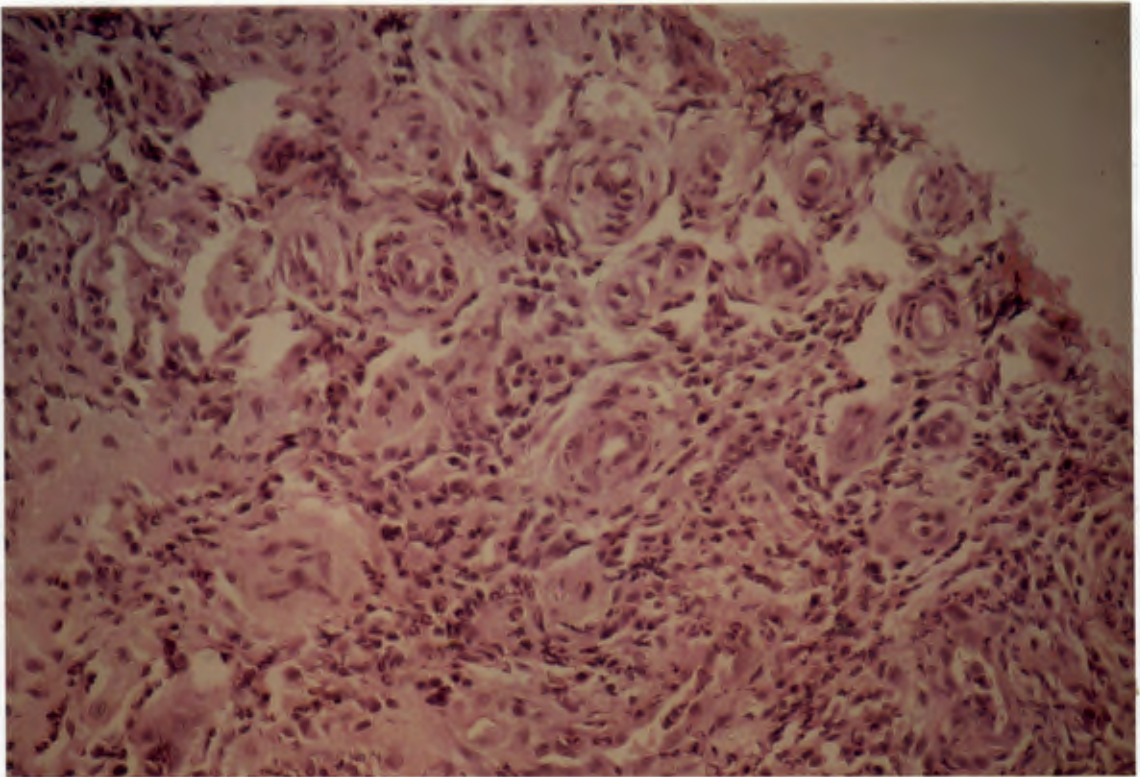


Fig. 30. Pathology of tropical ulcer showing vessel proliferation  
(haematoxylin and eosin) (x400)

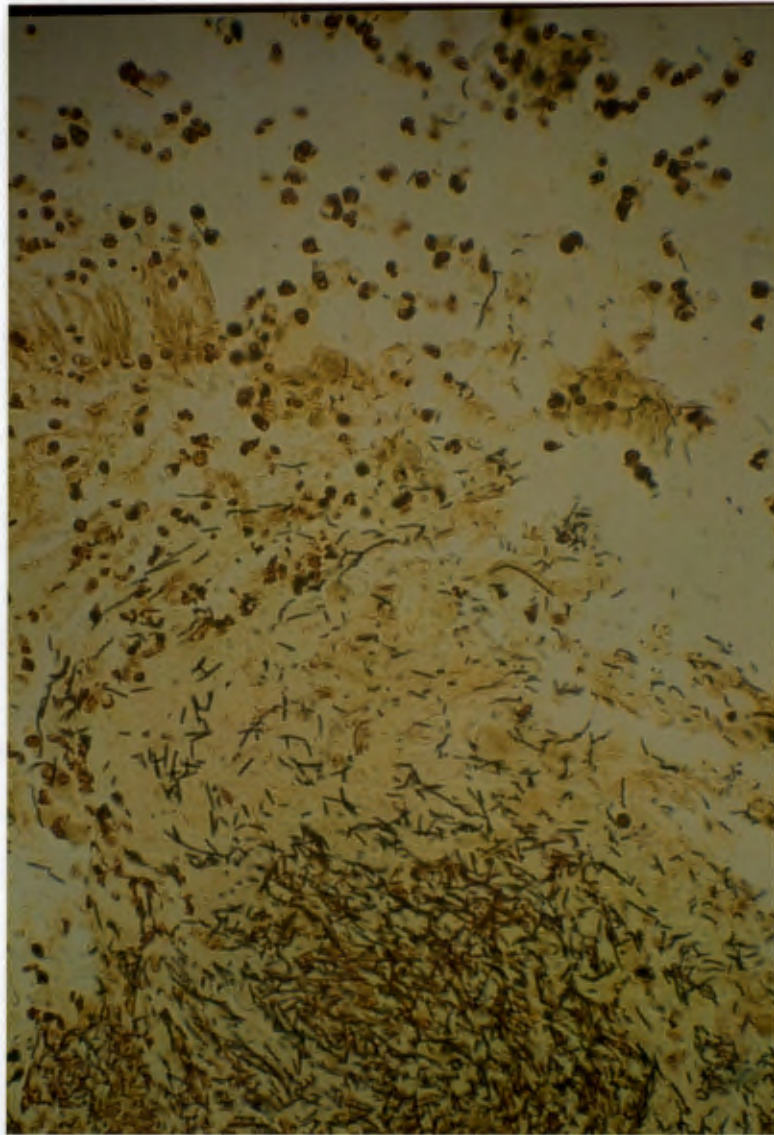


Fig. 31. Dieterle stain showing bacteria (x1000)

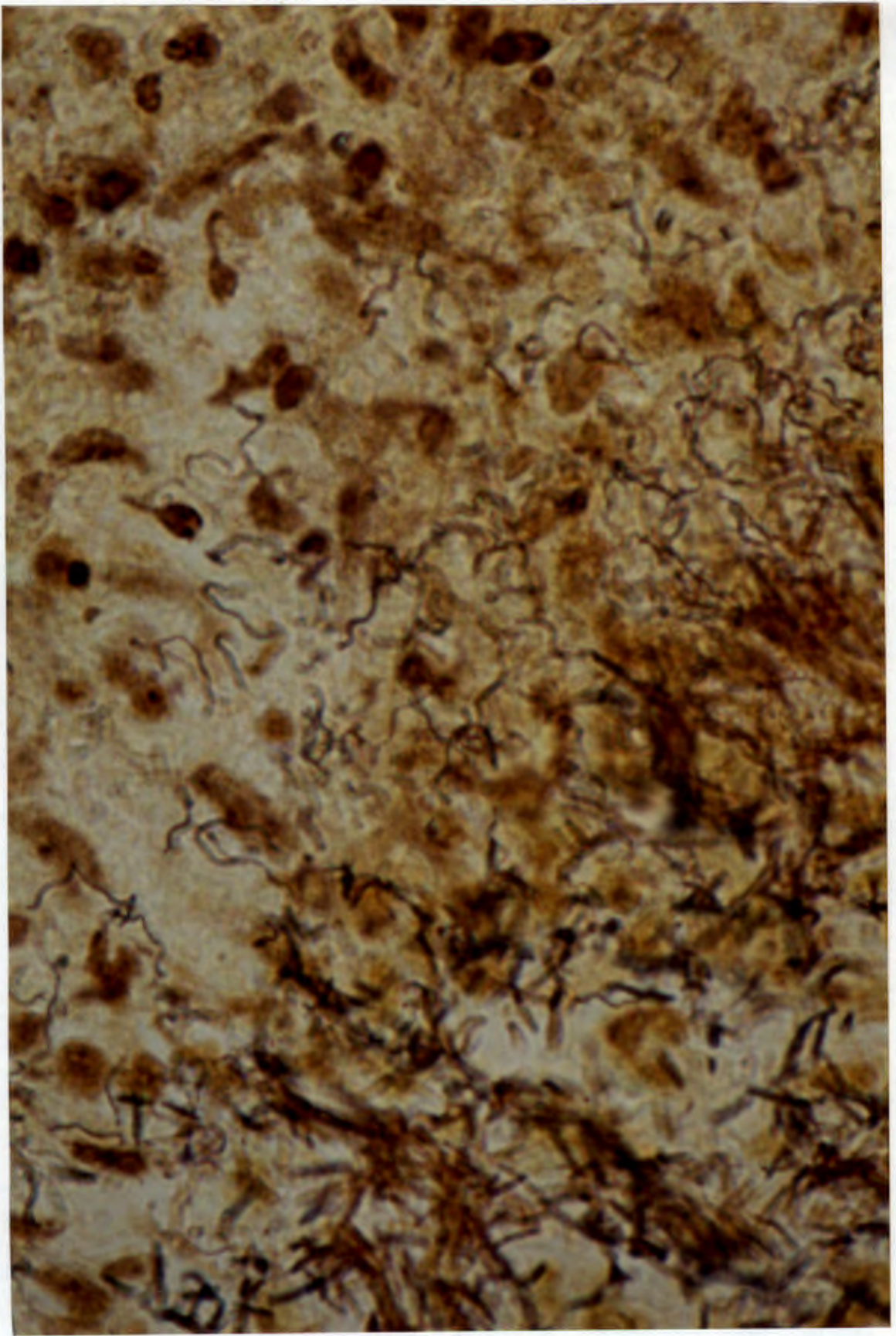


Fig. 32. Dieterle stain showing spirochaetes (x1000)

## Electron microscopy of tropical ulcer

Sections from seven patients were examined. The general features confirmed the light microscopic findings. In four acute cases the epidermal oedema was severe and the presence of polymorphonuclear leukocytes infiltrating the dermis was confirmed.

The predominating bacteria were rod shaped, round or elongated with cytoplasm containing ribosomes and a thin cell wall. The latter tended to be irregular (fig. 33) The appearances accorded most with the fusobacterial isolates from culture. By contrast organisms with the appearance of Bacteroides species examined in-vitro were only identified in two sections. These were rounder and had a more compact cytoplasm with thicker, more regular cell walls. The coliform organisms were not seen in the tissues sections. Small cocci were seen with dense staining cytoplasm and these were grouped around keratinocytes in some sections.

In two biopsies spirochaetes were observed in the deeper layers of the dermis and they were not associated with the other bacteria (fig. 34). These organisms showed sinusoidal periodicity and had three flagellae. Details of the insertion of the latter could not be adequately visualised in the sections. However these characteristics corresponded with those of spirochaetes (Hovind-Hougen 1976). Some pleomorphic bacteria were present in macrophages or polymorphonuclear leukocytes. From their appearance it seemed that they had resisted intracellular digestion. Many disrupted bundles of collagen were seen in the vicinity of the clusters of bacteria.

Those patients with leishmaniasis and ulceration showed definite changes of leishmaniasis in the histological sections as shown in figure 16. Several Leishman-Donovan bodies, together with a very mixed inflammatory cell infiltrate occupied the mid dermis. The inflammatory infiltrate consisted of lymphocytes and histiocytes mainly. In 2 sections, giant cells were seen. The overlying epidermis did not show any spongiosis.

#### Immunohistochemical studies

The skin biopsies of patients with tropical ulcers were studied by immunohistochemical markers to assess the inflammatory response in the dermis. The lymphocytic infiltrate in the dermis consisted of an equal number of T-cells and B-cells as shown in figures 35 and 36. The lymphocytic infiltrate was concentrated mostly at the periphery of the ulcer.

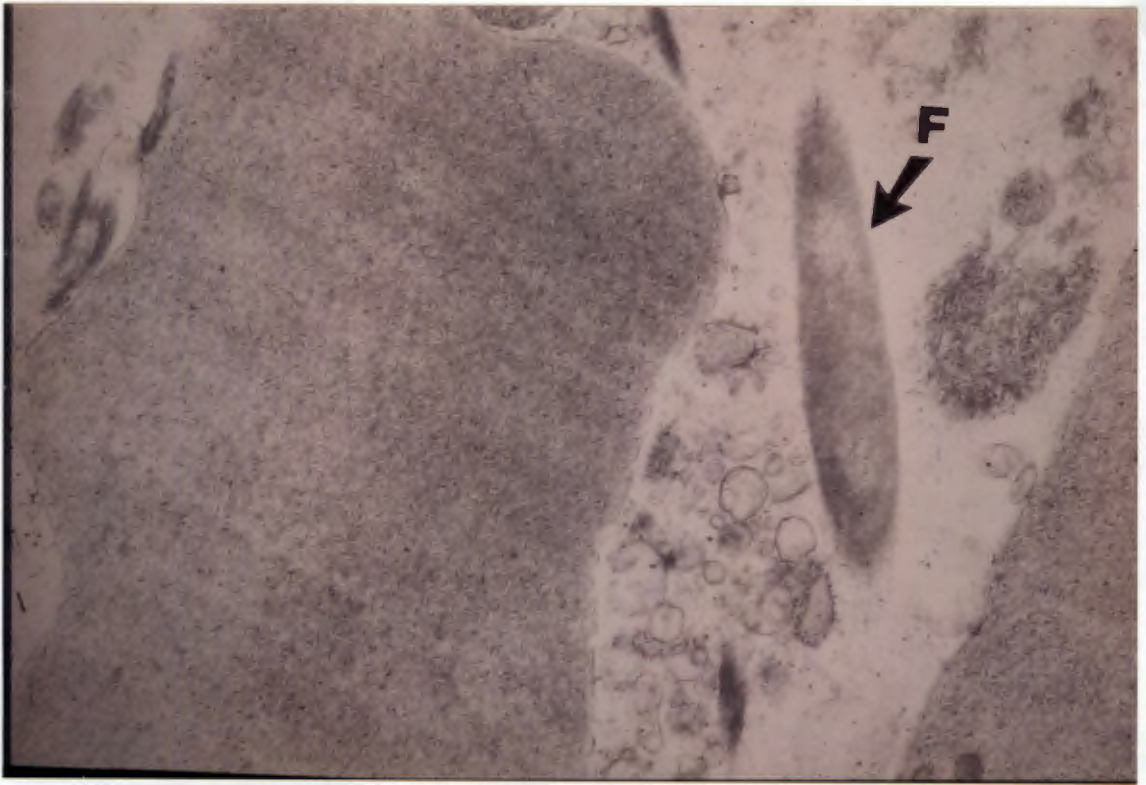


Fig. 33. Fusobacterium in the dermis (x10000)

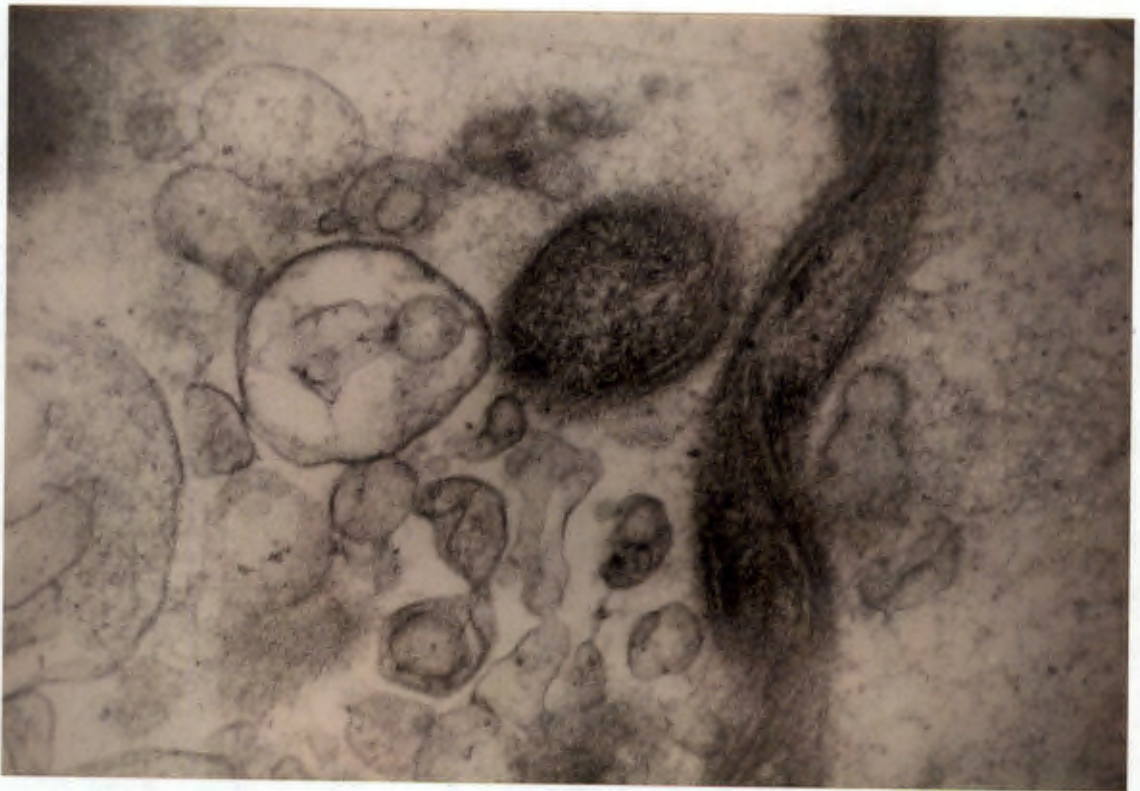


Fig. 34. Electron microscopy of treponemes in the dermis (x10000)



Fig. 35. T-cells in the dermis (x100)



Fig. 36. B-cells in the dermis (x100)

### Patient serology

The sera of 20 patients and 10 controls were tested to a whole cell preparation of fusobacteria antigen. In 8 sera (5 patients and 3 controls) there was bright fluorescence to the fusobacteria antigen visible (++) at a screening dilution of 1:16. Further dilution of these sera to cover a range of titres from 1:16 to 1:1024 failed to produce fluorescence at the higher dilutions. In two samples even the original fluorescence could not be reproduced. Further characterisation of the antibody was therefore not carried out. The results are shown in table 31.

### Antibody production in patients and controls

The ELISA test showed that the patients and controls had very low levels of antibody to most of the isolates from the ulcers. The results are shown in table 32.

### Treponemal serology

Sera of 20 patients and 10 controls were tested. All the sera showed a negative reaction to the test at a dilution of 1:4.

Table 31. Antibody production to fusobacteria.

Sample number	Antibody production (fluorescence)			
	Dilution of serum tested			
	1:16	1:64	1:128	1:256
Patient 1	++	+/-	-	-
2	++	+/-	-	-
3	+++	-	-	-
4	+	-	-	-
5	++	+/-	-	-
6	+	-	-	-
7	++	+/-	-	-
8	+	-	-	-
Control 1	+++	+/-	-	-
2	++	-	-	-
3	++	+	-	-

### Rabbit serology

A whole cell preparation of Fusobacterium was used as the antigen. The peroxidase labelled anti-rabbit IgG gave better readings at the 1:10,000 compared with the 1:5000 dilution. When the amount of IgG produced by the rabbit was compared with normal rabbit serum which served as the control only a small amount of antibody was produced. This amount was much lower than the positive control (against P.boydii ). The result is shown in table 33.

When the rabbit serum was tested for production of IgM, there was a small amount of IgM produced as is shown in table 34. As the amounts of antibody produced were small the rabbit serum was not used to detect the presence of fusobacteria in skin sections. The results of the ELISA test showed that only small amounts of antibody were produced in the rabbit in response to the repeated inoculations with fusobacterium.

Table 32 ELISA to bacteria isolated from ulcers

Antigen & dilution	NHS	1	2	3	4	5	6	7	8	8	10	Buffer
<u>Fusobacteria</u>												
1:10	0.000	-.021	-.061	0.071	0.078	0.308	0.085	0.166	UNDER	UNDER	-.065	UNDER
1:20	UNDER	UNDER	-.040	0.077	0.074	0.327	0.205	0.162	-.090	UNDER	UNDER	UNDER
1:40	UNDER	-.038	-.041	0.039	0.027	0.234	0.249	0.263	UNDER	UNDER	-.092	UNDER
1:80	UNDER	UNDER	UNDER	UNDER	-.098	0.204	0.299	0.240	UNDER	UNDER	-.057	UNDER
<u>Bacteroides</u>												
1:10	UNDER	UNDER	-.002	0.140	-.024	0.121	0.020	UNDER	UNDER	UNDER	0.006	UNDER
1:20	UNDER	UNDER	-.096	0.076	0.018	0.074	0.091	-.026	UNDER	UNDER	UNDER	UNDER
1:40	UNDER	UNDER	-.094	-.061	0.000	0.053	0.102	-.010	UNDER	UNDER	UNDER	UNDER
1:80	UNDER	UNDER	UNDER	UNDER	UNDER	0.069	0.185	0.031	UNDER	UNDER	-.021	UNDER

(NHS = Normal Human Serum)

Table 32 continued. ELISA to bacteria isolated from ulcers

Antigen & dilution	NHS	Patient Number										Buffer
		1	2	3	4	5	6	7	8	8	10	
<u>Coliforms</u>												
1:10	0.000	0.088	0.129	0.188	0.155	0.255	-0.023	UNDER	0.062	0.072	UNDER	UNDER
1:20	-0.069	0.075	0.157	0.174	0.246	0.269	0.143	-0.008	0.211	0.054	-0.102	UNDER
1:40	-0.094	0.064	0.077	0.177	0.098	0.246	0.192	0.058	0.193	-0.074	0.013	UNDER
1:80	UNDER	0.063	0.185	UNDER	0.115	0.278	0.215	0.092	0.033	UNDER	-0.014	UNDER
<u>Streptococcal</u>												
1:10	UNDER	0.024	0.121	0.225	0.258	0.243	0.200	-0.068	0.274	0.204	UNDER	UNDER
1:20	UNDER	0.047	0.249	0.241	0.154	0.199	0.231	0.218	0.167	-0.077	-0.080	UNDER
1:40	UNDER	0.022	0.192	0.311	0.162	0.058	0.148	0.066	0.242	-0.017	-0.014	UNDER
1:80	UNDER	-0.088	0.156	UNDER	0.071	0.158	0.273	0.127	UNDER	-0.054	-0.042	UNDER

(NHS = Normal Human Serum)

Table 33 ELISA - Rabbit IgG Levels

Antirabbit IgG tested at 1:5000

Antibody dilution	Fusobacterium antigen 2ug/100ul buffer				P. boydii 2ug/100ul							
	Buffer	Rabbit test serum	Normal rabbit serum	Buffer	RTS	P. boydii antiserum	RTS	P. boydii antiserum				
1:10	0.849	1.388	1.425	1.329	1.435	0.961	0.691	0.240	0.563	1.420	1.722	1.464
1:20	1.094	1.476	1.498	1.377	1.120	0.824	0.497	0.258	0.792	1.497	1.650	1.450
1:40	0.907	1.439	1.356	1.421	0.870	0.554	0.317	0.073	0.669	1.411	1.604	1.350
1:80	0.924	1.522	1.392	1.353	0.574	0.483	0.236	0.035	0.522	1.464	1.451	1.511
1:160	0.301	1.361	1.389	1.678	0.405	0.407	0.190	0.051	0.402	1.373	1.536	1.369
1:320	0.406	1.312	1.310	1.608	0.463	0.414	0.149	0.107	0.251	1.308	1.522	1.381
1:640	0.349	1.354	1.380	1.411	0.229	0.248	0.127	0.043	0.199	1.269	1.427	1.348
1:1280	0.229	1.329	1.293	1.235	0.184	0.208	0.108	0.036	0.030	1.235	1.408	1.276

(RTS = Rabbit test serum)

Table 34 ELISA - Rabbit IgM Levels

Antibody dilution	Fusobacterium antigen 2ug/100ul buffer				P.boydii 2ug/100ul						
	Buffer	Rabbit test serum	Normal rabbit serum	Buffer	Buffer	P.boydii antiserum	Buffer	Buffer			
1:10	0.000	0.394	0.402	0.023	0.028	0.029	-0.003	0.007	0.259	0.249	0.272
1:20	-0.003	0.371	0.372	0.004	0.002	0.004	-0.005	-0.004	0.188	0.193	0.210
1:40	-0.004	0.314	0.324	0.003	-0.004	-0.003	-0.006	-0.005	0.142	0.151	0.172
1:80	-0.004	0.274	0.266	0.005	-0.004	-0.004	-0.006	-0.006	0.102	0.102	0.121
1:160	-0.003	0.193	0.201	0.005	-0.005	-0.004	-0.005	-0.005	0.060	0.061	0.079
1:320	0.000	0.161	0.148	0.005	-0.005	-0.005	-0.005	-0.005	0.028	0.032	0.042
1:640	-0.003	0.097	0.101	0.003	-0.004	-0.004	-0.004	-0.005	0.011	0.013	0.034
1:1280	-0.001	0.081	0.073	0.001	0.011	0.001	0.000	-0.004	0.009	0.008	0.019

## Mechanisms of infection

### Cytotoxicity

Cytotoxicity was demonstrated by morphological changes in the cell monolayers. The affected cells showed long filamentous tendrils where damage was mild and rounding up of cells occurred when damage was more extensive. The nuclear-cytoplasmic ratio was 2:1 before exposure to the supernatants (fig.37) and 1:1 after cytotoxicity. The changes associated with the cytotoxicity were observed 24 hours after incubation. The rounded cells detached into the medium (figure 38). Of all the cell lines tested, Vero cells were affected most severely. The human embryonic intestine (Int 407) cell line showed similar but less severe changes. The percentage of Vero cells affected by culture filtrates of the test species is shown in table 35. The other cell lines namely, chimp liver, Chinese hamster ovary and MRC 5, were not affected. No similar cytotoxicity occurred when Vero cells were exposed to the uninoculated brain heart infusion broth, Bacteroides supernatants or the coliform supernatants. The growth medium had no effect on the cells. The results of the cytotoxicity tests using the cell lines other than Vero cells are shown in table 36.

Heating the supernatants to 55°C or 80°C had no additional effect on the cytotoxicity. The degree of cytotoxicity was the same as prior to heating.

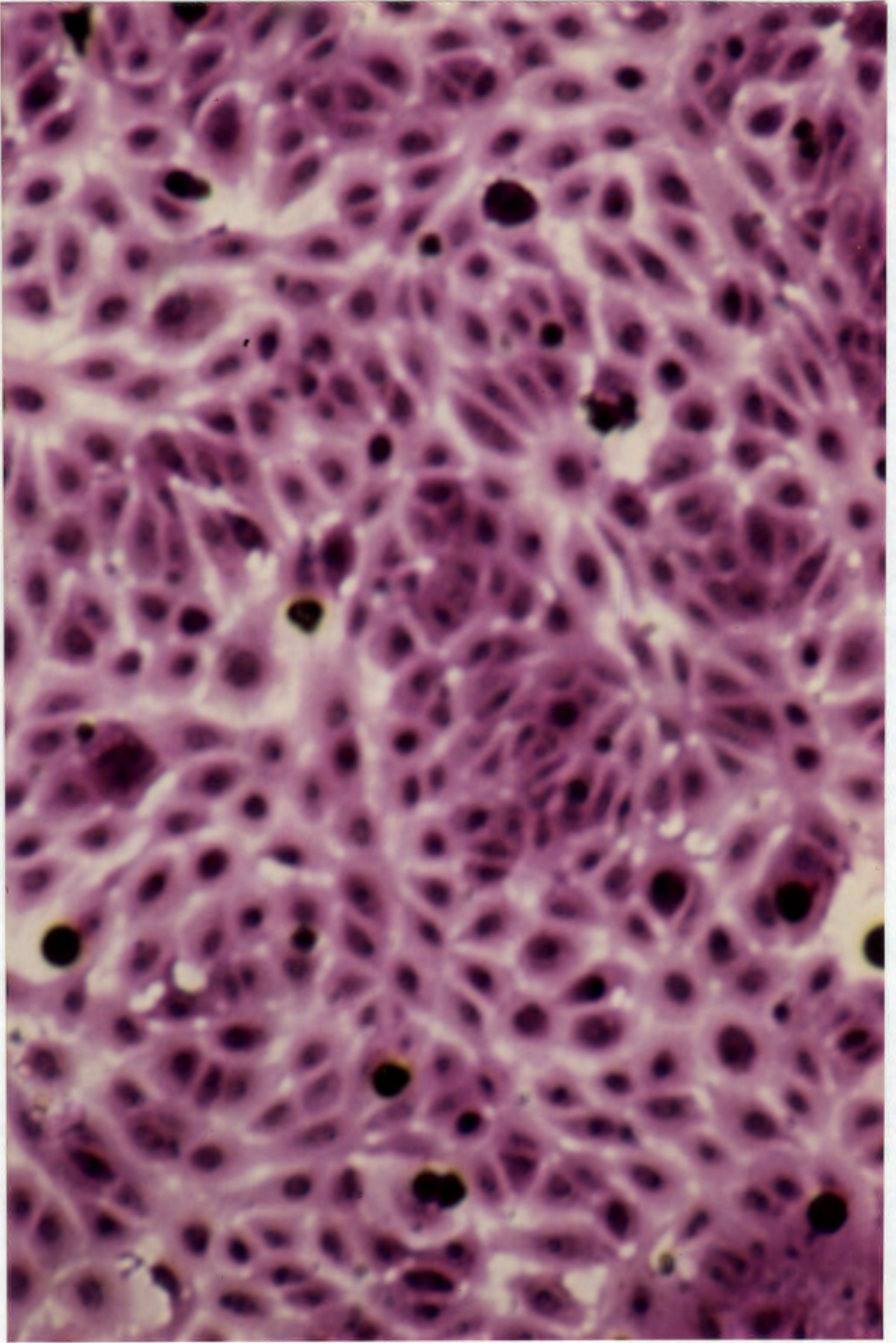


Fig. 37. Vero cells with growth medium

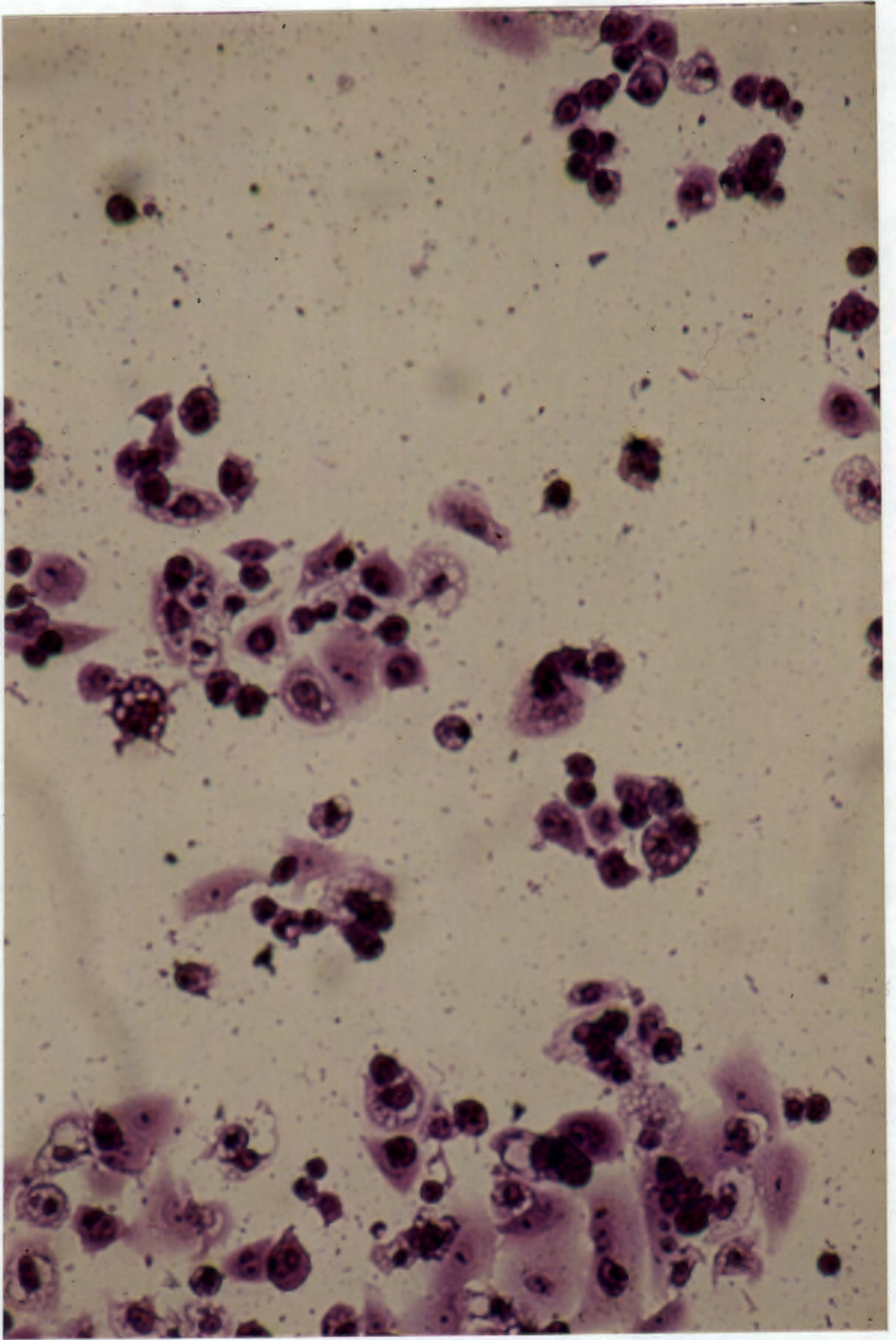


Fig. 38. Vero cells showing cytototoxicity

Dialysis of the supernatants against PBS resulted in loss of cytotoxicity. As the supernatants were produced under anaerobic conditions, anaerobiosis was thought to be important for the persistence of cytotoxicity. The supernatants were therefore dialysed against PBS with cysteine but cytotoxicity was still lost. Concentration of the supernatant against polyethylene glycol (PEG) increased the amount of cytotoxicity. The results are shown in table 37. This effect was not due simply to the concentration of proteins in the medium (BHI) as concentration of BHI against PEG failed to produce a similar result.

Equivalent dilutions of butyrate to that in the culture filtrates caused the same morphological effect on the Vero cells as the crude culture filtrates. The percentage of Vero cells affected by the dilutions of medium containing butyrate were the same as the corresponding culture filtrates of the fusobacteria. Butyrate concentrations of 30mM or more affected 100% of the Vero cells. These results are shown in table 38. Culture filtrate dilutions of strains of Bacteroides fragilis, Enterobacter cloacae or Streptococcus faecalis had no morphological effect on the Vero cells.

Table. 35. Percentage of Vero cells affected after 24 hours  
incubation with 1:8 dilution of test culture filtrates

Species	Vero cells affected
<u>F. ulcerans</u> (NCTC 12111)	100%
<u>F. ulcerans</u> (NCTC 12112)	100%
<u>Bacteroides fragilis</u>	5%
<u>Enterobacter cloacae</u>	5%
<u>Streptococcus faecalis</u>	5%

Table 36. Cytotoxicity in various cell lines excluding Vero cells

Sample & dilution	Cell Line			
	Chimp Liver	Hamster ovary	MRC 5	INT 407
<u>F. ulcerans</u>				
NCTC12111				
1:4	-	-	+	++
1:8	-	-	+/-	+
1:16	-	-	-	+/-
<u>F. ulcerans</u>				
NCTC 12112				
1:4	-	-	+	++
1:8	-	-	+/-	+
1:16	-	-	-	-
<u>B. thetaiotomicron</u>				
1:4	-	-	-	-
<u>E. cloacae</u>				
1:4	-	-	-	+
1:8	-	-	-	-
<u>S. faecalis</u>				
1:8	-	-	-	-

Table 37. Results of dialysis of supernatants

Sample	Dilution			
	1:2	1:4	1:8	1:16
<u>F. ulcerans</u> NCTC 12111	+++	+++	++	+
<u>F. ulcerans</u> NCTC 12112	+++	+++	++	+
<u>B. thetaiotomicron</u>	+/-	-	-	-
<u>E. cloacae</u>	+/-	-	-	-
Fusobacterium supn dialysed against PBS	+/-	-	-	-
<u>Fusobacterium</u> supn dialysed against PEG	+++	+++	+++	++
<u>Fusobacterium</u> supn dial. agnst. PBS+cysteine	+/-	+/-	-	-
BHI	-	-	-	-
BHI dial. agnst. PBS	-	-	-	-
BHI dial. agnst. PEG	-	-	-	-
<u>F. ulcerans</u> supn filtrates				
1-10	-	-	-	-
11-20	-	-	-	-
21-30	+	-	-	-
31-40	-	-	-	-

Table 38. Percentage of Vero cells affected after 24 hours incubation with varying concentrations of butyrate

Concentration in medium (M)	Cells affected (%)
0.625	100
0.500	75
0.375	50

## Protein precipitation and gel filtration

After ammonium sulphate precipitation, the supernatants caused severe damage to the Vero cells when the cytotoxicity experiments were repeated. A similar effect was observed when the ammonium sulphate was used as a control. Several fractions showed an increase in the solute concentration. These individual fractions were used in the cell culture experiments. However none of these produced changes similar to those of the supernatant. The fractions were thought to be too dilute so the gel filtration was repeated with supernatant fluid concentrated against PEG. Although several fractions again showed an increase in solute concentration, no cytotoxicity was observed when these fractions were tested.

### Animal studies

The potential for synergy between aerobic, facultative and anaerobic bacteria was studied with intradermal injections of mixtures of organisms isolated from tropical ulcers. Two experimental animal models were used to simulate the cutaneous ulceration of tropical ulcer. Synergy between two groups of bacteria was determined by titration of the aerobic bacterial strains in association with a single sublethal dose of one of the other anaerobic bacteria. The formation of an abscess after intradermal injection of bacteria was strongly dependent on the dose used.

### Experimental infection in mice

In the C57 black mouse, cutaneous necrosis occurred with inoculation of F. ulcerans NCTC 12111 (figure 39) at a dose of  $0.5 \times 10^9$  org/ml. The lesions became necrotic, crusted and the skin sloughed. This was not reproducible as some mice showed no reactions at all to the same dose of inoculation. No mice developed abscesses. In the other mouse strains no local reactions occurred. Mice from the two strains (Balb/c HeA 2 and C x S 12) became lethargic two days after the injections. The third day after the injection of the fusobacteria they died although there was no evidence of any local cutaneous reaction. The mouse model was therefore abandoned.

## Experimental infections in guinea pigs

The reactions to subcutaneous injections were recorded as;

- 1) Erythema: the area was measured and recorded in centimetres,
- 2) Induration: the induration was measured and recorded as above,
- 3) Abscess formation: this occurred when pus formed and ruptured through the skin.

### Determination of the minimal abscess forming dose

The minimal abscess forming dose was determined for each bacterium alone and in combinations of two or more bacteria. Only abscesses with pus formation were regarded as positive. The lowest dose producing an abscess in at least 50% of the injections was regarded as the minimal abscess forming dose (Brook and Walker 1983).

The results of the injections of each of the bacteria are shown in table 39. Of the anaerobic bacteria only B. thetaotomicron produced an abscess with an abscess forming dose of  $1 \times 10^9$ . F. ulcerans failed to produce an abscess after intradermal injection despite a high inoculum ( $1 \times 10^{10}$ ). At most erythema developed at the site of inoculation. The aerobic coliform bacterium E. cloacae produced an abscess with an abscess forming dose of  $1 \times 10^{10}$ .

Table 39. Reactions to intradermal injections of single bacteria

Organism injected	Dose	Result
<u>E. cloacae</u>	$1 \times 10^7$	no reaction
	$1 \times 10^8$	erythema
	$1 \times 10^9$	erythema + induration
	$1 \times 10^{10}$	abscess
<u>B. thetaiotomicron</u>	$1 \times 10^7$	erythema
	$1 \times 10^8$	erythema + induration
	$1 \times 10^9$	abscess
	$1 \times 10^{10}$	abscess
<u>F. ulcerans</u> NCTC12111	$1 \times 10^7$	no reaction
	$1 \times 10^8$	no reaction
	$1 \times 10^9$	no reaction
	$1 \times 10^{10}$	no reaction
	$1 \times 10^{12}$	erythema
<u>S. faecalis</u>	$1 \times 10^7$	no reaction
	$1 \times 10^8$	no reaction
	$1 \times 10^9$	slight erythema
	$1 \times 10^{10}$	erythema
Anaerobic cocci	$1 \times 10^6$	erythema
	$1 \times 10^7$	erythema
	$1 \times 10^8$	erythema
	$1 \times 10^9$	erythema

### Inoculations with a combination of organisms

The results after inoculation of a combination of bacteria are shown in table 40. The results can be expressed in groups according to their ability to cause subcutaneous abscesses in the guinea pig model.

Group 1 induced abscesses when injected by themselves. These included E. cloacae and B. fragilis .

Group 2 did not induce abscesses when injected by themselves and were never recovered from the abscesses when injected with other organisms. This group included S. faecalis .

Group 3 did not produce abscesses when injected by themselves but did survive and were associated with necrosis when injected with other organisms in at least 70% or more of the abscesses. F. ulcerans was included in this group.

Subcutaneous abscesses were formed after the inoculation of a combination of the two anaerobic test organisms ( F. ulcerans and B. thetaiotomicron ). Abscesses developed in 48 hours and reached a diameter of 10-20 mm in 3-5 days. They drained in that time then gradually healed. Injection of F. ulcerans and E. cloacae caused a subcutaneous abscess with pus formation which drained spontaneously through the skin after 2 days (fig. 40). All those abscesses which were not excised, drained freely and spontaneously healed over 6-7 days. The abscesses developed a superficial crust and gradually healed from the outsides inwards. No guinea pigs died after the inoculations.

The abscesses produced resembled those produced by E. cloacae alone except that the dose used ( $1 \times 10^8$ ) was less than the dose of the single organism ( $1 \times 10^{10}$ ) required to produce an abscess. The inoculations were repeated on the back of the guinea pig and produced similar results. The repeated inoculations were carried out at different sites away from previous abscesses. Despite repeated injections in the guinea pig model the clinical results were similar.

#### Recovery of organisms inoculated

Aerobic and anaerobic cultures of swabs from the abscesses showed the organisms originally inoculated in all cases.

#### Histology of experimental abscesses

The epidermis showed evidence of necrosis. Where early biopsies were done just prior to rupture and the overlying epidermis showed incipient rupture (fig. 41). Underneath the epidermis the dermis showed oedema and dilated blood vessels. The inflammatory infiltrate consisted mostly of polymorphonuclear leukocytes in the upper part of the dermis. In the superficial dermis the organisms could be seen on Gram stain. There was no evidence of vasculitis.



Fig. 39. Mouse model showing cutaneous necrosis

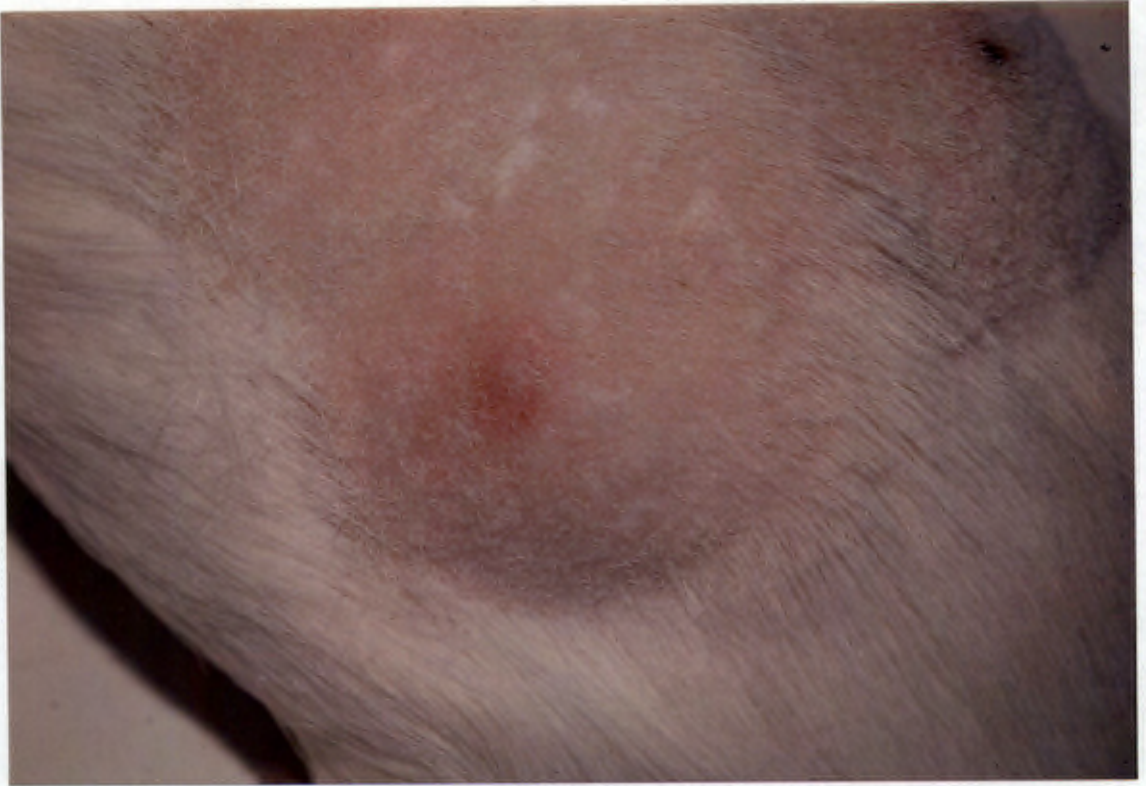


Fig. 40. Guinea pig model showing abscess formation

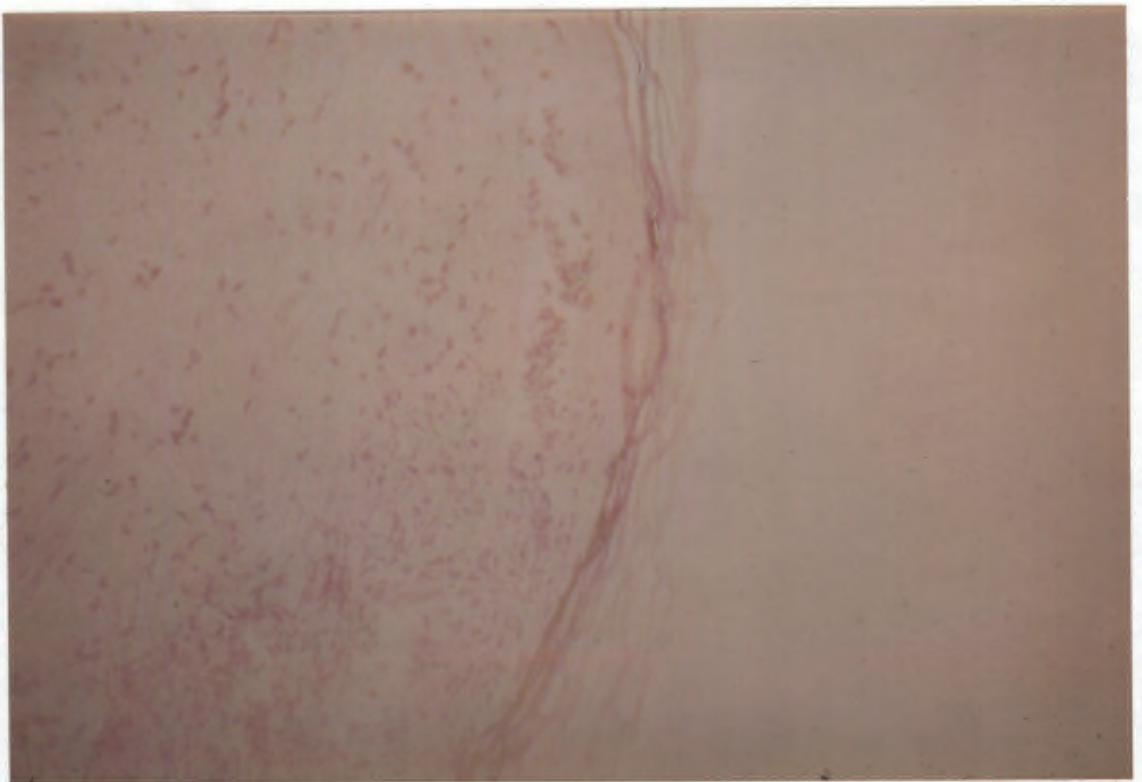


Fig. 41. Histology of experimental abscess  
(haematoxylin and eosin) (x400)

Table 40. Inoculations with a combination of organisms

Key: E = Erythema (cm) I = Induration (cm) A = Abscess

S. faecalis  $1 \times 10^9$  + F. ulcerans NCTC 12111  $1 \times 10^{10}$

Day	1	2	3	4	5	6
	E,8	E,7	E,5	E,1	-	-
	I,3	I,1				

E. cloacae  $1 \times 10^9$  + F. ulcerans NCTC 12111  $1 \times 10^{10}$

Day	1	2	3	4	5	6
	E,5	E,5	E,4	E,3	E,3	E,1
	I,2	I,4	A	A	A	A

E. cloacae  $1 \times 10^8$  + F. ulcerans NCTC 12111  $1 \times 10^{10}$

Day	1	2	3	4	5	6
	E,4	E,3	E,2	E,.2	E,2	E,1
	I,2	I,3	A	A	A	A

Anaerobic cocci  $1 \times 10^9$  + F. ulcerans NCTC 12111  $1 \times 10^{10}$

Day	1	2	3	4	5	6
	E,4	E,2	E,1	-	-	-

## DISCUSSION

Although tropical ulcer is endemic in certain parts of the tropics it is not confined to these areas. During this investigation, patients were studied in five tropical countries namely Zambia, Gambia, India, Papua New Guinea and Fiji. The prevalence of the disease varied from country to country and also from one part of a country to another. For instance in the Gambia the disease was more frequently seen in the rural areas than in the capital, Banjul. In Papua New Guinea ulcers were common in all areas studied. In Fiji the disease has been very common up to five years previously. Now few patients were developing ulcers, possibly a reflection of the impact of the Figian government's commitment to improvement of sanitation and living conditions in the rural areas. Nearly all villages have flush toilets and piped and regular visits from health inspectors ensure that toilets and water supplies are maintained.

The seasonal variation in the incidence of ulcers was also confirmed during this study particularly in Gambia and Zambia. The study in Fiji took place after the rainy season which could have accounted for the few cases observed. However this is unlikely as health workers in the area, who were well versed with the features of tropical ulcer, reported that the numbers of patients with ulcers were definitely decreasing. In Papua New Guinea (PNG) where the rainfall was similar throughout the year, ulcers occurred with equal frequency irrespective of the season.

The disease frequently affected children between the ages of 5 and 15 years although it was also seen in older patients. The disease was uncommon in patients over the age of 45 years. In Gambia and Papua New Guinea where more patients were over the age of 15 years, this increase in age could be attributed to the number of adult women who worked as farmers (in Gambia) and in fishing (in PNG) and who were thus exposed to the risk of disease while wading through the water to catch fish by line or trap nets (Robinson et al 1987).

While most cases were seen in rural communities, the patients in southern India were town dwellers. The clinical manifestations of the disease were similar in all the areas studied and most ulcers occurred on the lower legs. The same pattern of evolution was identified in all the areas. Only one patient developed a squamous cell carcinoma. The clinical manifestations were thus confirmed in all areas studied (Adriaans 1988).

The nutritional status of the patients was assessed objectively with three indices to determine whether or not tropical ulcers were associated with malnutrition. The results showed that nutritional status did not affect the onset of tropical ulcers. Several populations of children and adults were studied in different areas and poor nutritional status never correlated with the onset of tropical ulcers. Overt clinical malnutrition was rarely observed in these patients though many looked clinically malnourished, although not grossly so. Dietary histories did not suggest a critical shortage of first class protein. The majority of patients could recall eating meat or fish in the preceding week. Although fresh fish was less abundant in the rainy seasons, dried fish was freely available in all areas.

Ankle oedema was rare and when present was confined to the leg affected with the ulcer. The area of oedema was usually close to the confines of the ulcer. The haemoglobin levels in the 64 patients where this measurement was done were always above 10 G% even though many were clinically suspected of anaemia. Only one patient in the Gambia had a sickle cell trait with a normal haemoglobin. In this patient the ulcer was on the middle third of the leg and on the lateral border. Clinically this was more in keeping with a tropical ulcer than a sickle cell ulcer. There was no difference in the nutritional status of the ulcer patients and an age and sex matched control population in the same area although malnutrition was common in both groups studied. These findings suggest that malnutrition per se is not a major contributory factor in the aetiology of tropical ulcer.

The aetiology of tropical ulcer is obscure. While it is regarded by some workers as a non-specific response to injury, there is evidence that the disease is transmissible. McAdam (1966) for instance was able to induce tropical ulcers in normal healthy subjects by inoculating pus from an affected individual into normal skin. This suggested that infection played a role in the pathogenesis. Most previous reports on the bacteriology of ulcers have concentrated on aerobic cultures although several workers reported the presence of fusiform bacilli in smears. These had not been fully characterised. Despite this, many authors have alluded to the importance of these bacteria in the aetiology of tropical ulcers.

In this study the ulcers were investigated bacteriologically for both aerobic and anaerobic organisms. The successful isolation of anaerobic bacteria depended largely on obtaining good samples and then maintaining them under strict anaerobic conditions until the final analysis was done. Because the patients were seen a long way from the investigating centre, the swabs for bacteriological assessment had to be transported anaerobically until they were cultured. A suitable new system for transporting the samples was devised (Adriaans et al 1986). The Hungate tubes used for this purpose served as reliable individual culture systems and kept the samples in an anaerobic environment. The anaerobic organisms, including the fusobacteria which are believed to be important in the pathogenesis of this condition, remained viable under these conditions. The advantages of these tubes included the following:

- 1) they were reliable and maintained anaerobiasis
- 2) they were easy to use
- 3) they were convenient for storage

The weight of the tubes was their only disadvantage. Two tubes were used per patient. Each contained 10 ml of either transport fluid. The glass tubes were fragile, another potential disadvantage as the tubes always had to be transported by hand luggage throughout the field trips. However the advantages clearly outweighed the disadvantages.

In this study several anaerobic bacteria were isolated (Adriaans et al 1987a). Their successful isolation was possible because:

- 1) a reliable transport medium was used.
- 2) current techniques facilitated the isolation of anaerobic bacteria. These fastidious organisms could be recovered from the specimens with relative ease.
- 3) isolations were performed in an anaerobic cabinet
- 4) a number of selective media were used.

This study confirmed the presence of anaerobes in these wounds. The most frequently isolated anaerobic microorganisms were Fusobacterium species, Bacteroides species and various anaerobic cocci. Fusobacteria were isolated only from ulcers less than six week's old and none of the patients from whom these organisms were recovered had received any antibiotics.

From this study a previously unreported species of Fusobacterium was identified. It differed in its morphology, biochemical reactions and in its soluble cellular protein pattern from other previously described species of Fusobacterium.

This new species was isolated from several ulcers (Adriaans and Drasar 1987) and has been named Fusobacterium ulcerans in keeping with it's source of isolation (Adriaans and Shah 1988). The organism has been deposited with the National Collection of Type Cultures and is identified as NCTC 12111. This strain represents those bacteria described as type 1 fusobacteria which were the most frequently isolated species. A further representative strain has been deposited as NCTC 12112. This corresponds with type 2 fusobacteria.

No other species of Fusobacterium were recovered. Group 1 fusobacteria formed the majority and totalled 40 isolates. These isolates resembled Fusobacterium varium most closely but there were distinct biochemical differences. The second group of fusobacteria resembled F. mortiferum most closely. Yet they too showed several biochemical differences. Although the fusobacteria isolated in this study showed morphological and biochemical variations, the soluble cellular proteins of all the isolates were identical. Thus, despite interspecies variation, these organisms probably all belong to the same species.

The fusobacteria were isolated from swabs taken from the bases of ulcers. However the electron microscopic studies confirmed their presence in the dermis of the skin biopsies. These organisms therefore not only colonise the surface of the ulcers but also invade the dermis. The exact mechanism of invasion is not clear. However it seems likely that invasion follows a breach in the surface of the skin which allows these organisms to enter. The skin is a good barrier and extremely effective at keeping organisms out. The histories of trauma lend support the hypothesis that trauma is required to provide the portal of entry for these organisms. Those patients treated with antibiotics early in the disease recovered well and the ulcers healed easily. The fusobacteria isolated were sensitive to penicillin and metronidazole. The improvement after administration of either of these antibiotics may be related to removal of these organisms. This also supports the hypothesis that infection is important in the aetiology of tropical ulcer.

The source of these organisms was also investigated. Identical organisms were isolated from mud taken from the areas where patients with tropical ulcers lived. Whether these are human or animal derived bacteria is unknown. It is surprising that these organisms have not previously been documented if they were of human origin. In all areas studied animals roamed freely, sanitation was poor, stagnant water was commonly observed and it is likely that these bacteria are of animal origin.

The presence of anaerobes, particularly fusobacteria and spirochaetes, has been reported from several conditions such as cancrum oris, hospital gangrene and foot rot in sheep. The latter is a condition not too dissimilar from tropical ulcer. A necrotic lesion develops on the foot in wet or humid conditions. Fusobacterium necrophorum has been implicated in the aetiology of this disease (Hine 1984). F. necrophorum is known to be toxic to the epidermis in rats and has been shown to be toxic to Vero cells in culture (Grenier et al 1985). Likewise Treponema dysenterica is cytopathic to intestinal cells in combination with other bacteria (Lysons et al , 1978). It is thus likely that the combination of organisms is critical for the development of such diseases and synergy between anaerobes and aerobes may be mechanism whereby these organisms produce disease.

Fusobacteria are normally found in the faeces but they have been isolated from the gingiva in patients with gingival and periodontal disease (Moore et al 1984). Fusobacteria are also thought to play a role in cancrum oris, a mixed infection seen in malnourished children (Finegold 1977). In cancrum oris, extensive ulceration of the skin occurs predominantly around the mouth.

The tissue necrosis develops rapidly, in much the same way as tropical ulcer. However in cancrum oris the affected individuals are severely malnourished or have some underlying condition such as lymphoma. The fusobacteria involved in cancrum oris have not yet been characterised.

The pathogenicity of the fusobacteria isolated from the ulcers was tested in an in-vitro cell culture system. Of all the isolates tested only fusobacteria caused this effect. The significance of this finding may be interpreted in the light of the sudden breakdown of tissues at the onset of the ulcers. Damage to the cells may be due to toxic concentrations of butyrate which have previously been shown to be of aetiological significance in periodontal disease (Touw et al 1982). The fusobacteria do not produce haemolysin, nor do they digest casein which may contribute to the pathogenesis of the infection.

The histology showed that tropical ulcer was associated with large numbers of unusual bacteria. The predominant organisms in acute untreated lesions corresponded most closely to Fusobacterium ulcerans and some Bacteroides spp. Gram-positive bacteria were scantily distributed at the sites of tissue destruction. The ultrastructural appearances of spirochaetes were likely to be those of the Treponema spp. because of their flagellar pattern, length and periodicity. Healing was associated with acanthosis and loss of dermal oedema and the absence of bacteria was associated with antibiotic treatment. These features differed from those in Buruli ulcers where the disease affects the subcutaneous tissue more extensively and epidermal proliferation does not occur to the same extent as in tropical ulcer.

No gram-positive cocci nor crusting were seen as in ecthyma.

From the material examined, there was no evidence to implicate vasculitis or a thrombotic process in the pathogenesis of tropical ulcers. All the vascular changes were likely to have resulted from the healing process and were typical of those seen in other chronic ulcers (Adriaans et al 1987b). No spirochaetes were identified in arterioles or arteries which suggested that spirochaete associated endarteritis was not a factor. The destruction of collagen, the disruption of fibroblasts and inflammatory cells in the vicinity of large numbers of invading bacteria, suggest that the bacteria are cytotoxic. This was confirmed by the in-vitro cytotoxicity studies which showed that Fusobacterium ulcerans, but not Bacteroides spp. isolated from the ulcers, is cytotoxic to Vero cells in culture. Immunohistochemical stains showed a number of B-cells in the inflammatory infiltrate which suggests that the fusobacteria could stimulate the production of antibodies locally.

Although anaerobes are frequently associated with infections, information on the host's response to infection, especially with regard to antibody production against these organisms is limited. There are no previous reports on the host response to the infection in tropical ulcer. Various methods have been used to assess antibody response. These include gel diffusion, passive haemagglutination and immunofluorescence to demonstrate the presence of antibodies to bacteria such as members of the Bacteroidaceae in patients with infections caused by Bacteroides or Fusobacterium. The results of such investigations have frequently shown the presence of antibodies to these bacteria but the significance of these antibodies has not been clarified.

Sonnenwirth (1979) concluded that the increased titres of antibodies reported in association with infections were most often based on homologous isolates (the patient's own strains). This may reflect the strain and species specificity of antigens. With more sensitive tests (such as ELISA) even low titres of antibody may be detected. The sera from tropical ulcer patients were investigated for antibody production by an ELISA test. The same species of Fusobacterium was isolated from several ulcers which suggested it was of aetiological significance. The antigen was presumed to be constant and the antibody titre therefore comparable between patients. However no significant amount of antibody was produced in response to this infection. This may explain why patients develop recurrent infections over many years. This is the first report of antibody responses in patients with tropical ulcers.

## CONCLUSION

Tropical ulcer is a specific form of cutaneous infection which results from an infection with a mixture of organisms such as Fusobacterium ulcerans, coliform bacilli and spirochaetes. The most likely method of infection follows cutaneous trauma. When there is a breach in the skin the bacteria enter and multiply. The presence of a combination of organisms favours their growth. It seems likely that the infection not merely a polymicrobial infection but rather a synergistic infection as has been shown by in-vivo synergy experiments. Once the bacteria enter the skin they multiply and cause damage locally in the skin. This study has also shown that a combination of organisms is necessary for the development of a tropical ulcer.

Further work is needed to characterise the spirochaetes. Improved methods which will allow the growth of the spirochaetes, should provide details on their biochemical and further ultrastructural properties. Once the spirochaetes are maintained in-vitro their full role in the pathogenesis of tropical ulcer can be assessed. Similarly the new species of Fusobacterium isolated should be tested to see if in fact it stimulates the local antibody production in the skin (Mangan et al 1983). This would prove the hypothesis that local antibody production prevents dissemination of disease.

Tropical ulcers heal easily when the patients are treated with penicillin. The response is improved when treatment is started early. This corresponds to the stage when there are many organisms present as few are found in the late lesions.

This supports the hypothesis that infection is important and of the organisms isolated the fusobacteria are susceptible to penicillin. The improvement may thus coincide with removal of those bacteria.

Although the disease has a low mortality it is very debilitating and has a significant morbidity. With better sanitation, health care and housing conditions this disease should be eradicated.

## Appendix Ia. Tropical Ulcer Questionnaire

### Guide to Questionnaire

#### General points

If the answer to the question is not known leave blank. Put in as much or supplementary information as you like under "additional comments" in each section of the questionnaire if appropriate. Where answer to question is Yes/No or Sex M/F ring e.g. Yes/No.

#### SECTION I

Age if uncertain fill in box number of age category as indicated e.g. [5] means age 30-39 NOT 5 years old.

Household defined as those who normally eat together i.e. two wives in separate huts count as two households.  
Exclude visitors: include children away at school.

Town: 2,000 inhabitants or more.

Animals be as precise as possible about what animals are kept where.

Main activity describe e.g. desk, outdoor in fields etc.

#### SECTION II

To question "When did it start?" give interval from first medical contact if possible. This means orthodox medical contact e.g. community nurse, health centre, hospital NOT traditional or herbalist.

Family history of TPU if positive include simple family tree of first and second degree relatives if possible and arrow index case e.g.

Try to establish whether the relative was in contact with the patient at the time the ulcer developed.

#### SECTION III

Past medical history - leave blank unless certain. Ignore acute inter-current conditions e.g. measles, malaria.

#### SECTION IV

See instructions on sheet. Under "other obvious scars" ignore tribal markings.

#### SECTION V

Under distribution give approximate indication e.g. "anterior chest and abdomen" or "all over except face".

Dental D.M.F.: enter total number of decayed, missing or filled teeth.

Appendix 1a. continued

Tropical Ulcer Questionnaire

Case [1] Control [2] [ ]

Section 1: ENVIRONMENT

NAME: ..... SEX: M/F No. ....

DATE PATIENT FIRST SEEN (i.e. at health centre/hospital) .....

AGE: (Age given) ..... Confirmed by medical or birth record YES/NO

If uncertain, estimate: [ ]

[1] 0-4 yrs [2] 5-9 yrs [3] 10-19 yrs [4] 20-29 yrs [5] 30-39 yrs  
[6] 40 or over

HOUSEHOLD: (a) Number of adults [ ] (b) Number of children [ ]  
Exclude visitors, include children away at school

HOME: [ ] [1] In town [2] In village [3] Isolated dwelling

Number of houses within 50 yds [ ]

Name of nearest village/town .....

Number of rooms [ ] Building materials: walls .....

roof .....

Floor area [ ] sq yds

ANIMALS: What domestic animals are kept in close proximity (e.g. pigs, dogs, include poultry):

Where kept? By day ..... Any animals kept inside home?

By night .....

ACTIVITY:

Main activity, e.g. job/school .....

If seasonal, give details .....

Activity/job of head of house .....

HAZARD: Possible predisposing factors to TPU, e.g. trauma, wading, splash-  
ing mud .....

HYGIENE: Washing facilities at home or after main activity - river, pond,  
well, piped water:

Does availability of water vary with season? .....

How many buckets of water used by family each day .....

Drying: custom, practices eg after washing .....

Is soap readily available YES/NO Cost of bar of soap .....

Has the information on this sheet been obtained by home visit YES/NO

Additional comments to be made overleaf



Appendix 1a. continued

Section III: MEDICAL/NUTRITIONAL

NUTRITION: STAPLE

How often has animal protein (fish or meat) been eaten in the past week? .....

Does this vary according to season? YES/NO

What did you eat yesterday for

- (a) breakfast .....
- (b) lunch .....
- (c) evening meal .....
- (d) other times .....

HEALING: Are cuts etc, slow to heal? YES/NO

MEDICAL HISTORY:

Is there a definite history of past major medical condition, e.g. leprosy, Kwashiorkor, tuberculosis etc: .....

ADDITIONAL COMMENTS:

e.g. about staple/variations in diet, medical history, if thought relevant  
.....  
.....  
.....  
.....  
.....  
.....  
.....  
.....

Continue overleaf if necessary

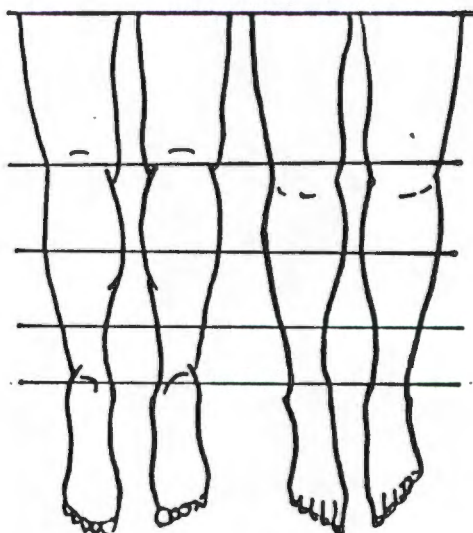
Appendix 1a. continued

SECTION IV

Map position of ulcer(s) on body diagram as an open circle, follow by number to indicate maximum diameter of ulcer in cms, e.g. 5 means ulcer of maximum diameter 5 cms.

Scars that you are confident are healed ulcers should be similarly mapped as closed circles, e.g. ● 5.

Indicate other obvious scars e.g. trauma, by lines to give approx. size



APPEARANCE:

Slough  
Edge  
Bleeding  
Surrounding oedema  
Tender  
Deep structure involvement  
Regional lymph nodes  
?Malignant change  
Smell

If there are multiple ulcers and you wish to refer to one of them, label the ulcers by letters, e.g. b ○ 5 on the diagram

Additional comments to be made overleaf



Appendix 1b. Peptone yeast glucose agar (transport fluid)

INGREDIENT	AMOUNT (gr)
peptone	0.5
trypticase	0.5
cysteine	0.05
yeast extract	1.0
sodium sulphoxalate	0.03
rezazurin	0.4 ml
distilled water	100.00 ml
hemin solution	1.0 ml
vitamin K solution	0.02 ml
salt solution	4.0 ml

Salt solution gr

$\text{CaCl}_2$ (anhydrous)	0.2
$\text{MgSO}_4$ (anhydrous)	0.2
$\text{K}_2\text{HPO}_4$	1.0
$\text{KH}_2\text{PO}_4$	1.0
$\text{NaCO}_3$	10.0
$\text{NaCl}$	2.0

Appendix 1c. Medium for spirochaete isolation.

NOS agar (Leschine et al 1980).

INGREDIENT	AMOUNT (gr)
Heart infusion broth	1.25
Trypticase	1.0
Yeast extract	0.25
Sodium thioglycollate	0.05
l-Cysteine HCL	0.1
l-Asparagine	0.025
Glucose	0.2
Noble agar	0.7
	ml/100 ml
Thiamine pyrophosphate	0.3
Volatile fatty acid	0.2
Normal rabbit serum	2.0
10% Sodium bicarbonate	2.0

Appendix 1d. Ingredients for polyacrylamide gel electrophoresis

Resolving gel:

a) Sterile distilled water	9.93 ml
b) Acrylamide/bis	6.8 ml
c) Resolving buffer	2.125 ml
d) APS 10%	100 ul
e) TEMED	10 ul

The first three ingredients were thoroughly mixed after each addition.

Stacking gel:

a) Sterile distilled water	18.3 ml
b) Acrylamide/bis	2.7 ml
c) Stacking buffer	3.0 ml
d) APS 10%	50 ul
e) TEMED	40 ul
f) Bromophenol blue dye (BPB)	7 drps

APS - Aqueous Ammonium persulphate solution  
10% wt/vol. Freshly prepared

TEMED - N,N,N',N' - tetramethylethylenediamine

BPB - 0.25% Bromophenol blue in water wt/vol

Appendix 1e. Medium used to support the growth of cells

1) Medium 199 (Gibco, U.K.).

90 ml sterile distilled water

10 ml 199 medium

1-5 drops 2N NaOH (to change the pH as indicated  
by a colour change to orange yellow)

5 % fetal calf serum

0.22% sodium bicarbonate

20mM Tricene pH 7.6

antibiotic solution 0.5%

2) Dulbecco's Modified Eagles Medium (Flow Lab. U.K.)

10% fetal calf serum

20 mM tricene

0.22% Sodium bicarbonate

25 ug/ml gentamicin

SUPPLIERS

Microscopes

Zeiss

PO Box 78

Welwyn Garden City

Herts

Leitz (cytotoxicity)

48 Park Street

Luton

Beds

Nikon

Richmond Road

Kingston-on-Thames

Surrey.

Homogeniser

Gallenkamp

Belton Road West

Loughborough

Leicestershire

Sonicator

MSE

Manor Royal

Crawley

Sussex

Multichannel pipette

Jencons Scientific (Titertek)

Leighton Buzzard

Beds

Filters

Millipore

Harrow

Middlesex

Whatman

Denmark

Oxygen free gas

B.O.C.

Special Gases Division

London

Culture media

Oxoid

Basingstoke

Hants

Difco

East Molesey

Surrey

Lab. M

Salford

Cheshire

Gibco

Uxbridge

Middlesex

Dessicator	Polysciences Ltd Moulton Park Northampton Northants
Hungate tubes	Bellco Glass Vinelands New Jersey U.S.A.
Anaerobic cabinet, Disposable loops & Multichannel cannula	Don Whitley Shipley Yorkshire
Electrophoresis tank	Biorad Watford Business Park Watford Herts
Glass beads	Ballotini Stanmore Middlesex
Sephadex beads	Pharmacia Chemicals Hounslow Middlesex
Anti-rabbit immunoglobulin	Dako High Wycombe Bucks

Antiseptic solution

I.C.I. Pharmaceuticals

Macclesfield

Cheshire

Anaesthetics

Astra Pharmaceuticals

Kings Langley

Bucks

Janssen Pharmaceuticals

Wantage

Oxfordshire

Biopsy punch

Stiefel

High Wycombe

Bucks

Scalpels

Gillette

Isleworth

Kent

Silk sutures

Ethicon (Mersilk)

McCarthy Supplies

Dagenham

Essex

Spiral plater

Spiral Systems

Clough Pike

Cincinnati

U.S.A.

Metronidazole discs

May and Baker

Dagenham

Kent

Streptex kit,

Wellcome' Diagnostics

Syfacard kit.

Dagenham

Kent

Petri dishes

Sterilin

Farnham

Hants

Microwell plates

Flow Laboratories

Rickmansworth

Herts

API kits

API Laboratories

Monralieu

France

Duolite resin

BDH Chemicals

Poole

Hants

Chromatograph

Philips

and computer

Cambridge

Cambs

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