OBSERVATIONS ON THE ONSET OF DELAYED TYPE HYPERSENSITIVITY REACTIONS TO *DERMATOPHILUS CONGOLENSIS* IN RATS AND GUINEA-PIGS.

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ABSTRACT

Dermatophilus congolensis infections applied to areas of skin which are already showing a cell mediated response to 1-chloro-2, 4-dinitrobenzene (D.N.C.B.) give rise to lesions in laboratory animals, from which zoospores may be recovered over significantly longer periods and in larger numbers than in control animals. Such an increased chronicity had been suggested as an important step in the search for a laboratory model of the economically and clinically significant chronic field condition. This project investigated the hypothesis that a delay in the onset of cell mediated responses to D. congolensis infections is caused by the preexisting hypersensitive reaction to D.N.C.B. at the site of infection. Any such delay could support this explanation for the increased recovery period of D. congolensis zoospores.

Experiments were carried out on groups of both rats and guinea-pigs, both D.N.C.B. sensitised and control animals. In guinea-pigs skin tests were used to monitor the cell mediated response to D. congolensis antigen. Skin tests proved unsatisfactory in rats and a direct Macrophage Migrations Inhibition test was developed and used in their place.

In neither species did the D.N.C.B. sensitisation have a significant effect on the course of the onset of cell mediated responses to D. congolensis antigens. It was however recorded that the lesions induced by scarification of the skin sites showing an hyperimmune response to D.N.C.B. were grossly more severe than those in control animals.

Delay in cell mediated responses to D. congolensis does not appear to be a factor in the increased "chronicity" of zoospore recovery from infection sites showing a hyperimmune response to D.N.C.B.

INTRODUCTION

Dermatophilosis is an acute, subacute or chronic exudative dermatitis, cosmopolitan in distribution which has been described not only in mammals including man, but also in a number of avian and reptile species. The first authenticated record of the infection was made by Van Saceghem (1915) in Zaire, then the Belgian Congo, in 1915 when he named the causative organism Dermatophilus congolensis. The bacteria has been classified in many ways and ascribed various names since then, but following work by particularly Gordon (1964) and Roberts (1965a), the genus Dermatophilus is now considered to have a single species, D. congolensis, which is of the family Dermatophilaceae within the order Actinomycetales.

The clinical signs of Dermatophilus infections vary widely within and between the animal species affected and with the environment in which they are recorded. However in many parts of the world, particularly the humid tropics, the disease is of considerable economic importance due not only to the effects on the indigenous livestock industry, but also the restriction it places on the introduction of highly productive exotic animals into regions where dermatophilosis is endemic.

One of the major enigmas of this disease is that infection can give rise to a wide variety of clinical syndromes. Thus in humid tropical Africa a chronic form of progressive exudative dermatitis is a frequent sequela to infection of cattle, causing a severe loss of condition and a consequent decrease in productivity and increased susceptibility to other pathogenic agents. In temperate regions,

however, the infection of cattle, although probably quite a widespread phenomenon (Lloyd 1981), is only rarely clinically apparent or of any economic significance. The reasons for the enormous difference between the nature of the infections are largely unknown. Too little information is available on the significance of the numerous reported predisposing factors which create the conditions for a serious clinical syndrome, and on the mechanisms involved in its pathogenesis. A major reason for this deficiency is the lack of a suitable laboratory animal model of the chronic disease. Such a model could make it possible to obtain much valuable information on the underlying mechanisms involved in the disease in an economically feasible way. Some progress has been made in this direction by the use of 1-chloro-2, 4-dinitrobenzene (D.N.C.B.). Animals infected with D. congolensis at a site showing delayed type hypersensitivity to D.N.C.B. appear to carry the infection longer than unsensitised animals (Davis & Philpott 1980). To further this end, this project is aimed at studying the effect D.N.C.B. delayed type hypersensitivity reactions have on the cell mediated immune responses to D. congolensis infection.

LITERATURE REVIEW

Dermatophilus infections have been reported from a large number of species both wild and domesticated, but since dermatophilosis is not an officially notifiable disease in any country the true prevalence may well be much higher than is at present assumed to be the case. The infections of wildlife species are, not surprisingly, even less well documented than the domesticated. A general review of the problem in non-domesticated species given by Richard and Shotts (1976) makes it clear that many species are both infected and affected. Other reports range from the individual isolations from zebra and polar bears (Smith & Cordes 1972) to the description of an outbreak of the disease in roan Hippotragus equinus and sable Hippotragus niger in the Kreuger National Park (de Vos & Innes 1976). Although the information is very patchy it emphasises the widespread nature of dermatophilosis and the possible role that wildlife may play in the epizootiology of the disease.

Dermatophilosis in Equids

Of rather more direct commercial significance is the range of dermatophilosis syndromes in the domestic equids. The nature of the disease and the clinical signs found in both natural and experimental infections has been thoroughly described in horses. There are both acute, chronic, widespread and limited syndromes. Most infections are found on the back, loin and rump, although those of the head and girth areas are also frequently recorded. They commonly follow exposure to heavy rainfall particularly where horses

are turned out, usually on rough pasture, in seasons when such rainfall is common. D. congolensis has also been suggested as a contributory factor in the aetiology of such blanket terms as heel bug, rain scald, mud fever and cracked heels (Bentnick-Smith, Fox & Baker 1961; Scarnell1961).

The domestic donkey Equus asinus asinus is the only other equid for which much information is available (Lloyd 1971; Green 1960). Lloyd (1971) described in detail an outbreak of dermatophilosis in these animals which showed the full range of symptoms ranging from mild subacute to the chronic debilitating, disease. The animals under study were brought into an area of endemic infection in Nigeria, and poor nutrition, a seasonally high rainfall and humidity, babesiosis histoplasmosis and strongylosis as well as high tick and biting fly challenge were all suggested as factors affecting the pathogenesis of the disease.

These descriptions of the disease in a large number of species should not be allowed to obscure the fact that the major commercial impact of dermatophilosis is the effect it has on cattle and sheep husbandry worldwide.

Dermatophilosis in Sheep and Cattle: Distribution and Economic Importance

Many different names are given to these infections: Kirci, Nasin Gishu, Serci, Senkobo skin disease, cutaneous actinomycosis, strawberry footrot, and lumpy wool disease to name but a few, reflecting the complex aetiology of the infection vide infra and the impact it has had on the large number of cultures in which it has been described.

In Australia the major losses are due to the infection in sheep. Lumpy wool disease causes a serious downgrading of fleeces due to the damage to and staining of the wool fibres and the increased processing costs when the trapped exudate is removed from the staple. Such losses are significant wherever the fleece is a major component of the economic return from sheep husbandry. Both cattle and, more frequently, sheep infections have been reported from all the states of Australia and from most of the countries that make up Oceania, particularly Papua New Guinea, Tasmania and New Zealand (Stewart 1972).

Dermatophilosis is reported as a disease of considerable economic importance in domestic ruminants in both central and tropical South America (Moreira & Barbosa 1976). Information from subtropical South America is too fragmentary to do more than state that the infection exists, but the large numbers of commercial animals in these regions certainly suggest at least a potential commercial threat (Londero 1976). There have also been many reports of the disease from the North American continent since it was first isolated there in 1961 (Bentnick-Smith et al. 1961) but at present it seems to be of little economic consequence, although some serum surveys indicate that the prevalence of subclinical infections may be high (Richard, Thurston & Pier 1976).

A similar state of affairs pertains in Europe and the Mediterranean countries. Prevalence of the infection is probably quite high (Lloyd 1981; Hart 1967) but because of the relatively mild and intermittent nature of the disease syndrome, it is paid little attention. Nonetheless a decrease in milk yield of

approximately 20% was reported from affected Israeli dairy cattle (Nobel, Klopfer & Neumann 1976) and with agricultural margins being squeezed even the "widespread but extremely mild" manifestations in British sheep described by Hart (1967) may become significant.

It is, however, in Africa where dermatophilosis was first described that it is most widely recognised as a problem. Not only is the prevalence of the clinical infection high, but in Africa the relative economic and social importance of cattle and sheep husbandry is possibly greater than in any other continent.

The disease is widely recognised in sheep, both in the tropical and subtropical zones. Reports were made as early as 1928 of a lumpy wool syndrome in sheep in South Africa (Becker 1928 op.cit. Stewart 1972) and a report from the Gambia (Macadam 1976) indicates that in that country sheep are the chief source of D. congolensis isolations with an incidence of 7.5% affected within his survey. Lower incidences have been widely reported from other tropical African countries.

It is the infection of cattle nonetheless that is of paramount economic importance. Since the time of Van Saceghem there have been countless reports of isolations from cattle particularly in tropical Africa, and it is the seriousness of the disease in this region which fuels much of the research interest in the subject. Oppong (1976) reported incidences of infection as high as 12.8% and 4.8% for the wet and dry season respectively in Ghana, and Oduye and Lloyd (1971) that the average incidence in the wet season in N. Nigeria was @ 6% accounting for approximately 50% of all the reported skin disease cases in cattle. Bida and Dennis

(1976), working in the same region in 1970-71, recorded an incidence of 11.6% during the wet season, with 85% of the herds in the area affected to a greater or lesser degree. Bwangomoi (1976) summarising data from a number of sources concluded that dermatophilosis was second only to demodicosis in the frequency with which it was reported as a skin disease in cattle in East Africa.

Categories of Economic Loss

The losses due to the disease are even more difficult to assess than the actual incidence but it is nonetheless important to consider them. A number of authors have attempted to categorise the problems and make some indication of the attendant economic consequences (Lloyd 1976; Coleman 1967; Stewart 1972; Hyslop 1980; Bwangomoi 1976). These may be summarised thus:-

(i) Hide damage is of great importance. Hides from affected animals are seriously downgraded and are of virtually no value in the crucial foreign exchange export market. This problem is often exacerbated by the widespread habit of line branding around lesions in a vain attempt to prevent their spread, thus further damaging the skin (Lloyd 1976).

(ii) Meat production. In some regions the loss of meat production is considered to be of as much consequence as that due to hide damage. Both moderate and severe infections can lead to emaciation particularly in cattle which are on a low or inadequate plane of nutrition.

(iii) Draught animals are fundamental as a source of power to the rural economies of many of the areas in which dermatophilosis is prevalent. Even mild infections, if they affect the parts of

the skin which chafe against the yoke or harness (common sites of infection), can render the animal useless for work. The problem may be amplified if normal farm equipment is geared to a team of two animals, infection in one can effectively prevent the use of either.

(iv) Exotic cattle which may form a valuable genetic pool for the upgrading of local stock may be excluded from an endemic area by their extreme susceptibility to the disease (Lloyd 1976; Mammerikx 1961 op.cit. Lloyd 1976). Or they may require more exacting, and therefore more expensive, husbandry than if the disease were absent.

(v) In the dairy industry infection has been reported to lead to up to a 20% loss of milk production. However damage to the udder, or to the animal as a whole, may lead to:-

(vi) Early culling.

All the above mentioned problems have been reported to lead to early culling and the consequent costs of lost potential production and support of increased replacement numbers.

Epizootiology and Epidemiology

The epizootiology and epidemiology of dermatophilosis provide an almost unparalleled complex of interactions between multiple predisposing and precipitating factors and a single infectious agent. The result is a legion of skin infections which vary according to the species of animal infected and the predominant predisposing agent.

A common denominator amongst these factors is that they combine to allow the Dermatophilus zoospores access to the

uncornified epithelial cells, by compromising the three broad physical skin barriers to such access described by Roberts (1963a): namely the hair or fleece, the sebaceous wax layer and the stratum corneum.

The cosmopolitan distribution of the disease and the frequency of both subclinical and mild infections has led many to suppose that D. congolensis is a true saprophyte in the soil (Pulliam, Kelley & Coles 1967; Vandemaele 1961) or commensal of the skin (Graber 1969; Fox, Campbell & Reed 1973). There is as yet no complete evidence that either is the case. D. congolensis has been isolated from soil during dry periods and from dried skin debris up to three and a half years post infection (Bida & Dennis 1976; Roberts 1963b) but the organism soon dies if exposed to moisture and it is during the wet season (vide infra) that the infection is most frequently transmitted. Consequently soil is probably not an important source of infection, and Macadam (1970,1976) from his observations on the epidemiology of the disease considered it unlikely that the organism existed as a skin commensal. The chronic and/or subclinically infected carrier animal is now considered to be the major source of dermatophilosis from one season to another (Amakiri 1976; Bida & Kelley 1976; Roberts & Graham 1966; Stewart 1972). This theory was lent weight by Graber (1969) who demonstrated that up to 50% of apparently healthy cattle in parts of West Africa were carriers of the organism; and by Abu-Samra (1980) who demonstrated that some chronically affected cattle showed extensive infected lesions for well over a year. Thus it can be seen that in any endemic area there is likely to be a ready source of infection in

any population of animals. It is also widely recognised that the incidence of clinical infections is much higher during the rainy/wet seasons in these endemic areas (Ainsworth & Austwick 1973; Bida & Dennis 1976; Chodnick 1956; Ford, Cairns & Short 1974; Lloyd 1976; Plowright 1956; Roberts 1967a; Scarnell 1961; Stewart 1972). This implies that during this season factors exist which lead to an increase in the severity of individual infections or an increase in the rate of transmission of the disease, or as seems most likely, both of these.

The physical effects of a high rainfall season, particularly high intensity precipitation and high relative humidity, have been considered by some authors to be of prime importance in the upsurge of the disease which occurs during these periods. Skin wetting, and thus wetting of dormant infected scabs, can bring about the release of the motile zoospores and their movement out of the scab down the CO_2 gradient. Wetting of the skin may also allow spread of the infection from one part of the skin to another by leaching out the sebum, and softening the stratum corneum enough to allow penetration by the zoospores (Roberts 1967a). Some authors also consider that a high relative humidity is an important factor in the development of the lesions (Vandemaele 1961). Chodnick (1956) considered that these physical effects were the sole precipitating factors of dermatophilosis in British West Africa, while observations on the incidence of lumpy wool disease in the UK show a strong correlation with weather trends, particularly the cumulative rainfall during the previous summer season (Hart 1967), although it is now recognised that it is the intensity of this rainfall that is

probably of most importance (Hyslop 1980). In contrast to this emphasis on climatic conditions Macadam (1961) demonstrated that dermatophilosis lesions far from being exacerbated by high relative humidity in fact resolved much more rapidly in moist air conditions than dry. It was therefore important to consider the other alterations in the environment which occur concurrently with the onset of the rainy season (Lloyd & McEwan Jenkinson 1980). Of these, that which has received the most emphasis is the increased activity and numbers of parasitic arthropods. It has been demonstrated that D. congolensis can be transmitted by a large number of haemophagous and non haemophagous species of arthropods, falling into two main categories, the flies and ticks. Plowright (1956) demonstrated that some of the common sites of dermatophilosis coincided with the predilection sites for Amblyomma variegatum. In his experimental work D. congolensis infection was found to be present in 70%-100% of cattle with a heavy tick burden but was not detected at all in cattle subjected to effective dippings. Animals that were receiving acaricidal treatment or which were otherwise kept tick free did not contract dermatophilosis even when they were exposed to prolonged heavy rainfall. Subsequently the importance of ticks as transmitters of dermatophilosis has been recorded by a number of authors and reviewed by Oduye (1976) and Macadam (1962, 1976), in conjunction with other factors. Although tick transmission was considered to be of prime importance by Plowright (1956) following the effective control of the disease by the use of acaricides, tick transmission is not readily reconciled with the frequent reports of lesions which occur predominantly

away from tick predilection sites.

Many of these patterns of infection can be, and have been, ascribed to transmission of the organism by sucking and, more especially, biting flies. Richard and Pier (1966) demonstrated that dermatophilosis could be transmitted by both Stomoxys calcitrans and Musca domestica, and Philpott and Ezeh (1978) that this form of transmission could occur in cattle. The organism itself has been isolated from the mouthparts of a large number of fly species in the field (Macadam 1964a). This information is backed up by the observation that dermatophilosis upsurges are associated with increases in the number of flies and that the distribution of dermatophilosis lesions sometimes closely follows the predilection feeding sites for flies (Ainsworth & Austwick 1973; Ford, Cairns & Short 1974; Macadam 1964a, 1964b, 1970, 1976; Oduye 1976; Roberts & Graham 1966; Smith & Cordes 1972; Stewart 1972; Gherardi, Monzu, Sutherland & Johnson 1981). Other arthropods, particularly lice and mites especially Demodex sp., have been implicated in the spread and pathogenesis of the disease but their significance is very incompletely understood (Oduye 1976; Oppong 1976; Roberts & Vallely 1962).

The protective skin barriers may also be breached by a large number of inanimate objects, and if that object is contaminated an infection may well be set up. The nature of these fomites is multifarious. The branches of trees or any sharp or thorny vegetation are the most frequently cited examples (Harriss 1948; Oduye 1976; Zlotnik 1955), but stony or rough concrete floors and shearing clippers are also commonly reported (Nobel et al. 1976;

Roberts 1967a). It should also be borne in mind that any injury to the skin, particularly if it releases blood or serum, is attractive to flies and hence increases the likelihood of their transmitting the infection from one animal to another. The ubiquitous ox pecker bird Buphagus erythrorhychus and biting flies themselves are important contributors to this type of injury (Bida & Dennis 1976; Macadam 1970).

In conclusion the spread of dermatophilosis requires the following conditions:-

(i) A source of D. congolensis, in most cases the lesions of a chronically infected animal.

(ii) A vector (although there is some evidence that passive transmission can occur) (Austwick 1976; Le Riche 1968).

(iii) Conditions suitable for the release of infective zoospores into the damaged skin site.

(iv) The breakdown of the protective skin barriers.

The combination of these is most frequently, although far from exclusively, seen during the wet season in the humid tropics.

Host Reactions and Defences: Non Specific Reactions

Dermatophilosis is an infection of the skin and except in some artificially induced cases (Abu-Samra 1978; Abu-Samra, Imbabi & Mahgoub 1976), its lesions are confined to that organ. Mammalian skin has a number of properties which are adapted to the non specific resistance to infection by surface pathogenic bacteria and these are of particular importance when considering the pathogenesis of D. congolensis. They are also of significance when studying the relative role of the more specific immune responses to the infection

by *D. congolensis*.

(a) Chemical.

(i) The pH of the skin surface of most mammals is acid although it has been demonstrated in cattle that the degree of this acidity varies from site to site on the animal. The acidity itself is effective against a number of types of organism but the maintenance of the pH is also essential to the maintenance of a stable physico-chemical environment at the skin surface. More alkaline skins have been shown to be more susceptible to infection, and possibly more germane to this essay if skin is continually wetted the pH buffering capacity of the skin is reduced.

(ii) Sebum: sebaceous fluid is produced in varying amounts by haired mammals and the composition differs widely. The importance of this sebaceous layer in the pathogenesis and epidemiology of dermatophilosis has been previously discussed but it is thought that the fatty acid component of this material is the major bactericidal agent. However Roberts (1967a) considered that in infections of sheep the sebaceous film acted only as a mechanical barrier to *D. congolensis* and Amakiri (1976) reported that the organism was frequently present in the sebaceous glands of chronically infected cattle.

(iii) The role of sweat is incompletely understood but it may well contain not only nutrients but also inhibitors to bacterial growth. Both the protein and non protein nitrogen and the dissolved salts will also have effects on the relative acidity of the skin surface.

(b) Physical.

The squamous cell layer and the process of keratinisation and desquamation are an important barrier to infection providing a relatively inert matrix for permeation by the skin secretions. The slow rate of squamae production is unlikely to produce marked alterations in the skin surface but seasonal variations, nutritional deficiencies and intercurrent disease can lead to an alteration in the rate of squamae production which could have an effect on the skin bacterial population. Failure to respond to Trychophyton rubrum infection in man has been ascribed to an inherently low rate of squamae production. Increased rate of squamae production following an hypersensitive reaction to some fungal infections has been implicated in the more acute course of such infections when compared to the more chronic non hypersensitive reaction inducing infections (McEwan Jenkinson 1976; Rothman & Lorincz 1962).

The deeper layers of the epidermis also act, by the physical continuity of their cells, as a barrier to invasion by skin bacteria. D. congolensis was only found in the deeper, papillary layer in chronically infected cattle when there had been enough mechanical trauma to disrupt the basement membrane (Amakiri 1976). Studies on the seasonal and regional variation in epidermal thickness in cattle indicate that it is the continuity of the cells rather than the thickness of epidermis that is important in maintaining this integrity (Amakiri 1974a, 1974b).

Host Reactions and Defences: Innate Resistance

A variation in susceptibility to infection in differing age, sex and genetic groupings of cattle has been observed, but these

are frequently contradictory and no overall generalisations emerge. That is with the exception of the dwarf West African Bos taurus animals of the Ndama and Muturu types which are frequently reported to be substantially more resistant to infection than Indian zebu and European breeds (Amakiri 1976; Bida & Dennis 1976; Coleman 1967; Le Riche 1968). The reasons for this resistance are not known. The higher proportion of lymphocytes and neutrophils in the superficial skin layers and a substantially different normal skin bacterial flora, which has a high proportion of subtilin producing bacillus species, have been suggested as possible effector mechanisms (Amakiri 1977; Nwofoh & Amakiri 1981). It has also been noted that the normal immunoglobulin profile in serum electrophoretic studies of these breeds is substantially different from others with a much reduced alpha-1 fraction (Amakiri 1977). The resistance of these breeds to possible predisposing causes of dermatophilosis, such as trypanosomiasis and heavy tick burdens, should, however, also be borne in mind.

Host Reactions and Defences: Acquired Resistance

Immunity to dermatophilosis, that is an increased resistance to the disease following infection or vaccination, is a complex subject on which there are many, seemingly conflicting, reports. Valid generalisations are few and there is considerable interspecies variation. It has long been recognised that cattle and other species may become infected repeatedly, if transiently (Bida & Dennis 1976; Bida & Kelley 1976; Bugyaki 1959; Philpott & Ezeh 1978; Richard et al. 1976). The role of the chronically infected animal in the epizootiology of the disease has already been

discussed. In most species, however, including cattle, some development of resistance has been reported, but this is not of a simple nature and is most usually recorded as an acceleration of the healing process. Roberts (1965a) noted that in guinea-pigs and sheep the dose of live zoospores required to give rise to a lesion of confluent scabs when applied to scarified skin was increased sixteenfold in previously challenged animals. This was provided the initial challenge had involved a breakdown of the "skin barriers" previously described. In rabbits, however, he found that there was no increase in the dose of zoospores required following either infection or vaccination, and Richard (1976) reported that he and others had infected rabbits repeatedly for at least a year at two week intervals with no apparent reduction in the severity of the disease. However, in preliminary work in vaccine development by Chamoiseau and Lefevre (1973) it was reported that intradermal injections of adjuvanted and non adjuvanted live young cultures of D. congolensis led to: "Une protection cutanée d'un niveau très élèvé surtout remarquable par sa régularitée." Following their success with use of vaccination in rabbits, this team reported a number of vaccination trials in cattle in Francophone West Africa, particularly Chad. From the observed incidence of dermatophilosis in vaccinated animals compared to unvaccinated controls, they concluded that a protective immune response could be induced in cattle by the strategic use of intradermal inoculations of live D. congolensis zoospores (Chamoiseau, Provost & Touade 1973; Provost, Touade, Guillaume, Peleton & Damsou 1976; Cheneau 1978). Others have also observed

that more rapid healing normally follows a secondary challenge and Makinde and Ezeh (1981) reported that in a small group of experimental white Fulani heifers the mean time for healing of a second infection was 10-15 days in comparison to the 20-25 days of the first. A similar accelerated healing process has been noted by Morrow in work on outbred Wistar white rats (personal communication). In conclusion an increase in resistance to dermatophilosis is seen in a number of species following either infection or vaccination, but this resistance, far from being absolute, is normally expressed as an accelerated healing of lesions.

Host Reactions and Defences: Immune Responses

The effector mechanisms of this immune response are not completely ascertained or understood but many observations have been made on both humoral and cell mediated responses. Seroconversion does occur following vaccination and infection. Roberts (1965a) reported that single infections in sheep gave rise to a low but detectable increase in flagellar agglutinin titres. An agglutinin and somatic precipitin response followed a second infection in guinea-pigs, rabbits and sheep. Repeated vaccination using adjuvanted killed zoospores led to a considerable rise in all groups of antibody in all three species examined. Richard et al. (1976) reported a similar seroconversion pattern in rabbits and deer, precipitin titres developing only after a second infection. Chronically infected cattle also show significant precipitin titres (Pulliam, Kelley & Coles 1967; Richard et al. 1976), and a classical anamestic response has been observed in the bovine following a second, experimental infection using an indirect haemagglutination

technique (Makinde & Ezeh 1981). Complement fixing antibodies have also been described (Kwapinski 1966; Pulliam et al. 1967), as have a number of different antigenic components of D. congolensis which have varying potencies for the induction of the different classes of humoral response. From these observations it has been concluded that D. congolensis as a species shows a remarkable antigenic homogeneity with little cross reaction with even closely related Actinomycetes, and that seroconversion, although it readily occurs, does not give rise to an immunity from infection, since even animals with high titres against D. congolensis may readily become infected.

Cell mediated hypersensitivity responses have also been reported following infection and/or vaccination. Roberts (1965b) recorded a delayed type hypersensitivity to skin tests, which developed within four or five days of a single infection. This hypersensitivity was associated with an accelerated infiltration of skin lesions by neutrophils accompanied by a decrease in hyphal penetration of the follicular sheaths. He also reported an immediate type hypersensitivity to skin tests but this did not develop until a second infection or repeated vaccination had taken place. Abu-Samra (1977), using skin tests, demonstrated a delayed type hypersensitivity response in rabbits, goats, sheep, donkeys, camels and calves, and also reported an immediate, delayed and Arthus type immune response in rabbits which had been infected by a deep footpad inoculation (Abu-Samra & Walton 1981). Makinde and Wilkie (1979) investigated both lymphocyte transformation and macrophage migration modulation and found that both formalised whole

cell and endo antigens induced a significant cell mediated response in rabbits. In cattle, however, Bida and Dennis (1976) did not observe any cell mediated response following their inoculation with killed D. congolensis and did not consider that delayed hypersensitivity played any role in the disease in cattle. However, histopathological changes in natural cattle infections showed accumulations of macrophages and lymphocytes and occasional giant cells "suggestive" of a delayed type hypersensitivity reaction (Abu-Samra et al. 1976).

Despite this plethora of records of immune responses to both artificial and natural infections, no clear view on their relative role in the pathogenesis and resistance to dermatophilosis emerges.

Possible Immuno Effector Mechanisms

Roberts (1965a, 1965b, 1965c, 1966) considered that enhanced killing of zoospores following phagocytosis by neutrophils lay at the heart of induced resistance to infection. He demonstrated that the ability of guinea-pig neutrophils to destroy engulfed zoospores could be made equal to that of the more resistant rabbit by:-

(i) previous exposure to D. congolensis either by infection or vaccination;

(ii) passive immunisation by the intraperitoneal injection of immune sera from such guinea-pigs into naive animals.

This enhanced zoosporicidal ability correlated well with a concomitant decrease in the penetration of the dermis by D. congolensis hyphae. If, however, rabbits were depleted of granulocytes by treatment with nitrogen mustard, hyphal penetration was at similar levels in both previously sensitised and unchallenged

animals, although delayed hypersensitivity reactions, as shown by skin tests, were not significantly reduced in the former group. This emphasis on granulocytes in the natural resistance of rabbits was not however confirmed by Merkal, Richard, Thurston and Ness (1972) who used methotrexate in place of nitrogen mustard as an antigranulocytic treatment. Roberts (1965b) himself observed that sheep appeared to rely on a different mechanism for healing from, and immunity to, dermatophilosis. In this species, resistance to invasion by D. congolensis hyphae occurred at the same time as the onset of delayed hypersensitivity reaction, that is @ 4 days post infection, in contrast to the forty-eight hours seen in guinea-pigs and rabbits.

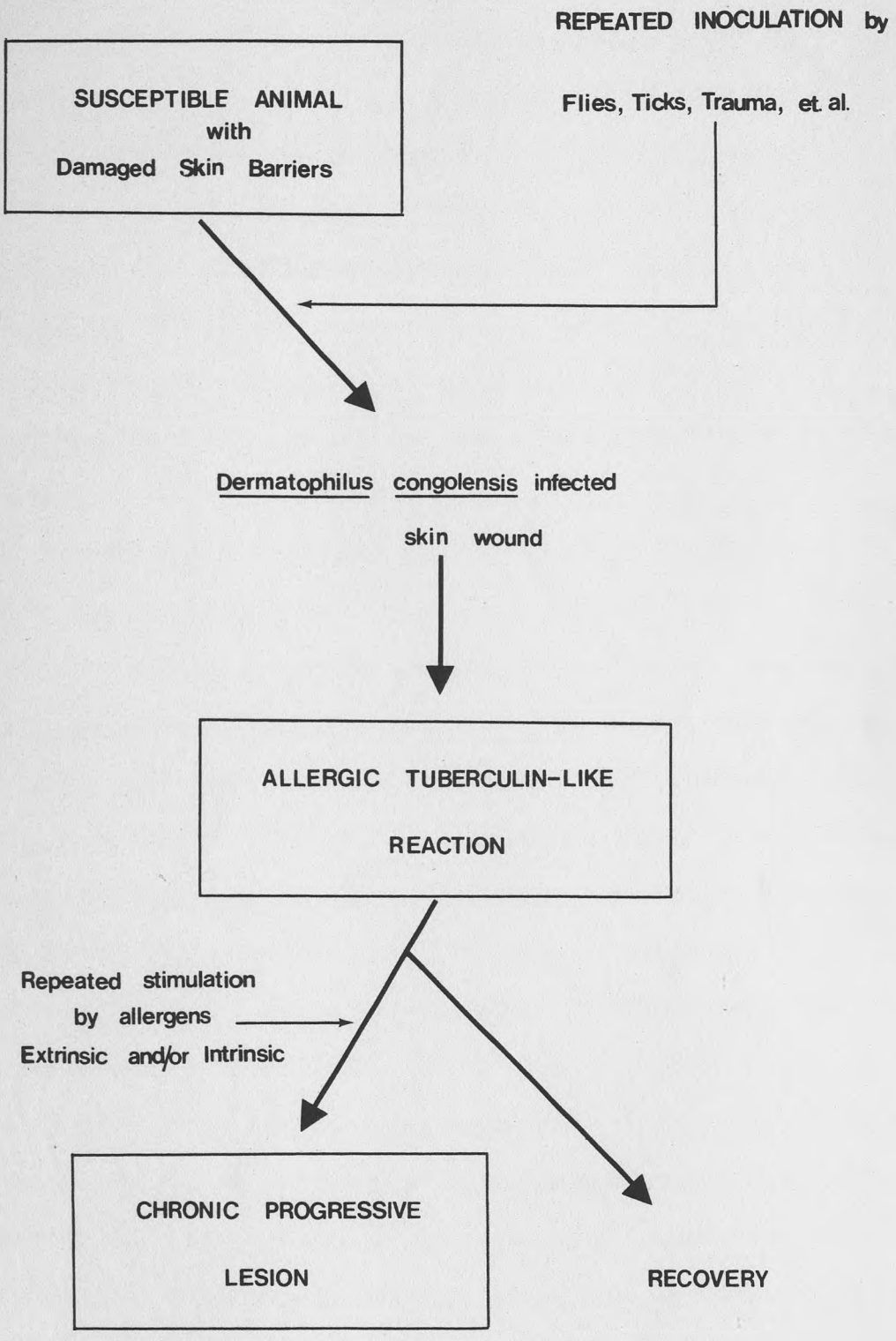
Since Roberts' work, many attempts have been made to enlarge on the mechanisms behind resistance to, and healing of, lesions caused by D. congolensis. More particularly, attempts have been made to interrupt this healing process in an effort to reproduce a chronic and, if possible, progressive lesion which would more closely resemble the real problem posed by field dermatophiloses and provide a model for the study of this syndrome. Various hypotheses have been put forward as to why a chronic lesion should develop, most postulating some breakdown or interference with the immune system.

Lesions in mice pretreated with prednisolone trimethylacetate and infected by scarification and intra and sub cutaneous injection with zoospores were reported as being much more severe and continuing over a longer time course than in untreated controls (Abu-Samra 1978). Similar effects are found in rats which are treated with

cyclophosphamide (Morrow, personal communication). Both these compounds have severe immunosuppressive effects and the modulation of the behaviour of the D. congolensis lesion could be due to this; however their physiological effects are widespread and not only the immune system is modulated. An intact immune system is not a prerequisite for the efficient healing of D. congolensis lesions since work by Morrow (unpublished) in nude mice indicates that these T. lymphocyte deficient animals heal as well and as rapidly as similarly infected immunocompetent controls.

Hypersensitivity reactions themselves, either to D. congolensis antigens or to other immunogens, have also been suggested as being important in the pathogenesis of dermatophilosis. Intradermal embedding of acacia thorns in the epidermis of cattle gives rise to a foreign body granuloma reaction, and if D. congolensis is applied to the same site simultaneously a more chronic lesion develops. Similarly the intradermal inoculation of D. congolensis zoospores with Freunds complete adjuvant leads to a reaction of the tuberculin type and a lesion of increased chronicity. Neither of these protocols leads to a progressive lesion (Abu-Samra et al. 1976).

In drawing on these and other findings it has been proposed that a progressive lesion could be caused by the presence of an extrinsic or intrinsic allergen introduced with the organism at the time of infection (see diagram over). An intrinsic factor has been considered unlikely since none of the experimental infections with D. congolensis alone have led to a chronic or progressive infection. This may however be due to the way in which these inoculations are administered, normally via a single very large dose of zoospores to


REPEATED INOCULATION by
SUSCEPTIBLE ANIMAL with Damaged Skin Barriers
Flies, Ticks, Trauma, et. al.
Dermatophilus congolensis infected skin wound
ALLERGIC TUBERCULIN-LIKE REACTION
Repeated stimulation by allergens Extrinsic and/or Intrinsic
CHRONIC PROGRESSIVE LESION
RECOVERY

a suitably prepared skin site. But in the field, given the correct predisposing factors for their initiation, repeated infection by small doses of D. congolensis, possibly in conjunction with another allergen, for example tick saliva or orf virus, could interfere with the healing process. The cell mediated hypersensitivity reactions to these and other allergens, but particularly tick saliva, have led to an interest in the effects that these or other extrinsic allergens might have on the course of D. congolensis infections and the immune responses to such an infection.

Some evidence of reactions of this sort giving rise to a chronic lesion was recorded by Davis and Philpott (1980). Using goats which had previously been sensitised to D.N.C.B. they applied D. congolensis zoospores to a scarified area of skin that had been previously challenged so as to exhibit a delayed hypersensitivity reaction in this case to D.N.C.B. This regime gave rise to a more chronic/extensive lesion than in the unsensitised control animals. In an effort to provide a means of objectively measuring the chronicity of dermatophilosis lesions Davis (1983) developed a technique based on quantifying the recovery of zoospores from the skin at known intervals following infection. Using this technique he demonstrated that zoospores were recoverable for a significantly longer period from the skins of rats and guinea-pigs if the site of infection was exhibiting an hypersensitive reaction to D.N.C.B. at the time of infection. This work led him to postulate that the reason for this increased chronicity could be a delay or modification of the onset of the cell mediated response to D. congolensis due to

the simultaneous presence of an hypersensitive response to D.N.C.B. It was the purpose of this project to investigate the onset of cell mediated responses to D. congolensis in rats and guinea-pigs that had been sensitised in this way.

MATERIALS AND METHODS

Selection of Antigens

Two antigen formulations were tested in guinea-pigs to ascertain which, if either, would give the most easily visualised skin reactions to intradermal injection. One, A, was composed mainly of the products of sonicated zoospores and was used at two concentrations expressed as the number of viable zoospores per ml. in the suspension before sonication. The second, B, was derived from predominantly the filamentous elements of D. congolensis (see Production of Antigens). Both antigens showed clear precipitation lines following gel diffusion against inactivated serum from rabbits hyperimmunised to D. congolensis.

These antigens were tested in a group of five guinea-pigs. One of these, I, had an uncertain history of exposure to D. congolensis, but had certainly been infected at least twice; the others (II, III, IV and V) had not previously been infected. Guinea-pigs (II-V) were infected by scarification (see Infection Technique) on the first day of the experiment. They were subsequently challenged by intradermal injections of the antigens and Phosphate Buffered Saline (P.B.S.), the results of these challenges being observed at 20 minutes, four hours, twenty-four hours and forty-eight hours after the time of injection. Each animal was tested in the same way: an area of skin on the opposite side to the site of infection was clipped and swabbed with alcohol; 0.05 ml. of P.B.S., Antigen A at 10^{10} zoospores per ml., Antigen A at 10^{12} zoospores per ml. and Antigen B were injected at four

different sites separated by approximately one centimetre. Skin reactions were assessed in two ways:-

(i) an approximate visual estimate of the size of weal produced was made, scoring it from 0 to +++;

(ii) measuring two diameters, at right angles to each other, of any resultant hyperaemic area using a pair of vernier callipers.

Attempts to measure comparative skin fold thicknesses were unsuccessful due to the fineness of the guinea-pigs' skin and the compressibility of the skin oedema produced following antigen challenge.

Bearing in mind that delayed hypersensitivity reactions to D. congolensis have been found to be first detectable in guinea-pigs four days after initial infection, the guinea-pigs were challenged as follows:-

No. I on the day the others were infected

No. II three days post infection

No. III four days post infection

No. IV seven days post infection

No. V seven days post infection

No reactions were recorded at 20 minutes following challenge. There was little difference in the reactions to the three antigens (see Table 1). In view of the relative ease with which large quantities of antigen B could be produced and the report by Makinde and Wilkie (1976) that cell mediated immune responses to D. congolensis were best elicited by whole cell antigen, it was decided to select antigen B (filament antigen) for further work.

A number of skin test experiments were attempted on outbred

TABLE 1

COMPARATIVE SKIN REACTIONS IN GUINEA-PIGS FOLLOWING CHALLENGE WITH VARIOUS ANTIGENS

Antigen	H.P.I.	Guinea-pig I		III		IV		V		II
	4	–	–	–	–	–	–	–	–	N
P.B.S.	24	–	–	–	–	–	–	–	–	O
	48	–	–	–	–	–	–	–	–	
	4	2 x 3	+	–	–	–	–	4 x 2	+	R
$A10^{12}$	24	12 x 7	+++	11 x 7	++	11 x 8	+++	16 x 7	+++	E
	48	4 x 3	+	6 x 4	+	–	–	–	–	A
	4	–	–	–	–	–	–	–	–	C
$A10^{10}$	24	10 x 4	++	12 x 6	+++	14 x 6	+++	8 x 5	++	T
	48	3 x 5	+	–	–	–	–	–	–	I
	4	6 x 3	++	–	–	4 x 3	+	–	–	O
B	24	11 x 7	+++	14 x 6	+++	16 x 7	+++	15 x 7	+++	N
	48	4 x 3	+	–	–	–	–	–	–	S

H.P.I. = hours post infection.

Wistar rats but despite work on numerous groups of animals, some infected only once and some which had been infected frequently enough to show significantly accelerated healing, no consistently measurable skin response was observed following challenge with either of the test antigens.

Production of Antigens and D. congolensis Zoospores

Production of D. congolensis Zoospores. Frozen samples of D. congolensis, originally isolated from a sheep, were taken from the deep freeze (-65°C), thawed, and then streaked out onto sheep's blood agar plates (Blood agar base Gibco 152,0600). These were then incubated at 37°C for three days in a candle-jar so as to produce individual colonies. A single loopful of pure D. congolensis from the edge of the streak was then inoculated into Brain Heart Infusion broth (B.H.I.) (Gibco M06800) and then incubated aerobically at 37°C for three days. The B.H.I. was then checked for pure flocculant growth and a clear medium. The medium and growth were then well mixed, and three drops of the mixture placed onto sheep's blood agar plates and evenly spread over the plate to within one centimetre of the edge. These plates were then incubated for 48 hours to produce a pure growth of D. congolensis that was rich in zoospores. These zoospores were harvested by adding 5 mls. of P.B.S. to the plates and rubbing the surface of the colonies with a bent pasteur pipette spreader thus releasing the zoospores from their surface. Highly concentrated zoospore recoveries could be made by transferring suspensions of zoospores in P.B.S. from one plate to another. The number of viable spores was ascertained by making tenfold serial dilutions of the

suspensions in P.B.S. Three 50 microlitre aliquots of each dilution were then placed on well dried sheep's blood agar plates and incubated aerobically for 48 hours. Individual colonies were then counted at the most suitable dilutions and the number of viable zoospores per millilitre calculated. Such quantified harvests of zoospores in P.B.S. were used directly to infect the rats and guinea-pigs in the described experiments.

Production of Antigen A (Zoospore Antigen). Zoospores harvested as described above were passed through a sterile 0.45 micron millipore filter to remove any larger filaments. The resultant filtrate was then sampled for zoospore numbers by colony counting on sheep's blood agar (see above). This filtrate was then sonicated for ten minutes at an amplitude of 8 microns peak to peak and the resultant sonicate passed through a 0.22 micron millipore filter to remove any undisrupted material. Samples from this secondary filtrate were cultured on sheep's blood agar to test for sterility. Non contaminated filtrate was stored in the deep freeze compartment of a commercial refrigerator at @ $-20^{\circ}C$ in 0.5 ml. aliquots. This technique, using an initial concentration of zoospores of 1×10^{12} per ml., gave rise to the antigen $A10^{12}$. The final filtered sonicate was also used at a dilution of 1 in 100 to give antigen $A10^{10}$.

Production of Antigen B (Filament Antigen). For this a profuse flocculant growth of D. congolensis in B.H.I., normally three to four days post inoculation, was used (see above). This flocculant growth was centrifuged for ten minutes at 3000 r.p.m. (2000G) and the resultant precipitate washed in P.B.S. and

recentrifuged. The washing and centrifuging was repeated three times. The washed, precipitated filaments were then resuspended in P.B.S. and sonicated at an amplitude of 8 microns peak to peak for thirty minutes. The resultant sonicate was then passed, first through a 0.45 micrometer and then a 0.22 micrometer millipore filter. The filtrate was checked for sterility, standardised by using a spectrophotometer and adjusting the absorbance at 250 nm. against a P.B.S. blank to 0.5, and stored until use in 2 ml. aliquots in the deep freeze compartment of a commercial refrigerator at @ -20°C.

Introduction to Macrophage Migration Inhibition

Since skin tests did not appear a promising avenue for the investigation of cell mediated immune responses in rats, the possibility of using Macrophage Migration Inhibition (M.M.I.) as a measure of such responses was considered.

Rich and Lewis (1932) were amongst the first to recognise that the migration of cells from tissue explants could be specifically inhibited by the incorporation of antigens to which the source animal had been sensitised. Landsteiner and Chase (1942) demonstrated that delayed type hypersensitivity could be conferred on a naive animal by the transfer of sensitised cells from an hypersensitive donor. It is now widely recognised that the major cell components of delayed type hypersensitive reactions are macrophages and lymphocytes, particularly T lymphocytes (Roitt 1974). The lymphocytes possess the immunological specificity to sensitising agents, while the macrophages are the main effector cells of the delayed type cell mediated immune response. On

stimulation by specific antigens, lymphocytes produce two broad groups of non antibody, multifactorial chemical amplifiers known as lymphokines. These are involved in the non specific regulation of circulating macrophages, lymphocytes and polymorphonuclear leukocytes. There are two main groups of lymphokines: the blastogenic factors and the macrophage migration inhibition factors (M.M.I.F.) (Bloom and Bennett1966). It is with these latter that this introduction is concerned.

Macrophage migration tests measure the effect that these inhibition factors have on test populations of macrophages or peritoneal exudate cells. George and Vaughn (1962) developed a technique in which peritoneal exudates from sensitised animals were placed in microcapillary tubes and the distance that they migrated from these tubes modified by the addition of a specific antigen to the surrounding medium. The cellular component of peritoneal exudate is composed of approximately 70% macrophages and 20% lymphocytes and thus provides a useful combination of specific receptor and effector cells. In a direct microcapillary M.M.I. test peritoneal exudates are obtained from an animal which has been exposed to a particular antigen. These exudate cells are then placed in microcapillary tubes which in turn are placed in wells bathed in a suitable maintenance medium. In the test wells, antigen is added to the medium, and the degree of migration which the macrophages perform is compared with the control wells to which no antigen has been added. If the animal that is the source of the cells is showing a cell mediated immune response to the test antigen, such antigen added to the maintenance medium in the test

wells will cause the lymphocytes to secrete M.M.I.F. and the migration of the macrophages will be reduced. Indirect tests are also possible since macrophage inhibition factors are not individual or species specific. Rocklin, Meyers and David (1970) demonstrated that human M.M.I.F. produced by peripheral blood lymphocytes was able to inhibit the migration of guinea-pig peritoneal exudates. Such microcapillary tube tests, both direct and indirect, have now been modified for the investigation of cell mediated immunity in a number of species against a variety of antigens.

For this project a direct test to study the onset of delayed type hypersensitivity to D. congolensis in rats was developed. The major problem in this development was to process the recovered peritoneal exudate in such a way as to give rise to a population of cells that would consistently migrate. The major problem areas appeared to be in removing the incomplete Freund's adjuvant, used to elicit a peritonitis, from the recovered cells, and in providing a sufficiently well buffered migration medium to fill the migration wells. After a number of failures the following modus operandi was contrived.

Macrophage Migration Technique

2 mls. of incomplete Freund's adjuvant (I.F.A.) were emulsified with 2 mls. of Hanks Balanced Salt Solution (H.B.S.S.) (Gibco 041402) to which heparin had been added at a rate of 5 units per ml. The resultant 4 mls. of emulsion were injected intraperitoneally into the test rat using a one inch 18 gauge needle. Forty-eight hours following this injection the resultant peritonitic exudate was

recovered. For this the rat was anaesthetised using di-ethyl-ether and exsanguinated by withdrawing blood direct from the heart. A volume of 8-10 mls. of whole blood was extracted on each occasion; if smaller quantities were all that could be removed percutaneously the chest cavity was opened to facilitate the process. Removal of smaller volumes of blood from the rat led to an unacceptably large proportion of erythrocytes in the recovered peritoneal exudate which interfered with the later migration.

Once the animal had been bled out, the skin was reflected from a midline abdominal incision and 10 mls. of H.B.S.S. plus heparin injected into the peritoneal cavity via the linea alba. The abdomen was then gently massaged to distribute the injected solution, and opened via a midline incision. The mixture of I.F.A., H.B.S.S. and peritoneal exudate was then drawn up into a sterile plastic 10 mls. syringe. On most occasions @ 8-12 mls. were recovered. This mixture was then allowed to stand in the syringe placed vertically on the bench nozzle downwards, for between 20 and 40 minutes.

At the end of this time the greater proportion of the I.F.A. had collected at the surface of the fluid. The relatively adjuvant free cell suspension was then dispensed into 3 mls. plastic, sterile, round bottomed tubes in @ 2½ mls. aliquots. These tubes were then centrifuged for five minutes at 800 r.p.m. (200 G) to bring the exudate cells out of suspension. The supernatant was then carefully removed using a sterile pasteur pipette, any traces of I.F.A. still on the surface being removed

first. The cells were then resuspended in 3 mls. of H.B.S.S. plus heparin per tube and centrifuged as before. This washing with H.B.S.S. plus heparin was repeated. After the second washing, the button of cells was suspended in 3 mls. of a solution based on Eagle's medium (Gibco 0421581 MEM. 10X) and composed as follows:-

85 mls.	distilled water
10 mls.	Eagle's medium MEM. 10X
1 ml.	L- glutamine (200 mM.) in 20 mls. (Gibco CTC 1327)
3 mls.	7.5% Sodium Bicarbonate solution
10 mls.	Newborne calf serum, virus and mycoplasm screened (Gibco 021-6010)
2.75 mls.	I molar Hepes buffer pH 7.3 (Gibco 043-5630)
0.1 ml.	Sodium benzylpenicillin 85 units/ml.
0.1 ml.	Streptomycin 55 x 10^{-6} g/ml.

(The pH of this migration medium was found to be critical and had to lie between 7.2 and 7.4 for consistent migration to take place.)

The suspension in Eagle's medium was then centrifuged as before. The resulting cells from each individual rat were then amalgamated into a single suspension in 3 mls. of Eagle's medium; this was then centrifuged as before. The supernatant was removed and discarded and the cells resuspended in 0.7 ml. of Eagle's medium. This final suspension was then equally distributed between twelve microcapillary tubes. The suspension was kept well agitated to ensure that each capillary tube when filled to the same level contained the same number of cells. When each tube was filled to the mark the end was sealed with a proprietary clay (Cristaseal Hawksley

& Sons Ltd. Cat. No.A1503). The dozen tubes were then bundled together with an elastic band and placed vertically in a suitable centrifuge tube, the sealed ends resting on a pad of cotton wool. The whole was centrifuged as before for five minutes at 800 r.p.m. (200G). Once centrifuged the capillary tubes were cut with a diamond just the cell side of the fluid cell interface and each resultant stub mounted on a blob of sterile silicone grease so that the cut end projected into the middle of a well of a macrophage migration plate (Sterilin Cat. No.132). The tops of the migration wells were then lightly smeared with the same silicone grease, the wells filled with 0.4 ml. of the Eagle's medium and each well made into an airtight migration chamber by sealing the surface with a cover slip. Care was taken to avoid trapping air bubbles in the chamber since this made readings of the cell migration difficult. Once all twelve chambers in the plate were filled and sealed, the plate was incubated at 37^{o}C for 24 hours.

After 24 hours the plates were examined for cell migration. A number of methods were attempted, the main problem being to decide on a reproducable way in which an interface between the area over which macrophages had and had not spread, could be demarcated. The most satisfactory employed an overhead projector. The projector was placed two metres from a white screen on which it was focussed. The migration plates were then placed on the glass plate of the projector and an image cast onto graph paper held against the screen. The image of the end of the capillary tube was marked, and the outline of the shadow cast by the migrated cells drawn on the graph paper. The amount of migration was recorded by measuring, with

vernier callipers, the perpendicular distance from the middle of a line joining the edges of the image of the cut end of the capillary tube, and the outline of the shadow cast by the migrated cells. Using the described technique, two control experiments were performed, one to investigate the effect of incorporating antigen B in the migration medium when cells from naive rats were used, and the other to see if such an antigen would cause a significant reduction in migration in cells from rats which had been infected with D. congolensis on more than one occasion, and which would therefore be expected to show some cell mediated response. The antigen was incorporated in test wells at a rate of one in ten. From the results obtained from these experiments (see Tables 2 and 3) it was concluded that antigen incorporated at this level gave a satisfactory degree of M.M.I. in cells from sensitised rats. The addition of antigen to migration media also appeared to have a lesser non specific effect upon the migration of non sensitised cells. However in a number of tests using sensitised rats incorporation of antigen at lesser rates did not lead to consisitent M.M.I.

Investigation of the Effect of D.N.C.B. Hypersensitive Reactions on the Cell Mediated Responses to D. congolensis Infection in Guinea-Pigs and Rats

This experiment was carried out on 60 guinea-pigs and 50 rats. Both rats and guinea-pigs were divided into two equal groups: one, group A, whose members were sensitised to D.N.C.B., the other, group B, were kept as controls.

Sensitisation. The procedure was the same for both species. Four weeks before infection the members of the A groups were

TABLE 2

PROJECTED MIGRATION DIAMETERS IN MILLIMETRES OF CELLS FROM UNINFECTED RATS

	Control Wells						Antigen Wells					
	1	2	3	4	5	6	1	2	3	4	5	6
Rat 1	10.6	11.4	14.3	14.1	8.7	10.4	8.9	7.6	10.5	12.3	8.7	10.4
Rat 2	10.3	18.4	14.3	14.5	5.5	16.1	15.3	12.1	13.3	10.8	11.0	16.1
Rat 3	9.6	9.9	15.4	11.6	12.8	11.9	15.1	12.1	7.6	8.2	10.7	11.1

CRUDE STATISTICAL ANALYSIS OF M.M.I. IN UNINFECTED RATS

	Control Wells			Antigen Wells						
	n	$\bar{x}$	s	n	$\bar{x}$	s	t	df	% red.	av. % red.
Rat 1	6	11.6	2.21	6	9.7	1.67	1.68	10	16.4	
Rat 2	6	13.2	4.61	6	13.1	2.22	0.05	10	0.8	8.8
Rat 3	6	11.9	2.12	6	18.8	2.73	1.10	10	9.2	

None of these reductions in macrophage migration is significant at P = 0.1.

TABLE 3

PROJECTED MIGRATION DIAMETERS IN MILLIMETRES FOR RATS INFECTED MORE THAN ONCE

	Control Wells						Antigen Wells					
	1	2	3	4	5	6	1	2	3	4	5	6
Rat 1	18.1	17.6	17.0	16.4	18.3	19.1	12.5	15.6	15.7	16.0	12.7	13.4
Rat 2	11.6	12.8	14.3	13.7	15.3	11.8	10.1	9.3	9.2	8.6	8.5	8.3
Rat 3	22.6	21.8	21.3	22.5	23.2	18.9	20.1	16.5	14.8	15.3	15.6	17.8

CRUDE STATISTICAL ANALYIS OF M.M.I. IN SENSITISED RATS

	Control Wells			Antigen Wells						
	n	$\bar{x}$	s	n	$\bar{x}$	s	t	df	% red.	av. % red.
Rat 1	6	17.8	0.97	6	14.3	1.62	4.45	10	19.3	
Rat 2	6	13.3	1.45	6	9.0	0.67	3.33	10	32.1	24.9
Rat 3	6	21.7	1.53	6	16.7	1.98	4.93	10	23.2	

These reductions are all significant at P = 0.01.

injected intradermally with 0.1 ml. of 3% D.N.C.B. in ethanol. These priming injections were administered to skin of the ventral surface of the animals which had previously been clipped and swabbed with ethanol. Sensitisation was completed by twice weekly topical applications of 15 microlitres of 1% D.N.C.B. in ethanol to alternate ears of the animals.

Infection. Both A and B groups of rats and guinea-pigs were infected using the same technique. The left flank of the animal was clipped and swabbed with ethanol. Fifty microlitres of 1% D.N.C.B. in ethanol was then dropped onto the prepared skin and spread over an area of approximately 2 centimetres square. Twenty-four hours later this area was thoroughly scarified using a sterile surgical stillette and 50 microlitres of Phosphate Buffered Saline, containing @ 17 x 10^{12} D. congolensis zoospores per millilitre (for production of D. congolensis zoospores see Production of Antigens) applied to it. Both group A and group B animals showed an area of hyperaemia at 24 hours on the skin to which D.N.C.B. had been applied, but this was more prominent in the case of the previously sensitised A group animals, where a raised, slightly moist plaque was formed. The infection gave rise to exudative and then scabby lesions, typical of D. congolensis infections in these species. However the impression was gained over the course of the experiment that the lesions were more severe in the A group (sensitised) animals of both species.

Cell mediated immune responses to D. congolensis were assessed on days three, four, five, seven and ten post infection. On each day, five rats and six guinea-pigs from the control group and the

same number from the D.N.C.B. sensitised group were tested.

In the case of the guinea-pigs this involved challenging the animals by intradermal injection of antigen and examining the skin reaction to challenge at 24 and 48 hours (see Selection of Antigens).

In the case of the rats the degree of M.M.I. harvested peritoneal cells demonstrated was assessed using the Macrophage Migration technique already described.

These experiments were carried out at the Centre for Tropical Veterinary Medicine, Roslin, between January and August 1983.

RESULTS

Rats

Migration diameters measured as described in the Materials and Methods section are recorded in Appendix 1. A two way analysis of variance was performed on the results for those rats tested three days following infection (see Appendices 2 (i) and 2 (ii)). This analysis indicated that the variation of migration diameters between individual rats was highly significant; treatment variance ratios being 48.2 and 62 for group A and group B rats respectively, both significant at the 1% level. This result indicated that such an analysis was inappropriate for examining the degree of migration inhibition that the cells from each rat underwent. Instead a Student "t" value, comparing the migration diameter readings for antigen treated and control cells from each rat, was calculated. These values, and the individual and group average percentage migration inhibition values, are recorded in Appendix 3 (Crude Stastical Analysis of M.M.I.). These results are summarised in Table 4, where the group average percentage migration inhibition, and the proportion of rats in each group showing a significant migration reduction, are recorded. This proportion is calculated according to the value of the Student's "t" statistics (see Appendix 3), and recorded at P = 0.1, 0.05 and 0.01. These results are displayed graphically as:-

(i) A Comparative Plot of Treated and Control Rats Showing the Proportion of M.M.I. Significant at the 0.05 Level.

(ii) A Comparative Plot of Average Percentage Macrophage Migration Reduction.

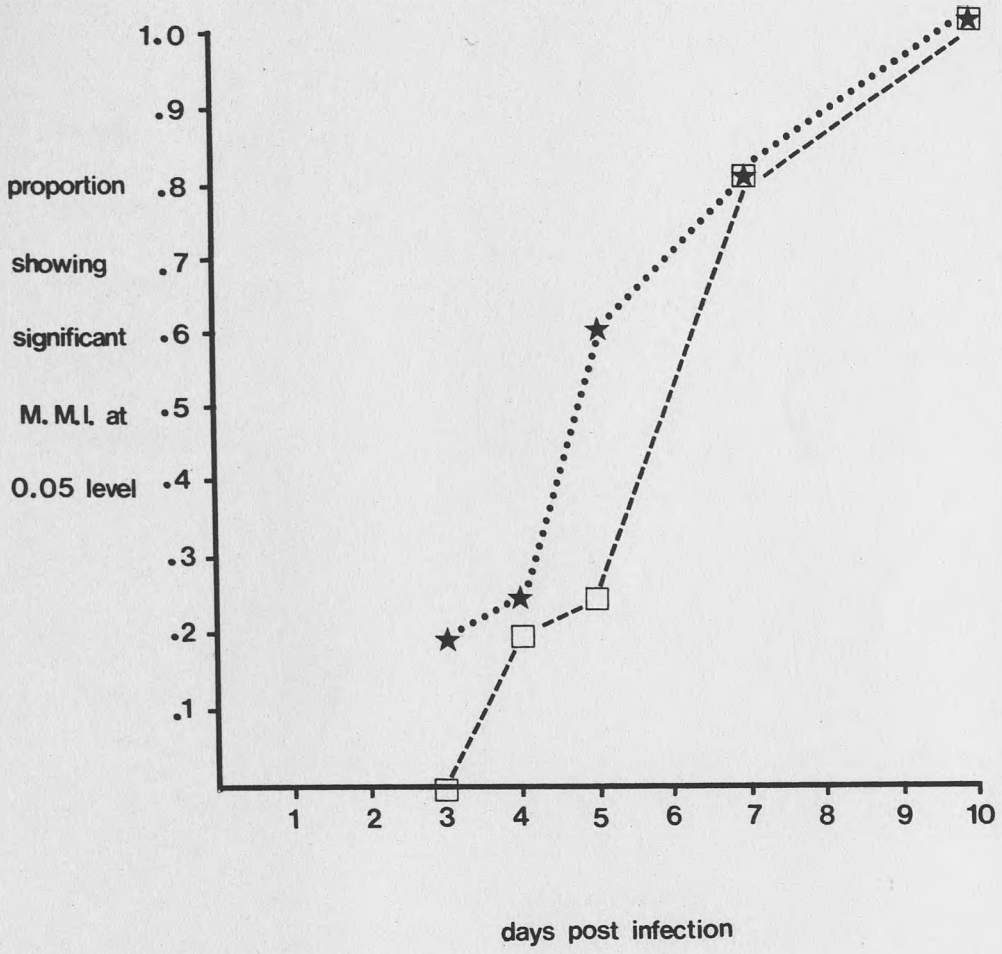
TABLE 4

PROPORTION OF RATS IN GROUP SHOWING M.M.I.

Group	Day	Level at which "t" Value is Significant			% Migration Inhibition
		0.1	0.05	0.01	
A1	3	0	0	0	8.7
A2	4	2/5	1/5	0	19.7
A3	5	2/4	1/4	1/4	22.1
A4	7	5/5	4/5	3/5	31.0
A5	10	4/4	4/4	2/4	40.5
B1	3	2/5	1/5	0	13.8
B2	4	1/4	1/4	1/4	7.0
B3	5	4/5	3/5	2/5	23.2
B4	7	4/5	4/5	4/5	25.8
B5	10	4/4	4/4	4/4	42.9

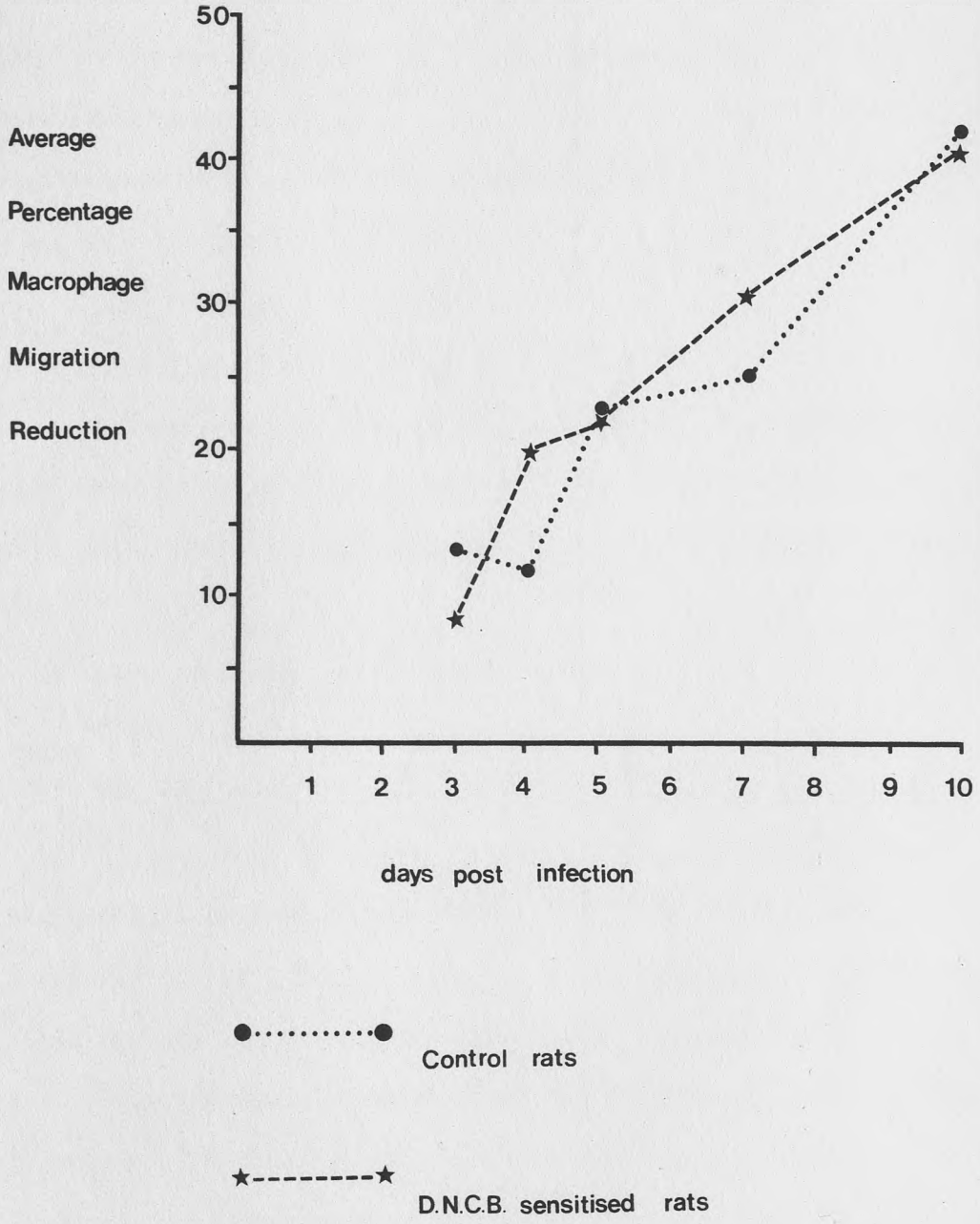
For source of "t" values see Appendix 3

COMPARATIVE PLOT OF TREATED AND CONTROL RATS



COMPARATIVE PLOT OF AVERAGE PERCENTAGE

MACROPHAGE MIGRATION REDUCTION



Non comparative graphs are to be found in Appendices 4 (i) and 4 (ii).

An analysis of variance of the linear regression was calculated for the average percentage macrophage migration reduction plotted against days post infection for both group A and group B rats (see Appendix 5 (i)). This showed that both regressions were significant at P = 0.05 with calculated Student's "t" values of:-

(i) A group t = 3.24;

(ii) B group t = 3.38;

with three degrees of freedom.

Similar results were obtained for plots of the proportion of rats showing M.M.I. significant at P = 0.05 (see Table 4) against days post infection (see Appendix 5 (ii)). Student's "t" values were:-

(i) A group t = 3.28;

(ii) B group t = 3.30.

Two Regression "t" tests between A and B groups (see Appendix 5 (iii)) show that for the percentage Reduction in Macrophage Migration lines there is no significant difference between the A and B populations (t = 0.1). For the lines plotting proportion showing M.M.I. at the P = 0.05 level against days post infection, there is a difference between the two populations calculated using the Two Regression "t" Test at the P = 0.1 level, but not at the P = 0.05 level (t = 2.4; see Appendix 5 (iii)).

From these results it is concluded that Macrophage Migration Inhibition gives a good measure of the onset of cell mediated responses to D. congolensis infections. It is also concluded that

infection of rats at a site showing a cell mediated response to D.N.C.B. did not significantly alter the time course of the cell mediated response to D. congolensis.

Guinea-Pigs

Skin reaction diameters were measured as described in the Materials and Methods section, and the results are recorded in Appendix 6 (i). The diameters measured at 24 hours were multiplied together to give a value directly proportional to the area of skin reaction (see Appendix 6 (ii)). A Student's "t" value was calculated comparing the A and B group animals' product values on days 4, 5, 7 and 10 post infection (see Appendix 7). (No skin reactions were recorded in any animals on day 3.) This showed a significant difference in the areas on days four and five post infection between the D.N.C.B. treated and untreated guinea-pigs (at levels P = 0.01 and P = 0.05, "t" values 6.8 and 2.6 respectively). A two way analysis of variance of the same values (see Appendix 7) also demonstrated variance ratios for Treatment (days post infection), Subclass (A or B group) and Interaction that were all significant at the 1% level.

In view of these results the means of the products of the diameters for each group were ranked and a one way analysis of variance carried out (see Appendix 8). The calculated variance ratio (8.5) was significant at the 1% level. A subsequent Duncan's Multiple Range test demonstrated a number of ranges which were significant (see Appendix 8 (ii)). However none of the adjacent means showed a difference significant at the 1% level.

Viewed differently, the individuals in groups A10, B10, A7, B7, B5 and A4, all showed a reaction that was measurable at 24 hours. The group with the lowest mean reaction value of these was B5, and the range value between it and group B4, the only group in which some animals reacted and some did not, was 38.2, which is significant but only at the 5% level (see Appendix 8 (ii)).

From these results it was concluded that there is possibly an acceleration of the cell mediated response to D. congolensis in guinea-pigs infected at a site showing an hyperimmune response to D.N.C.B. Further work concentrating on the period three to five days post infection would be required to confirm this.

DISCUSSION

The direct M.M.I. test on rats seemed to provide a useful, if time consuming, method for studying the onset of cell mediated responses to D. congolensis following infections in rats. The inhibition of peritoneal exudate cells from sensitised rats by the incorporation of antigen in the migration media seemed to differ markedly from the results reported by Makinde and Wilkie (1979). In an indirect test in which a guinea-pig peritoneal exudate migration system was treated with extracts from the lymphocytes of infected rabbits, these authors recorded a significant enhancement in the degree of migration paralleling the onset of other indicators of cell mediated responses. The results from the direct method described in this dissertation seem more in line with the commonly reported Migration Inhibition tests.

The significant linear regression lines (see Results) obtained for the plot of both the average percentage migration inhibition against time and the proportion of rats showing significant migration against time give an indication of the period over which the onset of cell mediated responses to D. congolensis occurs. When the peritoneal exudate is removed from the rat, it is simultaneously removed from the influence of the rat's immune system and the D. congolensis infection. In a skin test the immune system is in effect being provided with a secondary challenge for the period of the test whilst at the same time, in the case of this experiment, the primary lesion is still present. Skin tests are, however, quick and easy to perform and have been used

on a number of occasions (see Literature Review) in guinea-pigs as an indication of cell mediated immunity to D. congolensis infections.

The results from this experiment do not appear to show any great modification of the onset of cell mediated responses to D. congolensis by the presensitisation of the infection site to D.N.C.B. Not only is there little modification, but what there is differs in nature between the two species. In the treated rats there appears to be a small, and in this experiment a statistically insignificant, delay in the onset of the cell mediated response to D. congolensis. In guinea-pigs the treated animals show an accelerated response. The significance of this is questionable (see Results), particularly when one considers the work of Roberts (1965b) in which guinea-pigs showed a consistent reaction to skin tests four days following infection. He also recorded a consistent appearance of areas of hyperaemia around the sites of infection four days post infection, and a concomitant irritation to the animals. Either way the work of Philpott and Davis (see Literature Review) only indicated an increase in the chronicity of zoospore recovery in a number of species following D.N.C.B. treatment.

Hypersensitive reaction such as those induced by D.N.C.B. have other effects, particularly on the architecture and cell composition of the skin and the environment it provides to infective zoospores. In most experiments describing laboratory animals' infections, very large numbers of infective zoospores are employed. It would seem quite possible that in skin showing an hypersensitive reaction, the proportion of infecting zoospores that survive to divide and produce more zoospores might be substantially increased compared to normal

skin. Indeed in this project, it was recorded that the lesions appeared grossly more severe in the D.N.C.B. sensitised animals. This alone could possibly explain the increased chronicity of zoospore recovery and the increased numbers recovered at particular times post infection.

This is not to say that the economically and clinically significant chronicity of field infection is not intimately connected with hyperimmune responses to D. congolensis and possibly other allergens (see figure on page 23). In the human field a number of chronic dermatophytoses have been linked to aberrant cell mediated responses, particularly where the humoral response is of little protective value (Sorensen & Jones 1976). What it does mean however is that increased chronicity of the recovery of zoospores does not necessarily represent a lesion analogous to the chronic and often progressive lesion of the field case of chronic dermatophilosis. Much further work is required to establish a model chronic lesion and the mechanisms behind such a chronicity.

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APPENDIX 1

TABLE SHOWING PROJECTED MIGRATION DIAMETERS IN MILLIMETRES

GROUP		Control Migration Wells						Antigen Migration Wells					
		1	2	3	4	5	6	1	2	3	4	5	6
A1	1	9.1	12.8	10.5	9.8	9.8	10.8	8.9	8.2	10.7	9.5	9.2	11.5
	2	17.8	15.5	15.7	10.5	15.7	-	15.4	10.1	17.1	14.7	13.2	14.7
	3	26.8	24.0	24.2	21.0	23.7	21.0	18.5	15.9	22.6	15.6	26.7	20.3
	4	16.6	17.5	15.5	8.1	12.2	13.5	11.9	11.6	9.9	11.2	12.8	14.6
	5	20.7	19.4	22.5	19.4	18.8	17.2	19.2	20.0	21.0	17.9	18.1	20.6
B1	1	8.5	16.1	3.5	8.7	10.4	11.3	8.2	13.5	7.8	7.1	7.3	8.8
	2	11.4	14.8	8.5	9.3	11.3	15.8	12.1	10.5	11.2	11.2	12.1	12.9
	3	10.1	8.7	12.8	14.2	13.0	7.5	5.1	9.5	8.7	10.7	4.1	8.7
	4	12.6	10.9	12.0	12.8	13.3	13.0	9.3	11.5	10.6	7.4	12.0	13.2
	5	24.9	21.5	22.7	21.0	23.1	27.3	20.8	18.5	19.4	22.0	20.0	21.1

3 Days Post Infection

APPENDIX 1

TABLE SHOWING PROJECTED MIGRATION DIAMETERS IN MILLIMETRES

GROUP		Control Migration Wells						Antigen Migration Wells					
		1	2	3	4	5	6	1	2	3	4	5	6
A2	1	13.4	11.6	8.5	12.8	11.8	-	7.5	9.1	9.4	9.6	7.5	6.1
	2	10.4	12.3	14.0	8.7	5.3	11.4	9.3	6.5	6.6	7.8	5.7	8.5
	3	10.7	11.3	11.5	8.0	6.9	6.4	8.8	7.2	6.4	8.8	9.0	9.6
	4	15.1	16.8	16.9	16.8	19.7	16.8	12.2	15.2	12.5	11.0	10.6	19.2
	5	20.8	15.6	12.9	14.6	16.5	-	15.4	15.0	13.1	15.0	9.7	18.3
B2	1	11.5	10.9	13.9	16.2	14.5	10.7	14.2	15.6	15.1	13.7	14.7	11.4
	2	11.4	7.6	10.2	11.5	8.4	8.4	8.7	11.3	12.1	11.0	7.4	8.5
	3	16.8	19.2	16.6	15.1	22.9	14.9	18.7	9.4	15.3	16.9	18.7	16.7
	4	15.9	14.8	12.6	12.0	14.8	12.1	9.6	9.6	9.7	5.4	13.0	10.2
	5	-	-	-	-	-	-	-	-	-	-	-	-

4 Days Post Infection

APPENDIX 1

TABLE SHOWING PROJECTED MIGRATION DIAMETERS IN MILLIMETRES

GROUP		Control Migration Wells						Antigen Migration Wells					
		1	2	3	4	5	6	1	2	3	4	5	6
A3	1	17.7	20.7	11.5	-	-	-	9.6	12.5	10.9	-	-	-
	2	15.8	15.9	19.9	17.6	24.1	21.9	9.0	9.5	13.3	12.3	8.2	12.5
	3	11.8	8.4	6.9	9.3	10.3	-	9.4	5.6	8.5	7.4	7.4	6.4
	4	14.0	17.8	18.6	16.6	18.7	17.0	12.0	15.2	12.2	16.9	17.1	14.1
	5	-	-	-	-	-	-	-	-	-	-	-	-
B3	1	21.8	19.6	20.4	16.6	22.3	21.4	17.3	18.9	14.3	18.6	17.0	14.8
	2	12.1	12.3	10.6	12.0	16.1	18.0	7.7	8.7	6.6	9.8	8.9	7.7
	3	12.6	15.8	13.4	11.0	13.3	17.0	8.4	8.2	13.0	12.1	10.5	9.4
	4	10.5	10.4	17.7	-	-	-	12.4	13.7	9.6	-	-	-
	5	12.2	17.9	13.1	14.4	20.1	17.4	12.9	13.5	13.8	13.0	14.9	9.3

5 Days Post Infection

APPENDIX 1

TABLE SHOWING PROJECTED MIGRATION DIAMETERS IN MILLIMETRES

GROUP		Control Migration Wells						Antigen Migration Wells					
		1	2	3	4	5	6	1	2	3	4	5	6
A4	1	11.7	11.4	12.1	13.0	22.3	-	7.0	10.9	8.4	6.4	15.3	5.5
	2	14.5	10.5	11.1	12.6	19.0	16.3	13.4	14.5	6.3	6.7	5.8	3.9
	3	18.5	15.6	19.6	19.5	20.3	-	13.9	14.3	13.1	7.9	11.1	11.9
	4	22.0	25.5	22.7	23.4	25.0	22.4	20.9	19.2	20.1	20.6	22.1	17.4
	5	17.4	21.0	22.1	19.8	17.8	19.3	17.4	13.9	16.0	16.9	15.9	14.0
B4	1	23.7	24.0	24.5	29.9	27.7	20.4	20.9	16.9	20.7	18.6	21.1	18.9
	2	10.4	15.3	10.4	14.4	13.8	14.3	12.1	7.5	8.0	7.1	6.4	8.5
	3	14.3	7.4	9.8	7.3	5.4	6.5	5.1	7.2	4.2	7.6	5.2	6.2
	4	21.6	19.9	20.8	22.1	20.4	18.3	17.1	19.5	16.0	17.4	13.9	15.3
	5	19.3	21.7	18.4	20.6	19.1	19.4	16.4	17.2	16.4	15.5	15.8	12.6

7 Days Post Infection

APPENDIX 1

TABLE SHOWING PROJECTED MIGRATION DIAMETERS IN MILLIMETRES

		Control Migration Wells						Antigen Migration Wells					
GROUP		1	2	3	4	5	6	1	2	3	4	5	6
A5	1	16.5	24.6	24.0	19.7	23.7	13.2	6.8	15.9	9.0	8.7	11.2	5.5
	2	18.4	14.4	20.8	16.0	23.6	20.7	13.5	10.5	4.9	21.0	11.9	7.8
	3	19.6	12.9	22.6	14.4	21.6	13.9	16.4	8.6	6.8	7.0	6.3	7.2
	4	21.2	21.3	22.4	16.0	25.2	22.6	18.2	21.7	16.7	15.9	15.8	15.1
	5	-	-	-	-	-	-	-	-	-	-	-	-
B5	1	8.6	8.4	7.4	10.4	11.3	6.3	6.9	7.3	5.1	5.4	5.7	2.6
	2	13.2	13.4	16.6	16.5	17.0	16.9	9.2	7.9	9.9	9.4	7.9	9.3
	3	15.1	13.5	10.3	13.3	12.2	12.3	6.6	7.7	7.5	4.9	7.7	8.5
	4	8.8	12.0	7.7	17.6	13.1	13.1	5.3	6.0	7.4	7.0	5.5	6.7
	5	-	-	-	-	-	-	-	-	-	-	-	-

10 Days Post Infection

APPENDIX 2 (i)

TWO WAY ANALYSIS OF VARIANCE OF MIGRATION DIAMETERS FOR RATS (A GROUP) TESTED THREE DAYS POST INFECTION

	rat 1	rat 2	rat 3	rat 4	rat 5
Control Sub Group	9.1	17.8	26.8	16.6	20.7
	12.8	15.5	24.0	17.5	19.4
	10.5	15.7	24.2	15.5	22.5
	9.8	10.5	21.0	8.1	19.4
	9.8	15.7	23.7	12.2	18.8
	10.8	13.8*	21.0	13.5	17.2
Antigen Sub Group	8.9	15.4	18.5	11.9	19.2
	8.2	10.1	15.9	11.6	20.0
	10.7	17.1	22.6	9.9	21.0
	9.5	14.7	15.6	11.2	17.9
	9.2	13.2	26.7	12.8	18.1
	11.5	14.7	20.3	14.6	20.6

* Calculated using Yates modification of Allan and Wishart's formula.

For source of figures see Appendix 1.

APPENDIX 2 (i)

TWO WAY ANALYSIS OF VARIANCE OF MIGRATION DIAMETERS FOR RATS (A GROUP) TESTED THREE DAYS POST INFECTION

		rat 1	rat 2	rat 3	rat 4	rat 5		Totals
Control	Ex	62.8	88.96	140.7	83.4	118	Ex	493.86
Group	n	6	6	6	6	6	N	30
	Ex^2	665.6	1349.7	3323.6	1218.8	2336.7	Ex^2	8894.4
Antigen	Ex	58	85.2	119.6	72	116.8	Ex	451.6
Group	n	6	6	6	6	6	N	30
	Ex^2	568.1	1238	2474.2	876.6	2282	Ex^2	7438.9
Totals	EX	120.8	174.16	260.3	155.4	234.8	TEX	945.46
of	N	12	12	12	12	12	TN	60
Treatments	EX^2	1233.7	2587.7	5797.8	2094.9	4618.7	TEX^2	16332.8

APPENDIX 2 (i)

TWO WAY ANALYSIS OF VARIANCE OF MIGRATION DIAMETERS FOR RATS (A GROUP) TESTED THREE DAYS POST INFECTION

Correction Factor = 14898.2

	Treatment	Subclass	Interaction	Within	Total
ss	1098.5	31.1	20.1	284.9	1434.6
df	4	1	4	50	59
s^2	274.6	31.1	5.0	5.7	-

ss = Sums of Squares df = Degrees of Freedom

s^2 = Variance

Treatment Variance Ratio (F) = 48.2

calculated F > tabular F at 1% level.

Subclass Variance Ratio (F) = 5.5

calculated F < tabular F at 5% level.

Interaction Variance Ratio = 0.9

calculated F < tabular F at 5% level.

APPENDIX 2(ii)

TWO WAY ANALYSIS OF VARIANCE OF MIGRATION DIAMETERS FOR RATS (B GROUP) TESTED THREE DAYS POST INFECTION

	rat 1	rat 2	rat 3	rat 4	rat 5
Control Sub Group	8.5	11.4	10.1	12.6	24.9
	16.1	14.8	8.7	10.9	21.5
	3.5	8.5	12.8	12.0	22.7
	8.7	9.3	14.2	12.8	21.0
	10.4	11.3	13.0	13.3	23.1
	11.3	15.8	7.5	13.0	27.3
Antigen Sub Group	8.2	12.1	5.1	9.3	20.8
	13.5	10.5	9.5	11.5	18.5
	7.8	11.2	8.7	10.6	19.4
	7.1	11.2	10.7	7.4	22.0
	7.3	12.1	4.1	12.0	20.0
	8.8	12.9	8.7	13.2	21.1

For source of figures see Appendix 1.

APPENDIX 2 (ii)

TWO WAY ANALYSIS OF VARIANCE OF MIGRATION DIAMETERS FOR RATS (B GROUP) TESTED THREE DAYS POST INFECTION

		rat 1	rat 2	rat 3	rat 4	rat 5		Totals
Control	Ex	58.5	52.7	66.3	74.6	140.5	Ex	392.6
Group	n	6	6	6	6	6	N	30
	Ex^2	655.2	491.5	768.4	931.3	3317.5	Ex^2	6163.9
Antigen	Ex	52.7	70.0	46.8	64	121.8	Ex	355.3
Group	n	6	6	6	6	6	N	30
	Ex^2	491.5	820.4	398.9	704.1	2480.5	Ex^2	4895.4
Totals	EX	111.2	122.7	113.1	138.6	262.3	TEX	747.9
of	N	12	12	12	12	12	TN	60
Treatments	EX^2	1146.7	1311.9	1167.3	1635.4	5798.0	TEX^2	11059.3

APPENDIX 2 (ii)

TWO WAY ANALYSIS OF VARIANCE OF MIGRATION DIAMETERS FOR RATS (B GROUP) TESTED THREE DAYS POST INFECTION

Correction Factor = 9322.6

	Treatment	Subclass	Interaction	Within	Total
ss	1362.7	23.2	74.8	276	1736.7
df	4	1	4	50	59
s^2	340.7	23.2	18.7	5.5	-

Treatment Variance Ratio (F) = 62

calculated F $>$ tabular F at 1% level.

Subclass Variance Ratio (F) = 4.2

calculated F $<$ tabular F at 5% level.

Interaction Variance Ratio (F) = 3.4

calculated F $>$ tabular F at 5% level but $>$ tabular F at the 1% level.

APPENDIX 3

CRUDE STATISTICAL ANALYSIS OF M.M.I.

		Control Wells			Antigen Wells						
GROUP		n	$\bar{x}$	s	n	$\bar{x}$	s	t	df	% red.	av. % red.
A1	1	6	10.47	1.29	6	9.66	1.22	0.90	10	7.7	
	2	5	15.04	2.71	6	14.20	2.37	0.55	9	5.6	
	3	6	23.50	2.20	6	19.90	4.25	1.80	10	15.3	8.7
	4	6	13.90	3.45	6	12.00	1.59	1.23	10	13.7	
	5	6	19.70	1.79	6	19.50	1.29	0.21	10	1.0	
B1	1	6	9.75	4.12	6	8.78	2.39	0.50	10	9.9	
	2	6	11.85	2.92	6	11.66	0.86	0.15	10	1.6	
	3	6	11.05	2.68	6	7.80	2.60	2.13	10	29.4	13.8
	4	6	12.43	0.87	6	10.60	2.07	1.92	5	14.7	
	5	6	23.42	2.34	6	20.30	1.26	2.88	10	13.3	

n = number of migration chambers. $\bar{x}$ = mean migration diameter.

s = standard deviation. t = student's "t" statistic.

df = degrees of freedom. av. % red. = group average % migration reduction.

APPENDIX 3

CRUDE STATISTICAL ANALYSIS OF M.M.I.

		Control Wells			Antigen Wells						
GROUP		n	$\bar{x}$	s	n	$\bar{x}$	s	t	df	% red.	av. % red.
A2	1	5	11.62	1.89	6	8.20	1.39	1.70	9	29.4	
	2	6	10.35	3.05	6	7.40	1.36	2.16	10	28.5	
	3	6	9.13	2.30	6	8.30	1.22	0.25	10	9.1	19.65
	4	6	17.02	1.49	6	13.45	3.25	2.45	10	21.0	
	5	5	16.08	2.95	6	14.42	2.85	0.67	9	10.3	
B2	1	6	12.95	2.25	6	14.12	1.49	1.06	10	-9.0	
	2	6	9.58	1.68	6	9.83	1.87	0.24	10	-2.6	
	3	6	17.58	3.03	6	15.95	3.46	0.87	10	9.3	6.95
	4	6	13.70	1.67	6	9.58	2.43	3.42	10	30.1	
	5	-	-	-	-	-	-	-	-	-	

APPENDIX 3

CRUDE STATISTICAL ANALYSIS OF M.M.I.

GROUP		Control Wells n	$\bar{x}$	s	Antigen Wells n	$\bar{x}$	s	t	df	% red.	av. % red.
A3	1	3	16.63	4.69	3	11.00	1.45	1.99	2	33.9	
	2	6	19.20	3.37	6	10.80	2.15	5.15	10	43.8	
	3	5	9.34	1.86	6	7.45	1.37	1.35	9	20.2	22.1
	4	6	17.12	1.74	6	14.58	2.22	2.20	10	14.8	
	5	-	-	-	-	-	-	-	-	-	
B3	1	6	20.35	2.08	6	16.82	1.91	4.07	10	17.4	
	2	6	13.52	2.87	6	8.27	1.11	4.18	5	38.8	
	3	6	13.85	2.19	6	10.26	1.97	2.99	10	25.9	23.2
	4	3	12.87	4.19	3	11.90	2.09	0.28	2	7.5	
	5	6	15.85	3.09	6	12.90	1.91	1.99	10	18.6	

APPENDIX 3

CRUDE STATISTICAL ANALYSIS OF M.M.I.

GROUP		Control Wells n	$\bar{x}$	s	Antigen Wells n	$\bar{x}$	s	t	df	% red.	av. % red.
A4	1	5	14.10	4.62	6	8.92	3.62	1.99	9	36.7	
	2	6	14.00	3.26	6	8.43	4.39	2.49	10	39.8	
	3	5	18.70	1.85	6	12.03	2.36	3.68	9	35.7	31.0
	4	6	23.50	1.44	6	20.38	1.07	4.26	10	13.3	
	5	6	19.56	1.81	6	15.68	1.46	4.09	10	19.8	
B4	1	6	25.03	3.33	6	19.52	1.67	3.63	10	22.0	
	2	6	13.10	2.15	6	8.27	2.01	4.02	10	36.9	
	3	6	8.45	3.21	6	5.92	1.32	1.79	5	29.9	25.8
	4	6	20.52	1.35	6	16.53	1.93	4.15	10	19.4	
	5	6	19.75	1.19	6	15.65	1.61	5.03	10	20.8	

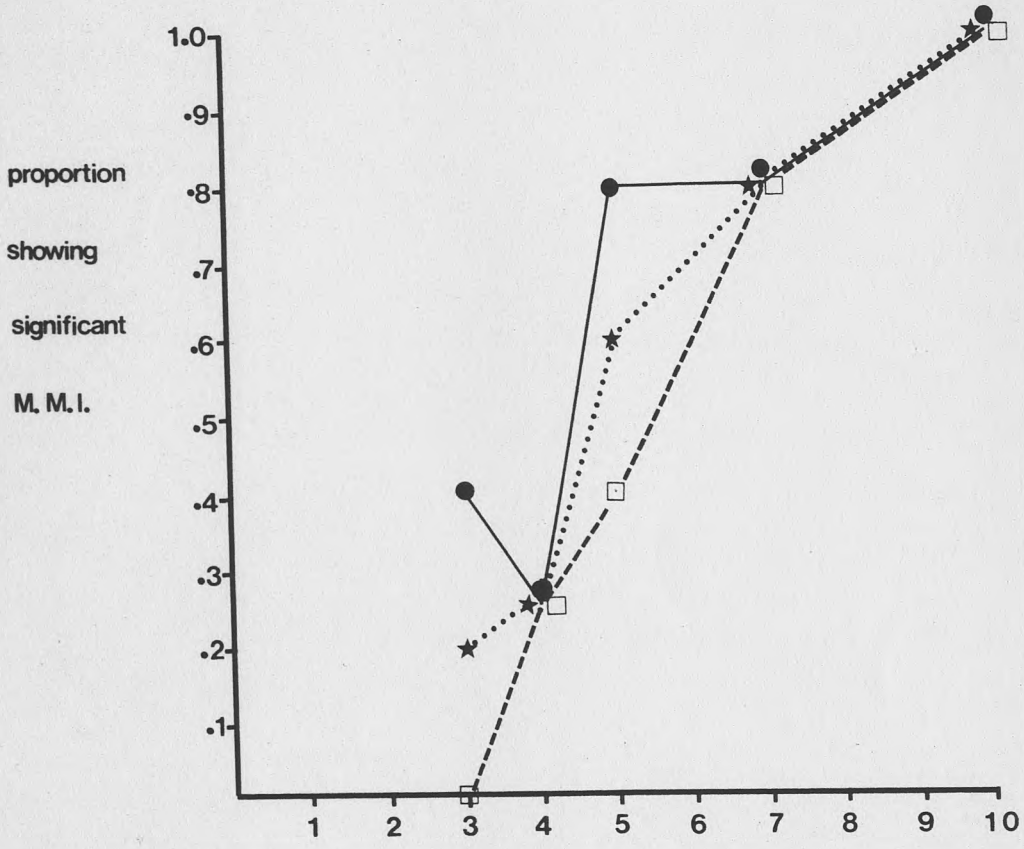
APPENDIX 3

CRUDE STATISTICAL ANALYSIS OF M.M.I.

		Control Wells			Antigen Wells						
GROUP		n	$\bar{x}$	s	n	$\bar{x}$	s	t	df	% red.	av. % red.
A5	1	6	20.28	4.67	6	9.52	3.69	4.43	10	53.1	
	2	6	18.98	3.40	6	11.60	5.53	2.31	10	38.9	
	3	6	17.50	4.27	6	8.72	3.84	3.75	10	50.2	40.5
	4	6	21.45	3.04	6	17.23	2.43	2.66	10	19.7	
	5	-	-	-	-	-	-	-	-	-	
B5	1	6	8.73	1.85	6	5.50	1.66	3.18	10	37.0	
	2	6	15.60	1.79	6	8.93	0.84	8.26	10	42.8	
	3	6	12.78	1.61	6	7.15	1.26	6.76	10	44.1	42.9
	4	6	12.05	3.54	6	6.32	0.85	3.86	10	47.6	
	5	-	-	-	-	-	-	-	-	-	

APPENDIX 4 (i)

CONTROL RATS



level at which Student "t" value is significant

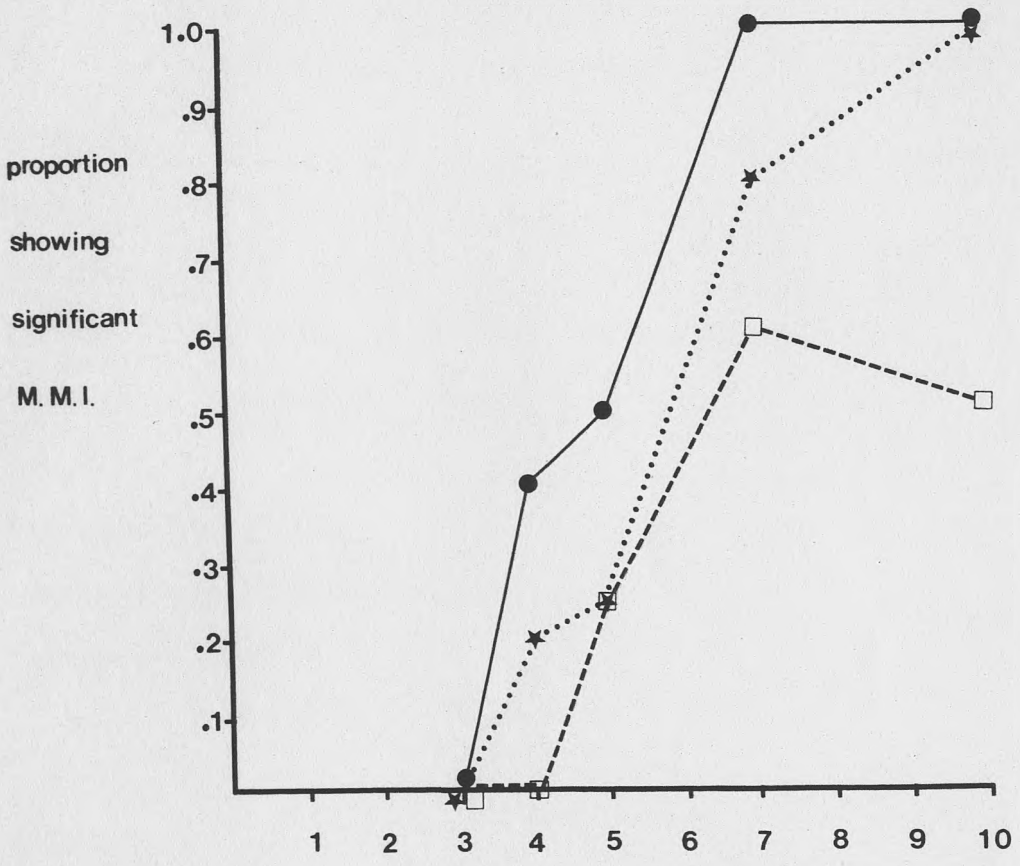
0.1

0.05

0.01

APPENDIX 4 (ii)

D.N.C.B. SENSITISED RATS



level at which Student "t" value is significant

0.1

0.05

0.01

APPENDIX 5 (i)

ANALYSIS OF VARIANCE OF LINEAR REGRESSION FOR AVERAGE % REDUCTION IN MACROPHAGE MIGRATION AGAINST DAYS POST INFECTION FOR RATS FROM A AND B GROUPS

A GROUPS

Days Post Infection	x	3	4	5	7	10
% Migration Reduction	y	8.7	19.7	22.1	31.0	40.5

$Ex = 29$, $Ex^2 = 199$, $Exy = 122$, $Ey = 122$, $Ey^2 = 3553.4$.

$SSx = 30.8$, $SSy = 576.6$, $SPxy = 129.8$.

$b = 4.2$.

Regression SS = 17.8, Within SS = 559.

Regression S^2 = 17.8, Within S^2 = 186.3.

Variance Ratio = 10.5.

$t = 3.24$ with three degrees of freedom.

B GROUPS

Days Post Infection	x	3	4	5	7	10
% Migration Reduction	y	13.8	6.9	23.2	25.8	42.9

$Ex = 29$, $Ex^2 = 199$, $Exy = 794.8$, $Ey = 112.7$, $Ey^2 = 3283.0$.

$SSx = 30.8$, $SSy = 745$, $SPxy = 141.4$.

$b = 4.6$.

Regression SS = 21.1, Within SS = 723.9.

Regression S^2 = 21.1, Within S^2 = 241.3.

Variance Ratio = 11.4.

$t = 3.4$.

APPENDIX 5 (ii)

ANALYSIS OF VARIANCE OF LINEAR REGRESSION FOR THE PROPORTION OF RATS SHOWING M.M.I. SIGNIFICANT AT P = 0.05 LEVEL AGAINST DAYS POST INFECTION FOR RATS FROM A AND B GROUPS

A GROUPS

Days Post Infection	x	3	4	5	7	10
Proportion of Significant M.M.I.	y	0	0.2	0.25	0.8	1.0

$Ex = 29$, $Ex^2 = 199$, $Exy = 17.7$, $Ey = 2.3$, $Ey^2 = 1.7$.

$SSx = 30.8$, $SSy = 0.7$, $SPxy = 4.6$.

$b = 0.15$.

Regression SS = 0.02, Within SS = 0.71.

Regression S^2 = 0.02, Within S^2 = 0.24.

Variance Ratio = 10.8.

$t = 3.3$

B GROUPS

Days Post Infection	x	3	4	5	7	10
Proportion of Significant M.M.I.	y	0.2	0.25	0.6	0.8	1.0

$Ex = 29$, $Ex^2 = 199$, $Exy = 20.2$, $Ey = 2.9$, $Ey^2 = 2.1$.

$SSx = 30.8$, $SSy = 0.48$, $SPxy = 3.7$.

$b = 0.12$.

Regression SS = 0.014, Within SS = 0.46.

Regression S^2 = 0.014, Within S^2 = 0.15

Variance Ratio = 10.9.

$t = 3.3$.

APPENDIX 5 (iii)

TWO REGRESSION "t" TESTS BETWEEN GROUP A AND GROUP B RATS

(a) % Reduction in Macrophage Migration from Appendix 5 (i)

Variance of difference between regression coefficients

$s^2 d = 217.3$

Standard error of the difference between slopes

$s\bar{d} = 14.1$

$t = 0.1$

(b) Proportion showing M.M.I. significant at P = 0.05 level

$s^2 d = 0.195$

$s\bar{d} = 0.013$

$t = 2.4$

APPENDIX 6 (i)

READINGS OF SKIN TESTS OF GUINEA-PIGS (DIAMETERS OF REACTIONS IN MILLIMETRES)

		D.N.C.B. Treated Group						Control Group					
		A						B					
Days P.I.	Hrs. P.T.	1	2	3	4	5	6	1	2	3	4	5	6
3	24	-	-	-	-	-	-	-	-	-	-	-	-
	48	-	-	-	-	-	-	-	-	-	-	-	-
4	24	7x9	13x9	11x13	10x8	9x8	9x14	4x6	2x3	-	-	-	-
	48	-	-	6x9	-	-	-	-	-	-	-	-	-
5	24	7x9	11x12	8x7	8x11	7x10	8x11	10x7	8x9	5x10	4x5	7x5	3x4
	48	3x5	4x6	5x7	5x6	3x5	4x4	5x5	3.5x4	-	-	3x4	3x4
7	24	11x12	10x9	8x12	9x8	9x12	7x8	9x11	9x10	11x13	8x8	6x7	9x8
	48	6x5	6x4	7x4	5x3	6x9	3x4	6x2	5x4	6x6	4x3	4x3	4x2
10	24	5x6	11x9	8x7	9x5	8x7	9x6	7x11	8x5	8x9	11x10	7x9	8x11
	48	3x5	4x6	4x5	7x4	3x6	2x4	3x4	4x2	4x3	7x2	6x2	5x1

P.I. = post infection Hrs. P.T. = hours post test

APPENDIX 6 (ii)

PRODUCTS OF READINGS OF SKIN TESTS OF GUINEA-PIGS AT 24 HOURS

	D.N.C.B. Treated Group A						Control Group B					
Days P.I.	1	2	3	4	5	6	1	2	3	4	5	6
3	-	-	-	-	-	-	-	-	-	-	-	-
4	63	117	143	80	72	126	24	6	-	-	-	-
5	63	132	56	88	70	88	70	72	50	20	35	12
7	132	90	96	72	108	56	99	90	143	64	42	72
10	30	99	56	45	56	54	77	40	72	110	63	88

APPENDIX 7

CRUDE STATISTICAL ANALYSIS OF GUINEA-PIG RESULTS:

PRODUCT OF TWO WAY ANALYSIS OF VARIANCE AND "t" STATISTICS FOR TWO MEASURED REACTION DIAMETERS (SEE APPENDIX 6ii)

	Days Post Infection			
	4	5	7	10
A Group	63	63	132	30
	117	132	90	99
	143	56	96	56
	80	88	72	45
	72	70	108	56
	126	88	56	54
B Group	24	70	99	77
	6	72	90	40
	0	50	143	72
	0	20	64	110
	0	35	42	63
	0	12	72	88
"t" Statistic	6.8	2.6	0.3	1.4

Subclass A	Ex	601	497	554	340	EX	1992
	n	6	6	6	6	N	24
	Ex^2	65567	44917	54724	21914	EX^2	187122
Subclass B	Ex	30	259	510	450	EX	1249
	n	6	6	6	6	N	24
	Ex^2	612	14353	49394	36526	EX^2	100885
Total	$E\underline{X}$	631	756	1064	790	TEX	3241
	N	12	12	12	12	TN	48
	EX^2	66503	59270	104118	58440	TEX^2	288007

APPENDIX 7

CRUDE STATISTICAL ANALYSIS OF GUINEA-PIG RESULTS

PRODUCT OF TWO WAY ANALYSIS OF VARIANCE AND "t" STATISTICS FOR TWO MEASURED SKIN REACTION DIAMETERS (SEE APPENDIX 6ii)

	Total	Treatment	Subclass	Interaction	Within
SS	69172	8323	11501	21559	27789
df	47	3	1	3	40
s^2	-	2774	11501	7186	695

SS = Sums of Squares

df = Degrees of Freedom

s^2 = Variance

Treatment Variance Ratio = 4

Subclass Variance Ratio = 17

Interaction Variance Ratio = 10

(All three Variance Ratios are significant at the 1% level.)

APPENDIX 8 (i)

ONE WAY ANALYSIS OF VARIANCE OF RANKED MEANS OF PRODUCTS OF DIAMETERS OF SKIN REACTIONS IN GUINEA-PIGS

Guinea-Pig Groups

	B4	B5	A10	B10
Ex	30	259	340	450
n	6	6	6	6
Ex^2	612	14353	36526	44917
$\bar{x}$	5	43.2	56.7	75

Guinea-Pig Groups

	A5	B7	A7	A4
Ex	497	510	554	601
n	6	6	6	6
Ex^2	44917	49394	54724	65567
$\bar{x}$	82.8	85	92.3	100.2

Correction Factor = 218835

Total SS = $TE^2 - C$

= 69172

Treatment SS = 41382.8

Within SS = 27789.2

Treatment s^2 = 5911.8

Within s^2 = 694.7

Variance Ratio = 8.5 (significant at 1% level)

APPENDIX 8 (ii)

DUNCAN'S MULTIPLE RANGE TEST ON RANKED MEANS OF PRODUCTS OF DIAMETERS OF SKIN REACTIONS

$$\text{Within Standard Error} = \sqrt{\frac{694.7}{6}} = 10.8$$

Least Significant Ranges at 1% Level

No. of Means	2	3	4	5	6	7	8
Least Significant Range	41.3	43.1	44.2	45.7	45.8	46.4	46.9

Ranges Between Means That Are Significant at 1% Level

A4 - A10 = 43.5, A4 - B5 = 57, A4 - B4 = 95.2

A7 - B5 = 49.1, A7 - B4 = 87.3

B7 - B4 = 80

A5 - B4 = 77.8

B10 - B4 = 70

A10 - B4 = 51.7

The Range B5 - B4 = 38.2 is significant at the 5% level.

(Least significant range = 34.6.)