# CHEMOTHERAPY OF AFRICAN BOVINE TRYPANOSOMIASIS: ASPECTS OF RESISTANCE OF *Trypanosoma congolense* TO ISOMETAMIDIUM

by

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## SUMMARY

The control of African bovine trypanosomiasis is likely to depend, to a large extent, on the use of trypanocidal drugs for the foreseeable future. Within the limited armoury of drugs currently available, Isometamidium occupies a position of prominence, and is probably the most widely used trypanocide. Drug resistance is commonly considered to be the major problem associated with the use of trypanocides, and therefore a number of aspects of resistance of Trypanosoma congolense to Isometamidium were investigated in the present study.

Three basic approaches to the assessment of Isometamidium sensitivity of strains of T. congolense were explored; tests conducted using mice or cattle as hosts, and tests carried out using an in vitro system. The mouse sensitivity test was shown to have limitations in the degree of correlation between mouse and cattle results, while the in vitro approach had the major disadvantage that most strains of T. congolense had to undergo a period of adaptation to in vitro culture conditions, before any in vitro assessment of sensitivity could be made. It was concluded that the method of choice was sensitivity tests performed in the definitive host, ie cattle, and it was also noted that tests to assess the duration of prophylaxis should be carried out in addition to those to test therapeutic activity.

An investigation of the apparent aparasitaemic interval following subcurative treatment with Isometamidium of T. congolense infections/

infections in a mouse model suggested that trypanosomes survived in the bloodstream in low numbers, rather than in cryptic foci, such as has been described for T.b. brucei.

Two aspects of resistance to Isometamidium, studied as part of this thesis, were of particular interest. First, deliberate attempts to induce changes in sensitivity by repeated subcurative treatment, failed to alter significantly the Isometamidium sensitivities of strains of T. congolense, both in mice and goats. Secondly, a relatively Isometamidium resistant strain of T. congolense was apparently unable to establish an infection in the face of an existing infection of a sensitive strain, although in the reverse situation the sensitive strain established an infection and suppressed the resistant strain. These findings may help to explain the apparent scarcity of reported incidences of Isometamidium resistance arising in the field.

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# CHAPTER ONE

## AFRICAN BOVINE TRYPANOSOMIASIS

# INTRODUCTION

The African animal trypanosomiases are a group of parasitic diseases which affect all the economically important livestock species. Of particular significance is the impact of tsetse-transmitted bovine trypanosomiasis; indeed, some authorities consider this to be the single most significant factor responsible for the continent's relatively poor output of foodstuffs, particularly animal protein (Trail, Sones, Jibbo, Durkin, Light and Murray, 1985). The potential benefits to be derived from effective control of the disease are enormous.

## The Organisms

Trypanosomes are parasitic protozoa which inhabit the plasma, and, in the case of some species, other body fluids and tissues, of a wide range of animal hosts throughout the world (Mulligan, 1970). The majority of trypanosome species are non-pathogenic, but some species are the causative organisms of the major disease complex, the trypanosomiases. Nagana, surra and dourine occur in domestic livestock and trypanosomiasis (sleeping sickness) and Chagas' disease in humans. The organisms belong to the order Kinetoplastidae, family Trypanosomatidae and genus Trypanosoma. In general bloodstream forms of trypanosomes are motile, unicellular organisms, usually spindle shaped, flattened and curved. They possess a single nucleus and a single flagellum, the latter being the organ of locomotion. The portion of the trypanosome which is directed forward during movement is conventionally regarded as the anterior end. The flagellum emerges at the basal body, posterior to the nucleus, and runs along the/

Table 1. Identification of *T.congolense*, *T.vivax* and *T.brucei* (after Morrison *et al.*, 1981).

| Species | Size (μm) | Kinetoplast | Flagellum | Behaviour |
|---|---|---|---|---|
| *T.congolense* | 9-18 | marginal | no free flagellum | sluggish; often attached to red cells. |
| *T.vivax* | 20-27 | terminal | free | very rapid movement across microscope field. |
| *T.b.brucei* | 15-39 | subterminal | free | rapid movement in confined areas. |

the outer margin of the pellicle to form an undulating membrane. In some species there is a free portion of flagellum, in others the flagellum terminates at the anterior end. In close proximity to the basal body is the kinetoplast, a disc-shaped organelle containing DNA. Morphologically important features which can be used to aid identification of different species include the position of the kinetoplast and presence or absence of a free portion of flagellum (Table 1). Species of Trypanosoma have been divided into two sub-groups, the Salivaria and the Stercoraria, on the basis of the site of development of the parasites in the vector species and the mode of transmission of infection.

Bloodstream, or trypomastigote, forms are ingested by the vector during the course of a blood meal from an infected host. In the tsetse fly for Salivarian species, or in the arthropod vector for Stercorarian species, the trypanosomes undergo a number of developmental stages before transforming into metacyclic trypanosomes which are the form capable of infecting a new host. Bloodstream forms ingested by the vector species transform into epimastigote forms, which develop further into infective metacyclic forms. The Stercorarian species develop entirely in the alimentary tract of the vector, with production of metacyclics occurring in the hind gut, from where they are voided in the faeces, and bring about infection either by contamination of skin abrasions or by oral ingestion by the host. This mode of development occurs in the important human pathogen T. cruzi.

The trypanosome species responsible for African bovine trypanosomiasis belong to the Salivarian sub-group. The initial stages/

stages of the development of metacyclics may occur in the alimentary tract of the vector but development is completed in the mouth parts. In the case of T. vivax development occurs exclusively in the proboscis. Cyclical development, that is the transformation through a number of morphological forms before infective metacyclics are produced, occurs in the principal vector of the Salivarian trypanosomes, species of the genus Glossina, the tsetse flies. Twenty-two species of tsetse are known to transmit trypanosomes (Murray, Morrison and Whitelaw, 1982) and, as each species is adapted to a slightly different habitat (Ford, 1971), the tsetse-transmitted trypanosomes have a widespread distribution south of the Sahara. Infection of new hosts occurs by inoculation of infective metacyclics by an infected tsetse taking a blood meal. Salivarian trypanosomes are also capable of being transmitted by mechanical transmission in which infected blood is simply transferred from one host to another via the mouth parts of a biting fly. With most species cyclical transmission is of much greater significance than mechanical, although, in the case of T. evansi, an important pathogen particularly of camels, transmission is entirely by the mechanical mode (Mulligan, 1970).

Three species of Salivarian trypanosome are implicated in tsetse-transmitted bovine trypanosomiasis, of which two are of particular importance. Most clinical cases of African bovine trypanosomiasis are due to infection of T. congolense or T. vivax with the former probably predominating in East Africa and the latter in West Africa (Leach and Roberts, 1981). Mixed infections of the two species are common and infections involving T. brucei brucei are also encountered.

The Disease in Cattle

The course and severity of the disease varies depending upon the species and strain of trypanosome and, to some extent, on the breed and prior experience of the bovine (Morrison, Murray and McIntyre, 1981). Infections due to T. vivax exhibit a particularly wide range of clinical manifestations, ranging from chronic, low grade infections to those which run an acute course, characterised by pyrexia, sustained parasitaemia, massive haemorrhage and death, often within two weeks of infection. T. congolense infections demonstrate a narrower range of virulence, infections ranging from relatively acute to the more usual chronic course. T.b. brucei usually gives rise to chronic, low grade infections.

The most common course for all three species, however, is a chronic condition associated with a series of waves of parasitaemia, generally at a relatively low level, with development of anaemia, seen as a pallor of the mucous membrane, as a major feature. In the early stages of the disease there is intermittent pyrexia and the superficial lymph nodes are enlarged. As the disease progresses lymph nodes revert to normal or may be reduced in size. The appetite is usually normal, except for short periods associated with pyrexia; animals have been reported which died with food still in the mouth. Anaemia develops, its severity dependent on the level of parasitaemia experienced, and is accompanied by loss of condition. This is especially marked if extensive foraging is necessary, as the animals become very weak and are eventually unable to stand.

The anaemia occurs in two distinct phases (Morrison, et al., 1981). Initially there is increased red cell destruction by extravascular phagocytosis (Jennings, Murray and Urquhart, 1974; Mamo and Holmes, 1975). Although bone marrow is responsive the erythropoietic response is probably less than the degree of anaemia dictates (Dargie, Murray and Murray, 1979). The role of haemodilution is equivocal, and haemorrhage is not usually a symptom except in the case of haemorrhagic T. vivax. Treatment of this initial phase with trypanocidal drugs generally results in a cure, with the packed cell volume (PCV) returning to normal levels. The second phase of anaemia which may occur after 4 to 6 months if the animal survives and is not successfully treated, is associated with progressively decreasing waves of parasitaemia: sometimes the presence of trypanosomes cannot be demonstrated. Red blood cell destruction by phagocytosis continues and femoral marrow is often inactive (Dargie, et al., 1979). In this second phase the condition usually responds poorly to trypanocidal drugs. The aetiology of the anaemia is multifactorial, with immune sensitisation of red cells, production of haemolysins by trypanosomes, fever and a more active mononuclear phagocytic system all having been implicated (Morrison, et al., 1981; Herbert and Inglis, 1973).

The outcome of the chronic disease is often fatal, death being caused by congestive heart failure brought about by a combination of anaemia, circulatory disturbance associated with increased vascular permeability and myocardial damage (Murray, 1974).

In some animals the PCV will stabilise at a low level; some individuals will die, others become aparasitaemic and the PCV gradually revert to normal, although as the duration of the infection increases recovery of PCV tends to be slight. Surviving animals tend to remain at a low level of productivity, with poor growth rates and poor reproductive performances.

The Extent of the Problem

Tsetse-transmitted bovine trypanosomiases have a devastating impact over an area of 10 million square kilometres of sub-Saharan Africa. Tsetse infest approximately 40% of the habitable land in an area embracing 37 countries (FAO/WHO/OIE, 1982); often these areas are the best watered and most fertile parts which otherwise would be suitable for mixed agricultural development or grazing. Thus, cattle and also sheep and goats, are excluded from vast tracts of tsetse-infested land. In other areas of less severe tsetse challenge enormous losses are incurred due to depressed levels of productivity - poor growth rates, low fertility, high incidence of abortion, long calving intervals, low milk yields, limited work potential - of animals exposed to trypanosomiasis. It has been estimated that 30% of the 147 million cattle in countries affected by tsetse are exposed to the disease (FAO/WHO/OIE, 1982).

Exclusion of cattle from tsetse-infected areas not only denies inhabitants of such areas access to livestock products such as milk, meat, skins, dung and for small-scale farmers, a saleable asset in times of need, but also means that draught power is not available. Since it has been estimated that possession of a single oxen can increase the food output of a family six-fold, the significance of this factor can readily be appreciated (McDowell, 1979).

Limited utilisation of tsetse-infested areas inevitably results in increased pressure on land use in tsetse-free regions. At a time of rapidly rising human population in Africa, this can result in overgrazing, pasture degradation and ultimately in desertification. Effective control of trypanosomiasis, resulting in the development of tsetse-infested land for mixed agriculture, would do much to relieve this pressure.

Some workers however take the contrary view point that the development of tsetse-infected land for grazing is environmentally detrimental. Omerod and Rickman (1988) for example, consider trypanosomiasis control to be 'one of the worst initiators of land degradation'. They suggest land cleared of tsetse and rapidly stocked with cattle is vulnerable to overgrazing, which can easily lead to disruption of the ecological balance of a relatively fragile environment.

Methods of Control

Approaches to the control of trypanosomiasis fall into one of two basic categories: those in which control of the disease is attempted by action against the vector and those in which direct action is taken against the parasite. Possibly the simplest approach to the problem in the past has been to avoid exposing livestock to the risk of infection by not keeping them in trypanosomiasis endemic areas. However, the extent of the area involved is enormous. Tsetse infest an area equivalent in size to the whole of the U.S.A. (Trail et al., 1985) and, with current trends in human population growth in Africa, the continuation of this approach in the future is unlikely to be tenable.

Vector control

Attempts to control tsetse have taken place for more than 60 years but despite this, territorial expansion of tsetse infested country is occurring throughout the continent (MacLennan, 1980). Initially reliance was placed on techniques such as selective bush clearance to remove breeding habitats, clearance of vegetation from tracts of land to act as barriers to the advance of tsetse and removal of wildlife as a source of infected blood meals. When synthetic insecticides became available after the Second World War, their use became the most commonly employed method of tsetse control. Residual insecticides such as DDT, or more recently dieldrin are generally applied by hand sprayer to likely tsetse resting sites, while non-residual insecticides are usually applied by fixed-wing aircraft or occasionally helicopters. Well planned campaigns using insecticides have succeeded in the elimination of tsetse from sizeable areas, for example, in Nigeria, Zimbabwe, Botswana and Zambia (Trail et al., 1985). The logistics involved, however, are complicated and the cost high. The danger of reinvasion of tsetse from adjacent areas is ever-present and, particularly when residual insecticides are used, the technique can be criticised on ecological grounds.

A number of biological methods of control are at various stages of experimental or field evaluation. The sterile insect technique (SIT) relies on the fact that a female tsetse mates only once, and if the male is sterile, the female will not produce any offspring. Sterilisation is achieved by exposure of colony reared males to sublethal irradiation or chemical sterilants. Normally the number

of/

of tsetse present in a target area are initially reduced by application of a non-residual insecticide, such as endosulphan, prior to release of the sterile males. A further requirement of the technique is an effective barrier, natural or man-made, to prevent reinvasion of tsetse from outside the area. This generally takes the form of a zone of sheer-cleared vegetation approximately 1 kilometre wide around the target zone. The difficulty of initially clearing this barrier and subsequently maintaining it is considerable. Disadvantages of SIT are its relative sophistication, expense and risk of reinvasion negating any benefits gained. The presence of more than one Glossina species in most infested areas also complicates this approach.

The use of biological control in the form of tsetse parasites, pathogens or predators has not progressed beyond the experimental stage.

A relatively simple approach to vector control is the use of strategically positioned tsetse traps. Originally traps were used mainly for sampling tsetse populations for survey purposes, but more recently the potential for using traps as means of tsetse control has been investigated. The latest developments in this area concern the use of insecticide-treated screens, or targets, baited with tsetse attractant odours such as acetone and octanol. The screen consists of a central rectangle of black cloth flanked by netting on either side. The cloth and netting are treated with/

with an insecticide such as meltamethrin, and tsetse, attracted by the odour plume, are killed when they either alight on the black cloth or collide with the netting. A trial conducted in the Rifa Triangle, Zimbabwe (Vale, Bursell and Hargrove, 1985) was apparently successful in eliminating previously abundant G.m. morsitans and G. pallidipes, although Tabanids and Muscids remained numerous. The technique has the advantage of being ecologically sound, but has yet to be shown to be effective against all Glossina species in all ecological zones.

Immunological control and the use of trypanotolerant livestock

A pronounced and rapid immune response follows infection of a host with trypanosomes, with humoral antibody being the main factor involved (Holmes, 1980). However, the parasites are able to overcome the host's response largely due to the phenomena of antigenic variation.

The pellicular surface of trypanosomes is covered with a glycoprotein. This surface is antigenic and by a process of repeatedly changing the dominant surface coat, or variable antigen type (VAT), the parasites are able to evade the antibody produced in response to them (Cross, 1975). The parasite in effect remains one step ahead of the host's immune response. The classical course of parasitaemia in a trypanosome infected animal is one of a series of waves of rising and falling parasite numbers. This is due to the rapid growth of a trypanosome population possessing

a/

a certain dominant VAT, the mounting of a successful immune response by the host, which is then defeated by the emergence of a new dominant VAT. This sequence of production of antibody against a specific VAT and emergence of a new dominant VAT is repeated indefinitely. The number of VATs in the repertoire of any given trypanosome strain is unknown but appears to be large. In the case of a clone of T. equiperdum, 101 distinct VATs have been isolated (Caphern, Giroud, Baltz and Maltern, 1977).

The development of an effective vaccine is, therefore, frustrated by a number of factors. Firstly, to be effective in the control of bovine trypanosomiasis, a vaccine must be active against T. congolense, T. vivax and ideally T.b. brucei as well. Secondly, within each trypanosome species there exists a large, but unknown number of antigenically distinct strains (van Meirvenne, Magnus and Vervoort, 1977) each with their own repertoire of VATs. Thirdly, the problem of VATs compounds an already complicated situation. It is relatively easy to produce immunological protection against homologous challenge in a laboratory environment (Holmes, 1980) but it seems difficult, if not impossible, to produce protection against heterologous strains or variants. In spite of enormous research effort in this area, the likelihood of a field vaccine becoming available in the foreseeable future seems slim (Murray and Gray, 1984).

It has long been recognised that there are certain breeds of cattle, sheep and goats which are able to survive in tsetse-infested regions where other breeds cannot (ILCA, 1979). These breeds tend to be confined to West and Central Africa due to a combination of historical factors and have been termed 'trypanotolerant'. Two major breeds of cattle are recognised as being trypanotolerant: the N'dama and the West African Shorthorn. The former are descended from the humpless Hamitic Longhorn which arrived in Africa around 5,000 B.C. and the latter are descendents of the humpless Hamitic Shorthorn which were imported into Africa around 3,000 B.C. Both breeds are classified as Bos taurus types, in contrast to the Zebu breeds which are Bos indicus and whose ancestors arrived on the continent around 700 A.D. (Murray et al., 1982).

Pagot (1974) defined trypanotolerance as a 'racial aptitude to maintain good condition and to reproduce while harbouring trypanosomes without showing clinical signs of the disease'. The term 'trypanotolerance' is in some ways misleading. The trypanotolerance of these breeds is not absolute (Stephen, 1966) and under some circumstances clinical disease can become manifest. A range of stress factors such as poor nutrition, overwork, intercurrent disease or simply a very high level of challenge may result in the breakdown of the trypanotolerance. The breeds in question, however, undoubtedly exhibit reduced susceptibility to the disease.

Several misconceptions have become associated with the trypanotolerant breeds, including the beliefs that the tolerance is an acquired trait and that the breeds are only capable of achieving a low level of productivity. Much experimental work, particularly with N'dama cattle, has shown that trypanotolerance is an innate characteristic (Murray et al., 1982). Comparative experiments with susceptible Zebu cattle have consistently shown that N'dama exhibit a lower level, prevalence and duration of parasitaemia; develop less severe anaemia; have greater survival rates and high reproductive performance in the face of challenge, whether that challenge was by syringe inoculation or naturally and was T. congolense, T. vivax or T.b. brucei (Murray, Murray, Wallace, Morrison and McIntyre, 1979).

The belief that trypanotolerant breeds are of a low productivity has arisen in the past due to prejudice against the relatively small mature bodyweight of these breeds and lack of convincing, comparative performance data. Recent investigations, however, have shown that trypanotolerant livestock are more productive than was previously thought (ILCA, 1979).

In spite of their apparent advantages over susceptible cattle, the major constraint that limits the contribution trypanotolerant cattle can make to the problem of trypanosomiasis on a continental scale is the relatively small numbers available. Latest population estimates of N'dama and West African Shorthorn are/

Table 2 Drugs currently used for the treatment and prophylaxis of African bovine trypanosomiasis. (Adapted from Leach and Roberts (1976).)

| Compound | Chemical class | Activity | | Principal Tradenames |
|---|---|---|---|---|
| Diminazene aceturate | Aromatic diamidine | Curative | T.congolense<br>T.vivax | Berenil (Hoescht, W Germany) |
| Homidium bromide | Phenanthridine | Curative | T.congolense<br>T.vivax | Ethidium (Camco, UK) |
| Homidium chloride | Phenanthridine | Curative | T.congolense<br>T.vivax | Novidium (May & Baker, UK) |
| Isometamidium chloride | Phenanthridine | Prophylatic | T.congolense<br>T.vivax | Samorin (May & Baker, UK)<br>Trypamidium (Rhone Merieux,Fra |
| Quinapyramine sulphate | Quinoline - pyrimidine | Curative | T.b.brucei<br>T.congolense<br>T.vivax<br>T.evansi | Trypacide (May & Baker, UK) |
| Quinapyramine sulphate: chloride (3:2 w/w) | Quinoline - pyrimidine | Prophylatic | T.congolense<br>T.vivax<br>T.evansi | Trypacide Prosalt (May & Baker |

are 3.4 million and 1.8 million respectively, or about 8% of the cattle population of the tsetse-infected countries (ILCA, 1979). Recent research suggests that even the trypanotolerant breeds can be more productive when maintained under trypanocidal drug regimes, although the frequency of treatment needed may be less than in the more susceptible breeds (Trail et al., 1985).

Trypanocidal drugs

The non-availability of an effective field vaccine, the difficulty of effective vector control and the relatively small numbers of trypanotolerant cattle currently available means that, in the foreseeable future, trypanocidal drugs will have to be relied upon to control the animal trypanosomiases.

There are two broad categories of drugs used against trypanosomes; those which bring about a sterile cure but have little or no residual activity and those which maintain trypanocidal levels in the blood or tissues of treated animals for extended periods. The former are termed therapeutic or curative and the latter prophylactic. Table 2 presents details of chemical class, activity and principal manufacturer and trade name for the currently available trypanocides used against African bovine trypanosomiases (Table 2).

Use of trypanocidal drugs is not without its problems, a topic reviewed by Holmes and Scott (1982). Initially the cost of drugs creates a strain on the foreign exchange reserves of the countries affected, a problem exacerbated by the fact that these countries tend to be the poorest and least developed nations.

Effective/

Effective use of trypanocidal drugs requires close veterinary supervision and the monitoring of treated cattle for signs of reappearance of trypanosomes. Shortages of trained manpower and materials makes such supervision difficult. Many authors consider the most significant problems to be that of drug resistance. To some extent, resistant strains of trypanosomes have emerged to all the trypanocidal drugs which have been, or still are being, used in the field. The problem of drug resistance is heightened by the small number of different trypanocidal drugs available and also the narrow therapeutic ratio of most of these products. No new drugs have become available since the introduction of Isometamidium in the early sixties. Williamson (1976) attributes this to the high cost of developing and marketing a compound for a market segment which, to the large pharmaceutical companies, is relatively small and which is made up of countries of uncertain economic stability, often with small veterinary budgets.

There are several categories of cattle exposed to trypanosomiasis risk and the desirability and practicability of providing effective therapeutic or prophylactic cover varies between these categories.

In West Africa it has been a comparatively common practice to trek cattle from up-country centres of production to the large urban markets on the coast. On route the cattle were often exposed to trypanosomiasis risk as they passed through tsetse-infected areas. In such a situation a chemoprophylactic treatment given at the start of/

of the trek was an effective method of providing protection. The period of protection conferred by the prophylactic drug was likely to exceed the period of risk, and several workers have conducted trials which have demonstrated that Isometamidium is effective in this situation (Jones-Davies, 1967; Na'Isa, 1969). More recently, a shift from trekking animals to transporting them more rapidly by lorry, has reduced the trypanosomiasis risk associated with this journey (Kilgour and Godfrey, 1978).

Susceptible Zebu cattle migrating into the tsetse belt in the dry season to gain access to grazing and water present a similar problem. Movement of this type is a common feature of cattle husbandry particularly in parts of West Africa. Animals exposed to this type of seasonal risk generally receive a prophylactic treatment before moving to the tsetse area, which should provide protection during their three or four month exposure to risk. A curative treatment is then usually administered at the end of the dry season grazing period to reduce the risk of drug resistant strains arising and to prevent the change of trypanosomes being transmitted mechanically by biting flies during the wet season grazing outside the tsetse belt (Anon, 1979).

Trypanocidal drug regimes for draught animals and cattle under individual ownership in trypanosomiasis endemic areas present particular problems. These include monitoring infection and ensuring that all animals receive adequate treatment at the appropriate interval, with due regard to correct dosage and with provision/

provision to safeguard against emergence of drug resistance. Bourn and Scott (1978) described a scheme in Ethiopia where 400 draught oxen were maintained in good health in a tsetse-infested area but emphasised the need for good veterinary supervision.

A recent major study, based at Muhaka on the Kenya coast, has demonstrated the potential benefit of Isometamidium prophylaxis, in terms of decreased infection rates and increased productivity even under village conditions (ILCA, 1986; Murray, Trail and Maloo, 1987).

It is far easier to provide chemoprophylactic protection for cattle maintained in endemic areas on ranches or large farms under permanent veterinary supervision. Provision must be made for regular monitoring of herds for signs of reinfection; individual animals must be ear-tagged to allow reliable identification; facilities must be available for handling and weighing animals and all animals must be presented for treatment. The trypanocidal regime used should be tailor-made to reflect the local challenge and, ideally, incorporate a curative drug at regular intervals.

Isometamidium has been successfully used to control trypanosomiasis in a number of field trails and commercial situations. Thus, Wilson, Le Roux, Paris, Davidson and Gray, (1975) reported a trial involving Zebu cattle in a tsetse infested area of Kenya. Three drug regimes were compared: Isometamidium administered on a herd basis when 5% of the herd was diagnosed as trypanosome positive; diminazene aceturate administered on the same basis and diminazene aceturate given to individuals shown to be clinically affected./

affected. Of the three regimes, Isometamidium prophylaxis was demonstrated to give the best economic return. potential emergence of resistant strains of trypanosomes was monitored by regular collection of isolates from tsetse or untreated control cattle. Stabilates were prepared and used to infect test cattle in a tsetse free area. Treatment was given 7 - 10 days after detection of parasites and jugular blood samples were monitored for 100 days. The absence of trypanosomes during this period was assumed to indicate that the isolates were sensitive to the drug under test. On the basis of this criteria, no drug resistance to either Isometamidium or diminazene aceturate was detected.

Logan, Goodwin, Tembely and Craig (1984) compared three monthly administration of Isometamidium with individual treatment with diminazene aceturate using Zebu-maure cattle in Mali. During the 21 month study, Isometamidium used prophylactically was shown to result in significantly greater weight gain and prevented losses more effectively than the diminazene regime, resulting in a herd with a greater market value. No evidence of drug resistance was encountered.

The results of an analysis of data from Mkwaja Ranch, Tanzania (Trail et al., 1985) indicated that a regime based on Isometamidium prophylaxis allowed a high level of productivity to be achieved. Indirect evidence suggested that even though Isometamidium had been used to protect the grade Boran cattle for more than 20 years, resistance had not emerged as a problem.

For most of the study period (1973 - 1980), Isometamidium was/

was administered on a herd basis at 0.5 mg/kg. The need for retreatment was determined by sampling 30 - 40 animals in each herd (225 - 300 cattle), beginning about 1 month after the last herd prophylaxis. The sample was biased to include any individuals which looked in poor condition. If 20% of the slides in a sample were shown to be trypanosome positive, by thick film examination, then the herd was retreated. If less than 20% of a sample were positive, then the need for retreatment was judged according to a subjective impression of the condition of the herd. If the herd's condition was considered to be poor, then the herd was retreated. If the herd's condition was judged to be generally good, individual trypanosome positive animals were treated with diminazene aceturate (Berenil, Hoechst, West Germany) at 3.5 mg/kg.

Use of trypanocidal drugs has increased dramatically in Africa in the past 10 years or so, and Tacher (1982) estimated usage at 25 million doses per year. He suggests that the use of trypanocidal drugs allows development of cattle production in areas where tsetse eradication is not currently possible, but, by using drugs, conditions could gradually be created under which operations against Glossina may eventually be undertaken. This was the original objective when Mkwaja Ranch was established in north-eastern Tanzania in 1954. It was anticipated that profits accrued from the beef ranch could be used to finance a bush clearance programme which would lead to tsetse eradication and pasture improvement. Rapid bush regeneration and less than anticipated profitability were/

were two factors which operated against this objective and 30 years later reliance upon trypanocidal drugs is absolute (Ford and Blaser, 1971; Trail et al., 1985).

Development of Drugs Against African Animal Trypanosomiases

The histories of the science of chemotherapy and of the development of synthetic trypanocides are closely linked, both having their roots in experiments carried out with trypanosomes in the early years of the present century.

Crude extracts of vegetable origin and other simple substances have long been used to treat a variety of disease; inorganic arsenic, for example, has been used since the time of Hippocrates. Livingstone, although ignorant of the aetiology of the condition, treated a 'fly-struck' mare with potassium arsenite during his travels in Africa in the 1840's (Hawking, 1963). However, the founding father of chemotherapy as a science is generally recognised as being Paul Ehrlich, a German physician.

Ehrlich recognised that in the same way that certain dyes were able to stain specific cellular elements, it may be possible to identify chemical substances which would specifically unite with, and destroy, disease-causing parasites, without at the same time destroying the cells of the host. As early as 1891 Ehrlich showed that methylene blue, which is specifically absorbed as a stain by Plasmodium species, also had a therapeutic action against malaria in humans. This observation apparently attracted little interest at the time. Interest in chemotherapy was, however, revived by the/

the need to find a method of controlling 'tsetse-fly disease', or nagana, which was seriously hindering the development of Africa in the late nineteenth and early twentieth century. Bruce (1895) demonstrated that the disease was caused by trypanosomes, similar to those seen in India, where they caused surra in horses and cattle (Evans, 1880). Laveran and Mesnil (1902) demonstrated that trypanosomes could be maintained in rats by continuous passage thereby providing a convenient laboratory model which made it possible to begin the search for effective remedies (Hawking, 1963).

Lingard had shown in 1893 that arsenious oxide had a temporary effect on surra. It was, however, toxic and treated animals eventually either died or relapsed. Ehrlich investigated hundreds of dyes and identified trypan red as being curative for T. equinum infections in mice. This work on the aniline dyes, which also led to the potent antispirochaetal agent salvarsan which revolutionised the treatment of syphilis, is often regarded as the first major breakthrough in chemotherapy. Davey (1958), however, suggests that Ehrlich would not have made his important discovery were it not for the earlier work of Thomas in Liverpool in 1904, who showed atoxyl to be curative against T. gambiense infections in mice.

Plimmer and Thompson (1908) first showed potassium antimony tartrate (tartar emetic) and its sodium analogue to be active against T. b. brucei and T. congolense in laboratory animls. The efficacy of tartar emetic was confirmed against T. congolense and T. vivax infections in cattle by Bevan (1928) and Curson (1928), but it was not active against T. b. brucei. A dose of 1 - 1.5 g per head

was/

was given in a 5% aqueous solution and treatment sometimes needed repeating daily. Severe local reactions occurred with intramuscular or subcutaneous injections, resulting in the need for intravenous administration which presented handling problems in relatively wild cattle. Despite a mortality rate of 6% in treated cattle, tartar emetic continued to be used up until the early 1950's. This was due to the even higher mortality rate of untreated animals and doubt as to the efficacy and toxicity of newer compounds (Leach and Roberts, 1981).

Chemists working on dyestuffs in the Bayer company during the first world war developed the synthetic compound suramin, having started with trypan red, trypan blue and African violet and investigating ureas of the aminophthalene-sulfonic type (Hawking, 1963). Initial activity was demonstrated against the human-infective trypanosomes and later against natural infections of T. evansi in camels (Knowles, 1925) horses and cattle (Edwards, 1926). Suramin was introduced under the tradenames Germanin (1921) and Naganol (1923). Considerably later Guimaraes and Lourie (1951) found that suramin and pentamidine (a drug used in the prophylaxis of human trypanosomiasis) produced a precipitate which reduced the toxicity of pentamidine without reducing its prophylactic activity. This finding led to considerable research effort and the examination of a wide variety of suramin complexes. Although some showed long periods of protection to cattle, local toxicity was a considerable problem and the complexes never came into general use for cattle.

Research interest in potassium antimony tartrate led to the synthesis of an analogous sulfonated pyrocatechol, antimosan, derived from trivalent antimony, and also the sodium salt, stibophen. Antimosan was shown to be active against T. congolense and T. vivax, although less so against T. b. brucei (Parkin, 1931; 1935). Repeated treatments at intervals of four weeks were, however, required at a dose of 3.6 g per 300 - 400 kg bodyweight.

Interest was also shown in the strylquinolines, some of which were found to have trypanocidal activity, particularly against the Trypanozoon subgenus (Browning, Cohen, Ellingworth and Gilbransen, 1926). The compounds, however, were found to be retained in the body for long periods and gave rise to local systemic toxicity, and did not act rapidly against trypanosomes.

Jensch (1937) reported a 4-aminoquinoline derivative, surfen C, with activity against T. congolense. Field trials in cattle demonstrated the drug to be toxic; Le Roux (1936) reported some animals dying of shock within minutes of treatment and also severe local reactions in surviving cattle. However, modification of the surfen C molecule, by chemists at Imperial Chemical Industries (ICI) in Britain, led to the synthesis of the quinapyramines (Barrett, Curd and Hepworth, 1953). Field trials by Davey (1950) demonstrated quinapyramine dimethosulphate to be active against T. congolense, T. vivax and T.b. brucei. Although introduced as a therapeutic drug in the early 1950's, quinapyramine dimethosulphate also had some prophylactic activity. Some authorities consider that this may have/

have been disadvantageous, and could have been a factor in the extensive field resistance which developed to the product (Davey, 1958). Quinapyramine chloride is much less soluble than the dimethosulphate and although subcutaneous injection resulted in the formation of a depot from which the drug was slowly released, the blood level attained was too low to be curative (Curd and Davey, 1950). A mixture of the two salts was, therefore, marketed under the name Antrycide Prosalt, initially as three parts dimethosulphate and four parts chloride, and then later, in 1958, as Prosalt RF with a half as much chloride. The latter formulation was cheaper to produce and was shown to be equally effective, providing three months protection to cattle in low tsetse challenge and two months in medium challenge situations (Davey, 1958). The revised formulation also resulted in less severe local reactions. Antrycide was withdrawn from the market in 1977, in the face of considerable field resistance. Quinapyramine was re-introduced, however, in 1984 under the tradename Trypacide (May and Baker) principally for the treatment and prevention of T. evansi infections in camels and equines.

Browning, Morgan, Robb and Walls (1938) reported activity against the Trypanozoon subgenus by phenidium chloride. Although this activity was subsequently confirmed in the field, the solubility of the compound was low and its therapeutic index narrow. The finding, however, focused attention on the phenanthridines, and soon another member of this group, dimidium bromide, was shown to be active against T. congolense in a field trial by Carmichael and Bell (1944)./

(1944). The drug was used for mass treatment campaigns in East and Central Africa, but unfortunately resistance was widespread by 1952, and the use of higher doses resulted in the development of photosensitization in cattle (Randall and Beveridge, 1946).

Watkins and Woolfe (1952) reported the synthesis of a new aminophenathridine compound, derived from dimidium. Homidium bromide was shown to be as effective as dimidium, but less toxic (Ford, Wilmshurst and Karib, 1953a; 1953b) and with limited prophylactic activity (Leach, Karib, Ford and Wilmshurst, 1955). Synthesis was achieved by the substitution of an ethyl for a methyl-group on the quaternary nitrogen heteroatom of dimidium. Wragg (1955), working at May and Baker, patented the chloride salt which had the advantage over homidium bromide of solubility in cold water. Homidium bromide was introduced as Ethidium (originally Boots Pure Drug Company; most recently Camco Ltd) and homidium chloride was marketed under the tradename Novidium by May and Baker. Mass treatment with homidium however, resulted in widespread development of resistant strains.

The introduction of homidium and quinapyramine in the 1950's meant that for the first time safe, mass treatment of cattle was possible. The number of trypanocidal treatments administered annually rose dramatically. In Kenya, for example, from 45,000 in 1951/52 to 641,000 in 1957/58 (Wilson, 1960). One of the major factors implicated in the decision to set up, in 1954, Mkwaja Ranch in an area of severe trypanosomiasis challenge in north-east Tanzania/

Tanzania, was the availability of Antrycide Prosalt which allowed, for the first time, susceptible cattle to be maintained in tsetse-infected areas (Ford and Blaser, 1971).

Interest in the diamidines originated from the observation that insulin, by lowering blood sugar levels, reduced the rate of replication of trypanosomes in vivo. Synthalin, a synthetic compound with hypoglycaemic activity, was also found to be trypanocidal in vivo (Jansco and Jansco, 1935). Lourie and Yorke (1937) investigated the activity of synthalin further and showed that it had trypanocidal activity in vitro. This demonstrated that synthalin was having a direct action on trypanosomes, not acting simply by reducing the amount of glucose available from the host. Synthetic activity then focused on the diamidines, and led to the synthesis of pentamine, and a number of experimental compounds; phenamidine, propamidine, stilbamidine and M & B 2242 (Williamson, 1970). Research chemists at Hoechst, West Germany, reported a new aromatic diamidine, diminazene aceturate (Berenil) (Bauer, 1955; Fussganger, 1955; Jensch, 1958). Jensch (1958) describes the development of diminazene aceturate from a systematic dissection of the surfen C molecule. It is also noteworthy that the diminazene aceturate molecule bears a close resemblance to M & B 2242, with only substitution of a triazine bridge for the aminotriazine ring of the latter compound. Berenil was rapidly taken into general use in the field, increasing from 2,000 doses in 1957 to 190,000 in 1961 in Kenya (Fairclough, 1963), and virtually replaced quinapyramine and/

and homidium in northern Nigeria (MacLennan, 1968). Limited stability of aqueous solutions led the manufacturers to add phenyldimethyl pyrazolone (antipyrine) as a stabilizer from 1959 onwards (Fairclough, 1963). Diminazene aceturate is also effective against babesiosis, which is an additional advantage of this product. Early hopes, however, that the short period of activity and rapid rate of excretion of this drug would avoid the development of resistant strains of trypanosomes have not been realised.

Watkins and Woolfe (1956) of the Boots company reported the synthesis of pyrithidium bromide, by the substitution of the pyramidyl moiety of quinapyramine into a phenanthridine which resembled phenidium. This compound, marketed as Prothidium, was shown to have therapeutic and prophylactic action (Watkins, 1958) although Leach and El Karib (1960) reported it to be less effective than quinapyramine, and also reported possible problems relating to systemic toxicity in cattle. Resistance rapidly developed to the drug when it came into general use. The product was withdrawn from the market in 1985.

Wragg, Washbourne, Brown and Hill (1958) described a new trypanocide formed by preparing the p-amidinophenylodiazoamino derivative of homidium chloride. This derivative was chosen because of the structural relationship the product would have to diminazene aceturate. The coupling reaction resulted in the formation of two isomers. Favourable results from the testing of these isomers resulted in an extensive investigation of structure/activity relationships of similar isomers, from which the/

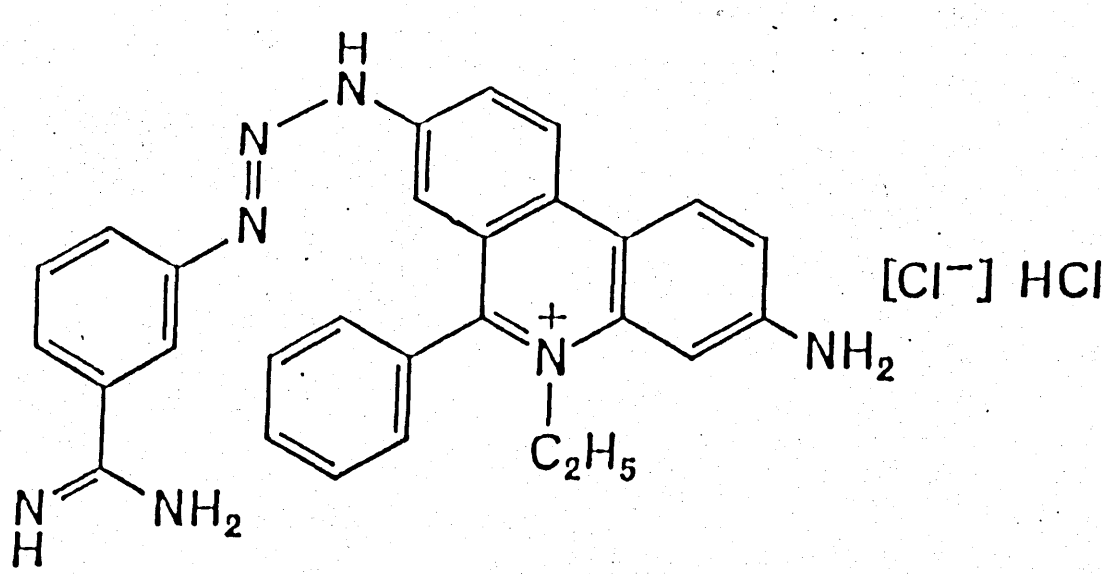


Figure 1. Structure of isometamidium (8-(m-amidinophenyldiazoamino)-3-amino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride).

the most promising product emerged as a mixture of two isomers which were m-amidino derivatives. This mixture of isomers was given the common name metamidium. Subsequently the red isomer present in metamidium, isometamidium chloride, was shown to be the more active of the two, and its chemical structure was described by Berg (1960) (Figure 1). After successful field trials isometamidium chloride was marketed in 1961 by May and Baker under the tradename Samorin, and subsequently it has also been marketed in francophone countries as Trypamidium (Specia, now Rhone-Merieux, France). The commercially available products Samorin and Trypamidium are not pure isometamidium chloride. They contain approximately 70% isometamidium chloride, with 20% of a purple isomer and 10% of a bis-compound (Hutchinson, 1981). In this thesis the term Isometamidium is taken to mean the commercially available product, and not pure isometamidium chloride.

A problem with the use of Isometamidium is that of local toxicity, particularly at the higher doses. Hope-Cawdery and Simmons (1964) experimented with formulations of Isometamidium in a high-viscosity base, but the results were disappointing. Several workers have experimented with Isometamidium-dextran complexes. James (1978) reported extended prophylaxis and reduced local toxicity in rodents. Aliu and Sannusi (1979) reported enhanced prophylaxis in preliminary experiments with rodents. They showed that subcutaneous administration of Isometamidium-dextran resulted in the formation of a small nodule at the site of injection, although no severe local reaction accompanied either subcutaneous or intramuscular injection.

No/

No advantage was demonstrated in the ability to cure infections; both Isometamidium and the dextran-complex failed to prevent the development of relapse infections when used at the 0.5 mg/kg dose level against a T. vivax challenge. Use of Isometamidium-dextran complexes has not progressed beyond the experimental stage.

Future Prospects for the Development of New Trypanocides

Up until the early 1960's several pharmaceutical companies were actively involved in the search for novel trypanocides. Programmes of lead-orientated chemical synthesis were carried out, and large numbers of compounds were fed into screening programmes. From the early 1960's however, the pharmaceutical companies have tended to reduce drastically or cease, their activities in this field. The reasons for this are complex, but include the escalating cost of developing new products - partly due to the increasingly rigorous registration requirements, the break-up of the old colonial territories and lack of confidence in the political and economic stability of the new independent countries. To the large multinational pharmaceutical companies the trypanocide market is not always an attractive one. The countries worst affected by the trypanosomiases are often the least-developed, poorest nations, with low and erratic veterinary budgets. To develop a new trypanocide in the prevailing economic environment would be particularly unfavourable. Even if the considerable development costs could be met and a product emerged which met all the registration requirements in the various intended markets, the financial rewards to the pharmaceutical company would be slight, because, to begin with at least, the product would be held in reserve

by/

by government veterinary services for use only when conventional trypanocides had failed. So, although a novel trypanocide, which could be used as an effective sanative in the face of resistance to the currently available products, would be of great value in the field, the profitability of such a product would be low.

Considerably cheaper and therefore more attractive to industry, is the development of improved formulations of, and new delivery systems for, existing products. Nearly all products currently used have undesirable local and systemic toxic side-effects. If the duration of prophylaxis achieved by a single administration could be lengthened, this also would be beneficial.

New Formulations of Isometamidium

Several possible approaches to new formulations of Isometamidium have been, or are being investigated. As stated earlier, dextran complexes have been examined, and although some authors reported reduced toxicity and enhanced prophylaxis, they did not progress beyond the experimental stage.

Another possibility currently being field tested in Zimbabwe is the incorporation of Isometamidium into liposomes. Liposomes are concentric shells of phospholipid bilayer membranes with aqueous interstices (Marr, 1984), which are prepared by emulsifying, under suitable conditions, phospholipids and other lipids in the presence of the compound which it is desired to trap within the lipid spherules. The use of liposome delivery systems was developed for the chemotherapy of leishmaniasis. It is known that liposomes injected into the bloodstream are taken up by the reticulo-endothelial/

reticulo-endothelial system. Since intracellular forms of leishmania live predominately within the cells of this system it seems an ideal means of selectively delivering the drug to those cells in which the parasite occurs. The same is not true for trypanosomes, which in the case of T. congolense and T. vivax inhabit the bloodstream but do not invade the blood cells, and in the case of T.b. brucei also occur in extravascular tissues. Liposomes also have some intrinsic drawbacks. It has been suggested that they have some adverse pharmacological effects themselves (Alving, Steck, Chapman, Waits, Hendricks, Swartz and Hansen, 1978), and there may be problems of stability at extremes of temperature and humidity. The economics of liposomal delivery systems may also be unfavourable.

It has recently been suggested that isometamidium may be metabolized by the bovine to a metabolite of greater trypanocidal activity than the starting material (Borowy, personal communication). The successful isolation and identification of such a metabolite, or metabolites, could give rise to a new trypanocide which may have advantages over isometamidium, e.g. enhanced activity, reduced toxicity.

Slow-release technologies are finding applications in many areas of veterinary and human medicine. A project has recently been initiated under the auspices of the FAO/IAEA to investigate possible slow-release formulations for the currently available trypanocides. Approaches being considered include trapping trypanocidal molecules in polymeric matrices, incorporating trypanocidal/

trypanocidal molecules into the structures of polymers and the use of slow-release glasses. By such techniques it may be possible to achieve extended periods of protection from compounds with prophylactic activity, and it may be feasable to use slow-release formations of curative drugs, e.g. diminazene aceturate, as prophylactics.

New Approaches to the Development of Trypanocides

The current situation then is one of a small and decreasing number of products being available for the prevention and treatment of nagana. This situation is exacerbated by the origin of these compounds from what Williamson (1976) has described as 'cannabilization synthesis', whereby a moiety of one compound is incorporated into another, giving rise to, not surprisingly, a high level of cross-resistance between products.

Against this unpromising background some potentially valuable new leads have emerged over the last two decades. During this time there has tended to be a shift in both the location of, and the approach to, the quest for new trypanocides. For 60 years or so the search for novel trypanocides was conducted by a process of 'intelligent empiricism', with large-scale screening of possible products. This was largely carried out by pharmaceutical companies in Britain and Germany - both countries with considerable colonial interest in Africa. From the early 1960's a shift has occurred whereby the bulk of trypanocidal research now takes place in academic institutes. A more rational approach has been pursued in/

in that basic research into trypanosome biochemistry has been carried out. This has led to the identification of a number of trypanosome-unique metabolic pathways, not found in the host. Clearly such pathways are vulnerable to attack by suitable inhibitors.

Drug Resistance

The phenomenon of resistance was probably first recognised by Kossiakoff (1887), who noted that by exposure to gradually increasing concentrations of the antiseptics boric or mercuric acid, bacteria could survive in concentrations which previously had been toxic. Franke and Rohl, working in Ehrlich's laboratory, were the first to observe the development of resistance to drugs in the course of treatment of the host. They noted that whereas, initially, treatment of T.b. brucei infected mice with parafuchsin resulted in the temporary disappearance of the parasites, treatment of the relapse infection and subsequent relapse infections led to a gradual loss of response. Significantly, they also subinoculated their relapse strains into new hosts, and demonstrated that the resultant infections also failed to respond to treatment with parafuchsin (Ehrlich, 1907). Browning (1907) demonstrated that the dyes trypan red and trypan blue could also give rise to resistant strains, and since then drug resistant strains have been demonstrated for all the economically important trypanosome species and over the full range of trypanocidal drugs (Schillinger, 1984).

Drug resistance has been defined as 'the ability of a former drug exposed trypanosome strain to survive, despite the administration of a drug given in doses equal to or higher than usually recommended' (Schillinger, 1984). Trypanosomes also exhibit natural tolerance, or natural resistance, to certain trypanocides, which is not dependant on prior drug exposure (Leach and Roberts, 1981). For example, T. simiae is naturally tolerant of most trypanocides. The fact that strains of T. congolense resistant to diminazene aceturate were difficult to produce in the laboratory, and yet diminazene aceturate resistant strains were isolated in the field within 10 months of the trypanocide first being used was considered by Jones-Davies (1968) to be evidence of the natural occurrence of strains resistant to diminazene aceturate, rather than due to the emergence of resistance as a consequence of under dosing. The above definition fails to embrace examples in the literature of drug resistance apparently occurring spontaneously, without prior exposure to the trypanocide to which resistance arose (Eagle and Magnusson, 1944). The situation is also complicated by cross-resistance, that is when a strain of trypanosome which is resistant to one trypanocide is also resistant to a second trypanocide, even though it may never have been exposed to the latter.

Much of the early work on resistance and cross-resistance in trypanosome infections of cattle was carried out by Whiteside in Kenya during the 1950's, using strains of T. congolense and T. vivax (Whiteside, 1960; 1962). Early work had produced conflicting results where, for example, in some experiments strains resistant

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to quinapyramine were shown to be cross-resistant to phenanthridines, while in other experiments cross-resistance was not demonstrated. Whiteside was able to show that much of this confusion was due to differences in the degree of resistance exhibited by the strains. He reported that resistant strains could be produced in the laboratory by exposure of infected cattle to repeated subcurative trypanocide dosages. The degree of resistance produced appeared to increase as the number of such exposures increased. Thus, Whiteside showed that whereas a strain of T. congolense made resistant to four times the minimum curative dose (MCD) of quinapyramine was sensitive to normal doses of either homidium or diminazene aceturate, if the level of resistance was increased further, cross-resistance to diminazene aceturate could be demonstrated. Whiteside, therefore, investigated cross-resistance by preparing in cattle strains of T. congolense which were highly resistant to each of the then available trypanocidal drugs, and tested each resistant strain against all the other trypanocides, thereby building up a table of cross-resistance.

Hopes that cross-resistance could be simply related to chemical structure were not entirely realised so, although cross-resistance could be demonstrated between the phenanthridines homidium, prothidium and metamidium, high doses of metamidium could still be effective against strains directly resistant to homidium. Cross-resistance was also demonstrated between chemically unrelated drugs, e.g. homidium and quinapyramine. Cross-resistance can also/

also occur in one direction only, e.g. strains resistant to diminazene aceturate showed no cross-resistance to quinapyramine, whereas cross-resistance can be demonstrated in the reverse direction.

Development of drug resistant strains in the laboratory

The ease with which drug-resistant trypanosomes can be produced in the laboratory varies considerably between strain and species of trypanosome, host species and trypanocidal drugs. The method generally used to produce resistant strains is to expose a population of trypanosomes to subcurative drug concentrations. This can either be done by allowing an infection to relapse to repeated subcurative treatment, or by rapid passage of trypanosomes through a series of drug-treated animals. Whiteside (1962) suggested that, while drug-resistant strains of T. congolense are relatively difficult to produce in mice, in cattle, except for diminazene aceturate, 'resistance never fails to develop with astonishing rapidity'. His method was to treat an infected steer with three to six successive subcurative treatments, and he reported that this resulted in a level of drug resistance 40 - 80 times the mediam curative dose ($CD_{50}$) for the initial strain. For example, a strain of T. congolense originally susceptible to 0.2 mg/kg pyrithidium was made resistant to 2.0 mg/kg by three successive treatments at 0.1,,0.1 and 0.5 mg/kg. Other workers have not found the production of resistant strains to be so easy. Stephen (1963) failed to produce resistance to homidium in a strain of T. vivax by a process of subcurative treatment and cyclical passage through Glossina palpalis. Folkers (1966) detected

no/

no decrease in sensitivity following repeated use of Isometamidium at the low dosage of 0.25 mg/kg, in naturally infected cattle in Nigeria.

Several authors have suggested that resistant strains emerge more rapidly in the absence of an immune response in the host. An advantage of the rapid passage method of producing resistant strains in the laboratory may be that it avoids the effect of the host's immune response (Jansco and Jansco, 1935; Schnitzer, Lafferty and Buck, 1946).

Limited work on cloned and natural populations of trypanosomes has produced equivocal results. Oehler (1913) found that resistance to salvarsan developed as quickly in a cloned population of T.b. brucei as in a normal population, although Prowazek (1913) noted some differences in sensitivity of cloned and normal populations of T.b. rhodesiense and T. equiperdum.

In some cases it does not seem possible to produce resistance directly by exposure of a normal trypanosome population to drug, but a strain already resistant to one drug can sometimes be readily induced to become resistant to another. For example, strains of T.b. brucei resistant to tartar emetic could not be produced directly, but were readily produced from atoxyl-resistant strains.

A decrease in sensitivity has apparently been brought about by a single exposure to drug, both in the laboratory and field, but usually multiple exposures are required. Bovet and Montezin (1937) demonstrated a marked decrease in sensitivity after a single dose/

dose of 3-hydoxymethyl-4-aminophenylarsonic acid, and Wilson (1949) found resistant strains of T. congolense and T. vivax after a single dose of quinapyramine.

In spite of inadequate culture techniques in the past, drug resistance has been produced in vitro. Inability to maintain blood stream forms for extended periods was overcome by exposing T.b. rhodesiense to gradually increasing drug concentrations in vitro for 50 minute periods, with alternating passages through mice. By this method no decrease in sensitivity was observed during the first four passages, but, thereafter resistance developed rapidly (Yorke, Murgatroyd and Hawking, 1931). Recent developments in the in vitro culture of trypanosomes, allowing the maintenance of bloodstream forms of T. congolense, T. vivax and T.b. brucei, open up new possibilities in this area.

Development of Resistance in the Field

Bayer introduced suramin, under the tradename Germanin, in 1923 and within a few years the first clinical consequences of drug resistance were encountered (Knowles, 1927). Resistance to the cattle trypanocides dimidium, quinapyramine and homidium emerged soon after their introduction, and more recently resistance has been encountered to both diminazene aceturate and isometamidium. It is not known how drug resistance arises in the field, although it is generally thought to occur as a result of underdosing, which can occur for a number of reasons: under estimation of bodyweight, error in preparation/

preparation of drug for injection or in the calculation of the correct dosage, solutions of drug kept for too long before use leading to loss of potency, use of counterfeit product - e.g. potassium permanganate fradulently substituted for Novidium in Nigeria. Exposure to high trypanosome challenge is also considered to be equivalent to underdosing by some authors (Whiteside, 1962).

It is a widely held belief, often repeated in the literature, that trypanocides having prophylactic activity are more likely to induce drug resistance than those with only curative activity. This is based on the theory that, following treatment with a trypanocidal drug, there eventually comes a critical point when the level of drug in the blood and tissues is sufficiently high to induce resistance, but not high enough to prevent infection. Those drugs with prophylactic activity are considered to have a longer 'critical period' than curative drugs, which are more rapidly excreted. Diminazene aceturate was originally held up as evidence of this hypothesis, it being thought that resistance to Berenil did not occur. However, although resistance to diminazene aceturate proved difficult to bring about in the laboratory, in the early 1960's the first cases of resistance in the field were reported from West Africa (Jones-Davies, 1967; Na'Isa, 1967), and more recently resistance has also been reported in East Africa (Mwambu and Mayende, 1971). Well documented cases of resistance to Isometamidium, a drug with pronounced prophylactic activity, are relatively few in number, which/

which fails to confirm this hypothesis. Evidence from Folkers (1966) and the Mkwaja Ranch, Tanzania (Trail et al., 1985) suggests that exposure to sub-curative levels of Isometamidium in the field does not necessarily lead to the emergence of resistant strains. A prophylactic regime based on Isometamidium has been in operation at Mkwaja Ranch, Tanzania, for more than 20 years. The need for retreatment was assessed by the detection of trypanosomes in the blood of a proportion of the herd, and, therefore, some animals must have had blood and tissue levels of isometamidium below the effective level, which is precisely the situation which is classically thought to ensure the development of resistant strains. There is, however, no evidence that resistant strains have emerged.

Mechanisms for the Development of Resistance

Drug resistance in micro-organisms is associated with either a change in the permeability of the organism to the drug in question, or a quantitative or qualitative alteration to a metabolic pathway, or inactivation of the drug. In some bacteria the biochemical basis of drug resistance is well understood. Resistance to penicillin is due to production by resistant organisms of an enzyme, β-lactamase, which inactivates penicillin by acting on the β-lactam ring. Recently this form of resistance has been overcome by the commercial production of β-lactamase inhibitors. The situation with trypanosomes is less clear. Mechanisms by which drug resistance could occur include section mutation and adaptation.

Hawking (1963a) suggests that a population of trypanosomes consists of individuals which exhibit a range of sensitivities to any given trypanocide, with the range of drug sensitivities describing a normal distribution. Exposure of that population to a subcurative drug concentration should result in the elimination of sensitive individuals, and to the selection of those individuals with drug sensitivities greater than the drug concentration used. This selection would lead to a downward shift in the sensitivity of the population. In practice, although repeated or even single exposure to a subcurative drug concentration can lead to a shift in sensitivity, particularly in large animal hosts, this is not always the case. If mice are infected with a strain of T. congolense, treated with a subcurative dose of Isometamidium which brings about temporary aparasitaemia followed by relapse, and are then retreated at the same dose rate, the second treatment again tends to cause a temporary aparasitaemia with subsequent relapse. If a simple selection mechanism was operating one might expect the first treatment to eliminate the majority of sensitive individuals in the population, leaving the most resistant tail of the distribution curve to replicate unhindered by the, perhaps, more viable sensitive trypanosomes. Repeat treatment with the same dose would then be expected to have little or no effect. A possible explanation for the observed response is that trypanosomes occupy drug inaccessible sites. Although such sites have been demonstrated for T.b. brucei in mice (Jennings, Whitelaw, Holmes, Chizyuka and Urquhart, 1979), they have rarely been demonstrated for T. congolense which is generally regarded as a strict haemoparasite (Losos, Paris, Wilson and Dar, 1973),/

1973), except for the initial replication of metacyclic forms in the connective tissue at the site of the bite of an infected tsetse (Luckins and Gray, 1978; 1979). There are, however, two reports in the literature which describe cerebral involvement of T. congolense. Haase, Bernard and Guidot (1981) infected four cattle with T. congolense, then T. vivax and finally T.b. brucei, and then treated them with diminazene aceturate. When no trypanosomes could be detected in the peripheral blood the cattle were killed and blood and organ macerates were subinoculated into rats. In the case of one Zebu, for which subinoculation of blood failed to result in the transfer of infection, subinoculation with brain homogenate resulted in the establishment of a T. congolense infection in the recipient rat. Masake, Nantulya, Akol and Musoke (1984) detected T. congolense in the cerebrospinal fluid of a goat which had an intercurrent, chronic T.b. brucei infection. In both cases it is possible that the presence of T. b. brucei resulted in damage to the blood-brain barrier, facilitating the entry of T. congolense.

Development of resistance generally proceeds by step-wise decreased in sensitivity, possibly indicating a series of mutations having a cumulative effect. The rate at which resistance develops would then depend on the rate at which mutation occurred.

## Characteristics of Resistant Strains

### Stability, infectivity and insect transmissibility

The stability of drug resistant strains may be an important factor in the spread of resistance. Stability in the absence of drug has been shown to vary with the strain and species of trypanosome, mode of transmission and drug. At one extreme Fulton and Grant (1955) describe an atoxyl resistant strain of T. rhodesiense still resistant 24 years after its last exposure to this drug and maintained by serial passage. At the other end of the scale, Unsworth (1954) describes a strain of T. congolense resistant to quinapyramine which showed a rapid decline in resistance after just 65 days.

Stability of drug resistance may be related to the mechanism by which resistance arose. If exposure to drug has been brought about by an adaptation, e.g. increased activity of an inducible enzyme enabling the organism to survive at higher drug concentration, then removal of the drug may result in a rapid return to pre-exposure sensitivity levels. If on the other hand, resistance was due to mutation, then removal of drug may be less important. Loss of resistance would then be dependant upon the rate of back mutation, and the relative competitive advantage of mutant and wild type individuals.

Several workers have demonstrated that resistant strains can be transmitted cyclically by tsetse flies in a laboratory environment, and that strains transmitted under such conditions are usually stable. Yorke, Murgatroyd and Hawking (1933) and

Murgatroyd/

Murgatroyd and Yorke (1937) demonstrated that tryparsamide resistance of T.b. brucei was unaffected by transmission through G. morsitans or G. palpalis, and Van Hoof and Henrard (1934) made a similar observation with a strain of T.b. gambiense. Similarly the quinapyramine resistance of a strain of T. vivax was unaffected by transmission through G. palpalis. Gray and Roberts (1971) transmitted a drug resistant strain of T. vivax through antelope and cattle by cyclical transmission. Resistance to diminazene aceturate (7.0 mg/kg) and quinapyramine sulphate (5.0 mg/kg) was retained for 7 months in tsetse and antelope, and 29 months in tsetse and cattle. Nyeko, Ssenyonga, Golder and Otieno (in press) detected no change in sensitivity of an Isometamidium resistant strain of T. congolense after 12 cyclical passages, although a slight, statistically significant reduction was detected with the same strain after 15 needle passages in mice.

There are numerous examples in the literature of cyclical transmission of drug resistant strains in the field (Fiennes, 1953, Williamson and Stephen, 1960; Jones-Davies, 1968). Studies of the stability of resistant strains in the field in the absence of drug have produced conflicting results. Fiennes (1953) isolated quinapyramine resistant strains of T. vivax and T. congolense in an area from which all drug treated cattle had been removed 5 - 7 months previously. Jones-Davies (1968) reported homidium resistant strains of T. congolense in tsetse flies in Nigeria, despite homidium having been withdrawn from use in the area two years earlier, to be replaced with diminazene aceturate. Other workers have found that resistant strains of T. vivax and T. congolense/

congolense tend to disappear from tsetse, 6 - 9 months after cattle are removed and the use of the drug in question suspended (Whiteside, 1960).

Viability of resistant strains

If continued exposure of trypanosomes to subcurative drug concentrations inevitably leads to the development of resistant strains, the problem of drug resistance might be expected to be more widespread than is the case. Prophylactic drugs, such as Isometamidium, are often used such that a proportion of the animals at risk are allowed to become reinfected, or to relapse, before repeat prophylaxis is carried out on a herd basis. This means that trypanosomes are regularly being exposed to subcurative drug concentrations as blood and tissue levels decline towards the end of the prophylactic period. As previously described, this was the basis on which prophylactic cover was provided for up to 12,000 cattle at Mkwaja Ranch, Tanzania for over 20 years from the introduction of Isometamidium on the ranch in 1963 (Trail et al., 1985). During this period no problems of drug resistance were encountered, and although diminazene aceturate was occasionally used in some animals it was not used in a regulated manner as a 'sanative'. The classical theories of the development of drug-resistant strains predict that in the conditions described, in an area of very high trypanosome challenge, resistant strains would have readily emerged.

At Galana Ranch, Kenya, during 1984/85, an outbreak of acute, haemorrhagic T. vivax occurred for which Isometamidium prophylaxis was ineffective (Rottcher and Schillinger, 1985; Njogu and Heath, 1986). Experimental herds of cattle were established in the area soon after, and despite weekly monitoring, no further evidence of drug resistance was subsequently detected.

One explanation for these observations is that some drug resistant strains of trypanosomes cannot successfully complete with strains of normal sensitivity. This could be because the resistant individuals replicate more slowly than normal individuals, and so eventually the latter simply overgrow the former (Hawking, 1963a).

Reduced growth rate of drug resistant, as compared to parent strains has been reported for both bacteria (Mayr-Harting, 1955) and Plasmodium galinaceum (Bishop and McConnachie, 1950). Limited investigation using mixtures of resistant and normal strains suggest that, for some strains at least, the resistant strains may be at a competitive disadvantage compared to a normal strain. Both a salvarsen resistant strain of T. gambiense in mice (Oehler, 1914) and an oxophenarsine resistant strain of T. equiperdum in rats (Cantrell, 1956) disappeared in the presence of intercurrent infections of normal sensitivity.

Drug uptake

One possible mechanism for reduced sensitivity of drug resistant strains of trypanosomes is reduced uptake of drug. Several workers have demonstrated that resistant strains take up drug more slowly than sensitive strains. Taking advantage of the/

the fluorescent properties of the drug, Hawking and Smiles (1941) looked at fluorescence of T. equiperdium after in vitro treatment with diamidinostilbene, and predicted that resistant strains would exhibit less fluorescence than sensitive ones. Fulton and Grant (1955) compared uptake of stilbamidine, and showed that the resistant strains absorbed less drug than the normal strain. Damper and Patton (1976) used $^{3}$H-labelled pentamidine to demonstrate a lower rate of pentamidine transport in drug resistant T.b. brucei strains compared to a strain of normal sensitivity, and also in T.b. rhodesiense which is naturally less sensitive to pentamidine than T.b. brucei.

Course of parasitaemia

Several authors have compared the course of parasitaemia of drug-resistant and sensitive strains. Tobie and Brand (1953) working with arsenic-resistant T. b. gambiense described a pattern of infection in rats infected with normal strains associated with continuous parasitaemia, compared with a predominance of intermittent relapsing parasitaemias in rats infected with resistant strains. Williamson and Stephen (1960) concluded from work on the development of resistant strains in rodents that an increase in resistance was manifest by a decrease in the period from subcurative treatment to subsequent relapse infection, and proposed a test for drug resistance based on this observation.

Stephen (1962) noted that breakthrough infections occurring towards the end of the protection period afforded by prophylactic drugs were often of a scanty and sporadic nature, particularly

for/

for T. congolense, with trypanosomes appearing at irregular intervals and in very low numbers. In cases where the relapse infection was T. vivax, however, parasites were present in moderately large numbers and appeared at quite regular intervals. Similarly with T. simiae infections in pigs, Stephen and Gray (1960) reported that breakthrough and relapse infections tended to be mild, and parasitaemias were usually, although not always scanty.

Hill (1962) investigated the nature of breakthrough strains of T. congolense in mice after treatment with experimental phenanthridines, and described a low grade, chronic relapsing course which was quite different from the acute or subacute course of the parent strain in untreated mice.

Pathogenicity

In experiments using wild caught tsetse as the source of infection, Stephen (1960) suggested that there was evidence that the trypanosomes occurring in breakthrough infections were less pathogenic than the trypanosomes infecting control animals that had not received a prophylactic drug. Cattle with breakthrough infections of T. congolense gained weight, did not become anaemic and did not die. Where the breakthrough infection was T. vivax two out of three cattle lost weight and developed a moderate anaemia although none died. In contrast three control animals that received no prophylactic drug died of mixed T. vivax/T. congolense infections.

In T. simiae infections in pigs, although the parasitaemias of breakthrough infections tended to be scanty, the trypanosomes causing/

causing those infections had not lost their pathogenicity (Stephen and Gray, 1960). Hill (1962) also reported that the low grade chronic infections he obtained with breakthrough T. congolense infections in mice were not due to reduced virulence of the strains. He demonstrated this by subinoculating mice with breakthough strains, and found the number of days to mortality was the same as the parent strain. He considered the difference in character of the breakthrough strains was probably due to the combined action of the drug and the immune system of the host. Recent experiments in cattle, however, have failed to demonstrate a detectable immune response in animals protected by Isometamidium prophylaxis (Whitelaw, Bell, Holmes, Moloo, Hirumi, Urquhart and Murray, 1986).

Resistance to Isometamidium

Relatively few reports have appeared in the literature regarding resistance, or reduced sensitivity, to Isometamidium. Lewis and Thomson (1974) mention two earlier reports of strains of T. vivax and T. congolense that were resistant to 0.25 and 0.5 mg/kg Isometamidium. The reports they cite, dated 1967 and 1970, are both annual reports of the Branch of Tsetse and Trypanosomiasis Control, Ministry of Agriculture, Rhodesia (Zimbabwe). The 1967 report seems to contain the earliest reference to resistance arising to Isometamidium in the field. Lewis and Thomson (1974) report a strain of T. congolense isolated from the Masumo and Logolo Valley region of Rhodesia where chemoprophylaxis had been in use since 1958. Initially quinapyramine prosalt was used

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with diminazene aceturate as a sanative. Isometamidium was first used in 1962/3, and replaced quinapyramine, due to resistance becoming a problem, in 1967.

Treatment was administered every 12 weeks at 1 mg/kg Isometamidium, with an extended period of 16 weeks when oxen were required for ploughing. Approximately 2,500 cattle were maintained in the area, which was within the flybelt. Up to 2.5% of animals failed to be presented for treatment. In 1968/69 no sanatives were employed, but the use of diminazene aceturate was reintroduced in 1970, 3.5 mg/kg being administered every 6 months, 4 weeks prior to a routine Isometamidium treatment.

From 1970 onwards there was evidence that a reduced period of protection was being achieved. In March 1972 strains of T. congolense were isolated from infected animals and stabilates prepared for storage in liquid nitrogen. Two isolates were used to infect two trypanosome naive bovines, which were maintained outside the tsetse-infested area. Bovine 1 developed patent parasitaemia 23 days post-infection and was treated with 1 mg/kg Isometamidium. A relapse infection was detected 50 days post-treatment, after which treatment was repeated at the same dose, resulting in a 150 day parasite-free period. Bovine 2 became parasitaemic 21 days post-infection, and relapsed 48 days after treatment at 1 mg/kg. Repeat treatment at 1 mg/kg resulted in a relapse infection 83 days post-treatment. Treatment at 2 mg/kg resulted in a 150 day parasite free period. It was concluded that resistance/

resistance of a strain of T. congolense to 1 mg/kg Isometamidium had been demonstrated after the drug had been used for prophylactic protection for 4 years.

Gitatha (1981) reports the isolation of a strain of T. congolense from the Shimba Hills Settlement Scheme, Kwale District, Coast Province, Kenya, which demonstrated resistance to quinapyramine, diminazene aceturate, homidium chloride and bromide and Isometamidium. The results of an Isometamidium sensitivity test in cattle were variable: of 5 animals infected with the strain, two were cured at 0.5 mg/kg, one at 2.0 mg/kg and two at 4.0 mg/kg. Although quinapyramine methylsulphate and prosalt, homidium bromide and diminazene aceturate were all used in the Shimba Hill Scheme between 1958 and 1971, Isometamidium has never been used. Irregular treatment of cattle in the scheme, with up to 50% not being presented for treatment on some occasions, is thought to have led to the development of multiple drug-resistance, and it is speculated that resistance to Isometamidium has arisen due to cross-resistance from the development of resistance to quinapyramine. An unusual feature was resistance to both diminazene aceturate and Isometamidium, two trypanocides normally considered to be an effective 'sanative pair'.

Bourn and Scott (1978) describe the use of Isometamidium in an agricultural development scheme in the Angar Valley, a tsetse infested area of Ethiopia. Initially diminazene aceturate was used to protect the draught oxen at a dosage of 3.5 mg/kg. However, in January 1973, an infection rate of 92% was detected,

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27 days post-treatment and so the move was made to a prophylactic drug, Isometamidium. The dosage was 1 mg/kg with repeat treatments scheduled every 8 weeks. In fact, the second treatment was given 89 days after the first, and 36 days after the third treatment 66% of the oxen were found to be infected, the majority with T. congolense. Emergence of Isometamidium resistance was suspected, and further evidence was obtained when it was shown in mice that T. congolense isolates from Augar Gutin were resistant to the phenanthridine drug homidium bromide (Scott and Pegram, 1974). It is significant, however, that despite the proven existence of phenanthridine-resistant trypanosomes, the work oxen in the area remained in good condition. Isometamidium continued to be used until January, 1974, after which diminazene aceturate and Isometamidium were used alternately for approximately 9 month periods, in an attempt to control development of drug resistant strains.

Kupper and Wolters (1983) investigated drug resistance in a feedlot in northern Ivory Coast. Many of the slaughter cattle in the Ivory Coast originate from Mali or Upper Volta (Burkina Faso) and are fattened in the Ivory Coast. Cattle are treated with trypanocides at the point of entry, and then in the case of cattle destined for the feedlot at Fencessendongon, are trekked through tsetse-infested country for 2 - 3 weeks. Cattle arriving at the feedlot generally became infected within 2 - 4 weeks of arrival, although tsetse are reported to be scarce. Diminazene aceturate had been used at 3.5 and 5.0 mg/kg, but within 8 days 48% of treated cattle had become reinfected with T. vivax and/or T. congolense./

congolense. A trial was therefore carried out in cattle to investigate drug efficacy. The results are difficult to interpret in the form presented, and are complicated by the late arrival of Isometamidium for inclusion in the trial. Rapid reinfection was demonstrated, however, following treatment at up to 1 mg/kg Isometamidium. The breakthrough infections were also resistant to homidium chloride at 1 mg/kg. Diminazene aceturate at 7 mg/kg eliminated trypanosomes for 28 days, and is concluded to be the drug of choice in this location. It is noted that swellings and abcesses occurred at the site of injection of Isometamidium, suggesting poor injection technique, and this may have influenced absorption of drug.

Pinder and Authie (1984) report the appearance of Isometamidium resistant T. congolense in cattle in Upper Volta (Burkina Faso). The reappearance of patent parasitaemia, mainly T. congolense, as early as two weeks after treatment with Isometamidium at 1 mg/kg suggested the existence of resistant strains. This was investigated using mouse sensitivity tests. Strains were also available from the areas in question, which had been isolated two or more years earlier, prior to the widespread use of the drug. The mouse tests showed that most T. congolense stocks isolated in 1982/83 were 4 - 8 times less sensitive to Isometamidium than those which had been isolated in 1979/80. Authie (1984) also reported that some of the strains shown to be resistant to Isometamidium were also resistant to diminazene aceturate.

Relapse Not Due to Resistance

When a therapeutic drug fails to achieve a sterile cure, or a prophylactic drug fails to confer the expected duration of protection, and after the possibility of accidental or fradulent underdosing has been eliminated, the usual conclusion is that a drug resistant strain of trypanosome has been encountered. There are, however, other explanations. Jennings and coworkers (1979) have demonstrated the importance of 'privileged sites' in experimental infection of T.b. brucei in mice, that is locations in the body which trypanosomes can invade, but which are inaccessible to drugs. In a series of experiments, they showed that the ability of diminazene aceturate to cure T.b. brucei infections in mice was related to the delay from infection to treatment. Treatment at 3 days post-infection resulted in a 100% cure rate, but if treatment was withheld until days 14 or 21, many mice went on to develop relapse infections. Carefully controlled organ transfer experiments demonstrated that the location of the cryptic trypanosome focus responsible for the relapse infection was the brain.

In the case of a prophylactic drug, even if trypanosomes could gain access to drug inaccessible sites, this should not affect the prophylactic period. In the curative situation it is envisaged that trypanosomes re-emerge from the privileged sites, and as soon as the trypanocide concentration decreased below the effective level, re-emerging trypanosomes can proliferate and become detectable. Trypanosomes re-emerging in an animal protected by a trypanocide with/

with prophylactic activity should still succumb. Jennings' work was with T.b. brucei, a trypanosome known for its ability to invade tissue as well as blood. T. congolense, however, is generally regarded as strictly vascular. Evidence for extra-vascular development of T. congolense has, until recently, been limited to reports of short-term development at the site of an infected tsetse bite, i.e. the chancre (Luckins and Gray, 1978). Masake et al., (1984), however, have demonstrated T. congolense in the cerebrospinal fluid of Boran steers inoculated with mixed T. brucei/congolense infections, but not in animals infected only with T. congolense. The authors point out that since mixed infections are commonly encountered in the field, invasion of the central nervous system poses a problem for chemotherapy since the drugs currently available do not penetrate the blood-brain barrier.

MacLennan (1973) has demonstrated a period of aparasitaemia in cattle infected with T. vivax and treated with diminazene aceturate, during which subinoculation of blood into naive cattle failed to result in the establishment of infection in recipients, suggesting the existence of a site inaccessible to drug. Haase et al (1981) demonstrated that homogenates of brain tissue obtained from cattle which had been infected with T. congolense and treated with diminazene aceturate, resulted in the establishment of infection in recipient rats, whereas subinoculation of blood failed to do so.

A further possibility to explain relapse infections is due to the presence of species of trypanosome naturally insensitive to the trypanocide in use. For example, it has been suggested that

some/

some cases attributed to drug resistant T. congolense may have been due to T. simiae infections. T. simiae is naturally insensitive to the range of currently available trypanocides, and also is indistinguishable from T. congolense morphologically. The two species can, however, be distinguished by the course of infection in pigs. T. simiae runs a rapid, fatal course in pigs, whereas T. congolense has a more chronic course. It is, therefore, desirable that suspected drug resistant strains of T. congolense are inoculated into pigs to eliminate this possibility, until such times as more convenient diagnostic techniques become available, such as species-specific DNA probes.

The control of African bovine trypanosomiasis is likely to remain heavily dependent on a small number of trypanocidal drugs for the foreseeable future. It is therefore important that those drugs which are available are used to the best effect, and that appropriate steps are taken to limit the development of drug resistant strains. Effective methods of monitoring the drug sensitivities of field isolates of trypanosomes are an important element in the overall trypanosomiasis control strategy. This thesis is therefore primarily concerned with the evaluation of several methods, both in vivo and in vitro, of assessing the Isometamidium sensitivities of strains of T. congolense. Experiments are also described relating to attempts to produce Isometamidium resistant strains of T. congolense in mice and goats, and to the relative viability of Isometamidium sensitive and resistant strains of T. congolense.

# CHAPTER TWO

## GENERAL MATERIALS AND METHODS

Experimental Animals

Mice: For most of the experiments described in this thesis, female CD1 strain mice, 6 to 8 weeks old at the beginning of each experiment were used (Charles River, England). Exceptions were the mouse sensitivity tests performed concurrently with the cattle sensitivity test (pp.85-95) for which female random-bred Swiss mice, 20 - 30 g bodyweight, were used. For routine subinoculation of cattle blood during this experiment, outbred mice from the Kenya Trypanosomiasis Research Institute (KETRI) closed colony were used.

The mice used as donors for the study of the aparasitaemic interval following subcurative treatment, were CD1 ex-breeder females. These larger mice were used because of the need to obtain daily 0.1 ml blood samples for subinoculation.

All mice were maintained under standard laboratory conditions.

Goats: Castrated male, British cross-bred goats were obtained from a commercial source, at 6 to 18 months of age. Prior to infection, goats were treated with an anthelmintic (Panacur, Hoechst, W. Germany), long acting oxytetracycline (Terramycin LA injectable solution, Pfizer, England) to prevent respiratory infections and an ectoparasitic dusting powder (Louse Powder, Cooper Animal Health, England). Goats were kept in fly-proof housing and maintained on a diet of hay, ad libitum, supplemented with concentrates (0.5 kg/d, 306 Ewbol Store Lamb Finisher Pellets, BOCM Silcock LTD., England) and occasionally small quantities of cabbage./

cabbage. Water, rock-salt and a mineral lick (Super Chelated Rockies, Tythebarn Ltd., England) were always available.

Cattle: Trypanosome-naive, Boran steers, originally obtained from a tsetse-free region of Kenya were housed in indivual stalls in fly-proof accommodation at KETRI. Cattle were maintained on a diet of hay, ad libitum, supplemented with concentrates (Ranchcubes, Unga Feeds Ltd., Kenya) at the rate of approximately 0.5 kg per head per day. Water and a mineral lick (MacLick, Twiga Chemicals Ltd., Kenya) were always available. Prior to the start of an experiments, cattle were treated with an anthelmintic (Nilzan, Wellcome Kenya Ltd., Kenya) and, as a precaution against tick-borne disease, cattle were sprayed with an acaricide (Delnav, Wellcome Kenya Ltd.,) once a week. One steer in the first phase of the experiment developed muscular dystrophy. All cattle in the second phase of the experiment were therefore treated with an injectable selenium/vitamin E preparation (Dystosel, Intervet, England) prior to the start of the experiment.

Trypanosomes

The following strains of trypanosomes were used in the experiments described in this thesis:

T. congolense KETRI 2880 (ILRAD 2856), a derivative of an isolate (Banankeledage/83/CRTA/67) prepared from a steer in Burkina Faso, and reported to be partially sensitive to Isometamidium at 4 mg/kg in mice (Pinder and Authie, 1984). The isolate was passaged eight times in rodents before being stabilated as KETRI 2880.

T. congolense KETRI 2883 (ILRAD 3035), a derivative of an isolate made from a steer maintained under Isometamidium prophylaxis at Muhaka, Coast Province, Kenya. The primary isolate was passaged in a goat and then further passaged nine times in rodents before being treated with Isometamidium at 2 mg/kg. The relapse infection was passaged twice in rodents before being stabilated as KETRI 2883.

T. congolense KETRI 2885 (Il Nat. 3.1), a doubly cloned derivative of an isolate originally made from a lion in Serengeti National Park (Geigy and Kauffman, 1973; Nantulya, Musoke, Rurangirwa and Moloo, 1984), known to be sensitive to 0.5 mg/kg Isometamidium in mice (Whitelaw, Bell, Holmes, Moloo, Hirumi, Urquhart and Murray, 1986).

T. congolense 12/1, originally obtained from Professor Lumsden, London School of Hygiene and Tropical Medicine, and passaged in mice, some of which had been treated with suramin (20 mg/kg).

T. congolense 82/1, originally obtained from Professor Vickerman, University of Glasgow (TC 1). The strain had been passaged in rodents and exposed to suramin (20 mg/kg)during some passages.

Cryopreservation of T. congolense

Trypanosomes for cryopreservation were harvested from mice or rats close to the first parasitaemic peak, by cardiac puncture under terminal anaesthesia. The volume of infected blood collected was then measured and 10% (v/v) glycerol was added dropwise. The blood/

Table 3 Composition of PBS and ESG buffers

PBS

Disodium hydrogen phophate

| | |
|---|---|
| $Na_2HPO_4$ (anhydrous) | 13.48 g |
| or if dodecahydrate | 34.01 g |

Sodium hydrogen phosphate

| | |
|---|---|
| $NaH_2PO_4 . 2H_2O$ | 0.78 g |

Sodium Chloride

| | |
|---|---|
| NaCl | 4.25 g |

made up to 1 litre with distilled water and used as 6 volumes PBS to 4 volumes distilled water. pH 8.0.

ESG

Sodium Chloride

| | |
|---|---|
| NaCl | 8.0 g |

Potassium dihydrogen orphophosphate

| | |
|---|---|
| $KH_2PO_4$ | 0.3 g |
| EDTA | 2.0 g |
| Glucose | 2.0 g |
| Indicator * | 5 ml |
| Distilled water | 1000 ml |

adjust pH to 7.4 with NaOH

* Indicator: 1g phenol red

4g NaCl

distilled water to 500 ml.

blood/glycerol emulsion was well mixed using a glass rod, and left to equilibriate for 5 minutes on ice, after which it was either placed in screw-top plastic vials, or was taken up in glass capillary tubes which were then flame sealed. The vials, or capillary tubes packed into screw-top plastic tubes, were suspended in the vapour phase of a liquid nitrogen Dewar flask, gradually lowering the containers closer to the liquid nitrogen surface. Finally 5 to 24 hours later, the stabilates were stored in liquid nitrogen.

Preparation of Inocula

Cryopreserved trypanosomes were rapidly thawed either by rolling the plastic vial between the fingers, or plunging capillary tubes into water. Thawed, infected blood was diluted with approximately 1 ml of phosphate buffered saline (PBS, see Table 3. EDTA-saline-glucose buffer (ESG) was substituted for PBS in the cattle/mouse sensitivity experiments), and the trypanosome density estimated using a haemocytometer.

For mice, $1 \times 10^5$ trypanosomes was the standard infective inoculum, administered in a volume of 0.2 ml PBS by intraperitoneal (ip) inoculation using a 25g, 5/8" needle, into the left side of the body to avoid the spleen. When intravenous (iv) inoculation was required, mice were anaesthetized (Hypnorm, Pfizer, England) approximately 15 minutes before inoculation, and were placed under heat lamps to ensure vasodilation. Intravenous inoculation was carried out using a 25g, 5/8" needle and syringe, into a tail-vein. The vein was occluded to raise the vessel and a volume of 0.2 ml of inoculum injected.

For cattle and goats the standard, infective inoculum was $1 \times 10^5$ trypanosomes administered in a volume of 4 ml PBS, by iv inoculation into the jugular vein.

Trypanocidal Treatment

Mice were treated with Isometamidium by ip injection. The selected dose was administered at the rate of 0.1 ml Isometamidium solution per 10 g bodyweight. A dose of x mg/kg was prepared by weighing x mg of Isometamidium, and making up to 10 ml with distilled water. If $x < 10$ mg, then the final concentration was achieved by serial dilution.

Goats were treated by deep intramuscular injection into the quadricep femoris muscle of the hind leg, using a 20 g, 1" needle and syringe. Successive treatments were administered alternately into the left and right leg.

Cattle were treated by deep intramuscular injection into the middle third of the neck. When the injection volume exceeded 15 ml, the dose was divided between the left and right sides.

For ruminants the Isometamidium concentration used varied with the dose administered. In goats, dosages up to 0.2 mg/kg were prepared at 0.25% (w/v) solutions using distilled water. Dosages of 0.5 mg/kg were prepared as 1% (w/v) solutions, while dosages of 1.0 mg/kg and above were prepared as 2% (w/v) solutions. All Isometamidium solutions were used within our hour of preparation.

Trypanosome Detection Methods

Mice

Wet-film: mice were bled by venesection at the tip of the tail, and collecting a drop of blood on to a microscope slide. The blood was covered by a coverslip (7 x 22 mm) and examined at x 400. Twenty microscope fields were examined and the number of trypanosomes counted was converted to trypanosomes per ml of blood by the method of Herbert and Lumsden (1976).

Capillary concentration: mice were bled by tail venesection and 50 µl of blood was collected using a micropipetter (Volac, England). The blood was dispensed into a well of a microtitre plate, 50 µl of diluent added (9% glycerol (v/v); 9% magnessium sulphate (w/v); 0.1% TRIS buffer (w/v), pH 8.0; phenol red 1:100,000 in distilled water) and mixed. The microtitre plates were kept at room temperature and 15 minutes later samples were taken up in plain capillary tubes, sealed at one end (Crystaseal, Hawksley, England) and centrifuged using a micro-haematocrit centrifuge (Hawksley, England) for 2 minutes. The capillary tubes were then cut, such that the retained portion contained approximately 1 mm of red blood cells, the buffy coat and the plasma. The red cells, buffy coat and approximately 10 mm of plasma were expelled on to a microscope slide, mixed and examined at x 400. Approximately 200 microscope fields, over the entire preparation, were searched for trypanosomes.

Goats

A jugular blood sample was obtained using a heparinised evacuated blood collection tube (75 x 13 mm Vacutainer, Becton Dickinson,/

Dickinson, England). Blood samples were placed on a rotary mixer for at least 5 minutes to ensure homogenous samples were obtained, and then plain capillary tubes were filled, sealed at one end with Crystaseal, and centrifuged for 5 minutes using a micro-haematocrit centrifuge. Examination of the buffy coat was carried out as described above.

Cattle

A peripheral blood sample was obtained by puncturing a marginal ear-vein with a sterile lancet, and collecting blood directly into a heparinised capillary tube, which was then sealed at one end with modelling clay. Samples were then processed as described above.

Packed Cell Volume

Packed cell volume (PCV) was determined using a jugular blood sample, prior to examination of the buffy coat in goats, or by a similar procedure, but using a specially collected jugular sample, for cattle. Blood samples were centrifuged in a micro-haematocrit centrifuge for 5 minutes and PCV's read using a microhaematocrit reader (Hawksley, England).

Rectal Temperature

Rectal temperatures were measured in goats and cattle at approximately 09.00 hours, using a standard clinical thermometer.

Euthanasia

Mice and rats were killed by inducation of deep anaesthesia using trichloroethylene (Trilene, ICI, England) followed by cervical dislocation.

Goats were killed by overdosing with pentobarbitone (Euthatal, May and Baker, England), administered by rapid injection into the jugular vein.

At the end of an experiment cattle were generally cured by chemotherapy (Isometamidium or diminazene aceturate) and then disposed of to commercial outlets for slaughter.

# CHAPTER THREE

## *In vivo* Approaches to the Assessment of Isometamidium Sensitivities of Strains *T. congolense*

## INTRODUCTION

Drug resistance is usually suspected in the field when either a prophylactic drug has apparently failed to confer the expected period of protection, or a curative drug has failed to achieve a sterile cure. The existence of drug resistant strains of trypanosomes, though important, is only one of many possible explanations for such failures. The animals may have been underdosed because bodyweight was underestimated. Very often weigh crates are not available, and in any case groups of cattle are often treated with a standard dose. Larger than average animals are, therefore, at particular risk from underdosing. The drug may have been prepared incorrectly or kept too long before use, or exposed to too high temperatures, leading to loss of potency. It may have been administered incorrectly; for example the trypanocide homidium chloride (Novidium, May and Baker, England) is supplied in tablet form, and it is intended that it should be broken up, dissolved in water and administered by deep intramuscular injection. Cattle owners in the north of Kenya favour this product, finding it more convenient than trypanocides in powder form, but have been known to misuse it by inserting whole tablets into bovine orifices or even placing tablets in incisions made in the neck which are then held in place by acacia thorn sutures (J. Waitumbi, personal communication). The material used may not have been trypanocide at all, but some similar looking material fradulently passed off

as/

as the authentic product: potassium permanganate has been made into tablets, embossed with the name Novidium and offered for sale in authentic looking packaging in Nigeria (E.W.G. Crouch, personal communication). Individual animals may have missed treatment, by failing to be presented for treatment, by escaping from the collecting pens, or crush, or accidental ommision.

There is also the possibility of relapse due to re-emergence from drug inaccessbile sites (see p. 55). Misdiagnosis of the original problem may explain the apparent failure of the trypanocides to have an effect: conditions such as liverfluke, heartwater or even starvation may be implicated (Jones-Davies and Folkers, 1966). The presence of mixed infections of trypanosome species or incorrectly identified species may also confuse the situation.

To prove that drug resistant trypanosomes are, in fact, involved then it is necessary to first discount these and other possibilities, and secondly to demonstrate the response of the trypanosomes involved to treatment under carefully controlled conditions. Ideally this should be carried out in the host species of interest, ie in Bos indicus cattle for strains isolated from East African zebu. Although this approach would allow a high degree of confidence in the results obtained, it would be very expensive in terms of animals, space, facilities and resources; prohibitively so for routine examination of field isolates. Most strains of T. congolense can be established and maintained in rodent hosts, and mice are most often used for trypanocide sensitivity testing. Mice clearly offer considerable advantages in terms of ease of handling and/

and overall economy, and because it is possible to use larger numbers of animals in a mouse test, experiments of superior experimental design can be carried out in terms of the number of treatment groups and number of replicates. There is also the advantage that whereas parasitaemias of T. congolense in cattle tend to be low-grade, parasitaemias in mice tend to be higher and, therefore, more easily detected: simple examination of blood wet-film preparations can be used in place of the more sophisticated concentration techniques which may be needed for cattle.

Drug resistant strains of T. vivax present a different problem because the vast majority of strains will not infect rodents. Williamson and Stephen (1960) reported that sheep were easily infected with tsetse-borne T. vivax, and, being more economical to use than cattle, were well suited to drug sensitivity investigations. Ilemobade, Leeflang, Buys and Blotkamp (1975) used a local breed of sheep for the isolation and assessment of the homidium sensitivity of isolates of T. vivax collected from cattle in Nigeria, but noted that exotic or cross-bred cattle were more readily infected than local breeds of sheep or goats. On the other hand, Schoenefeld, Rottcher, Schillinger and Gorton (1986) considered that small ruminants, even if splenectomized, were not suitable hosts because of their capacity to control parasitaemia and they concluded that drug sensitivities of T. vivax should be investigated in the bovine. Drug sensitivity studies of T. vivax are, therefore, of necessity relatively difficult and expensive to conduct.

A drug sensitivity test, whether conducted in laboratory or large animal hosts, requires a uniform batch of trypanosome-naive animals free from intercurrent disease. Infection with the strain of interest is usually effected by needle challenge using a standard sized inoculum. Groups of experimental animals are then treated at a predetermined stage of parasitaemia with one of a range of drug levels, or are left untreated as infection controls. After treatment the animals are monitored for a period of time long enough to be reasonably sure that all potential relapse infections have occurred. The two drug dosages identified in sensitivity tests are conventionally the lowest drug dose which will temporarily clear an infection (ie reduce the parasitaemia below the limit of detection of the method employed) from the blood of all, or a proportion, of the test groups, and secondly, the lowest dose which will achieve a sterile cure in all, or a proportion, of the test group. The terms minimum effective dose (MED) and minimum curative dose (MCD) are used to describe these two parameters. Different workers report varying proportions of test animals in their MCD and MED values, largely reflecting different experimental design. Hawking (1963a), for example, reported $ED_{80}$ and $CD_{80}$ values, ie the minimum dosage having the appropriate response in at least 80% of the test group, while Authie (1984) reported $ED_{75}$ values. The proportions selected seem to be less due to a consideration of statistical significance, but for convenience with regard to group size: Hawking tended to use five animals per treatment group, so that 80% represents four out of five responses, while/

while Authie used four animals per group. A dose which will bring about temporary clearance of trypanosomes from the blood is of no real practical significance, but is more readily determined than a curative dose.

The results of a mouse sensitivity test are only of value if they can be related in a predictable manner to the results achieved in cattle. There are obvious differences between the two species: a 10,000 fold difference in bodyweight and different routes of drug administration, usually intraperitoneal in the former, intramuscular or subcutaneous in the latter. The pharmacokinetics and metabolism of the drug may vary between the two species and host-parasite relationships may also differ.

Surprisingly little effort has gone into evaluating the mouse sensitivity test. Hawking (1963a) published comparative cattle and mouse sensitivity data for a number of strains of T. congolense and a range of trypanocides, although his work predated the availability of Isometamidium. The experimental design of his experiments can be criticized on the grounds that whereas the cattle work was carried out by Whiteside in Kenya, the mouse work was performed in London. Between the two sets of sensitivity tests the strains of T. congolense used were subjected to numerous bovine and murine passages. Hawking cautiously concluded that mice results will give a 'broad indication of the probably response of a strain in cattle'.

Several workers have adopted different approaches to assessing drug sensitivity in vivo, or have modified the basic drug/

drug sensitivity test. Jones-Davies and Folkers (1966) transported trypanosome-naive calves by lorry to treatment camps in Nigeria, and subinoculated them with blood collected from cattle presented for treatment. Large numbers of subinoculations were carried out simultaneously - up to 80 in one calf - although the site of the subcutaneous inoculations were varied over the body. Calves were then transported back to the laboratory and 24 hours later treated with 1 mg/kg homidium chloride (Novidium). Treated animals were then monitored for relapse infections, during which time they were deliberately maintained on a low plane of nutrition. The appearance of relapse infections in a calf was taken to be evidence of the existence of homidium resistant strains in one or more of the cattle from which blood had been subinoculated. They had previously shown that experimental cattle could be treated during the prepatent period, ie the period between infection and first detection of trypanosomes in the blood, and that it was not necessary to wait until parasitaemia had been established.

Williamson and Stephen (1960) had observed that in rodents the development of drug resistant strains was manifest by a decrease in the time taken for a relapse infection to develop following subcurative treatment. They went on to develop a test for homidium sensitivity of strains of T. vivax based on this observation. The method involved feeding laboratory-reared tsetse on cattle naturally infected with the trypanosome strain under investigation, transferring the infected flies back to the laboratory and allowing them/

them to feed on a trypanosome-naive sheep. When the sheep became parasitaemic, clean tsetse flies were allowed to feed, and were then used to infect a new batch of sheep. When trypanosomes had been observed on four consecutive mornings, treatment was carried out with a low (0.1 mg/kg) dose of homidium. The number of days from treatment to detection of a relapse infection was taken as an index of the homidium sensitivity of the strain under investigation: the longer the aparasitaemic intervals, the more sensitive the strain. Sheep were used as the host rather than cattle for reasons of economy.

Dukes,(1985) developed a mouse sensitivity test for T.b. brucei designed to overcome the complications of, firstly, sequestration by trypanosomes in drug inaccessible sites and, secondly, host immune response. In his test, sub-lethally irradiated mice were infected by the intravenous route with approximately $1 \times 10^5$ trypanosomes. Three hours later they were treated with one of a graded series of drug levels. Parasitaemias were monitored quantitatively by the rapid matching/counting method of Herbert and Lumsden (1976). $\text{Log}_{10}$ parasitaemias ($\log_{10}$ trypanosomes per ml) were then plotted against time, and the number of trypanosomes which notionally survived treatment and contributed to the relapse infection could be assessed. Thus, for a range of drug treated groups and an untreated control group a series of parallel lines would be obtained. The $\log_{10}$ of the percentage of trypanosomes present at t = 0 for each treatment group, related to the untreated control could then be calculated ($\log_{10}$ S) and be plotted against

$\log_{10}$/

$\log_{10}$ drug dose ($\log_{10}$D). For each strain tested a straight line was obtained, and the intercept with $\log_{10}S = 0.1$, ie the dose required to achieve 99.9% suppression of trypanosome numbers at t = 0, could be derived. This value, designated the $DS_{0.1}$, was the basis by which the relative sensitivities of different T.b. brucei strains were compared.

Frommel and Barber (1987) prepared drug resistant clones of T.b. rhodesiense and T.b. brucei from sensitive parent clones by passaging trypanosomes through irradiated mice treated with increasing concentrations of drug. They redefined MED as the minimum amount of drug required to cure mice infected with the sensitive clone, and introduced the term maximum ineffective dose (MID), which they defined as as the maximum amount of drug which had no effect on the population doubling time of the resistant clone during the 24 hour period following administration of drug in irradiated mice infected 36 hours previously. They calculated the resistance value (RV) as the ratio of MID to MED. RV values increase with relative resistance. The method is limited in application to studies involving laboratory derived resistant stocks. There is some risk of confusion since MED is conventionally considered to be the minimum dose which bring about temporary clearance of circulating trypanosomes with subsequent relapse.

Currently, assay methods sensitive enough to detect circulating concentrations of Isometamidium are not available. In the case of malaria, drug resistance is not considered proven until/

until plasma drug concentrations have been determined. When a reliable, sensitive assay procedure for Isometamidium becomes available, a simple definition of, and test for drug resistance, could be the demonstration of trypanosomes in a blood sample in the presence of a defined serum or plasma Isometamidium concentration.

## Isometamidium Sensitivities of Two Strains of T. congolense

### Introduction

The Isometamidium sensitivites of two strains of T. congolense, namely 82/1 and 12/1, were assessed using two different techniques in mice:

- ip infection and treatment at first parasitaemic peak,
- iv infection and treatment 3 hours post-infection.

The first is a standard method of carrying out sensitivity tests in mice similar to that adopted by Hawking (1963a), and the second is adapted from Dukes (1985). The latter method was specifically developed for T.b. brucei and was intended to overcome the complications of parasites penetrating drug-inaccessible sites and the effects of the host's immune response. The results obtained by the two methods were compared.

### Methods

Method 1: A batch of mice were infected ip with 0.2 ml of trypanosome suspension containing approximately $5 \times 10^5$ trypanosomes per ml, and the resulting parasitaemias monitored until the number of parasites was estimated to be $> \log_{10} 7.0$ per ml blood, using the rapid matching method (Herbert and Lumsden, 1976). Mice were then/

allocated to treatment groups, four per group, and treated ip with one of a range of Isometamidium dosages, or left untreated as infection controls.

Method 2: Groups of mice were anaesthetized to facilitate infection by iv inoculation into the tail vein. Approximately 0.2 ml of trypanosome suspension containing $5 \times 10^5$ trypanosomes per ml in PBS was inoculated, giving an inoculum of about $1 \times 10^5$ trypanosomes per mouse. Three hours post-infection, groups of five mice were treated with one of a range of Isometamidium dosages administered by ip injection, or were left untreated as infection controls.

In both cases parasitaemias were monitored by examination of tailblood wet-films, initially three times a week, decreasing to twice a week from day 21 post-treatment until termination of the experiment at day 100.

Results

T. congolense 12/1: For mice infected by ip inoculation with T. congolense 12/1, treatment with Isometamidium at 0.001 mg/kg at the first parasitaemic peak had no effect. No mice were cured and all mice remained continuously parasitaemic.

All four mice treated at 0.01 mg/kg experienced temporary remission of infection, with relapse infections first being detected between days 9 and 11 post-treatment. The MED was therefore 0.01 mg/kg.

Treatment at 0.1 mg/kg (the MCD) resulted in cures in all four mice.

For mice infected by iv inoculation with T. congolense 12/1, treatment 3 hours later with 0.001 mg/kg Isometamidium had no effect. No mice were cured at this dosage and the prepatent period was no different to the untreated, infection control mice.

Treatment at 0.01 mg/kg resulted in two of 10 mice being cured, and extended prepatent periods in the remaining eight mice.

Four out of five mice were cured at 0.025 mg/kg (the $CD_{80}$ value) and also at 0.05 and 0.075 mg/kg.

All five mice treated at 0.1 mg/kg (the MCD) and higher dosages were cured, ie never developed parasitaemia.

T. congolense 82/1: For mice infected by ip inoculation with T. congolense 82/1 and treated at the first parasitaemic peak, dosages up to 0.1 mg/kg had no effect.

Four out of five mice treated at 1 mg/kg ($ED_{80}$) experienced temporary remission of infection, and one of the five mice was cured.

All mice treated at 2 mg/kg (MCD) and above were cured.

For mice infected by iv inoculation with T. congolense 82/1 and treated 3 hours later with Isometamidium, treatment at dosages up to 0.1 mg/kg had no effect. Prepatent periods were similar to untreated, infection control mice.

Treatment at 0.25 mg/kg resulted in slightly extended prepatent periods: mice treated at this dosage were parasitaemic by day 8 compared to day 3 - 5 for infection control mice.

Treatment at 0.5 mg/kg resulted in a further extension in the prepatent period and two out of five mice were cured.

Table 4. Comparison of Isometamidium sensitivity data for T.congolense 12/1 and 82/1, using two techniques (mg/kg).

| T.congolense strain: | ED80 +3hr | ED80 1st peak | MED +3hr | MED 1st peak | CD80 +3 hr | CD80 1st peak | MCD +3hr | MCD 1st peak |
|---|---|---|---|---|---|---|---|---|
| 12/1 | - | - | - | 0.01 | 0.025 | - | 0.1 | 0.1 |
| 82/1 | - | 1.0 | - | [illegible] | 1.0 | - | 2.0 | 2.0 |

Three out of five mice treated at 0.75 mg/kg were cured, nine out of ten mice treated at 1.0 mg/kg ($CD_{80}$) were cured and all mice treated at 2.0 mg/kg (MCD) and above were cured.

Irrespective of the method used the MCD values determined for the strains were the same. T. congolense 12/1 was 20 times more sensitive than T. congolense 82/1 based on a comparison of MCD's. The results of the sensitivity test are shown in Table 4 .

Discussion

The two methods used to assess Isometamidium sensitivities have produced the same values for both the strains used. The method of Duke, whereby treatment is administered 3 hours after iv infection, was developed for use with T.b. brucei and was intended to minimise the influence of trypanosome penetration of drug inaccessible sites. The existence of cryptic foci have been demonstrated for T.b. brucei infections in mice, where the brain has been shown to be the source of the relapse infection (Jennings et al., 1979). T. congolense is however generally regarded as strictly vacular (Losos et al., 1973) and the fact that the two methods both produced the same MCD values suggests that drug inaccessible sites were not a factor in this experiment.

Treatment at the first parasitaemic peak has a number of practical advantages over treatment at 3 hours. Infection by the ip route is considerably easier in mice than the iv inoculation required by the latter method. The presence of infection can also be demonstrated prior to treatment: with treatment at 3 hours post-infection there is always the possibility that the mice did not receive an infective inoculum.

## Investigation of the Effect of the Number of Trypanosomes in the Initial Inoculum on the Assessment of Isometamidium Sensitivity of a Strain of T. congolense

### Introduction

Walker and Opiyo (1973) investigated the effect of the number of trypanosomes in the initial infective inoculum, on the curative dose of experimental T.b. brucei and T.b. rhodesiense infections in mice. Their method was to treat mice 2 days after infection with $10^6$, $10^5$, $10^4$ or $10^3$ trypanosomes. They reported that for a range of trypanocidal drugs, including Isometamidium, the MCD was markedly affected by the number of trypanosomes inoculated. It was therefore decided to investigate the effect of the number of parasites inoculated on drug sensitivity parameters of a strain of T. congolense, using the iv inoculation and treatment at 3 hours post-infection method.

### Methods

A stock suspension of T. congolense 12/1 was prepared from cryopreserved stabilate and the trypanosome density estimated by duplicate counts using a haemocytometer. Serial dilutions were then prepared containing $5 \times 10^6$, $5 \times 10^5$, $5 \times 10^4$ and $5 \times 10^3$ trypanosomes per ml PBS. Groups of five mice were anaesthetized and then infected by iv inoculation into the tail vein with 0.2 ml of inoculum. Three hours post-infection each group was treated with one of a range of Isometamidium dosages by ip injection, or left untreated as infection controls. Parasitaemias were monitored by examination of tail-blood wet-films, initially three times a week. Observation was continued for 100 days.

Table 5. Isometamidium sensitivity tests using a range of inital inocula.

| Isometamidium mg/kg | Initial inoclum (trypanasomes per mouse) | | | |
|---|---|---|---|---|
| | $1 \times 10^3$ | $1 \times 10^4$ | $1 \times 10^5$ | $1 \times 10^6$ |
| 0.01 | 4/5 * | 2/5 | 0/5 | 0/5 |
| 0.025 | 5/5 | 4/5 | 4/5 | 0/5 |
| 0.050 | 5/5 | 5/5 | 4/5 | 1/5 |
| 0.075 | 5/5 | 5/5 | 4/5 | 5/5 |
| 0.1 | 5/5 | 5/5 | 5/5 | 4/5 |
| 0.5 | 5/5 | 5/5 | 5/5 | 5/5 |

* number of mice cured/number of mice infected

Results

The prepatent period of untreated mice was found to be dependent on the size of the initial inoculum. With an initial inoculation of $1 \times 10^3$ trypanosomes, one of five mice did not become parasitaemic, while in the remaining four mice parasites were first detected in a mean of $9.8 \pm 1.5$ days post-infection. When the inoculum was increased to $1 \times 10^4$ parasites this period decreased to a mean of $8.0 \pm 2.2$ days, for $1 \times 10^5$ parasites it was $6.6 \pm 0.9$ days and for $1 \times 10^6$, $1.2 \pm 0.4$ days.

Three out of five infection control mice inoculated with $1 \times 10^6$ trypanosomes died during the initial 35 day post-infection. One mouse infected with $1 \times 10^5$ died, and there were no mortalities in the $1 \times 10^4$ or $1 \times 10^3$ trypanosomes per mouse groups.

The number of mice cured in each experimental group is shown in Table 5 . With an initial inoculum of $1 \times 10^3$ trypanosomes, four out of five mice were cured at a dosage of 0.01 mg/kg Isometamidium, and all mice were cured at dosages of 0.025 mg/kg or above.

With an initial inoculum of $1 \times 10^4$ trypanosomes two out of five mice were cured at 0.01 mg/kg, four out of five were cured at 0.025 mg/kg, and all mice were cured at dosages of 0.05 mg/kg and above.

A dose of 0.01 mg/kg failed to cure any mice infected with $1 \times 10^5$ trypanosomes. Four out of five mice were cured at dosages of 0.025, 0.05 or 0.075 mg/kg. Dosages of 0.1 mg/kg or above cured all mice.

With an initial inoculum of $1 \times 10^6$ trypanosomes, dosages up to 0.025 mg/kg were ineffective. Only one out of five mice

was/

was cured at 0.05 mg/kg. All five mice were cured at 0.075 and 0.5 mg/kg, although only four out of five were cured at 0.1 mg/kg.

Discussion

In the present experiment the MCD was shown to be dependent on the size of the initial inoculum. The MCD determined for the strain of T. congolense used varied from 0.025 mg/kg Isometamidium following an initial inoculum of $1 \times 10^3$ trypanosomes, to 0.1 mg/kg after an initial inoculum of $1 \times 10^5$ trypanosomes. Although all mice infected with $1 \times 10^6$ trypanosomes were cured after treatment at 0.075 mg/kg or 0.5 mg/kg, only four out of five were cured at 0.1 mg/kg. Thus, there was a range of at least a factor of four in the value of MCD's determined for the different size inocula.

This result is similar to the observations of Walker and Opiyo (1973) who worked with T.b. brucei and T.b. rhodesiense.

The reasons for the above findings are unclear. Davey (1958), who also reported similar findings in T.b. rhodesiense infections in rodents, ie that the curative dose was dependent upon the number of trypanosomes present at the time of treatment, considered the possibility that the drug was absorbed by the trypanosomes. Gilbert, Curtis and Newton (1979) used $^{14}C$-labelled homidium bromide in calves infected with T. congolense, and reported that one hour after intramuscular injection, when the amount of radio-activity in the blood reached a peak, 80% of that radioactivity

was/

was bound to trypanosomes. However, the tissue and blood levels, and excretion of radioactivity followed a similar course in infected and uninfected calves, suggesting that trypanosomes did not metabolise homidium.

It is also of interest that Whiteside (1962) was puzzled by his observation that the period of protection afforded by a prophylactic drug appeared to be dependent upon the degree of trypanosome challenge, and in particular queried how the number of trypanosomes entering the host could affect the duration of prophylaxis. More recently, Whitelaw et al., (1986) found no differences in the duration of prophylaxis of cattle treated with Isometamidium, and challenged either singly or repeatedly with trypanosome infected tsetse flies, or titrated doses of in vitro derived metacyclic T. congolense. The finding that the level of metacyclic challenge did not influence the duration of prophylaxis, was later confirmed by Peregrine, Ogunyemi, Whitelaw, Holmes, Moloo, Hirumi, Urquhart and Murray (in press).

The present experiment suggests that trypanosomes take up and metabolise Isometamidium, and thereby reduce effective plasma levels. It is difficult however to reconcile the marked differences in the efficacy of Isometamidium dosages with the range of inocula used to infect the mice. The difference in the biomass of the various levels of inocula used, would appear to be insignificant in comparison to the biomass of the mouse. This suggests a highly selective uptake of Isometamidium by the trypanosomes, as has previously been suggested by Gilbert et al., (1979) working with radiolabelled homidium (vide supra).

There are two practical implications of these findings. First, to permit comparisons to be made between drug sensitivity tests in which treatment is administered soon after infection, it is clearly of considerable importance that similar numbers of trypanosomes are inoculated into mice, and probably other species, used in such tests. Secondly, that in field situations animals infected with higher levels of parasitaemia may require higher doses of drug than animals with lower parasitaemias.

## Assessment of Sensitivity of *Trypanosoma congolense* to Isometamidium Chloride: A Comparison of Tests Using Cattle and Mice

### Introduction

Although mice are often routinely used to assess drug sensitivities of strains of trypanosomes, including *T. congolense*, there have been few attempts to correlate the results of such tests, to those obtained with the use species. Hawking's (1963a) work in this area, using strains of *T. congolense* in mice and cattle was flawed by the numerous bovine and murine passages the strains went through between being tested in cattle in Kenya and mice in London. It was therefore decided to carry out simultaneous mouse and cattle Isometamidium sensitivity tests using a number of strains of *T. congolense* of differing Isometamidium sensitivities.

### Materials and Methods

#### Experimental animals

Trypanosome-naive, Boran steers, and female random-bred Swiss mice were used for the drug sensitivity experiments. Female outbred mice, obtained from the KETRI closed colony, were used for routine subinoculation of cattle blood.

#### Trypanosomes

The three strains of trypanosomes used in this experiment were obtained as cryopreserved stabilates. Prior to use the strains were expanded in sub-lethally irradiated rats, and then stored as stabilates in liquid nitrogen until required. The strains were: *T. congolense* KETRI 2880, 2883 and 2885.

Methods

Cattle and mice were each infected with approximately $1 \times 10^5$ trypanosomes, the former by intravenous inoculation and the latter by intraperitoneal inoculation (ip). After infection cattle parasitaemias were monitored three times a week by the buffy coat method (Paris, Murray and Mcodimba 1982), but with direct rather than phase-contrast illumination, and using a peripheral blood sample collected from a marginal ear-vein. Packed cell volume (PCV), determined from a jugular blood sample, and rectal temperature were also recorded three times a week, and bodyweight recorded once a week. Mice were monitored by examination of tail-blood wet-films. In addition, at 2 weekly intervals, jugular blood samples were collected from all cattle judged trypanosome-negative by the buffy coat method and 0.5 ml aliquots were inoculated into mice (three per steer). After subinoculation mice were monitored by tail-blood wet-film examination for 8 weeks. No infections were detected by subinoculation of blood that were not also detected by the buffy coat technique, although in some cases infections were detected earlier by subinoculation.

For both cattle and mice, treatment with Isometamidium was administered at first detection of trypanosomes at the dose rates shown in Table 6. Initially 10 cattle were infected with each strain; three cattle were subsequently treated by deep intramuscular injection in the middle third of the neck, at each of 0.1, 0.5 or 1.0 mg/kg bodyweight, while one animal was left untreated as an infection control. In the case of KETRI 2885 for which 0.1 mg/kg Isometamidium proved to be curative, two further cattle were/

Table 6. Allocation of experimental animals to treatment groups (number of animals per group)

| | Cattle | | | Mice | | |
|---|---|---|---|---|---|---|
| | T.congolense KETRI; | | | T.congolense KETRI; | | |
| Isometamidium mg/kg | 2885 | 2883 | 2880 | 2885 | 2883 | 2880 |
| 0 | 1 | 1 | 1 | 5 | 5 | 5 |
| 0.001 | 1 | | | 5 | | |
| 0.01 | 1 | | | 5 | | |
| 0.1 | 3 | 3 | 3 | 5 | 5 | |
| 0.5 | 4 | 3 | 3 | 5 | 5 | |
| 1.0 | 3 | 3 | 3 | 5 | 5 | 5 |
| 2.0 | | | 2 | 5 | 5 | 5 |
| 5.0 | | | | 5 | 5 | 5 |
| 10.0 | | | | 5 | 5 | 5 |
| 20.0 | | | | 5 | 5 | 5 |

were infected and treated at either 0.01 or 0.001 mg/kg, and an additional steer was also infected with KETRI 2880 and treated at 2 mg/kg.

When the PCV of the infection control steers declined to 17%, they were withdrawn from the experiment and treated with diminazene aceturate (Berenil, Hoechst, W. Germany) initially at 3.5 mg/kg, increasing the dosage in increments of 3.5 mg/kg when relapse infections were encountered.

For all cattle in which relapse infections occurred after initial treatment with Isometamidium, parasitaemias were monitored for 7 days, and then the cattle were retreated with the same Isometamidium dose. When a second relapse infection occurred, cattle were again monitored for 7 days and then treated with diminazene aceturate. An exception was the steer infected with KETRI 2880 and treated at 1 mg/kg Isometamidium, which relapsed twice to this dose (Steer 895). This animal was then treated at 2 mg/kg Isometamidium.

Mice were treated at one of a range of Isometamidium doses up to 20 mg/kg by ip injection, with five mice in each treatment group. The parameters $ED_{80}$ and $CD_{80}$ were determined for mice, that is the minimum dose which brought about either temporary clearance of circulating trypanosomes ($ED_{80}$), or a permanent cure ($CD_{80}$), in at least 80 per cent of mice, i.e. four out of five. In the case of KETRI 2880 the assessment of $ED_{80}$ was complicated by characteristic remission of infection, even in untreated mice, following the first parasitaemic peak. The $ED_{80}$ value for KETRI 2880 was therefore taken to be the minimum dose which resulted in/

in temporary clearance of circulating trypanosomes in four or more mice, of at least twice the mean duration observed in the infection controls.

Cattle and mice were monitored for at least a 100 day post-treatment observation period.

Due to limited cattle accommodation, the experiment was carried out in two phases. Initially cattle and mice were infected with T. congolense KETRI 2885. Later new batches of cattle were infected with either T. congolense KETRI 2880 or KETRI 2883. One steer infected with T. congolense KETRI 2885 and treated at 0.5 mg/kg Isometamidium was included in the second batch as a control, so that the relative sensitivities of the three strains could be compared in the same conditions, at the same time.

Results

Prepatent period

For KETRI 2885 the mean prepatent periods (± SD), following needle challenge, were 7 (± 0) days in cattle and 5 (± 0) days in mice; for KETRI 2883, 7.1 (± 0.3) and 5.9 (± 0.5) days and for KETRI 2880, 10.6 (± 0.7) and 9.5 (± 1.4) days, respectively.

Virulence and pathogenicity

Sixty per cent (3/5) of mice infected with either KETRI 2885 or 2880 which received no treatment, died during a 100 day observation period. All mice (5/5) infected with KETRI 2883 and left untreated died, with a mean number of days to death of 28.6 (± 22.6). Deaths of mice which were infected with KETRI 2885

or/

Table 7. Responses of T.congolense-infected cattle to treatment with Isometamidium

| Strain | Dose mg/kg | Number in treatment group | Effect of treatment (number of cattle) | | |
|---|---|---|---|---|---|
| | | | No effect[a] | Temporary clearance[b] | Cure[c] |
| 2885 | 1.0 | 3 | 0 | 0 | 3 |
| | 0.5 | 4 | 0 | 0 | 4 |
| | 0.1 | 3 | 0 | 0 | 3 |
| | 0.01 | 1 | 0 | 0 | 1 |
| | 0.001 | 1 | 0 | 0 | 1 |
| | 0 | 1 | 1 | 0 | 0 |
| 2883 | 1.0 | 3 | 0 | 0 | 3 |
| | 0.5 | 3 | 0 | 0 | 3 |
| | 0.1 | 3 | 1 | 2 | 0 |
| | 0 | 1 | 1 | 0 | 0 |
| 2880 | 2.0 | 2 | 0 | 0 | 2 |
| | 1.0 | 3 | 0 | 3 | 0 |
| | 0.5 | 3 | 0 | 3 | 0 |
| | 0.1 | 3 | 1 | 2 | 0 |
| | 0 | 1 | 1 | 0 | 0 |

a Animals remained parasitaemic after treatment.

b Parasitaemia dropped below limit of detection following treatment, but trypanosomes reappeared during observation period.

c Trypanosomes disappeared from the circulation following treatment and did not reappear during a 100 day observation period.

or 2880 tended to be after a more chronic infection with mean numbers of days to death of 72.7 (± 19.6) and 84.3 (± 6.0), respectively.

Infection control cattle were prevented from dying by intervention with diminazene aceturate when their PCV had dropped to 17%. PCV values at infection were similar for all three cattle (33 - 34%). In the case of the KETRI 2880 infection control the PCV had declined to 17%, 33 days after infection. For the KETRI 2883 infection control this point had been reached 34 days post-infection and for the KETRI 2885 infection control, 38 days post-infection.

Responses to treatment with Isometamidium

Cattle: The responses of cattle infected with the three strains of T. congolense and treated with Isometamidium, are shown in Table 7. Cattle infected with KETRI 2885 were cured by treatment at 0.001 mg/kg Isometamidium, the lowest dose used.

KETRI 2883 was temporarily cleared from the circulation of two out of three cattle treated at 0.1 mg/kg. All cattle treated at 0.5 or 1.0 mg/kg were permanently cured.

No cattle infected with KETRI 2880 and treated with a single injection of Isometamidium at doses up to 1 mg/kg were cured, although two out of three steers treated at 0.1 mg/kg and all three treated at 0.5 or 1.0 mg/kg experienced temporary remission of infection.

Cattle which either relapsed after initial treatment with Isometamidium, or for which initial treatment had no effect, were retreated at the same dose (Table 8 ).

Table 8. Aparasitaemic intervals in cattle following treatment with Isometamidium.

| Steer number | Dose mg/kg | Days to relapse: First treatment | mean | Days to relapse: Repeat treatment | mean |
|---|---|---|---|---|---|
| KETRI 2883 | | | | | |
| 899 | 0.1 | 0 | | 7 | |
| 902 | 0.1 | 14 | 8.3 | 9 | 9.3 |
| 893 | 0.1 | 11 | | 12 | |
| | ≥0.5 | >100 | | | |
| KETRI 2880 | | | | | |
| 803 | 0.1 | 0 | | 0 | |
| 884 | 0.1 | 3 | 2.3 | 0 | 0 |
| 881 | 0.1 | 4 | | 0 | |
| 877 | 0.5 | 21 | | 16 | |
| 818 | 0.5 | 13 | 15.7 | 14 | 15.7 |
| 801 | 0.5 | 13 | | 17 | |
| 819 | 1.0 | 37 | | >100 | |
| 895 | 1.0 | 19 | 44.3 | 72 | |
| 870 | 1.0 | 77 | | >100 | |
| 895 | 2.0 | >100 | | | |
| 805 | 2.0 | >100 | | | |

Two out of three cattle infected with KETRI 2883 and treated at 0.1 mg/kg experienced a temporary clearance of circulating trypanosomes, and all three were temporarily cleared by repeat treatment at the same dose.

Although KETRI 2880 was temporarily cleared from the circulation of two out of three cattle treated at 0.1 mg/kg, repeat treatment at the same dose had no effect. Repeat treatment at the same dose for cattle initially treated at 0.5 mg/kg again resulted in temporary aparasitaemia. Of the three cattle originally treated at 1.0 mg/kg, one steer relapsed to repeat treatment at the same dose while the remaining two steers did not relapse during a 100 day post-retreatment observation period. The steer which relapsed twice after treatment at 1.0 mg/kg was then treated at 2 mg/kg, and did not relapse during the following 100 days. An additional steer infected with KETRI 2880 and treated at 2 mg/kg was also cured, corroborating this observation.

These results indicated that the MCD for KETRI 2885 was less than or equal to 0.001 mg/kg Isometamidium, for KETRI 2883 was 0.5 mg/kg and for KETRI 2880 was 2 mg/kg.

The intervals from treatment to relapse for KETRI 2880 and 2883 in cattle are shown in Table 8. Cattle infected with KETRI 2883 and treated with Isometamidium at a dose of 0.1 mg/kg relapsed in a mean of 8.3 days, and after retreatment at the same dose in a mean of 9.3 days.

For KETRI 2880 the mean number of days to relapse following treatment at 0.1 mg/kg was 2.3 days. Retreatment at the same dose/

Table 9. Responses of T. congolense-infected mice to treatment with Isometamidium (5 mice per treatment group).

| Strain | Dose mg/kg | Effect of treatment (number of mice) | | |
|---|---|---|---|---|
| | | No effect[a] | Temporary clearance[b] | Cure[c] |
| 2885 | > 0.1 | 0 | 0 | 5 |
| | 0.1 | 0 | 0 | 5 |
| | 0.01 | 0 | 4 | 1 |
| | 0.001 | 5 | 0 | 0 |
| | 0 | 5 | 0 | 0 |
| 2883 | 20.0 | 1 | 0 | 4 |
| | 10.0 | 0 | 1 | 4 |
| | 5.0 | 1 | 1 | 3 |
| | 2.0 | 0 | 5 | 0 |
| | 1.0 | 3 | 2 | 0 |
| | 0.5 | 4 | 1 | 0 |
| | 0.1 | 5 | 0 | 0 |
| | 0 | 5 | 0 | 0 |
| 2880 | 20.0 | 0 | 1 | 4 |
| | 10.0 | 0 | 4 | 1 |
| | 5.0 | 0 | 5 | 0 |
| | 2.0 | 0 | 5 | 0 |
| | 1.0 | 3 | 2 | 0 |
| | 0 | 0 | 4 | 1 |

a Animals remained parasitaemic after treatment.

b Parasitaemia dropped below limit of detection following treatment, but trypanosomes reappeared during observation period.

c Trypanosomes disappeared from the circulation following treatment and did not reappear during a 100 day observation period.

Table 10. Comparison of mouse $ED_{80}$ and $CD_{80}$ with cattle MCD values (mg/kg)

| Strain | $ED_{80}$ mouse A | $CD_{80}$ mouse B | MCD cattle C | Ratio A:C | Ratio B:C |
|---|---|---|---|---|---|
| 2885 | 0.01 | 0.1 | $\leqslant$ 0.001 | $\geqslant$10 | $\geqslant$100 |
| 2880 | 2 | 20 | 2 | 1 | 10 |
| 2883 | 2 | 10 | 0.5 | 4 | 20 |

dose had no effect. Mean number of days to relapse after both treatment and retreatment at 0.5 mg/kg was 15.7 days. Cattle treated at 1.0 mg/kg relapsed after a widely varying timespan (19 - 77 days post-treatment) with a mean of 44.3 days. Only one steer retreated at 1 mg/kg relapsed (day 72), and this animal remained aparasitaemic during a 100 day observation period following treatment at 2 mg/kg.

Mice: The responses of mice infected with one of the three strains of T. congolense and then treated with one of a range of Isometamidium dosages are shown in Table 9 . For KETRI 2885 the $ED_{80}$ was 0.01 mg/kg Isometamidium and the $CD_{80}$ value was 0.1 mg/kg. For KETRI 2883 and 2880 the corresponding values were 2 and 10 mg/kg and 2 and 20 mg/kg, respectively.

Comparison of mouse $ED_{80}$ or $CD_{80}$ and Cattle MCD values

Mice $ED_{80}$ and $CD_{80}$ values and cattle MCD values are compared in Table 10. The ratios in both cases show a wide range of variation ( > 10 fold) between the three different strains.

Responses to diminazene aceturate

Cattle which relapsed twice to their designated Isometamidium dosage, and also the untreated infection control animals when their PCV had dropped to 17%, were treated with diminazene aceturate. Treatment was initially attempted at 3.5 mg/kg, increasing the dosage in increments of 3.5 mg/kg if further relapse infections were encountered. Relapse infections were monitored for 7 days before treatment was attempted at a higher dose.

KETRI 2883: Four cattle infected with KETRI 2883 (three initially treated at 0.1 mg/kg Isometamidium, and the infection control), were treated with diminazene aceturate. All cattle relapsed to treatment at 3.5, 7.0 and 10.5 mg/kg diminazene aceturate,/

aceturate, at mean intervals from treatment of 7.5 (± 1.0), 14.3 (± 3.3) and 17.0 (± 6.0) days respectively. At the time the experiment was terminated - up to 35 days after treatment - no cattle had relapsed to treatment at 14.0 mg/kg.

KETRI 2880: Seven cattle infected with KETRI 2880 (three initially treated at 0.1 mg/kg Isometamidium; three initially treated at 0.5 mg/kg Isometamidium, and the infection control) were treated with diminazene aceturate. All cattle relapsed following treatment at 3.5 and 7.0 mg/kg diminazene aceturate, at mean intervals from treatment of 13.3 (± 4.5) and 28.1 (± 11.9) days respectively. At the time the experiment was terminated, no cattle had relapsed to 10.5 mg/kg: for five of the seven cattle this was in excess of 100 days from date of treatment.

Discussion

The three strains of T. congolense used in this experiment covered a wide range of sensitivities to the prophylactic drug Isometamidium, with a difference of at least 2000 fold in the cattle curative doses between the least and most sensitive strains. Thus KETRI 2885 was extremely sensitive to Isometamidium with a dose of 0.001 mg/kg proving to be curative in cattle, while KETRI 2880, the least sensitive strains, could be cured by treatment at less than the maximum tolerated dose.

Hawking (1963a) cautiously concluded that 'tests in mice apparently give a broad indication of the probable response of a strain in cattle'. Pinder and Authie (1984) suggested that his results showed a close relationship between mouse effective dose and cattle curative dose, with mouse curative doses being at least 10 times those for cattle.

The results of the present experiment show that there is considerable variation between strains in the relationship between mouse effective dose and cattle results. Thus while for KETRI 2880 and 2883 the mouse $ED_{80}$ values were close to cattle MCD values, there was at least a 10 fold difference in the two values for KETRI 2885. Similarly while mouse $CD_{80}$ values for KETRI 2880 and 2883 were approximately 10 times cattle MCD values, in the case of KETRI 2885 there was a difference of at least 100 times between the values for the two species. Based on these comparisons it is concluded that although the result of a mouse test may give a broad indication of the sensitivity of a strain, it cannot be reliably used to predict curative doses for cattle.

Mouse sensitivity tests can also be criticized on the grounds that they cannot be employed to investigate the sensitivity of strains of T. vivax and that not all cattle isolates of T. congolense are rodent infective. The variable infectivity of strains of T. congolense to rodents may be related to the morphological type of trypanosomes present (Godfrey, 1961) - greater rodent infectivity being associated with the longer dimorphon-type rather than the shorter congolense-type - and also, as has been reported for T. vivax, to the stage of infection in the donor (Desowitz and Watson, 1951).

Besides the obvious difference in bodysize between cattle and mice, there may also be differences in the pharmacokinetics and/

Table 11. Comparison of curative dose and subcurative treatment to relapse interval for cattle (treated at 0.1 mg/kg Isometamidium) and mice (treated at 2 mg/kg Isometamidium).

| Strain | Cattle | | Mice | |
|---|---|---|---|---|
| | MCD | Aparasitaemic interval (0.1 mg/kg) | CD80 | Aparasitaemic interval (2mg/kg) |
| 2885 | $\leq$ 0.001 | >100 | 0.1 | >100 |
| 2883 | 0.5 | 8.3 | 10 | 8.8 |
| 2880 | 2.0 | 2.3 | 20 | 6.0 |

and metabolism of trypanocidal drugs in the two species. Furthermore, even using the same stabilate for infection, the trypanosome population which becomes established in cattle may differ from that in mice. One, or more, of these factors may explain the observed differences in Isometamidium dose rates required to cure infections in the two species. The lack of correlation between drug sensitivity values determined in the two species for different strains of T. congolense could also be related to differences in parasite biology. For example, the propensity of a strain to sequester in drug inaccessible sites in one or other of the host species, or the relative susceptibility of a strain to bovine or murine products of drug metabolism.

If mouse sensitivity tests cannot be relied upon to predict curative dose rates of Isometamidium for cattle, then other approaches need to be investigated. Williamson and Stephen (1960) developed a method using sheep, to assess the sensitivity of strains of T. vivax to homidium, based on the interval from subcurative treatment to subsequent relapse (see pp.73/4). The interval from subcurative treatment to relapse was taken as an index of the degree of homidium resistance: the shorter the interval, the more resistant the strain. A good indication of the relative sensitivities of the three strains of T. congolense used in the present study could be obtained by a similar approach, but using needle challenge in place of the tsetse challenge used by Williamson and Stephen (1960) (Table 11). For both cattle and mice, the subcurative treatment to relapse interval was shorter for the more resistant strain.

In cattle, both KETRI 2880 and 2883 were resistant to diminazene aceturate, the former at dose rates up to 7 mg/kg and the latter at dose rates up to 10.5 mg/kg. Until recently (Authie, 1984) there has been no evidence of cross-resistance between Isometamidium and diminazene, and the two drugs have been regarded as a 'sanative pair' (Whiteside, 1960). Finelle (1974) recommended the alternate use of Isometamidium and diminazene, and, prompted by fears of drug resistance, the management of Mkwaja Ranch in Tanzania adopted a regime based on the alternate use of Isometamidium and diminazene aceturate in 1980 (Trail et al., 1985). In the present experiment KETRI 2880 has been shown to be resistant to the manufacturer's recommended doses of both Isometamidium (0.5 - 1.0 mg/kg) and diminazene aceturate (3.5 - 7.0 mg/kg). The occurrence of strains of this type clearly has serious implications for the effective control of trypanosomiasis by trypanocidal drugs, and emphasises the need for reliable techniques for the assessment of sensitivity.

# CHAPTER FOUR

# AN *in vitro* APPROACH TO THE ASSESSMENT OF ISOMETAMIDIUM SENSITIVITIES

# INTRODUCTION

The use of *in vitro* techniques for Salivarian trypanosomes has, until recently, been limited by inadequate culture techniques. Although most mammalian and lower vertebrate trypanosomes can be easily grown *in vitro* using relatively simple biphasic media, the African trypanosome species proved to be more difficult (Baker, 1970). Originally *in vitro* culture techniques were developed as methods for diagnosing sub-patent infections. This approach could be used for the Trypanozoon sub-group, and less reliably for *T. congolense*. However, since most Trypanozoon species readily infect laboratory animals, the *in vitro* technique was most applicable to suspected *T.b. gambiense* infections. This species is non-infective to most laboratory animals, except young *Cerocopithecus* spp. monkeys (vervet), although Heisch, Killick-Kendrick, Dorrell and Marsden (1968), demonstrated that *T.b. gambiense* will also develop when injected into the testes of rabbits.

Early attempts to use *in vitro* cultured trypanosomes for biochemical or drug-related investigations, relied upon two basic approaches. Short duration incubations at 37°C of trypomastigote forms harvested from infected animal hosts, or longer duration experiments, usually conducted at 26°C. The usefulness of the latter was limited by the fact that, under the conditions used, bloodstream form (trypomastigote) trypanosomes transformed to forms morphologically and biochemically resembling insect mid-gut forms, which were not animal infective.

Short duration *in vitro* incubations of *T. congolense* or *T.b. brucei* isolates, in bovine serum containing 0, 1 or 2 μg/ml diminazene aceturate were carried out by Van Hoeve and Grainge (1966) to assess drug sensitivity. Trypanosomes were incubated at 37°C for 7 hours in serum, with or without diminazene aceturate, before being inoculated into mice for assessment of infectivity.

Yorke, Adams and Murgatroyd (1929) developed a medium which enabled them to maintain trypanosomes *in vitro* at 37°C for 24 hours. Using this medium they produced drug-resistant *T.b. rhodesiense* by a procedure of 60 minute *in vitro* exposures to gradually increasing drug concentrations, alternating with passaging in mice (Yorke, Murgatroyd and Hawking, 1931).

Attempts at longer term *in vitro* culture, generally at 26°C, resulted in transformation to procyclic forms. Hawking (1963) noted that these culture forms were much less sensitive to trypanocidal drugs than the same strains in laboratory animals. For example, for two strains of *T. congolense* the highest concentration of Isometamidium used *in vitro* (0.1 mg/ml) proved to be inactive, although in mice the strains were sensitive at a calculated blood concentration 500,000 times smaller. Hawking (1963) suggests that the insensitivity of these culture forms is due to the possession of alternative metabolic pathways. The culture forms can be maintained in more simple media than bloodstream forms because they may possess these additional metabolic pathways and, therefore, the specific enzymes blocked by a trypanocide can be bypassed.

Hirumi, Doyle and Hirumi (1977) investigated 96 combinations of culture conditions for T.b. brucei, the most successful of which proved to be RPMI 1640 medium, supplemented with 20% foetal bovine serum in the presence of a monolayer of fibroblast-like cells acting as a feeder layer, and maintained at 37°C. Under these conditions, for the first time, T.b. brucei isolated from infected rodents could be maintained and propogated in vitro, without transformation to insect mid-gut forms. In vitro derived trypanosomes retained the morphology, biochemistry, specific surface antigens and animal infectivity characteristic of bloodstream forms.

In vitro culture of T.b. brucei was further refined by the development of a cell-free system using a semi-defined media (Baltz, Giroud and Crockett, 1985). The presence of 2-mercaptoethanol was found to be essential for growth. Eliminating the need for a feeder layer considerably simplifies in vitro culture of T.b. brucei, and is of particular benefit in biochemical and drug-related studies.

Prior to 1984, in vitro culture of T. congolense was limited to non-infective procyclic and epimastigote forms, other forms which retained animal infectivity for limited periods only, and metacyclic-like forms (Hirumi and Hirumi, 1984). However, Hirumi and Hirumi (1984) described a system for the continuous cultivation of animal infective bloodstream forms of T. congolense. The system comprised a monolayer of bovine aorta endothelial (BAE) cells and Hepes-buffered RPMI 1640 medium supplemented with adult and foetal goat serum. Cultures were maintained at 37°C in a $CO_2$ gassed, humidified incubator. Trypanosomes propogated using this method were/

were morphologically indistinguishable from trypomastigotes from animal hosts, were covered with an electron-opaque surface coat and were animal infective.

Baltz et al., (1985) considered that trypanosomes pass through three stages in becoming established in vitro: an initiation phase, an adaptation phase and finally a maintenance phase. Of the 14 strains of T. equiperdum, T.b. evansi, T.b. brucei, T.b. rhodesiense and T.b. gambiense investigated, 10 were successfully established in culture, eight adapting to culture very rapidly. These workers recognised, however, that particularly for those strains which experienced a severe decrease in numbers of trypanosomes during the initiation phase, the population established was probably due to selection of variants that could adapt to the prevailing culture conditions, and may not be representative of the trypanosomes in the original inoculum.

Ross, Gray, Taylor and Luckins (1985) successfully initiated four cloned stocks of T. congolense, of West African origin, into culture. Only with one stock could bloodstream forms be introduced directly on to BAE cell monolayer. For the other three stocks, initiation was achieved by the use of bovine dermal explants. Only very small proportions of the trypanosomes in the inoculum adapted to growth in the conditions employed at 28$^{o}$C, again suggesting that a sub-population may have been established in vitro, not necessarily representative of the starting material. In contrast, Hirumi and Hirumi (1984) established a strain of T. congolense in culture, with 25% of the inoculum adapting to the conditions used and, therefore, possibly representing a less drastic selection procedure.

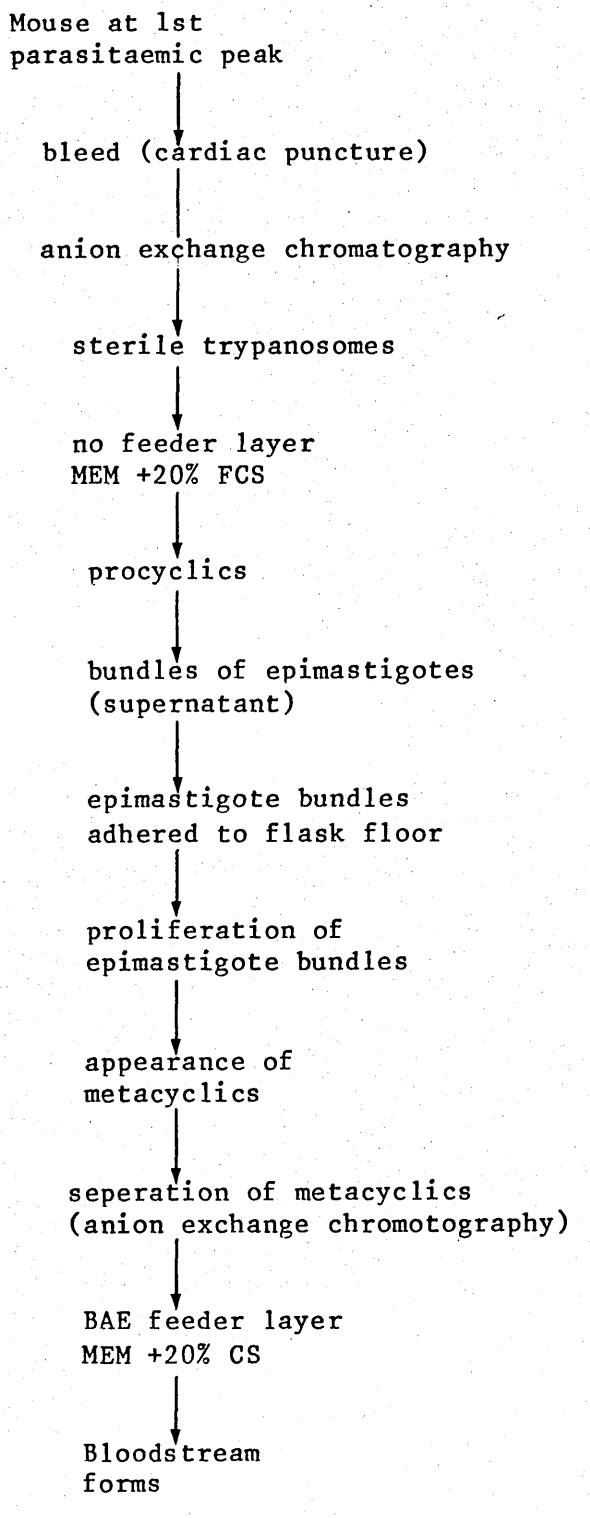
Borowy, Fink and Hirumi (1985) used an *in vitro* system incorporating a bovine fibroblast feeder layer to assess the effect of a number of trypanocides on a strain of *T.b. brucei*. Concentrations identified as effective were within the range of drug concentrations estimated to be in body fluids of animals receiving curative doses. This was in contrast to earlier workers, for example Hawking (1963) who found that drug concentrations much greater than used *in vivo* were required *in vitro*. This difference may be because Hawking was using culture form (procyclic) trypanosomes, while Borowy and co-workers were using trypomastigotes, or it may relate to the fact that in Borowy's assay actively multiplying populations were used and the trypanocides used may specifically act on these organisms.

*Adaptation to in vitro Culture*

Prior to carrying out *in vitro* sensitivity tests, it was necessary to adapt the three strains of *T. congolense* previously used in the cattle/mouse Isometamidium sensitivity experiment, to *in vitro* culture.

*T. congolense* KETRI 2885 were harvested from a mouse near to the first parasitaemic peak and introduced directly on to a BAE feeder layer. Using Eagles' minimum essential medium (MEM) with Hepes buffer (25 mM) supplemented with 20% calf serum (CS), and maintained in a 5% $CO_2$ gassed incubator at 37°C, trypanosomes survived and actively replicated for at least 7 days. This was in accordance with observations made at the laboratories of ILRAD (Nairobi, Kenya) (personal communication, A. Peregrine). It was therefore/

Fig.2. Adaptation of T.congolense via insect mid-gut forms.



therefore decided to use bloodstream forms of T. congolense KETRI 2885 isolated from infected mice in subsequent in vitro drug experiments, thereby avoiding the need to produce an in vitro adapted population.

T. congolense KETRI 2880 and KETRI 2883 proved to be less amenable to in vitro culture than T. congolense KETRI 2885. When bloodstream forms of either of these strains were introduced directly on to BAE feeder layer, all trypanosomes eventually died. Successful adaptation to in vitro culture was eventually achieved by a process involving transformation through the insect mid-gut forms. The procedure used is depicted in Figure 2. Initially trypanosomes were harvested from a mouse with a high parasitaemia within a few days of infection, and separated from blood components by sterile minature anion exchange chromatography (Lanham and Godfrey, 1970). Approximately 1 - 2 x $10^7$ trypanosomes were then introduced into culture flasks without BAE feeder layer, using MEM supplemented with 20% foetal calf serum (FCS) and maintained at 28°C. These conditions induced the transformation of bloodstream forms to procyclic forms. Continued maintenance in these conditions, with changes of media every 2 - 3 days, resulted in the appearance of small bundles of epimastigote forms which adhered to the base of the flask. Proliferation of epimastigote bundles eventually led to the appearance of metacyclic forms, which were then separated from the epimastigote forms by miniature anion exchange chromatography. Metacyclic forms were then introduced on to BAE feeder layer with MEM supplemented with 20% FCS and maintained at 28°C.

This/

This resulted in the transformation of metacyclics to forms which resembled bloodstream forms and which were mouse infective. These *in vitro* derived bloodstream forms were then further passaged under similar conditions and were harvested as required for *in vitro* drug experiments. The adaptation procedure was particularly difficult for *T. congolense* KETRI 2883, and took 3 months to complete the sequence from the initial separation of trypanosomes from mouse blood to final transformation of metacyclics to *in vitro* adapted bloodstream forms.

## The Effect of Isometamidium on the Glucose Utilisation Rate of Bovine Aorta Endothelial Cell Feeder Layer

### Introduction

*In vitro* culture of bloodstream forms of *T. congolense* is currently dependent on systems incorporating a monolayer of BAE coating the base of the culture vessel. Although cell-free systems have been developed for the Trypanozoon sub-group, such systems have not yet been successfully developed for *T. congolense*. The monolayer of BAE cells, or feeder layer as it is often referred to, is believed to have a number of functions:

supply of essential nutrients not provided by the medium, and which the trypanosomes cannot synthesize themselves:

removal of toxic metabolites produced by trypanosomes, e.g. peroxides. (Until recently trypanosomes were thought to be deficient in enzymes involved in the removal of peroxide. Recent research however suggests that although glutathione peroxidase/

peroxidase and catalyse are absent, or present only in trace amounts, trypanothione peroxidase plays a role in the removal of peroxide, at least in T.b. brucei (Fairlamb and Henderson, 1987).):

provides a suitable surface for the trypanosomes to attach by their flagella.

Interpretation of in vitro experiments involving trypanocidal drugs is complicated by the presence of the BAE feeder layer. It is possible that any observed effect of a drug on trypanosomes is due to an indirect effect mediated via the feeder layer, rather than a direct effect on the trypanosomes themselves. If the concentration of drug used advserely affects the cells making up the feeder layer, this could impair its ability to support trypanosome growth. It was therefore decided to investigate the effect of a range of Isometamidium concentrations on the metabolism of the BAE feeder layer. The metabolic rate of feeder layer cells was estimated by measurement of the rate of disappearance of glucose over a 48 hour incubation period.

Methods

Wells of tissue culture plates (Nunc, Denmark) were seeded with BAE cells and were maintained at 37°C using MEM supplemented with 20% CS, until the feeder layer was confluent, usually in 4 - 5 days. The overlying media was then removed from each well and was replaced with 990 µl MEM supplemented with 20% CS and 10 µl of the appropriate Isometamidium solution, to provide a final Isometamidium concentration in the well of 100, 30, 10, 3, 1 or 0 µg/ml. Isometamidium solutions were prepared in sterile distilled water and filter sterilized. Tissue culture plates were then maintained at/

Table 12. Effect of Isometamidium on glucose concentration after 48 hours incubation.

| | Isometamidium concentration (μg/ml) | n | Glucose concentration after 48 hours (mmol/l) | Glucose disappearance rate (mmol/l/48hr) |
|---|---|---|---|---|
| i No feeder layer | 0 | 4 | 5.9(0.4)* | - |
| ii Feeder layer | 0 | 5 | 3.6(0.3) | 2.3 |
| iii | 1 | 4 | 3.3(0.2)a | 2.6 |
| iv | 3 | 5 | 3.3(0.4)a | 2.6 |
| v | 10 | 5 | 3.7(0.3)a | 2.2 |
| vi | 30 | 5 | 3.5(0.5)a | 2.4 |
| vii | 100 | 5 | 4.3(0.4)b | 1.6 |

* mean and (S.D.)

a Not signifantly different from group ii

b Significantly different from group ii ($p < 0.05$)

n number of wells per concentration

at 28°C for 48 hours in a 5% $CO_2$ gassed incubator, after which the media overlying the feeder layer was sampled and the glucose concentration estimated using a commercial glucose kit (Sigma Diagnostics, procedure 45) based upon the hexokinase method. Control wells consisting of 990 µl media with 10 µl of distilled water, but without feeder layer were also incubated for 48 hours prior to estimation of glucose concentrations.

Five replicate wells were set up for each Isometamidium concentration. Glucose disappearance was calculated from the difference in glucose concentrations of the media without feeder layer and the test wells with feeder layer.

Results

The mean glucose concentration measured after the 48 hour incubation period, and the mean glucose disappearance rate for each Isometamidium concentration is shown in Table 12. A Student t-test was carried out to compare the glucose concentration measured for each Isometamidium concentration with the untreated feeder layer wells. The glucose concentration of wells treated at 1, 3, 10 or 30 µg/ml Isometamidium was not significantly different from the untreated BAE wells. The glucose concentration in the wells treated at 100 µg/ml was significantly different ($p < 0.05$), indicating that 100 µg/ml Isometamidium had caused a significantly decreased rate of glucose utilisation.

Discussion

This experiment indicates that Isometamidium concentrations up to 30 µg/ml do not adversely affect BAE cell metabolism, as determined by glucose utilisation rates. This suggests that,

up/

up to this concentration, any observed effects of drugs on trypanosome survival or replication rates are due to direct effects on the trypanosomes, and are not mediated via an indirect effect on the feeder layer. However at a dose of 100 μg/ml Isometamidium the BAE feeder layer is adversely affected, and therefore the maximum concentration that should be used in this in vitro system is 30 μg/ml.

Borowy et al., (1985) noted that an Isometamidium concentration of 100 μg/ml also destroyed foetal bovine fibroblast feeder layers, although at 10 μg/ml feeder layer cells were unaffected.

## In vitro Isometamidium Sensitivity Testing of Strains of T. congolense

### Methods

The in vitro drug experiments were based on the methods developed by Borowy et al., (1985) for T.b. brucei. His method involved incubating trypanosomes in the presence of a foetal bovine fibroblast feeder layer, using 24 well tissue culture plates. Approximately 1 x $10^5$ in vitro derived trypanosomes were introduced on to confluent feeder layers. Each well contained 1 ml of medium and drug was added in a volume of 10 µl to maintain the osmolarity of the medium. The effect of the drug was assessed at intervals of up to 36 hours, by sampling the wells and making counts of trypanosomes using a haemocytometer. Waithaka, Borowy, Gettinby and Hirumi (1986) expressed the in vitro activity of trypanocidal drugs as the concentration required to inhibit growth by 50% compared to untreated controls ($IC_{50}$).

For T. congolense in the present study this method was adapted by using BAE cell feeder layer and MEM supplemented with 20% CS. Trypanosomes were maintained exposed to a range of Isometamidium concentrations for 48 hour periods. Several different modifications to the basic method were investigated.

Basic method: The central eight wells of a 24 well tissue culture plate were seeded with BAE cells, and were maintained in a 5% $CO_2$ gassed incubator at 37°C using MEM supplemented with 20% FCS. When a confluent feeder layer had been achieved, usually in 4 - 5 days, the overlying medium was removed and replaced with/

with 1 ml of MEM supplemented with 20% CS. Trypanosomes were harvested from a tissue culture flask with a heavy population of healthy motile trypanosomes on the feeder layer, by vigorous pipetting, and a sample of trypanosome suspension was counted using a haemocytometer. The volume of trypanosome suspension required to contain $5 \times 10^5$ trypanosomes could then be calculated. This volume, plus an additional 10 µl, was then removed from each well. A stock solution of Isometamidium (10 mg/ml) was prepared using sterile distilled water and filter sterilized using a 0.2 µm membrane filter (Flow Laboratories, W. Germany), and a range of Isometamidium concentrations were prepared by serial dilution using sterile distilled water, such that the final Isometamidium concentration required in each well could be achieved by the addition of 10 µl of solution. Approximately $1 \times 10^5$ trypanosomes were then added to each well, and the contents of each well mixed by gentle pipetting. The tissue culture plates were incubated at 28°C for 48 hours, in a 5% $CO_2$ gassed incubator.

This basic procedure was varied in some situations:

For T. congolense KETRI 2885 trypanosomes were harvested from parasitaemic mouse blood and not from in vitro culture. Trypanosomes were seperated from blood components by differential centrifugation. In this process a mouse with a parasitaemia close to the first parasitaemic peak was bled by cardiac puncture, taking aseptic precautions. Approximately 1 ml of heparinised blood was added to 10 ml MEM in a sterile plastic universal bottle and centrifuged in a refrigerated centrifuge (40 g, 10 minutes) to sediment the red/

red blood cells. The supernatant was carefully decanted and centrifuged (100 g, 10 minutes) to sediment the trypanosomes. The supernatant was discarded and the pelleted trypanosomes resuspended in a few ml of MEM supplemented with 20% CS. If necessary the first centrifugation step was repeated, although the presence of a few red blood cells did not seem to adversely affect the in vitro culture. In vitro drug tests for T. congolense KETRI 2885 were conducted at 37°C, while those for T. congolense KETRI 2880 and KETRI 2883 were carried out at 28°C. For the latter two strains, 28°C was the temperature at which the adaptation and subsequent maintenance of in vitro cultures was carried out.

Different periods of incubation of trypanosomes in the presence of the BAE feeder layer, prior to the addition of Isometamidium to the wells, were also used. Thus, Isometamidium was added after 0, 4 or 24 hours incubation. In the latter case, the medium was changed prior to the addition of Isometamidium.

Enumeration methods

Borowy's method involved removal of samples of medium and trypanosomes by gentle pipetting, and then counting trypanosome densities using a haemocytometer. It was however thought that this approach might be unsuitable for T. congolense which attaches to the feeder layer by the flagellum, because it was possible that different strains would be more or less readily detached, thereby distorting the results. Also, since dead or dying trypanosomes were likely to be more easily detached from the feeder layer/

layer than healthy trypanosomes, this might also influence the results. Initially therefore a method was devised which relied upon a direct estimate of numbers of trypanosomes still attached to the feeder layer, using an inverted microscope (X200). In wells containing the higher drug concentrations, where relatively few trypanosomes survived after 48 hours incubation, the number of trypanosomes in each of three microscope fields near to the centre of each well are counted. In those wells in which the number of trypanosomes per field was too high to count, an eye-piece graticule made up of 25 squares was used. The number of trypanosomes in each of five squares, one at each corner and one in the centre, of each of three microscope fields was counted. The five squares together were estimated to represent one eighth of the total microscope field, and therefore the total number of trypanosomes per field was estimated by multiplying the total number of trypanosomes counted in the five squares by eight. The number of trypanosomes per field of drug treated wells was related to the untreated wells, and the percentage inhibition calculated:

$$\frac{(\text{Mean No.tryp's per well})_{\text{control}} - (\text{Mean No.tryp's per well})_{\text{test}}}{(\text{Mean No.tryp's per well})_{\text{control}}} \times 100$$

= % inhibition

The disadvantages of this method were that it was time consuming and that the results were vulnerable to uneven distribution of trypanosomes throughout the well. Clumping of trypanosomes also made counting difficult. It was therefore decided to try removing/

Table 13. Effect of 48 hour incubations with Isometamidium on T.congolense 2885.

| Test number | n | Isometamidium concentration (µg/ml) 0.03 | 0.01 | 0 |
|---|---|---|---|---|
| 1 | 3 | 0 | Few individuals | Heavy |
| 2 | 3 | Few individuals | Heavy | Very heavy |

trypanosomes from the feeder layer by vigorous pipetting, and then estimating trypanosome densities by haemocytometer counts. It was found that although the different strains of trypanosomes did attach to the feeder layer with differing degrees of firmness, vigorous pipetting removed virtually all trypanosomes from the feeder layer. Although this technique was still time consuming, it did overcome the problem of uneven distribution of trypanosomes over the well.

Results

T. congolense KETRI 2885

Bloodstream forms were isolated directly from parasitaemic mice and incubated in the presence of each of a range of Isometamidium concentrations (0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 μg/ml) for 48 hours at 37°C. Trypanosomes and Isometamidium were introduced on to the feeder layer at the same time. The results of two experiments are presented in Table 13. In both experiments counting of the untreated wells was difficult due to the large number of trypanosomes present and the presence of large clumps of trypanosomes. In experiment 1 there was a marked response to Isometamidium concentrations down to 0.01 μg/ml, the lowest concentration used. In experiment 2, numbers of trypanosomes in the untreated wells and 0.01 μg/ml treated wells were too heavy to count and consisted of large clumps of trypanosomes. In the latter trypanosome densities were heavy, but noticably less dense than the untreated wells. The effect of Isometamidium concentrations down to 0.03 μg/ml was very marked, with very few trypanosomes in these wells after 48 hours.

Table 14. Effect of 48 hour incubations with Isometamidium on T.congolense KETRI 2883
% inhibition compared to untreated controls.

| | | Isometamidium concentration (µg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Test number | n | 30 | 10 | 3 | 1 | 0.3 | 0.1 | 0 |
| | | % inhibition | | | | | | |
| 1 | 2 | 100 | 99.8 | 89.5 | 56.6 | 30.0 | 11.1 | 0 |
| 2 | 2 | 94.5 | 62.4 | 33.5 | - 0.5 | 2.0 | - 2.7 | 0 |
| 3 | 3 | 99.4 | 50.5 | 11.3 | 2.6 | 0 | 3.7 | 0 |
| 4 | 3 | 99.4 | 67.3 | 37.0 | 36.5 | 4.5 | 6.4 | 0 |
| 5 | 3 | 81.2 | 38.8 | - 4.4 | 7.1 | - 0.8 | - 1.3 | 0 |

n: number of wells per concentration

Table 15: Effect of 48 hour incubations with Isometamidium on T.congolense KETRI 2883 % inhibition compared to untreated controls. Haemocytometer counting method

| Test number | n | Pre-incubation (hr) | Isometamidium concentration (µg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 30 | 10 | 3 | 1 | 0.3 | 0.1 | 0 |
| | | | % inhibition | | | | | | |
| 1 | 4 | 24 | 92 | 60 | 38 | 29 | 29 | 22 | 0 |
| 2 | 4 | 4 | 93 | 61 | 17 | 27 | 15 | 15 | 0 |

T. congolense KETRI 2883

Method 1: Bloodstream forms of trypanosomes were harvested from in vitro cultures, and were incubated in the presence of a range of Isometamidium concentrations (0, 0.1, 0.3, 1, 3, 10, 30 μg/ml). Trypanosomes and Isometamidium were introduced on to the feeder layer at the same time. The effect of Isometamidium was determined by counting trypanosomes attached to the feeder layer using an inverted microscope. The percentage inhibition for each Isometamidium concentration was calculated in relation to untreated wells. The results of five experiments are shown in Table 14.

In three tests the $IC_{50}$ value, ie the Isometamidium concentration which resulted in a 50% inhibition of growth compared to untreated wells, lay between 3 and 10 pg/ml Isometamidium, while in one test the $IC_{50}$ value lay between 0.3 and 1 μg/ml, and in one test the value lay between 10 and 30 μg/ml.

This variation between tests could have arisen due to uneven distribution of trypanosomes on the feeder layer, or may have resulted from differences in the populations of trypanosomes harvested from the culture flasks, e.g. trypanosomes originating from more rapidly dividing populations may be more susceptible to Isometamidium.

Method 2: In these tests the basic method was varied by incubating the trypanosomes for either 4 or 24 hours prior to addition of Isometamidium and then incubating for a further 48 hours. The effect of Isometamidium was assessed by removing trypanosomes from each well by vigorous pipetting, and counting with a haemocytometer. The results of two tests carried out using this method are shown in Table 15. Incubation for 4 or 24 hours prior to the addition of Isometamidium does not appear to affect the outcome of the tests. In both cases the $IC_{50}$ value fell between 3 and 10 μg/ml.

Table 16. Effect of 48 hour incubations with Isometamidium on T.congolense KETRI 2880 % inhibition compared to untreated controls.

| | | Isometamidium concentration (µg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Test number | n | 30 | 10 | 3 | 1 | 0.3 | 0.1 | 0 |
| | | % inhibition | | | | | | |
| 1 | 4 | 93 | 50 | 16 | 11 | 2 | 11 | 0 |
| 2 | 4 | 100 | 81 | 61 | 23 | 30 | 23 | 0 |
| 3 | 4 | 96 | 79 | 32 | 18 | 21 | 11 | 0 |

n: number of wells per concentration

Of the seven tests carried out with this strain, in five the $IC_{50}$ was between 3 and 10 μg/ml.

T. congolense KETRI 2880

Bloodstream forms of trypanosomes were harvested from *in vitro* cultures, and were incubated at 28°C in the presence of BAE feeder layer and one of a range of Isometamidium concentrations (0, 0.1, 0.3, 1, 3, 10, 30 (μg/ml). Trypanosomes were incubated for 4 or 24 hours prior to the addition of Isometamidium and the effect of drug was assessed by removing trypanosomes from the feeder layer by vigorous pipetting and estimating trypanosome densities by the use of a haemocytometer. The results of three tests are shown in Table 16.

The $IC_{50}$ value lay between 3 and 10 μg/ml in two of the three tests and between 1 and 3 μg/ml in the third. Again incubation of trypanosomes for 4 or 24 hours prior to addition of Isometamidium does not seem to have influenced the outcome of the test.

Even though slightly different methods were used in the different drug sensitivity tests performed, the basic approach was the same. Clear differences were demonstrated between the Isometamidium sensitivities of T. congolense KETRI 2885 and the two more resistant strains, namely KETRI 2880 and KETRI 2883. The $IC_{50}$ values for T. congolense KETRI 2880 and KETRI 2883 were both in the range 3 - 10 μg/ml, while for T. congolense KETRI 2885 this value was $\leqslant$ 0.01 μg/ml.

It was not possible to differentiate between T. congolense KETRI 2880 and KETRI 2883 using this approach, although the MCD's in cattle for these strains were 2.0 and 0.5 mg/kg respectively. Since one of these strains could be cured by treatment at the manufacturers' recommended dose of Isometamidium, and one could not, this degree of difference in Isometamidium sensitivity in vivo is of practical significance.

Discussion

An in vitro approach to the assessment of Isometamidium sensitivities of three strains of T. congolense has been explored. Although the method employed could detect broad differences in Isometamidium sensitivities, it was unable to demonstrate relatively narrow differences. Thus, a strain of T. congolense with a MCD in cattle of $\leqslant$ 0.001 mg/kg Isometamidium could readily be distinguished from either of two relatively resistant strains by the in vitro method. However the two latter strains, with MCD's of 0.5 and 2.0 mg/kg respectively, could not be differentiated.

A number of problems associated with the in vitro approach became apparent, of which the most significant is adaptation of strains to in vitro culture. It is important that the population used in any in vitro drug experiments is a healthy, replicating one. Two of the three strains used in this study, namely T. congolense KETRI 2880 and KETRI 2883 rapidly died when introduced directly on to BAE feeder layer. A similar finding was reported by Ross et al., (1985) who found that three out of four cloned stocks of T. congolense died when introduced directly on to feeder layer. These strains therefore required more complicated and time consuming periods/

periods of adaptation to *in vitro* culture, prior to use in *in vitro* drug tests. Both *T. congolense* KETRI 2880 and KETRI 2883 were successfully adapted to *in vitro* culture by transformation of blood-stream forms harvested from mice, through the insect mid-gut stages to metacyclic forms using a cell-free culture system, and then introducing the *in vitro* derived metacyclics on to BAE feeder layer where transformation back to blood stream forms occurred. In the case of *T. congolense* KETRI 2883 this adaptation phase required several attempts stretching over 6 months. For *T. congolense* KETRI 2880, even after the adaptation phase, *in vitro* cultures tended to replicate slowly. Of the three strains used, *T. congolense* KETRI 2880 had the longest pre-patent period in cattle, goats and mice, and resulted in characteristically intermittent parasit-aemias in mice. It therefore seems likely that both *in vivo* and *in vitro* this strain is inherently slow at replicating.

The difficulty of getting field isolates, or even laboratory strains of *T. congolense* to adapt to *in vitro* culture is clearly a major constraint on the wider use of this approach to drug sensitivity testing.

Having successfully adapted strains of *T. congolense* to *in vitro* culture, the question arises, how representative of the original trypanosome population is the *in vitro* adapted population? The adaptation procedure presumably selects those individuals best able to survive in the *in vitro* conditions employed. A recent study has compared, in mice, the drug sensitivities of three strains of *T. congolense* both before and after adaptation to *in vitro* culture. It was concluded that the adaptation process had not altered the drug/

drug sensitivities of the strains used (Brown, Ross, Holmes, Luckins and Taylor, 1987).

An additional disadvantage of *in vitro* systems for assessing the effects of drugs is that the influence of the host metabolism, and the effect of distribution and pharmacokinetics of the drug, are eliminated. If the metabolites of a drug play an important role in the *in vivo* activity of that drug, the results of *in vitro* work based on the use of 'raw' drug may be misleading. In this context, it is interesting to note that Isometamidium concentrations that were active in the present study, are similar to calculated and reported values for blood concentrations soon after drug administration. For example, the results of the *in vitro* experiments indicate that the $IC_{50}$ value for *T. congolense* KETRI 2883 is in the range 3 - 10 µg/ml. The maximum calculated blood concentration for a 250 kg steer treated at 0.5 mg/kg Isometamidium (the MCD for this strain) is approximately 9 µg/ml. Currently available methods for estimating Isometamidium concentrations in tissues are relatively poor, but Braide and Eghianruwa (1980) using a spectrophotometric technique report a serum concentration of 2 µg/ml, 24 hours after a single administration of 0.5 mg/kg to a goat by intramuscular injection.

The interaction of the hosts' immune response with trypanocidal treatment can also be important, and clearly such interactions cannot occur *in vitro*. Whitelaw (1982) noted that a combination of diminazene aceturate treatment and maternal antibody provided greater protection from infection in young mice than either/

either chemoprophylaxis or immunoprophylaxis alone. de Gee, McCann and Mansfield (1983) and Bitonti, McCann and Sjoerdsma (1986) both reported that an antibody response was necessary for the rapid elimination of trypanosomes in rodents treated with α-difluoromethylanithine.

The facilities and resources required for the in vitro culture of trypanosomes are relatively sophisticated and expensive, and are not readily available in many African countries. Although in vivo methods tend to be expensive in developed countries, in developing countries where labour costs are lower, in vivo methods are probably more attractive, relying on locally available materials and labour. While the anti-vivisection lobby is an increasingly powerful force in Europe and America, with considerable pressure to use fewer animals in research, it is as yet, of little significance in Africa.

An alternative to using bloodstream forms for in vitro drug experiments is to use procyclic forms. This approach has the advantage that in vitro derived procyclic forms are relatively easy to produce from mouse harvested bloodstream forms, and also eliminates the need for a feeder layer. Procyclic forms of T.b. brucei have been used for in vitro studies of resistance to α-difluoromethylornithine (Phillips and Wang, 1987; Bellofalto, Fairlamb, Henderson and Cross, 1987). However, it remains to be demonstrated whether or not drug sensitivities determined in vitro using procyclic forms of T. congolense correlate well with values obtained using bloodstream forms.

In conclusion, the relevance of *in vitro* methods for the assessment of drug sensitivities of strains of *T. congolense* is limited, firstly by the difficulty of adapting strains to culture, and secondly, by the facilities and resources required. Improvements in *in vitro* culture techniques, e.g. the development of cell-free systems for bloodstream forms of *T. congolense*, may make this approach more applicable in the future, but at the present time an *in vitro* approach is not appropriate to routine monitoring of drug sensitivities in Africa.

# CHAPTER FIVE

## STUDIES OF THE APPARENT APARASITAEMIC INTERVAL FOLLOWING SUBCURATIVE TREATMENT WITH ISOMETAMIDIUM OF T. congolense INFECTIONS IN MICE

INTRODUCTION

Treatment of animals infected with T. congolense, with an effective but subcurative dosage of Isometamidium results in a period of apparent aparasitaemia, followed by a relapse infection. This raises the questions, what is the source of the relapse infection and what happens to the trypanosomes during the apparent aparasitaemic interval? In the case of T.b. brucei infections in mice, Jennings et al. (1979) demonstrated that the brain was the source of the relapse infection following subcurative treatment with diminazene aceturate. MacLennan (1973) demonstrated a temporary period of aparasitaemia in cattle infected with T. vivax and treated with diminazene aceturate, during which subinoculation of blood into naive cattle failed to result in the establishment of infection in recipients. Cattle subinoculated with blood collected 12 hours, or 3, 6 or 9 days after treatment with 3.5 mg/kg diminazene aceturate did not become parasitaemic, while cattle subinoculated 6 hours or 12 days post-treatment became parasitaemic. He suggested this was evidence that following treatment with diminazene aceturate, T. vivax 'survives in treated animals other than in the bloodstream'.

With few exceptions, T. congolense is regarded as strictly vascular (Losos et al., 1973). Two groups of workers have demonstrated T. congolense in the brain or CSF of cattle (Haase et al., 1981; Masake et al., 1984) although in both cases the presence of intercurrent infections with T.b. brucei was a complicating factor, and may have facilitated the breaching of the blood-brain barrier by T. congolense.

In the present study mice were examined during the aparasitaemic interval following effective but subcurative treatment with Isometamidium of T. congolense infections.

The Relapsing Mouse Model

A preliminary experiment in mice infected with T. congolense 82/1, indicated that 1.0 mg/kg Isometamidium administered on day 21 post-infection resulted in an aparasitaemic period, as demonstrated by examination of tail-blood wet-film preparations, of 12 - 15 days duration, followed by recrudescense of parasitaemia. Increasing the dosage to 2.0 mg/kg resulted in all mice being cured. A dosage of 1.0 mg/kg administered on day 21 was therefore selected as a suitable treatment regime to investigate the apparent aparasitaemic interval following subcurative treatment with Isometamidium.

Organ Transfer Experiments

Methods

Donor mice were infected with approximately 1 x $10^5$ trypanosomes, T. congolense 82/1, administered by ip inoculation. Twenty-one days post-infection donor mice were checked to confirm the presence of circulating trypanosomes by examination of tail-blood wet-film preparations, and were then treated with Isometamidium (1 mg/kg) administered by ip injection. Seven days after treatment mice were again checked to confirm absence of circulating trypanosomes, and then deep anaesthesia was induced using trichloroethylene. Approximately 0.5 ml of blood was obtained from each mouse by cardiac puncture using a heparinised syringe and needle. Mice were then killed/

Table 17. Results of the subinoculation of blood or organ macerates to irradiated recipient mice.

| Donor mouse number | Blood | Heart | Spleen | Liver | Kidney | Brain | Reprod. organs |
|---|---|---|---|---|---|---|---|
| 1 | + | + | | | + | | |
| 2 | | | | | +i | | |
| 3 | | | | | | | |
| 4 | + | + | | | | | |
| 5 | | | | | | | |
| 6 | + | + | | | | | |
| 7 | | D(17) | | | | | |
| 8 | D(2) | + | | + | | D(2) | + |
| 9 | + | + | | | | | +ii |
| 10 | + | + | | D(2) | + | + | + |
| 11 | | | | | D(2) | | |
| 12 | | | | | D(10) | | |
| 13 | + | + | | | | + | |
| 14 | | | D(8) | | | | |
| 15 | | | D(3) | | | | |
| 16 | | | | | | | |
| 17 | | | | | | | |
| 18 | | + | D(24) | | | | |
| 19 | | + | | | | | |
| 20 | | + | | | | | |

+ infection developed in recipient.

D mouse died on day post-subinoculation indicated, without development of infection.

i transient parasitaemia: one trypanosome per 20 fields on day 15 post-subinoculation.

ii transient parasitaemia: one trypanosome per 20 fields on day 24 post-subinoculation.

killed by cervical dislocation, and heart, brain, liver, kidneys, spleen and reproductive organs (ovaries, Fallopian tubes and uterus) dissected out. Each organ was carefully macerated by forcing through a wire gauze with a syringe plunger, and suspended in approximately 1 ml of sterile, isotonic saline. The blood samples and macerated organ suspensions were inoculated by the ip route into mice which had been irradiated (600 rads) 24 hours earlier, one organ per mouse. In the case of the liver, only half the tissue was sub-inoculated. Recipient mice were maintained on oxytetracycline-treated drinking water (Terramycin, Pfizer, England) to control adventitious bacterial infections, and were monitored for the development of parasitaemia by examination of wet-films, three times a week. Observations were continued for 28 days. Control mice, which had been infected and treated at the same time as the donors, were retained to monitor recrudescense of parasitaemia after treatment.

Results

Control mice which had been infected and treated exactly as the donors were retained to monitor development of the relapse infection. Of nine control mice, eight developed relapse infections, with parasites first being detected in tail-blood wet-films between 8 and 10 days post-treatment. One mouse remained clear of circulating trypanosomes throughout the 35 day post-treatment period.

The results of the subinoculation of blood or organ macerates are shown in Table 17. Although all donor mice were shown to

be/

be free of circulating trypanosomes by examination of tail-blood wet-film on the day the subinoculations were carried out, six of 20 recipient mice which received subinoculations of blood developed parasitaemias. Heart macerates obtained from donors in which the blood gave rise to infection, also resulted in infection being transferred to recipient mice. In addition, a further four mice receiving subinoculations of heart macerate became infected, although subinoculations of blood from the same donors failed to transfer infection.

No mice subinoculated with spleen macerate became infected. One mouse subinoculated with liver macerate became parasitaemic. The receipient of blood from the same donor died shortly after subinoculation.

Three mice subinoculated with kidney macerate became parasitaemic. Of these, two mice subinoculated with blood from the same donor developed parasitaemias. A third mouse developed a transient parasitaemia, one motile trypanosome being observed on one occasion 15 days after subinoculation. Two new irradiated mice were subinoculated with 0.1 ml of blood from this mouse, 9 days after the single trypanosome had been observed. Both remained trypanosome-free throughout the 28 day observation period. Blood from the same donor mouse did not result in transmission of infection; neither did any other organ macerate.

Three mice subinoculated with brain macerate became infected. Two of the recipients of blood from the same donors became infected. In/

In a third mouse the corresponding subinoculation of blood failed to transfer infection, although the heart macerate resulted in parasitaemia.

Three mice subinoculated with reproductive organ macerate became infected, one only transiently. Two of the matching blood subinoculations also resulted in infection. The recipient of blood from the third mouse died soon after subinoculation.

Discussion

Of the twenty apparently aparasitaemic donor mice used in this experiment, blood or organ macerates from 11 of them resulted in the transfer of infection to subinoculated recipient mice, although at the time of subinoculation donor mice were aparasitaemic, as judged by examination of wet-film preparations. In six of the 11, subinoculation of blood resulted in the transfer of infection to recipient mice, and in another mouse the recipient subinoculated with blood died without development of parasitaemia. Clearly if blood from a donor mouse caused transmission of infection, then it is impossible to say whether an organ macerate from the same donor has resulted in a parasitaemia due to a cryptic trypanosome infection, or due to blood incidentally subinoculated along with the tissue. In four donor mice however, subinoculation of organ macerates resulted in transfer of infection to recipients, when matching blood samples did not. In two cases this occurred with heart macerate, in one case heart and brain macerate and in one case kidney macerate resulted in a low-grade, transient parasitaemia. T. congolense are known to/

to preferentially inhabit capillaries rather than larger vessels, and therefore it is possible that the latter examples can be explained by very low numbers of circulating trypanosomes failing to transfer infection in the blood, but larger numbers of trypanosomes inhabiting the capillaries of the heart, brain or kidney, resulting in transfer of infection in the organ macerates.

The finding that blood subinoculated from six of the 20, apparently aparasitaemic, mice resulted in establishment of parasitaemias in irradiated recipient mice, indicated that a more intensive investigation of the apparent aparasitaemia following subcurative treatment, was warranted.

Investigation of the Aparasitaemic Period Following Subcurative Treatment with Isometamidium, Using Three Trypanosome Detection Methods

Method

Donor mice (ex-breeder females, mean weight 37.3 ± 2.2 g) were infected by ip inoculation with approximately 1 x $10^5$ trypanosomes, T. congolense 82/1, prepared from cryopreserved stabilate. Twenty-one days post-infection, donors were checked to confirm the presence of circulating trypanosomes by examination of tail-blood wet-films, and then treated with Isometamidium (1 mg/kg) administered by ip injection. After treatment donors were monitored by examination of tail-blood wet-film preparations. On the first occasion when trypanosomes could not be detected by the wet-film technique, Walkers' (1972) modification of the capillary concentration technique was carried out (see p 64). If the capillary concentration method also failed to demonstrate trypanosomes, then a recipient mouse was irradiated (600/

Table 18. Results of three trypanosome detection methods employed during the apparent aparasitaemic period (Experiment 1.)

| Donor mouse number | | Day post-treatment 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Wet film | 4.3 | | | | | | | 4.3 | 4.6 | |
| | Cap.conc. | + | | | | | | | + | | |
| | Subinoc. | | | K | + | - | + | - | + | | |
| 2 | Wet film | 4.6 | 4.3 | | | | | | 4.3 | 4.3 | 6.1 |
| | Cap.conc. | | + | - | - | - | - | - | + | + | |
| | Subinoc. | | | | - | + | + | - | + | | |
| 3 | Wet film | 7.3 | 4.3 | | | | | | 4.3 | 4.3 | 4.6 |
| | Cap.conc. | | + | + | - | - | - | - | - | + | |
| | Subinoc. | | | | | - | + | - | + | + | |
| 4 | Wet film | 6.1 | 4.3 | | | | | | 4.3 | 4.9 | 6.4 |
| | Cap.conc. | | + | - | - | - | - | - | + | | |
| | Subinoc. | | | | + | + | + | - | + | | |
| 5 | Wet film | 4.3 | | | | | | | 4.3 | 5.2 | 7.1 |
| | Cap.conc. | + | + | - | - | - | - | - | + | | |
| | Subinoc. | | | | + | + | + | + | + | | |
| 6 | Wet film | 5.2 | 4.3 | | | | | | 4.3 | 5.8 | 7.2 |
| | Cap.conc. | | + | - | - | - | - | - | + | | |
| | Subinoc. | | | | + | + | + | + | + | | |
| 7 | Wet film | 4.3 | | | | | | | 5.2 | 6.1 | 6.7 |
| | Cap.conc. | + | - | - | - | - | - | - | + | | |
| | Subinoc. | | | K | + | + | + | - | + | | |
| 9 | Wet film | 7.1 | 4.3 | | | | | | 4.3 | 4.3 | 4.6 |
| | Cap.conc. | | - | - | - | - | - | - | - | + | |
| | Subinoc. | | | K | - | + | + | - | + | + | |
| 10 | Wet film | 4.3 | | | | | | | 4.3 | 4.9 | 7.0 |
| | Cap.conc. | + | - | - | - | - | - | - | + | | |
| | Subinoc. | | | - | + | + | + | + | + | | |

Wet film: $\log_{10}$ trypanosomes per ml.

Cap.conc: + indicates trypanosomes observed
- indicates no trypanosomes observed

Subinoc. + indicates infection developed in recipient mouse
- indicates no infection developed in recipient mouse
K indicates mouse killed *in extremis* without developing infection

(600 rads) and 24 hours later subinoculated with 0.1 ml of tail-blood collected from the apparently aparasitaemic donor mouse. Irradiated recipient mice were maintained on oxytetracycline-treated water to control adventitious bacterial infections. Observation was continued for up to 40 days (Experiment 1).

In Experiment 2, the same basic method was followed, except that new recipient mice were subinoculated each day beginning on day 2 post-treatment throughout the apparent aparasitaemic period. Recipient mice were monitored for up to 30 days.

Results

Experiment 1: The results, presented in Table 18, clearly demonstrated the relative sensitivities of wet-film, capillary concentration and subinoculation to immunosuppressed mice. In cases where examination of 20 microscope fields of a tail-blood wet-film preparation failed to reveal any trypanosomes ($< \log_{10} 4.3$ trypanosomes per ml blood), the capillary concentration technique frequently demonstrated the presence of parasites (mice 1, 5, 7 and 10 on day 2; mice 1, 2, 4, 5, 6 and 10 on day 9; mice 2, 3 and 9 on day 10). Likewise, subinoculation of 0.1 ml aliquots of blood to irradiated recipients often resulted in transmission of infection when the capillary concentration technique showed no trypanosomes (mice 1, 4, 5, 6, 7 and 10 on day 5; mice 2, 4, 5, 6, 7, 9 and 10 on day 6; mice 1, 2, 3, 4, 5, 6, 7, 9 and 10 on day 7; mice 5, 6 and 10 on day 8; mouse 3 on day 9).

Of 10 donor mice used in the experiment, nine developed a relapse infection as determined by either the wet-film or capillary concentration/

Table 19. Results of three trypanosome detection methods employed during the apparent aparasitaemic period (Experiment 2).

| Donor mouse number | | Day post-treatment | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| 1 | Wet film | 7.0 | 4.9 | | | | | | | | | | | 4.9 |
| | Cap.conc. | | | - | - | - | - | - | - | - | + | + | | |
| | Subinoc. | | + | + | - | - | - | + | + | + | + | + | | |
| 2 | Wet film | 7.1 | 4.6 | | | | | | | | | | | 6.1 |
| | Cap.conc. | | | - | - | - | - | - | - | - | + | + | | |
| | Subinoc. | | - | - | - | - | - | + | + | + | + | + | | |
| 3 | Wet film | 6.9 | 6.4 | 4.3 | | | | | | | 5.5 | | | 7.7 |
| | Cap.conc. | | | - | - | - | - | - | + | + | | | | |
| | Subinoc. | | + | - | + | - | + | + | + | + | | | | |
| 4 | Wet film | 6.9 | 4.6 | | | | | | | 4.6 | 6.1 | | | 7.0 |
| | Cap.conc. | | | + | + | - | - | + | + | | | | | |
| | Subinoc. | | + | + | + | - | - | + | + | | | | | |
| 5 | Wet film | 7.1 | | | | | | | | | 4.9 | | | 6.7 |
| | Cap.conc. | | + | + | - | - | - | - | + | + | | | | |
| | Subinoc. | | + | - | + | + | + | + | + | + | | | | |
| 6 | Wet film | 6.9 | 7.0 | | | | | | | | | | | 6.7 |
| | Cap.conc. | | | + | - | - | - | - | - | + | + | | | |
| | Subinoc. | | + | - | - | - | + | + | + | + | | | | |
| 7 | Wet film | 7.0 | | | | | | | | | 5.8 | | | 7.3 |
| | Cap.conc. | | + | - | - | - | - | - | + | + | | | | |
| | Subinoc. | | - | - | - | - | + | + | + | + | | | | |
| 8,9,10 | Cap.conc. | + | + | + | + | + | + | + | + | + | + | | | |

concentration techniques, with trypanosomes disappearing from the bloodstream between days three and five post-treatment and reappearing on day 9 or 10. However successful transmission of infection by subinoculation of blood during this apparent aparasitaemic period, suggests that the level of circulating trypanosomes may have merely dropped below the limit of detection of the less sensitive trypanosome detection methods used. The results are incomplete due to a 24 hour delay from first demonstration of a negative capillary concentration preparation, to subinoculation of blood to an immunosuppressed recipient mouse, and also because some mice were killed in extremis without the development of parasitaemia. These individuals may have developed a bacterial infection in spite of being maintained on oxytetracycline-treated water, or they may have been affected by the irradiation process. Experiment 2 attempted to overcome the former problem by performing subinoculations daily from day 2 post-treatment until detection of the relapse infection by the wet-film or capillary concentration techniques.

Experiment 2: Of the 10 donor mice used in the experiment, seven developed a relapse infection, as demonstrated by either the wet-film or capillary concentration techniques, with trypanosomes disappearing from the bloodstream between day 3 and 5 post-treatment and reappearing between days 7 and 10. The results for the three trypanosome detection methods are shown in Table 19 . As in Experiment 1, the relative sensitivities of the three detection methods were clearly demonstrated. For the seven mice which experienced temporary remission of infection, followed by relapse, the mean aparasitaemic periods,/

periods, as determined by the different detection methods were; wet-film 8.4 days, capillary concentration technique 5.0 days and mouse subinoculation 2.7 days. Two mice which were subinoculated with blood which was shown to be trypanosome-positive by the wet-film or capillary concentration techniques, failed to become infected. In both cases this occurred 2 days after Isometamidium treatment, when the number of circulating trypanosomes had already been reduced to a low level.

Discussion

The estimate of the duration of the apparent aparasitaemic period following subcurative treatment with Isometamidium, has been shown to be markedly influenced by the trypanosome detection method used. The most sensitive detection method used, subinoculation of 0.1 ml of blood into immunosuppressed mice, demonstrated the shortest intervals and in some mice no aparasitaemic interval could be demonstrated using this method.

The apparent aparasitaemic interval could be due to either complete elimination of circulating trypanosomes, or a reduction in the number of circulating parasites to below the limit of detection of the method used. Since a detectable relapse infection eventually occurs, it follows that if trypanosomes are eliminated from the bloodstream, a cryptic focus of infection must exist which is drug inaccessible, and from which trypanosomes re-emerge when circulating levels of trypanocide have dropped below an effective level. These experiments, whilst not being conclusive, suggest that numbers of trypanosomes may merely have dropped below the limit of detection.

Although/

Although in theory, transfer of a single trypanosome is enough to initiate an infection, in practise it can be difficult to establish an infection of T. congolense from a single trypanosome, eg in cloning.

The relapse phenomenon described by Jennings et al. (1979) in which T.b. brucei infections in mice undergo a demonstrable aparasitaemic period following subcurative treatment with diminazene aceturate, during which subinoculation of brain macerates, but not other tissues or blood, consistently resulted in transfer of infection, does not seem to be paralleled in the T. congolense model used in the present experiment.

Isometamidium Sensitivities of the Parent and Relapse Population of T. congolense 82/1

Introduction

If the apparent aparasitaemic interval is due to a reduction of numbers of circulating trypanosomes to a level below the limit of detection of the trypanosome detection method employed, then one might expect the trypanosomes which survive treatment and contribute to the relapse infection to differ from the pre-treatment population. It was therefore decided to compare the Isometamidium sensitivites of the relapse population of T. congolense 82/1 with the parent infection.

Method

A group of nine mice was infected with $1 \times 10^5$ trypanosomes, T. congolense 82/1, prepared from cryopreserved stabilate and administered by ip inoculation. Twenty-one days post-infection the/

Table 20 Comparison of Isometamidium sensitivities of the parent and relapse population of T.congolense 82/1.

| Isometamidium (mg/kg) | Parent | | Relapse | |
|---|---|---|---|---|
| 0.1 | 4.2±1.1 a | | 5.0±0 | |
| 0.25 | 7.0±1.4 | | 7.6±2.5 | |
| 0.5 | 12.0±1.7 | 2/5 b | 13.0±0 | |
| 0.75 | 11.5±2.1 | 3/5 | 13 | 4/5 |
| 1.0 | 13 | 4/5 | | 5/5 |
| 2.0 | | 5/5 | | 4/4 |

a Parasitaemic by day : mean ± S.D

b number of mice cured/number of mice infected

the mice were treated with Isometamidium, 1 mg/kg by ip injection. A stabilate was prepared from the resultant relapse infection, by pooling blood from mice close to the first parasitaemic peak. This stabilate was called T. congolense 82R and was stored in liquid nitrogen until required.

Later groups of five mice were anaesthetized and then infected via the tail vein with 1 x $10^5$ trypanosomes of either the relapse infection (T. congolense 82/R) or the parent infection (T. congolense 82/1). Three hours post-infection mice were treated with one of a series of Isometamidium doses, or left untreated as infection controls. After infection, development of parasitaemias were monitored by examination of tail-blood wet-films, initially three times a week. Observation was continued for 100 days.

Results

The results of the Isometamidium sensitivity test are shown in Table 20. The minimum Isometamidium dose which cured all infected mice (MCD) was 2.0 mg/kg for T. congolense 82/1 and 1.0 mg/kg for T. congolense 82R, although this difference is in fact due to just one mouse relapsing in the parent strain/1.0 mg/kg treatment group. $CD_{80}$ values for the parent and relapse infections were 1.0 and 0.75 mg/kg respectively.

Discussion

Only minor differences were observed in the drug sensitivities of the parent and relapse populations of T. congolense 82/1, both in terms of number of cures at each dose, and in the delay in appearance of trypanosomes in the blood. If the period of apparent aparasitaemia/

aparasitaemia following subcurative treatment with Isometamidium is due to a drop in the number of circulating trypanosomes below the limit of detection of the method used, then one would have thought that the parent and relapse populations of trypanosomes would have responded differently to treatment. No such differences were demonstrated in this experiment. The question of how some trypanosomes survive treatment to contribute to the relapse population remains unanswered.

# CHAPTER SIX

## ATTEMPTS TO INDUCE CHANGES IN ISOMETAMIDIUM SENSITIVITY BY REPEATED SUBCURATIVE TREATMENT OF T. congolense INFECTIONS IN MICE AND GOATS

Introduction

It is frequently suggested that exposure to subcurative levels of trypanocidal drugs enhances the likelihood of drug resistant strains of trypanosomes developing, and that drug resistance is more likely to develop to drugs with extended prophylactic activity, such as Isometamidium (Whiteside, 1962). Exposure of trypanosomes to subcurative levels of trypanocidal drugs has been successfully used by many workers as a means of inducing drug resistant strains of trypanosomes in laboratory conditions. Although there are numerous examples in the literature of the production of resistant strains of a number of trypanosome species by this technique (Wilson, 1949; Fulton and Grant, 1955; Hawking, 1963a), and for a range of trypanocidal drugs, there are very few examples of Isometamidium resistant strains being created. Hawking (1963a) found that resistance to metamidium developed more slowly than resistance to quinapyramine or pyrithidium, but slightly faster than resistance to diminazene aceturate. He passaged a metamidium-sensitive strain of T. congolense through metamidium treated guinea-pigs or irradiated mice, and found that it took 33 months to produce maximal resistance. The strain was initially sensitive, with an MED of 0.005 mg/kg and a MCD of 0.1 mg/kg. The resistant strain he produced had a MED of 12 mg/kg, and the curative dose was greater than the maximum tolerated dose (25 mg/kg). Metamidium is a precussor of Isometamidium, and is a mixture of two isomers, in the proportions 55% of a purple isomer and 45% of a red isomer. The water soluble red isomer is the more active of the two, and was later given the name Isometamidium.

Whiteside/

Whiteside (1960) mentions that he made a strain of T. congolense resistant to 2.0 mg/kg metamidium, by successive treatment in cattle with 0.05, 0.05 and 0.5 mg/kg metamidium. Folkers (1962) noted no decrease in Isometamidium sensitivity following repeated treatment at 0.25 mg/kg, in naturally infected cattle in Nigeria.

Whiteside (1962) considered that drug resistant strains of T. congolense were difficult to produce in mice. In contrast however, in cattle and adopting a method of repeated subcurative treatment and relapse, he noted 'excepting only ... berenil, resistance never fails to develop with astonishing rapidity'. He found that three to six successive treatments were all that was needed to make strains resistant to 40 to 80 times the median curative dose ($CD_{50}$) and that the procedure could be carried out in a single steer.

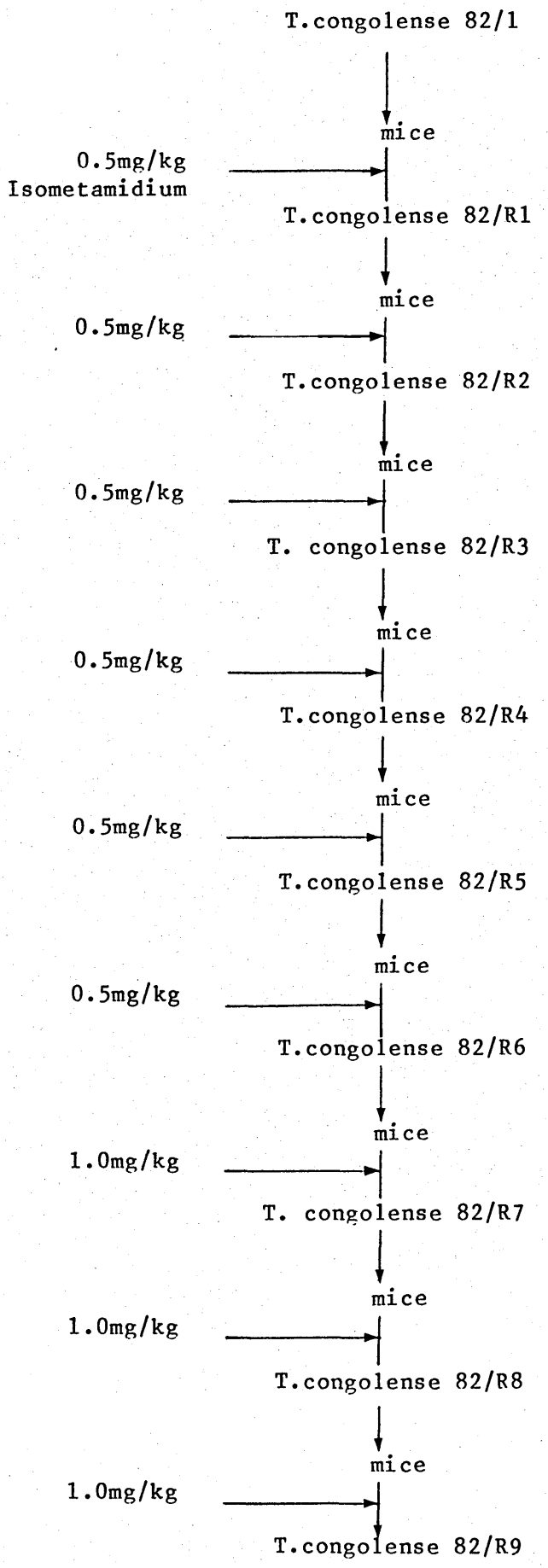
In view of the lack of data relating to the ease with which Isometamidium resistant strains of T. congolense can be produced in the laboratory, it was decided to attempt to induce changes in sensitivity by repeated subcurative treatment using mice and goats as hosts.

Experiments with Mice

Methods

Groups of 10 mice were infected with approximately 1 x $10^5$ trypanosomes per mouse, T. congolense 82/1 (parent strain), prepared from cryopreserved stabilate. On day 7 post-infection, mice were checked to ensure that they were parasitaemic by examination of tail-blood wet-film preparations, and were then treated by ip inoculation/

Fig. 3. Derivation of relapse populations of T.congolense 82/1.



inoculation with Isometamidium at 0.5 mg/kg. After treatment parasitaemias were monitored, again by examination of wet-films, three times per week. A stabilate was prepared of the relapse population, by pooling blood from mice close to the first parasitaemic peak. This stabilate was designated T. congolense 82/R1. A new batch of mice was then infected with T. congolense 82/R1, and the process repeated to derive T. congolense 82/R2. The procedure was repeated several times, and in this way a series of trypanosome populations, designated T. congolense 82/R1 through to T. congolense 82/R6, was derived. A new batch of mice was then infected with T. congolense 82/R6 and the procedure outlined above was followed, except that the Isometamidium dosage was increased to 1.0 mg/kg. Using this new dosage trypanosome populations T. congolense 82/R7, 82/R8 and 82/R9 were derived (Fig 3 ).

The Isometamidium sensitivities of the parent, R3, R6 and R9 populations were then compared in a mouse sensitivity test. Batches of mice were infected by ip inoculation of approximately $1 \times 10^5$ trypanosomes of either T. congolense 82/1 (parent strain), or the R3, R6 or R9 relapse population. One week later mice were checked to ensure they were parasitaemic by examination of wet-films, and were then allocated to treatment groups receiving 0, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 or 2.0 mg/kg Isometamidium by ip injection. Parasitaemias were monitored for 60 days by examination of tail-blood wet-films and also by the capillary concentration method.

Table 21. Comparison of Isometamidium sensitivities of the parent and relapse populations of T.congolense 82/1

| | Isometamidium (mg/kg) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.5 | 1.0 | 1.25 | 1.50 | 1.75 | 2.0 | $\leq$1.75 |
| Parent | 5/5 * | 4/5 | 4/5 | 1/5 | 1/5 | 0/5 | 15/25 |
| R3 | 5/5 | 3/5 | 3/5 | 2/5 | 1/5 | 0/5 | 14/25 |
| R6 | 5/5 | 5/5 | 2/5 | 2/5 | 1/5 | 0/5 | 15/25 |
| R9 | 5/5 | 5/5 | 2/4 | 2/5 | 0/5 | 0/5 | 14/24 |

* number of mice relapsed/number of mice infected

Results

The results of the Isometamidium sensitivity test carried out in mice infected with the parent or relapse populations of T. congolense 82/1, are presented in Table 21. These fail to show any evidence of a decrease in Isometamidium sensitivity as a result of up to nine exposures to subcurative Isometamidium treatments.

Experiments with Goats

Prior to any attempt to produce Isometamidium resistant strains of T. congolense using goats as hosts, basic information on the infectivity, course of infection and pathogenicity of a number of strains of T. congolense in this host species was required. Goats were therefore infected with one of a number of strains of T. congolense.

Infectivity and pre-patent periods: All the strains of T. congolense used, KETRI 2880, 2883, 2885 and 82/1, proved to be goat infective. After infection by the iv route with approximately $1 \times 10^5$ trypanosomes, prepared from cryopreserved stabilate, trypanosomes were first detected, using the capillary concentration/buffy coat technique, in a mean of $13.7 \pm 1.6$ days for T. congolense KETRI 2880. Corresponding values for KETRI 2883 and KETRI 2885 were $7 \pm 0$ and $8.1 \pm 0.4$ days respectively. For T. congolense 82/1, trypanosomes were first detected in a mean of $8.7 \pm 2.5$ days. A comparison of the pre-patent periods for T. congolense KETRI 2880, 2883 and 2885 in cross-bred British goats in the present experiment and/

Table 22. Comparison of pre-patent periods in goats and cattle for three strains of T.congolense.

| | T.congolense: | | | | | |
|---|---|---|---|---|---|---|
| | KETRI 2880 | | KETRI 2883 | | KETRI 2885 | |
| | n | mean ± S.D. | n | mean ± S.D. | n | mean ± S.D. |
| Goats | 6 | 13.7 ± 1.6 | 3 | 7.0 ± 0 | 7 | 8.1 ± 0.4 |
| Cattle | 11 | 10.6 ± 0.7 | 10 | 7.1 ± 0.3 | 12 | 7.0 ± 0 |

Table 23. Effect of infection with T.congolense on PCV during first 21 days of infection.

| | | Mean PCV (± S.D.) | | |
|---|---|---|---|---|
| T.congolense: | n | Day 0 | Day 21 | Decrease 0-21 days |
| KETRI 2880 | 4 | 36.1 (3.1) | 27.4 (5.4) | 8.8 (4.9) |
| KETRI 2883 | 1 | 34 | 18 | 16 |
| KETRI 2885 | 4 | 35.3 (2.4) | 22.0 (3.7) | 13.3 (4.6) |
| 82/1 | 1 | 31 | 25 | 6 |

and zebu Boran cattle determined in a previous experiment (see Chapter 3), is presented in Table 22. *T. congolense* KETRI 2880 had the longest pre-patent period in both host species.

Pathogenicity: The effect of infection with strains of *T. congolense* on PCV during the first 21 days post-infection is shown in Table 23. The limited number of animals available, and the wide variation within the strains, makes it difficult to compare the relative pathogenicities of the strains in goats.

Goat Y98, infected with *T. congolense* 82/1, developed severe clinical signs around day 30 post-infection. Although it had a PCV of 22, the animal was dull and lethargic with drooping ears and dilated pupils, and prolonged pyrexia with rectal temperatures of up to 41.9°C being recorded.

Course of parasitaemia: Two goats were infected with *T. congolense* KETRI 2880 and maintained without treatment. One of the goats remained continuously parasitaemic from first detection of trypanosomes through to day 70 post-infection, when observation was discontinued. The second goat became parasitaemic on day 14. No trypanosomes could be detected on day 15 or day 29 post-infection, but otherwise it was continuously parasitaemic up to day 35, when treatment was administered.

One goat was infected with *T. congolense* KETRI 2883 and maintained without treatment up until day 26 post-infection. From first detection of trypanosomes on day 7, until treatment on day 26, it was not possible to detect trypanosomes on only one occasion (day 12).

Four goats were infected with T. congolense KETRI 2885 and maintained without treatment. In three of the goats trypanosomes could not always be detected: goat Y97 was apparently aparasitaemic on four occasions during a 70 day observation period (days 40, 44, 54 and 78); goat F185 was apparently aparasitaemic on day 24 post-infection and goat F195 on days 22 and 24. Both F185 and F195 were monitored up until day 27 post-infection.

A single goat, infected with T. congolense 82/1, was maintained until day 33 post-infection before intervention with trypanocidal drugs. After the pre-patent period, on one occasion (day 16) trypanosomes could not be detected.

Selection of Strains of T. congolense

Three strains of T. congolense were selected for attempted induction of changes in Isometamidium sensitivity in goats; T. congolense KETRI 2880, KETRI 2883 and T. congolense 82/1. The former two strains had previously been used in the cattle/mouse sensitivity test in Kenya, and therefore Isometamidium dose rates likely to be subcurative in ruminants were already known: T. congolense KETRI 2880 had a MCD in cattle of 2.0 mg/kg, and cattle were temporarily cleared of circulating trypanosomes by treatment at 0.1 (2/3 cattle), 0.5 or 1.0 mg/kg. T. congolense KETRI 2883 had a MCD in cattle of 0.5 mg/kg, and treatment at 0.1 mg/kg resulted in temporary remission of infection in two out of three cattle. T. congolense KETRI 2885 could not be used for attempted production of Isometamidium resistance, because previous work in cattle had shown the lowest dose administered, 0.001 mg/kg to be curative. Comparative data for T. congolense 82/1 was only available from mice: the MCD in this species was 2.0 mg/kg.

Attempted Induction of Changes in Isometamidium Sensitivity in Goats

Methods

A single goat was infected with the appropriate strain of T. congolense, with an inoculum of approximately 1 x $10^5$ trypanosomes being administered by iv inoculation. Rectal temperature was recorded daily at 09.00 hrs and at the same time a jugular blood sample was collected for estimation of PCV and parasitaemic status. Bodyweight was measured once a week. When trypanosomes were first detected, by the capillary concentration/buffy coat method, the goat was treated with the selected dose of Isometamidium, administered by deep intramuscular injection into the quadricep femoris muscle of the hind leg. Dosages of 0.05, 0.1 or 0.2 mg/kg Isometamidium were prepared as 0.25% (w/v) solutions using distilled water. A dosage of 0.5 mg/kg was prepared as a 1% (w/v) solution. Repeated treatment was administered alternately in the left and right leg. After treatment the goat was again monitored daily. When a relapse infection was detected, the parasitaemia was monitored for 7 days. On day 7 a jugular blood sample was collected and 0.5 ml aliquots of blood were subinoculated into each of five mice which had been sublethally irradiated 24 hours previously (600 rads). The goat was then retreated with the selected dose of Isometamidium, monitoring was continued, and the above procedure repeated. Stabilates of the subinoculated relapse infections were prepared by pooling blood from the five mice close to the first parasitaemic peak. In this way a series of relapse populations was isolated and stabilated for each strain.

Table 24. The interval to relapse following treatment and retreatment with Isometamidium.

| Day post-infection | Isometamidium mg/kg | Treatment-relapse interval days |
|---|---|---|
| KETRI 2880 (Y92) | | |
| 12 | 0.1 | 9 |
| 27 | 0.1 | 6 |
| 38 | 0.2 | 8 |
| 53 | 0.5 | 15 |
| 76 | 0.5 | 38 |
| 124 | 0.5 | >46 |
| KETRI 2883 (Y95) | | |
| 7 | 0.1 | 7 |
| 20 | 0.1 | 13 |
| 39 | Killed in extremis | |
| KETRI 2883 (Y100) | | |
| 8 | 0.1 | 4 |
| 19 | 0.1 | 11 |
| 36 | 0.1 | 12 |
| 55 | 0.1 | 14 |
| 79 | 0.1 | 13 |
| 99 | 0.1 | 15 |
| 82/1 (Y91) | | |
| 7 | 0.1 | >42 |
| 82/1 (Y197) | | |
| 11 | 0.05 | 22 |
| 41 | 0.05 | >46 |

Results

T. congolense KETRI 2880: The single goat (Y92) infected with T. congolense KETRI 2880 became parasitaemic on day 12 post-infection, and was treated with Isometamidium at 0.1 mg/kg. The interval from treatment to relapse following this and subsequent treatments with Isometamidium, are shown in Table 24. The results indicate that repeated subcurative treatment with Isometamidium did not result in a significant change in sensitivity.

T. congolense KETRI 2883: Goat Y95, infected with T. congolense KETRI 2883, became parasitaemic on day 7 post-infection, and was then treated with Isometamidium at 0.1 mg/kg. The intervals to relapse following treatment and retreatment with this dose are shown in Table 24. Goat Y95 relapsed to repeat treatment with 0.1 mg/kg Isometamidium on day 33 post-infection. The relapse infection was accompanied by pyrexia, with a peak rectal temperature of 40.3°C being recorded. On day 38 post-infection the animal was sternally recumbent: although it could stand with assistance it was ataxic. It was also noticed to be drinking more than normal. On day 39 the condition had deteriorated to lateral recumbency, with opisthotonus, paddling of the limbs, bleating and a sub-normal temperature (37.6°C), and the goat was killed by administration of an overdose of pentobarbitone. An autopsy was performed and the principal findings were; congested intestine, haemorrhage on the surface of the lung and old Fasciola lesions in the liver. An attempt to collect a blood-free sample of cerebrospinal fluid (CSF) for mouse subinoculation was unsuccessful, because/

because the CSF was very viscous: this may have been a post-mortem change. These findings seem consistent with trypanosomiasis with cerebral involvement (Whitelaw, Moulton, Morrison and Murray, 1985), but without demonstration of trypanosomes in the CSF this can only be a speculative diagnosis. A second goat (Y100) was therefore infected with this strain, and became parasitaemic on day 7. Treatment with Isometamidium was accidentally delayed until day 8. The intervals to relapse following treatment and retreatment with 0.1 mg/kg Isometamidium are shown in Table 24. Relapse after initial treatment at this dose occurred in 4 days, but thereafter relapses occurred in 11 and 15 days. The consistent interval between treatment and relapse suggests that no decrease in sensitivity had occurred during repeated (6) exposure to sub-curative Isometamidium doses in goats.

T. congolense 82/1: Goat Y91, infected with T. congolense 82/1, became parasitaemic by day 6 post-infection, and was treated with Isometamidium at 0.1 mg/kg. The dose was selected based on mouse data, and represented 1/20th of the mouse MCD. Following treatment no parasites were detected during a 42 day observation period. A second goat was therefore infected with this strain, and became parasitaemic by day 11 post-infection. Treatment was then administered at a dosage of 0.05 mg/kg, and a relapse infection was first detected 22 days later. No relapse infection was detected following retreatment at this dosage during a 46 day observation period, after which the experiment was terminated.

Discussion

The present experiment demonstrated that in both mice and goats, there was no significant change in Isometamidium sensitivity following repeated subcurative treatment. In mice, after nine successive exposures to subcurative doses of Isometamidium, no change in sensitivity, compared to the parent strain, could be demonstrated. Similarly, up to six successive subcurative treatments in goats, failed significantly to alter the Isometamidium sensitivity of a number of strains of T. congolense. These findings are in some respects similar to those of Whiteside (1962) who reported that drug resistant strains of T. congolense were difficult to produce in mice. However, he suggested that drug resistant strains were much more easily produced in cattle. During the course of the present study a facility became available in which trypanosome infected goats could be housed, and so it was decided to attempt to induce drug resistance using goats as hosts.

The strains of T. congolense used in cattle and goats seemed to behave similarly in the two species in terms of infectivity, prepatent periods and response to Isometamidium. For example, cattle infected with KETRI 2880 relapsed to treatment with Isometamidium at 0.5 mg/kg in a mean of 15.7 days: a goat infected with the same strain relapsed to initial treatment at 0.5 mg/kg in 15 days. Similarly, cattle infected with KETRI 2883 and treated at 0.1 mg/kg Isometamidium, relapsed in a mean of 8.3 days: of three cattle, one did not experience remission of infection following treatment, while the other two relapsed after 11 and 14 days, respectively. A goat infected with the same strain, relapsed to repeat treatment with 0.1 mg/kg in 11 to 15 days.

Goat Y100, infected with T. congolense KETRI 2883, was treated with six successive doses of 0.1 mg/kg Isometamidium. The intervals from treatment to first detection of the relapse infections were remarkably consistent. Williamson and Stephen (1960) suggested the use of the subcurative treatment to relapse interval as an index of relative sensitivity: the shorter the interval, the more resistant the strain. On this basis it would appear that for this strain, repeated subcurative treatment has not resulted in any shift in Isometamidium sensitivity.

Goat Y92, infected with T. congolense KETRI 2880 was treated with successive Isometamidium dosages of 0.1, 0.1, 0.2, 0.5 and 0.5 mg/kg. On the basis of the intervals from treatment to relapse, no major decrease in Isometamidium sensitivity has occurred. Ideally the sixth relapse infection, ie the relapse infection which occurred after the third treatment at 0.5 mg/kg, would have been used to infect a new goat which would then have been treated at 0.1 mg/kg Isometamidium, to see if this dose was still effective. Demand for the fly-proof animal facility precluded this possibility.

The increased interval from treatment to relapse following successive treatment with 0.5 mg/kg Isometamidium, was probably due to the combined effect of the latest Isometamidium treatment together with the residual effect of previous treatments. Adoption of an experimental design similar to that used for mice would overcome this complication, but the increased number of animals required would stretch animal facilities and be prohibitively expensive.

These experiments suggest that changes in Isometamidium sensitivity of strains of T. congolense do not readily occur as a result of deliberate underdosing.

# CHAPTER SEVEN

## STUDIES WITH MIXED INFECTIONS OF ISOMETAMIDIUM SENSITIVE AND RESISTANT STRAINS OF T.congolense

Introduction

Documented incidences of Isometamidium resistance in the field are relatively few in number. This is surprising in view of the often expressed opinion that prophylactic drugs are more likely to give rise to drug resistance than curative drugs (Whiteside, 1962), and that Isometamidium has been widely used, and abused, for nearly 30 years. One explanation for this paradoxical situation is that most drug resistant strains of trypanosomes, and other microorganisms, are less viable than normal strains, and that in mixed infections the former tend to die out (Cantrell, 1956), possibly due to their lower replication rates.

This experiment investigates the ability of two unrelated strains of T. congolense, one of which is highly sensitive to Isometamidium (T. congolense KETRI 2885) and one of which is resistant to the manufacturer's recommended dosage in cattle (KETRI 2880), to become established in the face of an existing infection with the other strain.

Methods

Three goats were infected by the intravenous route with approximately $1 \times 10^5$ trypanosomes T. congolense KETRI 2885. Rectal temperatures were recorded three times a week and at the same time a jugular blood sample was obtained for measurement of PCV and to assess parasitaemia by the capillary concentration technique and examination of the buffy coat. Seven days after the first detection of trypanosomes two of the goats were superinfected by/

Table 25 Experimental design and response to treatment with Isometamidium.

| Goat number | Primary infection | Day | Secondary infection | Day | Isometamidium (mg/kg) | Day | Response to treatment |
|---|---|---|---|---|---|---|---|
| F186 | KETRI 2880 | 0 | KETRI 2885 a | 21 | 0.1 | 35 | Temporary remission |
| Y96 | KETRI 2880 | 0 | KETRI 2885 a | 21 | 0.1 | 35 | Temporary remission |
| F188 | KETRI 2880 | 0 | | | 0.1 | 35 | No effect |
| F194 | KETRI 2885 | 21 a | | | 0.1 | 35 | Cure |
| F187 | KETRI 2885 | 0 | KETRI 2880 b | | 0.1 | 35 | Cure |
| F178 | KETRI 2885 | 0 | KETRI 2880 b | | 0.1 | 35 | Cure |
| F200 | KETRI 2885 | 0 | | | 0.1 | 35 | Cure |
| F184 | KETRI 2880 | 15 b | | | 0.1 | 35 | No effect |

a Same inoculum

b Same inoculum

by iv inoculation with approximately 1 x $10^5$ trypanosomes T. congolense KETRI 2880, and an additional goat was also infected with the same inoculum to serve as a challenge control. Twenty days after the superinfection, all four goats were treated with Isometamidium at 0.1 mg/kg by deep intramuscular injection in the quadricep femoris muscle of the hind leg.

Three more goats were infected iv with approximately 1 x $10^5$ trypanosomes, T. congolense KETRI 2880, and seven days after first detection of parasites two of the goats were superinfected with T. congolense KETRI 2885. At the time of the superinfection an additional goat was also infected with T. congolense KETRI 2885 as a challenge control. Fourteen days after the superinfection all four goats were treated with Isometamidium at 0.1 mg/kg.

After treatment with Isometamdium, all goats were monitored three times a week for rectal temperature, PCV and parasitaemia. The experimental design and outcome of treatment with Isometamidium are shown in Table 25.

Results

For the three goats infected on day 0 with T. congolense KETRI 2880, parasites were first detected on day 14 post-infection. Two of these goats were then superinfected on day 21 with a second strain of T. congolense KETRI 2885. An additional, previously uninfected, goat was also infected with T. congolense KETRI 2885 on day 21, to serve as a challenge control, and it became parasitaemic 8 days later. This demonstrated that the inoculum used for the superinfection/

superinfection was infective. The two goats which received the superinfection experienced temporary remission of infection following treatment with Isometamidium at 0.1 mg/kg on day 35, with trypanosomes again being detected 5 and 8 days after treatment, respectively. In contrast, the goat which received only the primary infection of T. congolense KETRI 2880 remained continuously parasitaemic following treatment. The challenge control, infected with the same inoculum used for the superinfection, was cured by treatment at 0.1 mg/kg: no parasites were detected during a 40 day post-treatment observation period, after which the experiment was terminated.

The three goats infected on day 0 with T. congolense KETRI 2885 became parasitaemic on day 8. Two of the three goats were then superinfected on day 15 with T. congolense KETRI 2880. An additional, previously uninfected, goat infected at the same time with T. congolense KETRI 2880 to serve as a challenge control, became parasitaemic 13 days later, thus demonstrating that the inoculum used for the superinfection was infective. All three goats infected on day 0 with T. congolense KETRI 2885 were apparently cured by treatment on day 35 with 0.1 mg/kg Isometamidium irrespective of whether or not they had been superinfected with T. congolense KETRI 2880: no trypanosomes were detected during a 22 day post-treatment observation period. The challenge control goat, infected with the same inoculum as was used for the superinfection (T. congolense KETRI 2880) remained parasitaemic after treatment at 0.1 mg/kg.

The different intervals from superinfection to treatment with Isometamidium were selected to reflect the different prepatent periods of the two strains used in the experiment. In both cases treatment was administered approximately 7 days after parasites were first detected in the goats used to test the infectivity of the superinfection inoculum.

Discussion

The two strains of T. congolense used in this experiment were of different Isometamidium sensitivities. T. congolense KETRI 2885 was highly sensitive to Isometamidium (MCD in cattle $\leqslant$ 0.001 mg/kg), while KETRI 2880 was relatively resistant (MCD in cattle 2 mg/kg) (Sones, Njogu and Holmes, in press). Goats which were initially infected with the sensitive strain, and were then challenged with the resistant strain were cured by treatment at 0.1 mg/kg Isometamidium, indicating that the resistant strain did not establish an infection. Goats initially infected with the resistant strain, which were then challenged with the sensitive strain experienced temporary remission of infection followed by relapse after treatment at 0.1 mg/kg Isometamidium. In contrast, the goat infected only with the resistant strain remained parasitaemic following treatment at 0.1 mg/kg. This suggests that superinfection with the sensitive strain resulted in the establishment of infection, which suppressed the resistant strain to below the limit of detection of the method used. Only after treatment, at 0.1 mg/kg, which eliminated the sensitive strain, could the resistant strain re-establish itself.

This experiment lends support to the theory expressed by Cantrell (1956) that 'most drug-resistant strains ... are less well adapted to life in the absence of the drug than their unmodified parents'. Cantrell worked with an oxophenarsine resistant strain of T. equiperdum and its unmodified parent strain, and demonstrated that the resistant daughter strain was unable to maintain itself in a mixture with the parent during passaging in rats. However in one rat passaged line the resistant strain reverted to sensitive, 6 - 8 months after the last exposure to drug, hinting that a change in the sensitivity of the resistant strain may have contributed to its disappearance.

Oehler (1914) also reported that an arsphenamide resistant strain of T.b. brucei was displaced by the parent strain during mouse passage in the absence of drug, and he attributed this to differences in growth rates of the two strains. Other workers however have found the resistant strain to displace the sensitive parent (Laveren and Roudsky, 1912), or the results to be variable (Teichman, 1918).

More recently several workers have reported an interference phenomenon operating in the establishment of superinfections of T. congolense in ruminants, whereby the presence of an active infection of one serodeme inhibited the establishment of a second infection with a different serodeme (Morrison, Wells, Moloo, Paris and Murray, 1982; Dwinger, Luckins, Murray, Rae and Moloo, 1986). The interference phenomenon appeared to be independent of specific antibody/

antibody and required the presence of an active infection, and occurred whether infection was brought about by inoculation of bloodstream forms, or by the bite of infective tsetse flies. With different combinations of serodemes the interference effect observed varied from insignificant to marked. Morrison et al. (1982) postulate that competition between the trypanosome populations may be implicated, and it is possible that the relative competitive advantage of a strain is related to its drug sensitivity. Dwinger et al. (1986) found the interference phenomenon to be dependent on the interval between the primary and secondary infections, both being administered by the bite of infective tsetse flies. The interference phenomenon was maximal at day 7, and diminished from day 14 onwards, and no interference was demonstrated when infection with the two unrelated serodemes occurred simultaneously. However, Morrison and co-workers were able to demonstrate a marked interference when superinfection was administered by either needle challenge with bloodstream forms or by infective tsetse flies, 35 days after the primary infection. The intervals between the primary and secondary infections in the present experiment were dependent on the prepatent period of the primary infection: superinfection was carried out approximately seven days after trypanosomes of the primary infection were first detected. Although it is possible that the observed differences were due to the timing of super-infection, this seems unlikely, in view of the fact that Morrison et al. (1982) observed interference when superinfection was delayed

to/

to day 35 using needle challenge of bloodstream forms, a similar protocol to the present experiment in which superinfection was carried out by this method on day 15 (primary infection sensitive; secondary infection resistant) or day 21 (primary infection resistant; secondary infection sensitive).

In contrast to the findings of Morrison et al. (1982) and Dwinger et al. (1986), in which interference was solely restricted to the development of a superinfection, in the present study the sensitive strain was dominant, regardless of whether it was the primary or secondary infection.

It is possible that the observed differences in viability of the two strains in mixed infections is incidental to the differences in Isometamidium sensitivity, and may, for example, simply reflect differences in replication rates. In some respects it would have been preferable to perform the experiment using a laboratory produced, resistant daughter strain, and the unmodified parent strain. Attempts to produce such strains by a process of repeated subcurative treatment of strains of T. congolense in both mice and goats failed to induce any change in sensitivity to Isometamidium. It should be borne in mind however that antigenic similarities between parent and daughter strains may influence the results of such an experiment because of immunological responses.

If drug resistant strains which arise in the field tend to be less viable than normal strains, this could explain the relative scarcity of Isometamidium resistance. Following treatment with Isometamidium,/

Isometamidium, the period of prophylaxis against drug sensitive strains can extend to six months (Whitelaw et al., 1986). However during this period, as the level of circulating trypanocide declines, challenge with Isometamidium resistant strains may result in successful establishment of infection. It has been suggested that drug resistant strains may be of low pathogenicity (Stephen, 1960) and they may also have low replication rates (Hawking, 1963a), and therefore this infection may be of little clinical significance. As the level of circulating trypanocide continues to wane, treated animals will eventually also become susceptible to challenge with drug sensitive strains. It is possible that at this stage, infection with trypanosomes of normal sensitivity could overgrow, and eventually eliminate, the resistant trypanosomes.

The results of this experiment may have interesting practical implications for the control of drug resistance, and may even indicate that complete prophylaxis is not always necessary or desirable. Further work in this interesting field is clearly warranted.

# CHAPTER EIGHT

## GENERAL DISCUSSION

Bovine trypanosomiasis is acknowledged to be one of the major constraints limiting livestock production in sub-Saharan Africa (Trail et al. 1985). In the absence of an effective vaccine and lacking a coherent strategy for controlling the insect vectors, the livestock industry is highly dependent on the small number of drugs currently available for the treatment and prevention of the disease. Amongst that limited range of drugs, Isometamidium is perhaps the most widely used trypanocide.

Although examples do occur in the literature of demonstrable field resistance to Isometamidium, and indeed to the other trypanocidal drugs, the term 'drug resistance' is frequently used to explain any therapeutic or prophylactic failure, often with little or no supporting evidence. The existence of drug resistance strains, though important, is only one of many possible explanations for such failures. This makes it particularly important to be able to investigate reported incidences of drug resistance from the field, and ideally to be able to evaluate the sensitivities of the strains of trypanosomes implicated.

Three basic approaches to the assessment of drug sensitivities of strains of T. congolense have been explored in the present studies; tests conducted using mice or cattle as hosts and tests carried out using an in vitro system. The mouse sensitivity test has the advantage of being inexpensive in terms of experimental animals, animal housing and maintenance. Laboratory mice are widely available, and the method has the additional advantage of being

low/

low tech, not reliant upon imported facilities and supplies, and therefore is capable of being carried out on the spot in Africa. This is important because disease control regulations make it difficult to export isolates of trypanosomes, for example to the UK, and therefore tests need to be applicable to local African conditions. The mouse model, however has been shown to have limitations in the degree of correlation between mouse and cattle results, and can therefore only give a broad indication of the sensitivity of a strain, and cannot be relied on to predict curative doses for cattle.

Sensitivity tests carried out using in vitro systems have the potential advantages of being quick, and of eliminating much of the biological variation encountered in animal experiments. However, with the present state of the art, the approach has the major disadvantage that most strains of T. congolense need to undergo adaptation to in vitro culture before an in vitro sensitivity test can be carried out. This adaptation procedure can take several months for some strains, and it is possible that the in vitro adapted population varies from the initial population in important respects, such as drug sensitivity. A similar criticism can also be levelled at mouse sensitivity tests: the population which becomes established in mice may vary from the population which occurred in the original bovine host. An in vitro approach requires sophisticated, imported equipment, such as microbiological cabinets and incubators, all of which need regular, expert servicing, and regular inputs of media/

media, sera and sterile laboratory ware are also required. The equipment, servicing and supplies are all expensive and are unlikely to be locally available in most African countries.

The most appropriate and meaningful approach to sensitivity testing of strains of trypanosomes in an African context is undoubtedly tests using the definitive host, cattle. Such tests are more expensive to conduct than mouse tests in terms of animals and feedstuffs, but there is no reason why the animals used cannot be cured by chemotherapy at the end of an experiment and disposed of through commercial outlets for slaughter, thus recouping some of the expenditure. A possible problem in some locations may be the supply of trypanosome-naive cattle. In Kenya, for example, there are tsetse-free areas from which trypanosome-naive cattle can readily be obtained, but in some African countries this may not be the case. The provision of fly-proof accommodation for experimental cattle is a prerequisite. Such housing is relatively expensive to build, and is difficult to maintain intact, but is important, even outside the tsetse-infested areas. Biting flies could transfer infection from infected to uninfected cattle, which would confuse the outcome of an experiment, and there is also the risk of biting flies carrying the infection to neighbouring cattle. The consequences of the latter could be especially serious if drug resistant strains were involved.

Probably the best, most practical approach to the assessment of drug sensitivities in most circumstances is to collect blood from/

from animals naturally infected with the trypanosome strain of interest; transfer the blood to the laboratory and expand the primary isolate by infecting a trypanosome-naive calf to provide sufficient trypanosomes to infect the required number of cattle, and to prepare a stabilate for future use; infect a minimum of three cattle with approximately $1 \times 10^5$ trypanosomes per head by intravenous inoculation; treat some animals at first detection of trypanosomes and retain others as untreated controls; and monitor cattle for response to treatment and occurrence of relapse infections. If the minimum of three cattle are used, one should be left untreated and the other two treated with the dosage of drug used in the field. Isometamidium is usually used at 0.5 or 1.0 mg/kg, so treatment at either of these doses would allow the sensitivity to the recommended dosage to be assessed using a therapeutic protocol. This range covers the level of sensitivity of practical significance.

The duration of the observation period following treatment is a contentious issue. In the present study cattle were observed for 100 days post-treatment. The longer the period selected, the more confident one can be that all potential relapse infections have occurred, but excessively long periods increase the cost of an experiment and limit the throughput of strains in a given facility. A 100 day post-treatment monitoring period is probably a reasonable compromise between experimental design and more practical considerations.

Another practical problem is the delay between collecting blood samples from naturally infected cattle in the field, and subinoculating the material into a naive calf in the laboratory. In the past Jones-Davies and Folkers (1966) overcame this problem by transporting calves by lorry to cattle treatment camps, and subinoculating blood samples as they were collected. Up to 80 blood samples were subinoculated into a calf, and animal welfare considerations apart, it would be difficult to ensure that the calf was not bitten by tsetse infected with other strains.

Ilemobade et al. (1975) collected blood from T. vivax infected cattle, with 20 ml samples being collected into chilled, heparinised Universal bottles, which were then stored in ice. Samples kept under these conditions for up to 4 hours retained their infectivity. Although it would be possible to prepare stabilates of infected blood in the field, and transport them back to the laboratory in liquid nitrogen or solid carbon dioxide, this is unlikely to be practicable in most circumstances.

All the drug sensitivity work carried out in this thesis was performed adopting a curative protocol. Isometamidium is most often used as a prophylactic agent, but it has generally been considered in the past that drug sensitivity assessed by a therapeutic protocol would be a reasonably good indicator of sensitivity to the prophylactic activity of a trypanocide. Recent work by Peregrine, Moloo and Whitelaw (1987), however, brings this assumption into doubt, at least for T. vivax. They found that, whereas treatment of a clone of T. vivax with 0.5 mg/kg Isometamidium resulted/

Table 26. Some examples of the duration of Isometamidium prophylaxis.

| Cattle | Challenge | Isometamidium (mg/kg) | Mean period of protection (days) | Ref: |
|---|---|---|---|---|
| Cattle | - | 1.0 | 186.5 | Boyt (1963) |
| Steers | med./high | 0/5 | 99.4 | Fairclough (1963) |
| Zebu cattle | high | 1.0 | 97.0 | Kirkby (1964) |
| Zebu steers | - | 0.5 | 168[a] | Wiesenhuetter (1968) |
| | | 1.0 | 217[a] | |
| Grade dairy cattle | - | 0.5 | 66.5 | Wiesenhuetter (1968a) |
| | - | 1.0 | 87.5 | |
| Work oxen | high | 1.0 | 35[b] | Scott & Pegram (1974) |
| Boran cattle | high | 0.5/1.0[c] | 79.3 | Trail et al., (1985) |
| Zebu cattle | - | 1.0 | 14-42[d] | Pinder & Authie (1984) |

a
10% infection rate.

b
66% infection rate.

c
See pp. 19-20.

d
Some animals trypanosome positive by 14 days; many by 42 days.

resulted in cures in all five cattle, the same dosage resulted in less than one month's prophylaxis. They concluded that the level of sensitivity of a T. vivax population to the prophylactic activity of Isometamidium, could not be derived from sensitivity tests conducted using a therapeutic protocol, and suggested that investigations of putative Isometamidium resistant trypanosomes should be conducted using prophylactic protocols.

Adoption of prophylactic protocols to test drug sensitivity will be more difficult than therpaeutic tests. The tests are likely to take longer, and the point at which it is decided a prophylactic dose is ineffective depends on the expectation of the experimenter. Duration of prophylaxis achieved under laboratory or field conditions varies considerably. Some examples of the duration of protection achieved using Isometamidium under different field conditions are shown in Table 26. The range is wide, varying from less than 14 days to more than 200 days.

Recently a number of laboratory studies have examined the influence of various factors on the duration of Isometamidium prophylaxis. Complete protection from either single or monthly challenge with five infected tsetse flies, of at least five months duration, was reported by Whitelaw et al. (1986) following treatment of cattle with 1 mg/kg. The serodeme used was T. congolense IL Nat. 3.1, from which KETRI 2885, used in this thesis, was derived. Two thirds of the cattle also resisted challenge with up to 500,000 in vitro derived metacyclics, or multiple tsetse challenge at six months. Peregrine et al. (in press) found

treatment/

treatment at 1.0 mg/kg, followed by challenge with the same serodeme, or a second unrelated serodeme, provided complete protection for three months. Significant differences between serodemes were demonstrated by Peregrine et al. (1987). They showed that whereas for a serodeme of T. vivax from Nigeria, treatment at 0.5 mg/kg provided at least two months protection against challenge, for a serodeme originating in Kenya, complete protection was afforded for less than one month.

Any period of prophylaxis which is selected as being 'normal' for the purposes of a sensitivity test will be an arbitrary choice, but protection of less than one month would generally be considered suboptimal, and more than two months would be desirable. One possible protocol for the routine testing of the prophylactic sensitivity of isolates of trypanosomes would be to treat a minimum of two cattle with 1 mg/kg Isometamidium and challenge them 30 days later with, ideally, tsetse infected with the trypanosome strain under investigation. Less satisfactory, but easier, would be needle challenge with bloodstream forms. At the same time, an untreated steer would also be challenged in a similar manner to act as a challenge control. If challenge was successfully resisted, and no trypanosomes were detected in treated animals during the next 30 days, challenge would be repeated on day 60 post-treatment, again including an untreated animal. If challenge at 30 days resulted in establishment of parasitaemia, then the strain in question could be considered, for practical purposes, resistant to the prophylactic/

prophylactic activity of Isometamidium. Complete protection at 60 days would indicate sensitivity, and resistance to challenge at 30 days, but not 60 days, would indicate intermediate sensitivity.

In summary, to obtain a complete picture of the Isometamidium sensitivity of a strain of trypanosome, tests in cattle adopting both therapeutic and prophylactic protocols should be performed.

The minimum effective dose (MED), ie the minimum dose which will cause a temporary clearance of circulating trypanosomes, is a parameter of drug sensitivity commonly reported in the past. Consideration of MED's prompted studies of the apparent aparasitaemic interval following subcurative treatment with Isometamidium of T. congolense infections in a mouse model. Though inconclusive, these studies suggested that trypanosomes survive in the bloodstream in low numbers, rather in cryptic foci, such as has been described for T.b. brucei infections in mice. Further work would be of value in this field, using both the mouse model and ruminants, to improve our understanding of host-parasite and parasite-trypanocide interactions.

Drug resistance is frequently considered to be the major problem associated with the use of trypanocidal drugs. The use of some drugs has undoubtedly been influenced by the emergence of drug resistant strains. In 1965, for example, the use of homidium was discontinued in Nigeria in favour of diminazene aceturate, due to the discovery of homidium resistant strains of T. congolense (Na'Isa, 1967). Reported incidences of resistance to Isometamidium are/

are relatively scarce, with only a handful of documented accounts in the literature. This is surprising since Isometamidium has been in use for approaching 30 years, and is frequently misused, and also in view of the often expressed opinion that drugs with prophylactic activity are more likely to induce drug resistance in the field than those with only curative activity. Two aspects of resistance to Isometamidium, studied as part of this thesis, may help to explain this situation.

First, deliberate attempts to induce changes in sensitivity by repeated, subcurative treatment, failed to alter significantly the Isometamidium sensitivities of strains of T. congolense, both in mice and goats. The ease with which Whiteside (1962) reportedly produced marked decreases in sensitivity by a few subcurative treatments in cattle, could not be repeated for Isometamidium in goats in the present studies. Secondly, a relatively Isometamidium resistant strain of T. congolense was apparently unable to establish an infection in the face of an existing infection with a sensitive strain, although in the reverse situation the sensitive strain established an infection and suppressed the resistant strain. More work in this important and interesting area is clearly indicated, but these early observations suggest that at least some Isometamidium resistant strains of T. congolense cannot compete in mixed infections with sensitive strains.

Although the importance of drug resistance may sometimes have been overstressed, this does not mean that reasonable steps should not be taken to reduce the likelihood of resistant strains from/

from arising. In particular the use of an effective sanative, ie a drug which is effective against strains resistant to the trypanocide in use, should be central to any chemoprophylactic regime. The narrow range of trypanocides available makes the choice of a trypanocide to fulfil this role difficult. Until recently, cross-resistance between Isometamidium and diminazene aceturate was thought not to occur, but Authie (1984) reported strains of T. congolense resistant to both these trypanocides, and in the present studies a strain of T. congolense isolated from the same area was shown to be resistant to the manufacturer's recommended doses of the two trypanocides. In spite of this, diminazene aceturate is the sanative of choice for most prophylactic regimes based on Isometamidium. For example the regime currently in use at Mkwaja Ranch, Tanzania consists of all animals in a herd receiving diminazene aceturate at 3.5 mg/kg, when monitoring of a herd indicates relapse or reinfection has occurred, followed one week later by Isometamidium at 1 mg/kg.

Isometamidium is likely to continue to play a major role in the control of African bovine trypanosomiasis for the foreseeable future. Even if alternative control strategies which seem to have considerable potential, such as increased use of trypanotolerant cattle, or the deployment of insecticide treated, tsetse attractant targets, fulfil their promise, trypanocidal drugs will still have an important part to play. Novel trypanocidal drugs are unlikely to become available within the next few years, indeed no new trypanocides have/

have been introduced since Isometamidium in the early 1960's, and therefore it is important that the maximum benefit is derived from the available products. Monitoring the incidence of drug resistance is an important element of any trypanosomiasis control programme which includes the use of trypanocidal drugs, and at the present time there is no substitute for sensitivity tests carried out in cattle. Although drug resistance is potentially an important constraint on the use of trypanocidal drugs, its impact on some products, including Isometamidium, may have been over-emphasised in the past. For a variety of reasons, widespread surveys to determine the extent of drug resistance have not been carried out in post-colonial Africa. There is clearly a need for more work to be done in this area, so that an accurate appraisal of the current situation can be made. The facts that in the present studies repeated, deliberate underdosing did not result in decreased sensitivity to Isometamidium of strains of T. congolense, and that some Isometamidium resistant strains appear to be less viable than strains of normal sensitivity, may help to explain why Isometamidium remains effective in most field situations after nearly 30 years use.

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