Rock Hyraxes and Cutaneous Leishmaniasis

in

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ABSTRACT

Seventy rock hyraxes of the genera Procavia and Heterohyrax were trapped from CL endemic (18) and non-endemic areas (52). Natural infection with Leishmania aethiopica were not found either on smear or culture from noses or ears of captured animals. Experimental infections were initiated in 26 hyraxes, 3 baboons and a grivet monkey. Inoculation of hyraxes resulted in either occult symptomless infections, or failure to establish an infection. Two of the baboons and the grivet monkey proved to be susceptible and produced clinical lesions following infection. Cultures of hyrax macrophages were made and successfully infected with L. aethiopica promastigotes.

Delayed type hypersensitivity reactions were measured by skin testing and were significantly higher in infected animals and animals immunised with leishmania, when compared to uninfected animals. However, skin test responses could only be elicited with a dose seven times higher than that routinely used in humans. DTH lesions were never erythematous.

Normal serum of hyraxes contains both natural agglutinins and cytotoxic factors although these were lower than those found in man and other animals. The direct agglutination assay showed that antibodies to leishmania promastigotes progressively increase following infection. It is possible that this assay could be used for epidemiological detection of infections in wild hyraxes. Cytotoxic antibodies could not be demonstrated in hyraxes after infection although they were produced following artificial immunisation.

Sandfly biting experiments did not reveal any infections although they did reveal that the eyelids, nose, lips and nostrils were common feeding sites.

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CHAPTER I

INTRODUCTION

INTRODUCTION

The leishmania parasite that most commonly causes cutaneous leishmaniasis (CL) in Ethiopia is Leishmania aethiopica. The disease is known to be zoonotic among the rock hyraxes of two species, Procavia habessenica and Heterohyrax brucei (Ashford, et al., 1973). Man is normally infected only secondarily. Transmission is by two species of Phlebotomine sandflies- Phlebotomus longipes and P.pedifer (Foster, 1972; Ashford et al., 1973). These sandflies are highland species (Ashford,- 1977). Also the disease is the ailment of highlanders of Ethiopia. Although L. aethiopica is the parasite responsible for most CL in Ethiopia, other leishmania parasites could well be responsible for the hitherto undescribed cutaneous lesions especially in the lowland areas. For instance, the existence of L. major has been reported from Ethiopia (Personal communication from D. Humber).

Cutaneous disease due to L. aethiopica has many unique epidemiological features (Lemma et al., 1969; Bray et al., 1973; Ashford et al., 1973; Ashford, 1977) including:

a) unlike most other species of leishmania the host reservoirs of L. aethiopica are not rodents or dogs but members of the family Procavidae in the order Hyracoidea.

b) The insect vectors are two species of highland sandflies which have not been reported elsewhere to be vectors of leishmaniasis, except in mount Elgon Kenya (Mutinga, 1975).

c) The parasite shows marked resistance to the conventional antimony compounds used for treating other types of Leishmania (Bray, et al., 1973)

d) The disease has two polar types, localized self-healing

cutaneous leishmaniasis (LCL) and diffuse cutaneous leishmaniasis (DCL) associated with a specific immunological unresponsiveness to leishmanial antigens.

The epidemiology of CL in Ethiopia is governed by the conventional four factor complex: the vector, the natural host, man and the environment. It is restricted to highland areas between 1700 and 2700 meters which receive an annual rainfall above 800 mm. (Ashford, 1977). Although the animal hosts, hyraxes, are not limited by altitude the sandflies are, since they are strictly highland species occupying diverse habitats within this attitudinal range (Ashford, 1977).

The exact distribution of CL within the highlands of Ethiopia is not well known although it has been reported from nine of the 15 administrative regions of the country. Much of the information concerning the epidemiology of CL has been obtained from studying a number of endemic foci that have been described such as Ocholo, Aleku, Kutaber and Meta Abo (Sebeta). It is apparently restricted to rural areas, although a small micro-focus is present at the fringes of Addis Ababa (unpublished observations).

Natural L. aethiopica infections are almost entirely restricted to three genera of the order Hyracoidea with a single report of this parasite being isolated from the Giant Rat, Cricetomys sp. (Mutinga, 1975). In addition experimental infections in laboratory rodents has been unsuccessful and clinical lesions have only been produced in primates such as baboons and vervet monkeys and the Golden Mole rat (Lemma and colleagues unpublished results; Ashford, 1977). This apparent narrow specificity of L. aethiopica to hyraxes may be considered as an indicator of an old system in which the co-evolution of both host and parasite are reflected.

There are a number of criteria which determine the zoonotic potential of reservoirs of Leishmaniasis in the Old World (Bray, 1983):

a) constant contact with man via the vector,
b) good presentation of the organism to the vector,
c) intimate contact with the vector,
d) major source of blood meals for the vector, and
e) chronic susceptibility and a considerable infection rate (greater than 10%).

The rock hyraxes in Ethiopia fulfill many of the above criteria. They have an infection rate between 13 and 20% (Ashford, et al., 1973; Ashford, 1977 and Bray, 1983), they appear to have a chronic symptomless infection and live in intimate contact with both sandflies and man.

In order to develop an effective control programme for leishmaniasis it is vital that the biology of the host reservoir, the hyrax is studied. Todate there is little information regarding the ecology, biology and behavior of hyraxes in Ethiopia

Distribution, Life History and Taxonomy of the Hyracoidea.

Hyraxes belong to the order Hyracoidea in which two extinct, and one living families are found. There is a single extant family, the Procavidae, which is comprised of three genera - the rock hyraxes (Procavia and Heterohyrax)and the tree hyraxes (Dendrohyrax). The order is considered to have evolved in Africa sometime before the Oligocene (Walker, 1975) and they now cover a large part of Africa, Arabia and the Mediterranean region (Walker, 1975; Kingdom, 1971; Corbet, 1979). Hyraxes are found in a wide variety of habitats and altitudinal ranges (above 4650m on Mount Kenya to sea level).

The rock hyraxes present considerable taxonomic problems within the genera since they are comprised of allopatric forms due to

the high degree of variation resulting from the isolation of habitats within Africa (Corbet, 1979).

There are two genera of rock hyraxes in Ethiopia which are represented by Procavia habessenica and Heterohyrax brucei. The former is the larger of the two, its taxonomic position is at present confused and Ashford (1977) considers that it includes more than one species. Corbet (1979) recognizes subspecific ranks for the genus Procavia for what he considers conspecific variants in which reproductive isolation has not been described. The specific ranks used in his reference is Procavia capensis to which Procavia habessinica and others are assigned to subspecific ranks. All the members in the genus found in Africa or elsewhere are placed in this same specific taxa by Walker (1975). In contrast four distinct species are recognised by other authorities such as Kingdon (1971). These are P ruficeps, P. capensis, P. habessinica and P. johnstoni. In addition, Ashford (1977) identifies a third species together with Heterohyrax brucei and Procavia habessinica at the Bale mountains where he observed a variant which has been assigned a subspecific rank (P. capensis capillosa) by Corbet (1979). As can be seen there is considerable confusion and disagreement regarding the taxonomy of hyraxes within the genus Procavia. There is, therefore, an urgent need to apply modern taxonomic methods to this group in order to clarify the situation.

In the genus Heterohyrax eight subspecies have been recorded by Kingdon (1971), although Walker (1975) recognizes six species with Heterohyrax syriacus as the type species of the genus. Kingdon (1971) gave the following list of subspecies with H. brucei:

H. b. bakeri	H. b. munzneri
H.b. kempi	H. b. prittwitzi
H. b. hindei (albipes)	H. b. songeae
H. b. diesneri	H. b. lademanni

The members of the genus Heterohyrax, like those of Procavia, exhibit allopatric variation (Corbet, 1979)

Although the life style of the hyraxes is similar to many rodents it is in fact related to the ungulate mammals. Fossil evidence shows that they are direct relatives of the Proboscideans (Sale, 1970) and because of biological characteristics such as a very long gestation period, they are thought to have evolved from a much larger ungulate stock. Sale (1966a) considers that the elephant is the largest, and the hyrax the smallest members of this evolutionary line who had a common ancestor in the Late Eocene. The hyraxes remained plantigrade and left the plains for rockier habitats which provided protection from predators. This change of habitat was accompanied by a reduction in body size.

Habitat, Habits and Adaptive Features

Rock hyraxes are largely diurnal and are not fossorial, but inhabit cavities and rock crevices in rocky outcrops, cliffs and boulders of rock formations, mountains and escarpments. Selection of suitable colony sites appears to depend on a variety of biological requirements, including, protection from predators and shelter from severe climatic conditions. A feature of hyrax colonies is that they avoid isolated holes and prefer cavities and holes which interconnect and have relatively narrow entrances to provide protection from predators (Sale, 1966b). In addition the caves and crevices provide a stable microclimate for the hyrax which has a poor thermoregulatory system. Although hyraxes occur in habitats with a wide temperature range

(-5°C to over 41°C) their cave temperature only ranges between about 3°C and 10°C (Sale, 1966b)

While providing an ideal habitat for the hyrax the system of interconnecting tunnels makes investigations of sandfly breeding sites, colony size and behavioral studies extremely difficult. Members of the genus, Heterohyrax have undergone a secondary adaption to arboreal life. This is especially true in parts of Ethiopia (e.g. Zway and Aleku) where H. brucei live entirely in large trees and are independent of rocky habitat (Ashford, 1977). The tree hyrax, Dendrohyrax, leads an arboreal life completely independent of rock shelters but is believed to have evolved from Heterohyrax (Kingdon, 1971). Cavities, rock crevices, and holes not only provide suitable habitat for hyraxes but also for the sandflies, and in highland areas hyraxes are probably the main blood meal sources for P. pedifer and P. longipes (Ashford et al., 1973).

Both Heterohyrax and Procavia display similar ecological characteristics living in colonies and might even co-exist except that Heterohyrax has the ability to use more heavily vegetated, smaller rock formations and trees (Ashford, 1977; Kingdon, 1971).

Some of the adaptive features in hyraxes include the dorsal glandular spot which has both social and reproductive functions (Sale, 1970); and the camouflaging body colorations for escape from predators. The pinnae of the ears are small and the soles of the feet have a special clinging power. Tails are vestigial; the body is covered by long tactile hairs.

The vernacular name for the Hyrax in Ethiopia is "Shikoko" which according to Bruce in 1790 (cited by Sale, 1970) is derived from the word for thorn "Ashok" representing the long herinaceous hairs on the fur of the Hyrax. An alternative derivation is from

the word "Shikuk" (Hide and seek) which amply describes their life style.

Feeding

Feeding in hyraxes is a very fast and intensive process and is mostly a group activity with a pattern and periodicity that helps to avoid predation (Sale, 1966a). Feeding is also normally confined to areas very close to shelter in which refuge can be taken. The continuous threat of predation has resulted in modest feed requirements, and hyraxes can survive in relatively dry areas with poor vegetation. This low food intake in hyraxes is augmented by decreased activity and compensatory activities such as sunbathing. Hyraxes are generally catholic in their food selection and if a food is regularly taken it is normally because it is locally abundant (Sale, 1966a; Kingdon, 1971), for example, at Zway and Ocholo hyraxes regularly feed on fig leaves as these trees are relatively common. As far as is known hyraxes do not chew the cud, accumulate food reserves, refect or exhibit coprophagy (Sale,1966a; Walker, 1975).

Thermoregulation

Thermoregulation is poorly developed in hyraxes and the young are especially thermosensitive (Sale, 1965; reviewed by Sale, 1969) and both adult and young hyraxes regularly sunbathe outside their caves. In captivity mortality due to both hyper and hypothermia is very high. This is especially true for young animals and the smaller species in the genus Heterohyrax. Since sweat glands are absent in hyraxes (Kingdon, 1971) temperature regulation can presumably only be achieved through panting, changes in coat thickness (through the errector pili muscles attached to the hair follicles) and behavioral activities such as sheltering and sunbathing.

Reproductive Functions and Colony Size

The reproductive adaptability and plasticity in hyraxes is

thought to have significantly contributed to their widespread distribution in Africa and the Middle East (Sale, 1969). The timing of birth appears to be adjusted depending on environmental factors such as availability of food and ambient temperature (Sale, 1969). The gestation period of hyraxes is long ranging between 28 and 30 weeks and the young have a high birth weight (Walker, 1975; Kayanja and Sale, 1973). Although the average litter size for both Procavia and Heterohyrax is two (Kayanja and Sale, 1973), litter sizes up to six have been recorded (Walker, 1975). Hyraxes are relatively long lived with a life span in excess of 7 years during which time they may attain a body weight of over 4 kgs.

Since hyraxes spend a great deal of their time in small underground caves, estimation of colony size is difficult. Estimates given by various authors are variable, for example, 5 to 50 is given by Walker (1975), and 25 to 60 by Kingdon (1971). This is not only a reflection of the variations of colony size, but also the difficulty of carrying out accurate counts. Colony size might be expected to vary considerably depending on the specific ecological setup of the rocky habitats or the inhabitation of fig trees by Heterohyrax which plays a part in determining colony size in Ethiopia.

Ecological Importance

Rock hyraxes make up part of the grazing/browsing trophic level in a relatively unoccupied niche of rocky habitats. Among the ungulates, only the Klipspringer depends on this habitat (Kingdon, 1971; Sale, 1965; reviewed by Sale, 1970). Hyraxes also makeup one of the major food sources for a variety of carnivores such as leopards, pythons, weasels, mongooses, foxes, etc. living in rocky habitats (Walker, 1975; Sale, 1966b, 1969). In addition, avian predators like the eagles and other birds of prey also feed on them. Where the balance between predators and hyraxes is disturbed as has happened in parts of South Africa,

hyraxes have become agricultural pests (Kingdon, 1971; Sale, 1969).

Hyraxes have thick fur and support ectoparasites such as lice and fleas and it is possible that they may play a role in disease transmission since hyraxes are known to be susceptible to bubonic plague (Kingdon, 1971). Other insects also depend on hyraxes for blood meals, especially sandflies. Ashford (1974, 1977) reported capturing 9 species of sandflies from hyrax caves. This association between sandflies and hyraxes provides the basis for their role as animal reservoirs for cutaneous leishmaniasis in Ethiopia.

General remarks and aims

The interaction between L. aethiopica and the rock hyraxes has resulted in a parasitic system whose nature of interaction is described only from the theoretical point of view. The pathology and immune responses of infections in these hosts, and therefore the fate of the parasite in this system are not well known. The degree of susceptibility of these animals and the extent to which parasites are presented to the vectors is also unclear. It is well known that chronic susceptibility encourages long-lived patent infections in the host ensuring transmission of the parasite form host to host. This is true for host-parasite systems in which equilibrium has been established and the host experiences minimal damage or pathology (Moulder, 1974). Cutaneous leishmaniasis, as a disease in man, is a reflection of, and manifested by the accompanying immune phenomenon.

The nature of immune responses and the resulting immuno-pathological processes in chronic infections of wild animal reservoirs has been poorly studied. In L. aethiopica infections no suitable animal model has so far been described. It infects the hamster only with difficulty and mice not at all (Bray et al.,

1973) although Childs et al., (1984) demonstrated symptomless infections in 12 strains of inbred mice. In the wild, where an intimate host-parasite relationship exists, animal leishmaniasis, including Ethiopian CL, presents itself more as an occult infection than as a disease (Mauel and Behin, 1982; Ashford et al., 1973; Lemma et al., unpublished data). In Ethiopia the absence of active lesions or ulcers in wild caught hyraxes from endemic areas is suggestive of a stable host-parasite relationship free from immunopathological consequences. An understanding of the immunological basis underlying the natural susceptibility and subclinical infections would help to:

a) assess diagnostic methods in determining natural infection rates in the wild.

b) provide data on leishmania infections in the animal reservoir so that comparisons can be made with the infection in man.

The aims of this study are to capture hyraxes from areas endemic and non-endemic for cutaneous leishmaniasis and to:

a) Study the biology parameters of hyraxes so as to provide the beginnings of a data base on hyraxes in Ethiopia.

b) Study the differences in immune responses between hyraxes from endemic and non-endemic areas.

c) Initiate experimental infections in hyraxes in order to determine the fate of L. aethiopica parasites in these animals and to study the way in which the immune system of hyraxes responds to these infections.

CHAPTER II

MATERIALS AND METHODS

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Study Areas: Areas endemic and non-endemic for cutaneous leishmaniasis were chosen for the study including, Sebeta (endemic), Addis Ababa (probably non-endemic), Debre Berhan (endemic) and Wef Washa (non-endemic) in Shoa and Ocholo (endemic) and Konso (non-endemic) in Gamo gofa.

Animal Trapping: Locally made snare traps and commercially made live traps were set at the entrances of caves, rock holes or tree burrows, and at feeding grounds. The types of traps used is shown below:

Harvart trap (Ossining, USA)
Tomahawk trap (USA)
Spring door trap (make unknown)
Hollow wooden traps (France)
Steel wire snares (Locally made)

The most effective method of capture was unbaited steel wire snare traps used by the local people and most of the animals used in this study were captured using them. Commercially manufactured, and baited traps were much less successful and no hyrax were captured in either the Harvart traps or the Hollow wooden traps and only one hyrax in each of the other makes. However, snare traps occasionally killed or injured the hyraxes. Animals were also captured by hand and also flushed into traps using smoke cartridges (Tradoc, U.K.). In general, baiting was unsuccessful and no bait was identified that attracted hyrax.

After capture all animals were sexed, weighed and given identification ear tags. The animals were examined for the presence of ectoparasites and then treated with a dip insecticide (Asuntol, Germany).

Maintenance of Hyrax: Initial experience with both large (7m x 2m) and small outdoor cages showed that these were unsuitable for maintenance of hyraxes since the death rate after capture was exceedingly high. Subsequently, all animals were kept indoors in heated rooms maintained at between 18 and 25oC for the highland Procavia and between 25 and 32oC for the lowland Heterohyrax. During the day cages were often taken outside and placed in a sunny location so that animals could sunbathe. The regular feed was cabbage, supplemented with french beans, carrots, and occasionally grass. Initially drinking water was provided but since animals were never observed drinking this was discontinued (except when young were born). However, despite this, copious urine was produced (presumably from the large quantities of cabbage consumed - approximately 12 kg per hyrax per week).

Once animals were subjected to laboratory infection, their cages were covered with a fine mesh cloth in order to avoid any uncontrolled transmission between animals and to humans.

Parasitology: All animals were screened for infection with endoparasites.

a) Peripheral blood films were stained with Geimsa and examined for the presence of haemo-parasites.
b) Fecal pellets were processed according to the methods described in Technical bulletin No. 18 by the Ministry of Agriculture, Fisheries, and Food Development, London (undated). Fecal cultures were set up to study the third stage larvae of nematode infections, although unsuccessful.
c) Normal skin from the bare patches on the nose and ear were sterilized with 70% alcohol and smears and samples for culture were taken with a 23 gauge needle (Hendricks, et al., 1979) and either fixed and stained with Geimsa or inoculated into NNNN medium. Cultures were incubated at

24°C and examined weekly for 4 weeks before discarding.

Hematology: Haemoglobin levels were determined using Sahli's (Acid Hematin) method (Palla and Mehta, 1982). Total white cell counts were made and differential counts performed on Giemsa stained preparations.

Histology: Skin biopsies were taken from normal skin, inoculated sites and skin test sites using a 3 mm biopsy punch (Baker-Commins, USA). Necropsies were also obtained from lymph node, spleen, and liver. Tissues were fixed in 10% buffered formalin and sectioned at 5 um after routine processing. Tissue sections were stained with hematoxylin and eosin.

Parasite Culture: The diphasic medium NNNN with Locke's solution as an overlay was routinely used for parasite maintenance and cultivation. One hundred units of penicillin and 100 ug of streptomycin were added to 1.0 ml of the overlay to control bacterial contaminants. The stationary phase promastigotes were harvested from 9 to 11 day old cultures incubated at 24°C. Parasites were also routinely subpassaged every two weeks in this medium.

Antigen Preparation: Stationary phase promastigotes derived from a patient with localized cutaneous leishmaniasis were grown on NNNN medium, harvested by centrifugation (2225 g) at 4°C and washed 3 times with saline. The washed promastigotes were killed with 0.5% phenol-saline and adjusted to 1.5×10^8/ml.

Skin testing: Cell mediated immune responses were assayed by routine skin testing using the antigen preparation described above and the diameter of the skin reaction was measured at 24, 48, and 72 hours after testing. However, since there is no established dose of promastigotes for use either in humans or other animals dose response curves were constructed (Figure 2).

Appropriate dilutions of the skin test antigen were prepared in 0.5% phenol saline and 0.1ml of the suspension was injected intra-dermally into the shaved rump or thigh (in initial experiments the hind foot pad was used but this was found to be unsatisfactory since the CV was up to 175%). Following this experiment a dose of 7.5×10^6 promastigotes was adopted as the dose of antigen in all subsequent experiments together with the standard human dose (0.5 to 1.0×10^6 promastigotes).

Antibody Titrations: Doubling dilutions (final volume 50 ul) of heat inactivated (56°C for 20 minutes) test antisera were made with phosphate buffered saline in flat bottomed microtitre plates (Flow Laboratories, U.K.). To each well containing diluted antisera 50 ul of a live, stationary phase, promastigote suspension (5×10^6/ml) was added. For the determination of agglutinating antibodies these plates were incubated for 1-2 hours at 24°C (although agglutination was still present in positive wells after 24 hours). After incubation agglutination was scored by micro and macroscopic observation of the promastigote suspension. Wells containing no test antisera and antisera from immunized hyrax and rabbits were routinely included as controls. The agglutination end point was taken as the dilution of the last well which contained agglutinated promastigotes.

In experiments designed to determine the presence of complement fixing antibody the assay (IAEA, 1982) was modified by the addition of a complement source (see results section) followed by a one hour incubation at 24°C and a further hour at 37oC to allow the complement to become activated. Immunized hyrax and rabbit serum were included in all assays as a positive control. The end point was taken as the dilution of the last well in which 50% of the promastigotes were lysed.

Immunization: Hyraxes and rabbits were immunized with 5×10^7 promastigotes which had been fixed with 1% formalin, washed, and

emulsified with Complete Freund's Adjuvant (Difco, U.S.A.). The animals were immunized subcutaneously in 3 sites (neck, flank and rump). Weekly booster injections were given with whole killed promastigotes (5×10^7) or the same number sonicated for 3 to 5 minutes at 110 to 150 watts at 4°C (Labline Ultratip Labsonic system, U.K.). The two preparations of antigen were used on alternate weeks. Booster injections were given together with Incomplete Freunds Adjuvant.

Species Identification: Hyrax skins and skulls have been sent to the Natural History Museum (mammal section) U.K. for confirmation of identification. In addition, live animals and museum specimens were examined by Dr. Dick Ashford, Liverpool Tropical Medicine and Hygiene during his recent visit to Ethiopia.

Ectoparasite and endoparsites were also sent to the Natural History Museum, U.K. for full identification.

Experimental Animal Inoculation: Experimental animals, hyraxes (18 Procavia and 6 Heterohyrax), 3 gelada baboons, one olive baboon and a grivet monkey were infected by subcutaneous injection of 5×10^6 stationary phase promastigotes of L. aethiopica which had been recently isolated from patients with LCL (Isolates 1336/86, 1627/86 kindly provided by Dr. Genene Mengistu, Armauer Hansen Research Institute).

Experimental Macrophage Infection: Mononuclear macrophages were obtained from hyrax spleens cells (2.5×10^6/ml) cultured on coverslips in 24 well tissue culture plates (Flow Laboratories, U.K.) in RPMI 1640 (Flow Laboratories, U.K.) supplemented with 10% foetal calf serum and penicillin and streptomycin. After 3 days of culture at 37°C in an atmosphere of 5% CO_2 in air, the non-adherent cells were washed off and fresh medium added to the macrophages. Stationary phase promastigotes of (isolate 1336/86) were then added at an approximate parasite to macroph-

age ratio of 18:1. The cultures were incubated at 37°C and samples were taken every 25 minutes for fixing and staining.

Sandfly Biting Experiments: Laboratory reared and wild collected (from Addis Ababa) P. longipes were either forced or allowed to feed on the noses of hyraxes that had been experimentally infected with L. aethiopica using previously described methods (Foster, 1972) and using a modified version of Barraud's cage. Engorged sandflies were maintained under conditions described by Foster, et al. (1970) and dissected and examined for the presence of promastigotes 2 to 8 days later.

Field Observations: In the field hyrax colonies were initially located by pellet heaps or urine sentinels in rock formations. Tracks, paths, caves and feeding areas were identified by whole day observation periods. Colony sizes were estimated by total counts of flocks led by family heads. This was especially possible when colonies were less isolated and when there were few lone stray hyraxes. Counts were also made by forcing herds to evacuate their caves as a result of excessive human activity during trapping.

CHAPTER III

RESULTS AND DISCUSSION

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Trapping and Maintenance

The number of hyraxes captured at the different sites between October, 1986 and May, 1987 is shown in Table 1. A total of 70 hyraxes were captured most of which were caught using steel snare traps (73%). It is apparent from the table that fewer animals were caught in endemic areas and this reflects the difficulty of the terrain in these areas, higher human habitation densities and possibly lower densities of hyrax. In areas such as Wef Washa where hyrax density was high and human interference was minimal the hyrax were relatively tame and up to eight hyrax a day were captured. In contrast, 24 days trapping at Ochelo, Gamo Gofa, only resulted in the capture of two animals. In general, Heterohyrax were more difficult to trap as members of this genus were more timid and were easily alarmed by human activity.

Table 1 - Numbers of hyraxes captured at different localities

Location	Males	Females	Not Sexed[1]	Genus
Wef Washa (3600 m)	15	14	5	Procavia
Debre Berhan (2750 m)	4	12	3	Procavia
Konso (1400 m)	10	5	-	Heterohyrax
Ocholo (2100 m)	1	1	-	Heterohyrax

Figures in parenthesis represent the altitude in meters a.s.l.
Hyraxes could not be captured at Sebeta and Addis Ababa.
[1] Animals escaped before sexing

The overall male to female sex ratio of the captured animals was 1:1.1 and there was therefore no trapping bias in favor of one particular sex.

General Hyrax Biology

The mean values for haemoglobin and total and differential white cell count is shown in Table 2. The major feature of the hematological picture of the hyrax is the absence of basophils, a feature that is also present in laboratory mice (Hudson and Hay, 1980). It is not likely that the basophils were missed since in the course of the investigation over 4000 white cells were examined.

The hematological changes during leishmania infections described previously are variable and depend on whether the parasite is restricted to cutaneous lesions, metastasizing nodules or is a visceralizing strain (Meischer and Belehu, 1982). In this study the only significant change was an increase in the proportion of eosinophils from a range of 0% to 4% in uninfected animals to 1.5% to 26.5% in infected animals ($p < 0.05$). However, it is possible that this increase is not directly due to infection with leishmania, but may be the result of increased helminth infection due to captive conditions.

The body weights of each animal were recorded soon after capture and the mean values and ranges are shown in Table 3. The mean adult weight for Procavia was approximately 2.5 kg and that of Heterohyrax 1.0 kg. Although a number of Procavia young were born in captivity none of them survived more than 35 days. The mean body weights of newborn Procavia was 238 g for females and 207 g for males and up to 35 days there was a linear increase in weight with time (correlation coefficient = 0.82).

Hyraxes kept in rooms where the overnight temperature fell below

Table 2 - Hematological data of *Heterohyrax* and *Procavia*

Parameter	*Heterohyrax*		*Procavia*	
	Males	Females	Males	Females
Hgb in g%	9.8 ± 2.5	9.6 ± 0.9	11.4 ± 1.1	10.2 ± 1.7
WBC count	8413 ± 3809	10400 ± 0.0	10856 ± 4551	8795 ± 2715
Differential Count (%)				
Neutrophil	12 - 85	25 - 51	47 - 79	45 - 82
Lymphocyte	14 - 88	NA	13 - 44	16 - 52
Monocyte	1 - 3	1 - 4	1 - 10	2 - 6
Eosinophil	NA	0 - 4	0 - 4	0 - 3

Values shown ± 1 standard deviation.

NA = Results not available

Table 3 - Body weights and temperatures in *Heterohyrax* and *Procavia*

Parameter	*Heterohyrax*		*Procavia*	
	Males	Females	Males	Females
Weight in g	756 ± 247	1004 ± 134	2652 ± 608	2449 ± 850
Temperature[1]	37.5°C		37.5°C	

[1]Rectal temperature did not differ between sexes and the results have been pooled. Body weight values are shown ± 1 standard deviation.

7°C had extremely high mortality rates (over 20%) especially when daytime temperatures did not rise sufficiently or the animals were unable to sunbathe. In both Heterohyrax and Procavia, the normal body temperature was 37.5°C (Table 3), however, in animals dying of hypothermia the body temperature steadily fell as low as 27°C, although in one case a hyrax recovered after its body temperature had fallen to 32°C. It appeared that the hyraxes coming from hotter areas such as Konso were more sensitive to low temperatures especially so for the smaller species of Heterohyrax.

Colony size was an extremely difficult parameter to determine and only rough estimates could be made from day long observations of individual colonies. The estimated colony sizes were variable, ranging from about 12 hyraxes (Heterohyrax) per colony at Ocholo to 40 hyraxes (Procavia) per colony at Wef Washa. Depending on the local density of the human population hyraxes colonies may be within a few meters of human habitation (e.g. Ocholo).

The hyraxes observed in this study did not appear to show any food preferences but fed on any locally abundant vegetation. Limited examination of hyrax caves at Wef Washa did not reveal the presence of any sandflies.

Hyrax Parasitology

The results of endo, and ectoparasite examinations are shown in Table 4. No information has yet been received from the British Natural History Museum and therefore no firm identification to the species level is given.

Examination of stool material and/or gut contents of 18 hyraxes (2 Heterohyrax and 16 Procavia) and from the autopsy material of 4 of these hyraxes revealed that the commonest parasite was Trichostrongyloides which was present in both Heterohyrax and

Procavia from all of the locations studied. This is a common

Table 4 - Endoparasites and ectoparasite of Heterohyrax and Procavia

Parasite	Stages present	Number positive
Ascaris	eggs/larva/adults	2/18[1]
Trichostrongyloides	eggs/larva	8/18[1,2]
Tapeworms	scolex/proglottids	2/18[1,2]
Toxocara	eggs	2/18[1]
Fleas	adults	26/35[1,2]
Lice	adults	21/30[2]

1 - Heterohyrax 2 - Procavia

parasite of herbivorous animals (Beaver et al., 1984). The two Heterohyrax examined had Ascaris ova, larva and adults and one was multiply infected with Trichostongyloides, Ascaris, tapeworms and Toxocara. Cyclophyllidean tapeworm infections were also found; the scolices, and proglottids were recovered from the gut of both genera (the scolices had four suckers and rostellum without hooklets). The tapeworms recovered from the two genera are probably two different species.

Repeated smear and culture examination of skin from the noses and ears for natural leishmania infection did not reveal any infected animals, even in animals from endemic areas. It is possible that infections were not detected in the two animals captured from the endemic area of Ocholo, because of the small number involved; and although an infection rate of between 13 and 20% has been reported for hyraxes from this area, by chance these two animals might have been uninfected. The other endemic

area from which animals were captured was Debre Berhan. However, in the trapping area over a two year period, only two cases of human leishmaniasis were recorded (one active lesion and one scar) and it is therefore unlikely that the infection rate of hyraxes was high.

Clinical Aspects of Experimental Inoculation

Experimental inoculation with L. aethiopica was carried out in 26 hyraxes (8 Heterohyrax and 18 Procavia) 4 baboons and one grivet monkey. The animals were examined for gross pathological changes at regular intervals up to 160 days after inoculation. The results are summarized in Table 5 and it is clear from the data that inoculation with L. aethiopica parasites caused very little clinical changes in the hyraxes in contrast to the changes observed in the primates. This apparent situation of sub-clinical infection in the hyrax confirms previous reports on natural L. aethiopica infections in hyrax (Ashford, et al., 1973). Lemma, et al., unpublished report) was also unable to produce obvious clinical symptoms in experimentally infected captive animals.

It is evident from Table 5 that only benign or unnoticeable pathology developed in the experimentally inoculated hyraxes. In the experimental inoculations, tiny papules, bumps, scars, and patches developed; and since parasites could not be isolated or identified in these lesions it is possible that they were due to other causes. This is in parallel with the observations made on naturally infected hyraxes (Ashford et al., 1973).

It is very difficult to explain why parasite could not be identified or isolated following experimental inoculations although previous investigators were also unable to isolate Leishmania parasites by smear and histology techniques (Ashford et al., 1973). The same inoculum was used to infect two baboons and one monkey, and lesions were produced as early as 81 days in

Table 5. Summary of clinical features

Animal Sp.	NAD	Crusts, flakes & tiny scars	Induration	Sunken/raised patches	Bumps	Tiny papules (<2.5mms)	Larger papules (2.5-5mms)	Ulcers
Hetero-hyrax Sp. (8)*	7	0	1	1	1	0	0	0
Procavia Sp.(18)*	8	4	1	3	1	2	0	0
Papio anubis (1)*	0	0	0	0	1	0	0	0
Theropi-thecus gelada (2)*	0	0	0	0	1	1	1	0
Cercopi-thecus aethiops (1)*	0	0	0	0	0	1	1	1

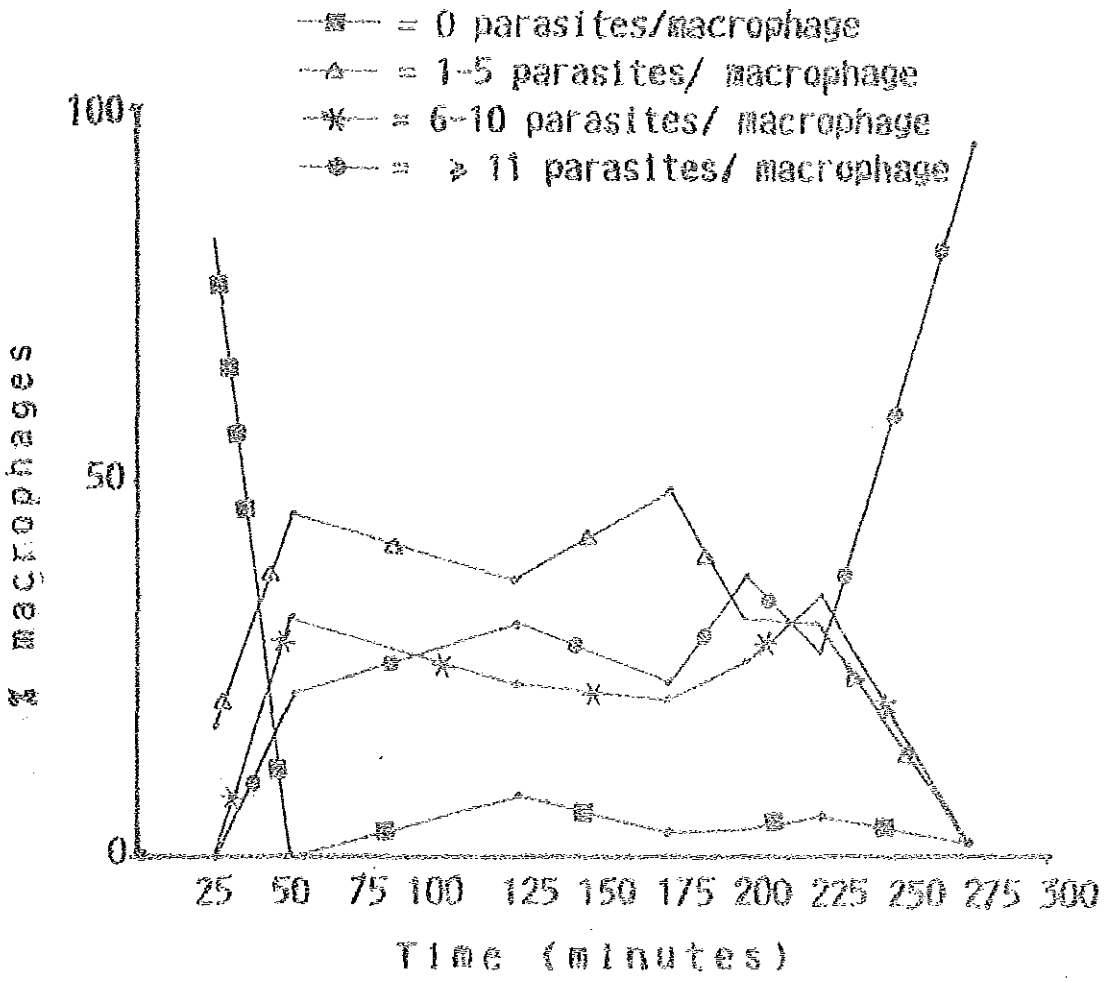
( )* = total number infected
NAD = No Abnormality Detected

baboons and 112 days in a grivet monkey. The slightly longer time taken for the development of the lesion in the grivet monkey is probably due to the fact that it was given a divided dose of 5×10^6 in three sites. The lesion on the grivet monkey formed a crust within two weeks of the lesion appearing.

Smears and cultures from these lesions were positive for leishmania parasites. The nodular lesions from the baboons started to regress within 8 weeks without forming ulcers or sores. During and after healing the lesions were smear and culture negative. The immunological changes in these animals have not yet been investigated.

Since no parasites could be identified or isolated from the lesions in hyraxes an in vitro approach was used to examine the initial interaction between the parasite and the host. Macrophage cultures derived from spleen cells were infected with stationary phase L. aethiopica promastigotes at an approximate parasite to macrophage ratio of 18:1. Samples were taken every 25 minutes for a series of 12 periods and then fixed and stained in Geimsa stain. The percentage of macrophages infected during the experiment is shown in Figure 1. Most macrophages were infected between 25 and 50 minutes and then infection stabilized until the macrophages began to die off. Only a small proportion of the macrophages (< 10%) remained uninfected after the first 25 minutes. At the end of the experiment (275 minutes) the majority (98%) were packed with amastigotes and many had burst releasing amastigotes into the medium. The rapid increase in the percentage of infected macrophages towards the end of the experiment may be due to reinvasion by released amastigotes. The rate of uptake of the promastigotes by macrophages was very rapid and clearly demonstrates that hyrax macrophages are capable of supporting the amastigote stage of L. aethiopica in vitro. Hyrax macrophages take up L. aethiopica promastigotes much more rapidly than human macrophages since even after 2

Fig. 1 Parasite load in macrophages over a period of 300 minutes.



hours incubation at a similar parasite to macrophage ratio over 40% of human peripheral blood macrophages remain uninfected (D. Humber, personal communication).

It is apparent therefore that the failure to isolate parasites from the site of inoculation of hyraxes is probably not due to a failure of hyrax macrophages to take up the parasites.

Histological Features

Histological material obtained from experimental inoculation sites was examined after fixation processing and staining in hematoxylin and eosin. In hyraxes, the specific features of tissue responses to leishmania that are found in man were not shown throughout the first 15 weeks of inoculation.Only non-specific inflammatory cells were seen in the infiltrate. In addition, the inoculated promastigotes could only be detected extracellularly up to 1 hour after inoculation ; after this period neither promastigotes nor amastigotes could be detected. Dermal scrapings and cultures from sites of experimental inoculation didnotreveal the presence of Leishmania parasites. It is possible that L. aethiopica infections in hyraxes do not localize superficially in the skin at the site of inoculation but that they are disseminated elsewhere in the body, possibly by blood born macrophages. Alternatively localized infection may result from repeated inoculation in the same area by infected sandflies.

In contrast to the findings in experimentally inoculated hyraxes, materials obtained from infected monkey and baboons showed either lymphocyte in the infiltrate or amastigotes inside macrophages. The histological findings are summarized in Table 6.

Table 6. Summary of Histological Features

Animals	Route of infection	Time after infection	Plasma cells	Lymphocytes	Histiocytes	Epitheliod cells	Giant cells	Parasites	Remark
Hyraxes	id	30-60'	+/-	+	-	-	-	+	promas-tigotes
	sc	2hrs.	+/-	+	-	-	-	-	
	id+sc	24hrs.	+/-	+	-	-	-	-	
	sc	1-15wks.	+/-	+	-	-	-	-	
	id	48hrs.	+/-	+	-	-	-	-	DTH site
Grivet monkey	sc	124days	++	++	-	+	+/-	+	ulcerative
Gelada Baboon	sc	135days	+/-	+/-	+++	-	-	+++	nodular
Olive baboon	sc	135days	++	++	-	+	+/-	-	Regressing nodule

+++ = Abudant
++ = Many
+ = Few
+/- = Scanty
- = Absent

One interesting histological observation is the presence of sweat glands in the skin of hyraxes, since in previous investigations these have been reported as absent in the hyrax (Kingdon, 1971).

Delayed Type Hypersensitivity

Optimization of skin test dose - The data obtained using different doses of killed promastigotes is shown in Figure 2. Analysis of variance between the means of the variable doses showed significant differences ($F = 8.5$, $CV = 27\%$). The optimum dose was determined to be 7.5×10^6 promastigotes per test since reactions plateau above this dose. Throughout these experiments two doses were used, the standard human dose ($0.5\text{-}1.0 \times 106$) and the higher optimal hyrax dose determined in this study.

DTH in Hyraxes - Animals were repeatedly tested (biweekly) over the entire observation period. The time course of the reactions presents a typical DTH reaction with the size of the lesion remaining relatively constant between 24 and 72 hours. No immediate reactions were observed. A characteristic feature of the skin test reactions was that induration was rarely accompanied by erythema.

The maximum mean induration in promastigote inoculated hyraxes was 2.5 ± 0.2 and 4.1 ± 0.1 millimeters at the low and high doses respectively. Although in humans, indurations of 5 mm and above are generally taken as positive (Manson-Bahr, et al., 1959; Manson-Bahr, 1961; Fuller et al., 1980; Leeuwenburg et al., 1983) these small responses were significantly different from uninoculated controls ($p < 0.05$ - Figure 3 - high dose). In comparison with response to leishmania antigens in humans these results appear to be very low, although it is possible that DTH responses in general in hyraxes are not well pronounced.

Fig. 2 · Dose - response curve of DTH reaction in promastigote inoculated hyraxes

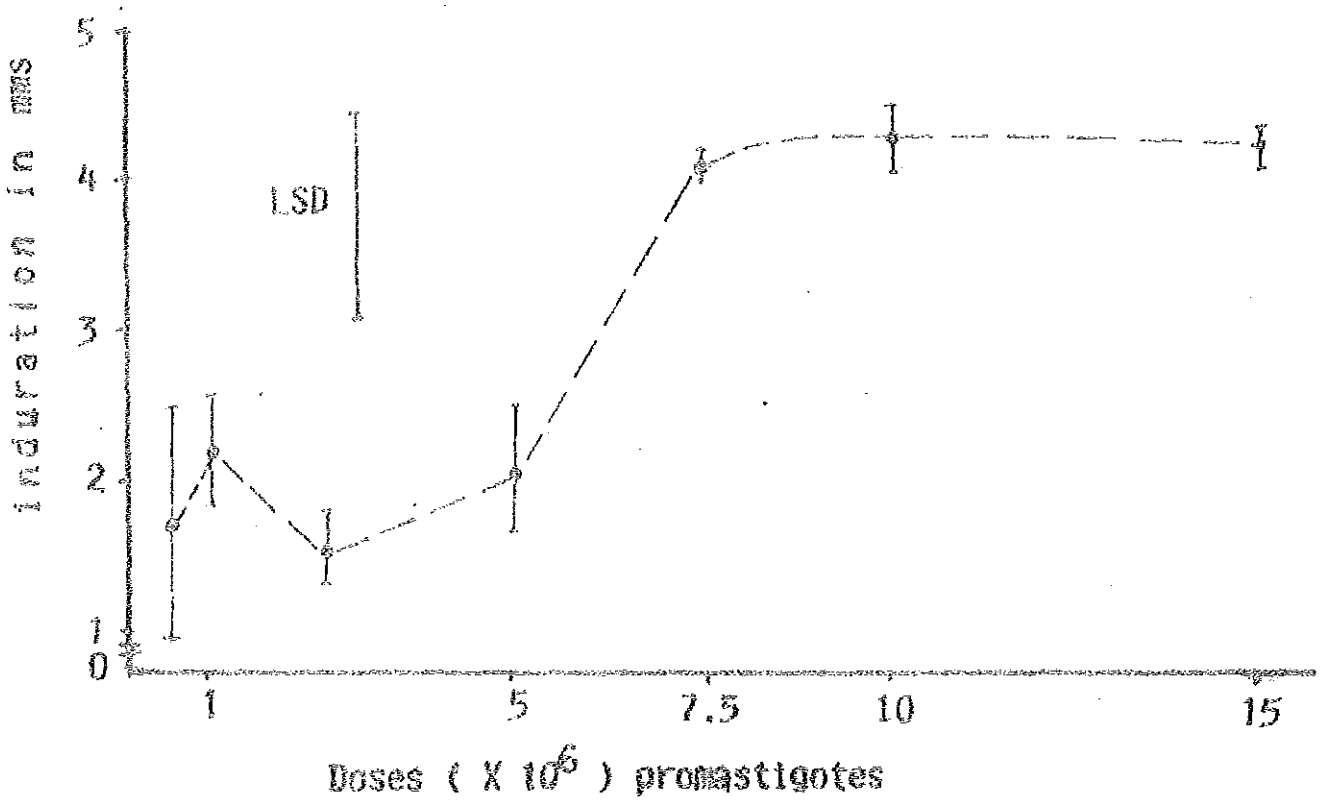
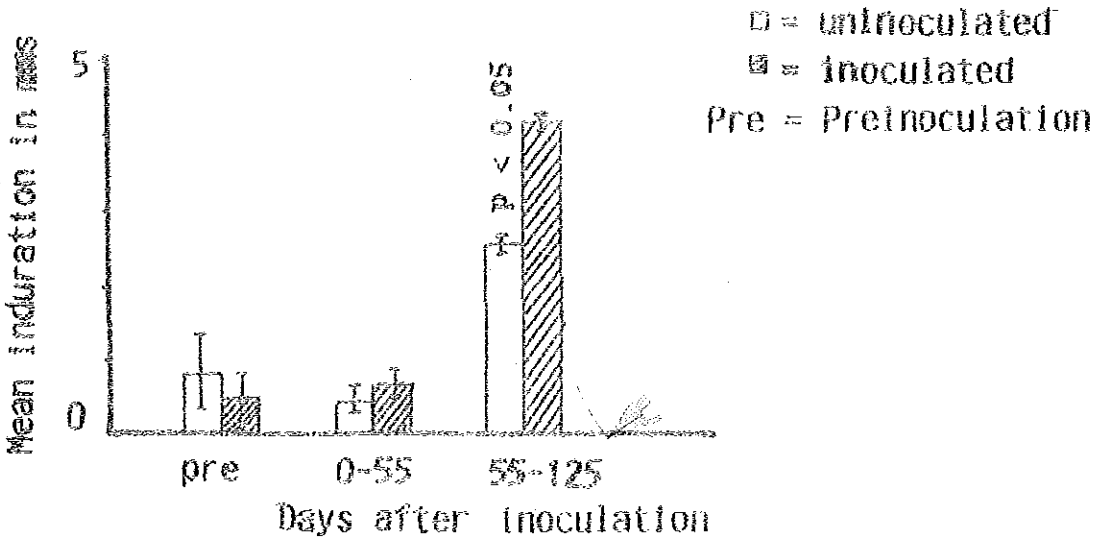
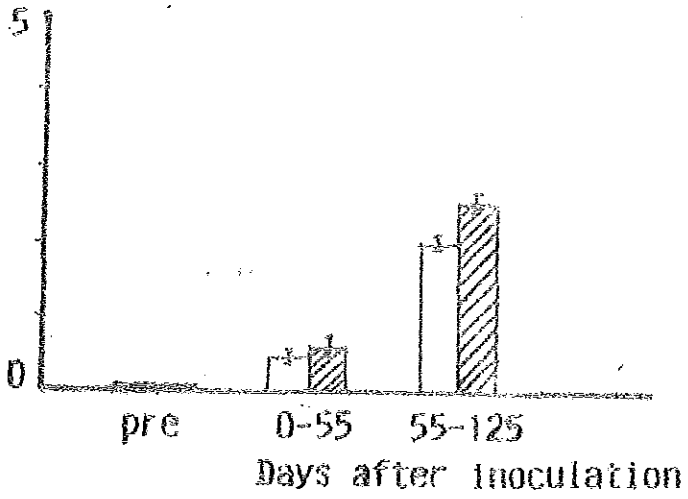


Fig.3 DTH In mms of mean induration for inoculated and uninoculated hyraxes over a period of 125 days

A. Using high dose (mean ± S.E)



B. Using low dose



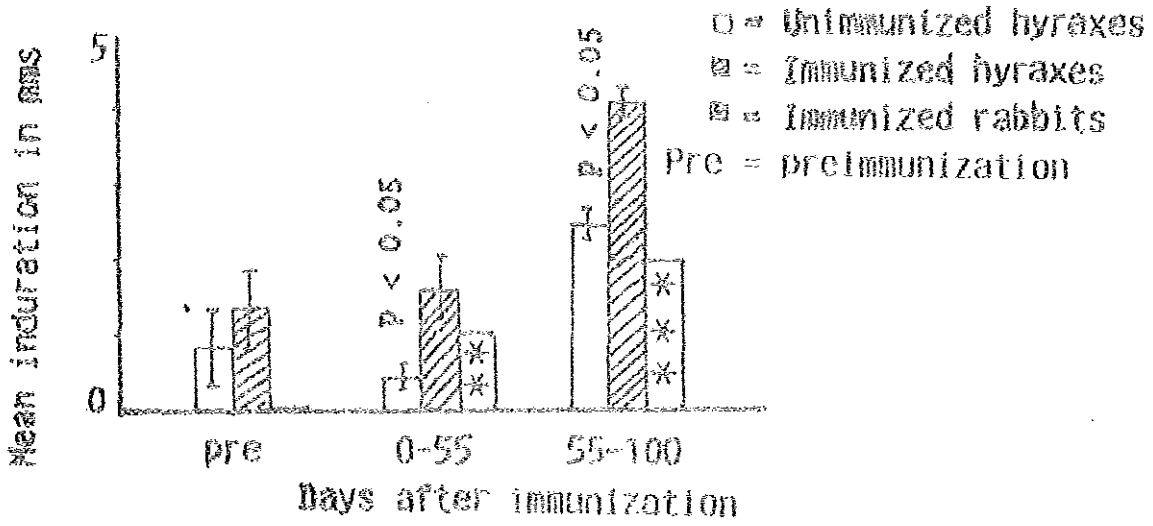
It is difficult to explain the abrupt change in the DTH responses late after inoculation since there is also a concomitant change in the uninoculated controls. One possible explanation could arise from batch differences in the skin test antigen. Another possible explanation is that these animals were progressively sensitized through repeated (biweekly) antigen administration. However, despite the changes in the control animals there is still a significant difference resulting from parasite inoculation. In contrast to the findings in this study, Ashford et al., (1973) were unable to demonstrate positive DTH reactions in hyraxes collected from endemic areas, including animals from which parasites were isolated. This difference in results may be due to the low dose of skin test antigen used by Ashford and his colleagues.

Immunization of both rabbits and hyraxes with leishmanial antigens resulted in the development of strong DTH reactions (Figure 4).

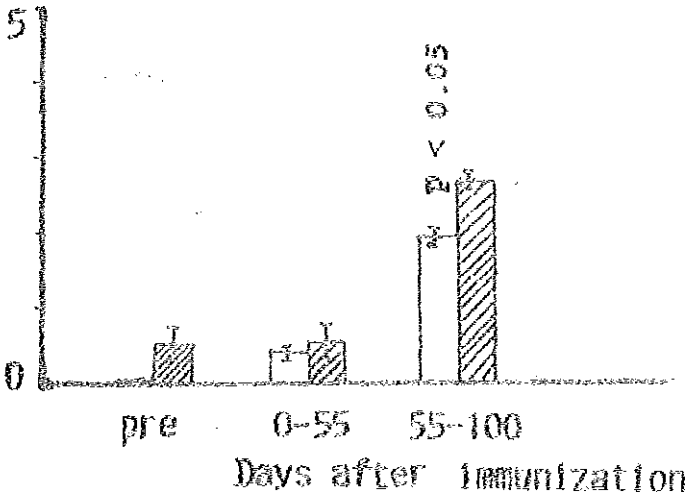
Positive DTH reactions in leishmania patients are generally strong (although less so in L. aethiopica infections) and indurations may be as large as 18 mm (Wyler, et al., 1979). In the infected grivet monkey an induration of 14 mm was recorded at the ulcerative phase of the lesion. The nature of the DTH response to leishmania antigens following inoculation therefore appears to be different in hyraxes and primates. Whether this is an intrinsic difference in the immune responses of these two groups or whether it is specific to leishmania infections is not clear. Ashford et al., (1973) claimed that the immune processes to leishmanial infections were unique in hyraxes, but it is not clear whether this assumption is valid since CMI was not carefully assessed.

Fig. 4 DTH in mms of mean induration for artificially immunized hyraxes and rabbits for a period of 100 days

A. using high dose (Mean ± S.E)



B. Using low dose



Humoral Immunity

Cytolytic assay - Fresh or frozen (-70°C) hyrax serum contained a heat-labile factor which when incubated with promastigotes resulted in lysis. The cytolytic effects of normal hyrax sera were compared with sera of other animals and man. The dilutions of the various sera causing 50% lysis are shown in Table 7 and it is apparent that in all other animals tested, that the natural cytolytic activity was greater than that in hyraxes.

Failure or success to infect host cells probably depends onthe balance between extracellular lysis and opsonization (Mosser and Delson, 1984), both of which are mediated by complement at high and low concentrations respectively. The lower titre of toxicity in hyrax serum might be relevant in discussing the

Table 7 Summary of comparative serum cytotoxicity.

Serum source	Log 2 reciprocal titre (50%lysis)
Human (4)	8.5 ± 0.6
Baboon (2)	7.0 ± 0.0
Rabbit (3)	6.0 ± 0.0
Guinea Pig(3)	6.0 ± 0.0
Hyrax (5)	4.0 ± 0.0
Control (PBS)	No lysis

Numbers in parentheses are numbers of animals.

equilibrium within the host-parasite system of L. aethiopica and rock hyraxes.

Although naturally occurring cytolyic factors (possibly complement factors) were present in hyrax serum, it was not

Table 8 Summary of cytolytic assays with hyrax and rabbit immune sera

C^1 Source and test dose	Immune Serum	Antisera dilutions Low	High
Killing C^1 dose			
*NGS (1/64 - 1/32)	infected hyrax	-	+
*NRS (1/32 - 1/16)	immunized hyrax	+	+
*NHrS (1/8 - 1/4)	immunized rabbit	+	+
Non killing C^1 dose			
NGS (1/128)	infected hyrax	-	-
NRS (1/64)	immunized hyrax	+	-
NHrS (1/16)	immunized rabbit	+	-

*NGS = Normal Guineapig serum; NRS = Normal rabbit serum; NHrS = Normal hyrax serum + = lysis; - = no lysis

Table 9 Antibody titres of immune sera from hyraxes and rabbits.

Antiserum source	Complement source and dose	Antibody titres Agglutinating	Cytolytic
Immunized Rabbit	NGS (1/128) NRS (1/64) NHrS (1/16)	1/512	1/128
Infected hyrax	NGS (1/128) NRS (1/64) NHrS (1/16)	1/512	trace
Immunized hyrax	NGS (1/128) NRS (1/64) NHrS (1/16)	1/256	1/64

possible to demonstrate complement fixing antibodies to L. aethiopica parasites after inoculation (Table 8). In contrast, immunization of hyraxes did produce cytolytic anti-leishmanial antibodies that would lyse promastigotes in the presence of Guinea pig complement, although the titres were lower than the agglutination titres and only about half of the cytolytic titres found in immunized rabbits (Table 9).

Agglutination Assay - Naturally occurring, heat stable, agglutination factors were present in normal hyrax serum and the titres of 44 hyrax sera ranged between 1:16 and 1:64, with a mean log 2 reciprocal titre of 5.1 $\pm$ 0.7. The titre was much lower in newborn hyraxes (2.0 $\pm$ 1.7).

Antibody titrations increased during the first 12 weeks after inoculation and the time curve of antibody responses after inoculation is shown in Figure 5. Analysis of variance indicates a significant difference among the mean titres of sera collected at 3 week intervals (F = 17.95, CV = 12.8%). Using an LSD at 5%, significant increases in antibody titres occurred during the 6th week of inoculation. From the data presented in Figure 5 it is possible to estimate a cut-off titre (at a titre of 1:85) which might be useful in diagnosing leishmania infections in hyraxes.

Titration of three sera obtained from two human cases of LCL and one DCL showed titres of 1:256, 1:512 and 1:1024 respectively. Mean titres as high as these were found in hyraxes 9 weeks post inoculation. Immunized hyraxes also produce agglutinating antibodies(Fig.6). Titres comparable to 6 weeks post inoculation were attained after the third immunization (3 weeks).

The role of antibodies in cutaneous leishmaniasis is not clear although various properties of antibodies have been demonstrated

Fig.5 Reciprocal agglutinating antibody titres before and after inoculation of promastigotes

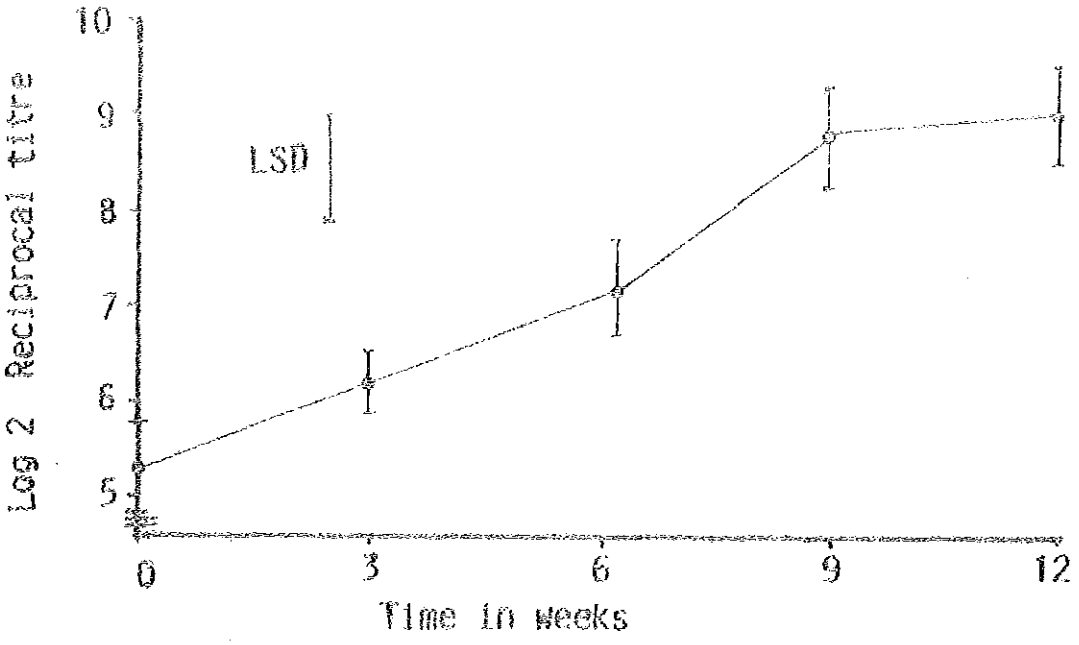
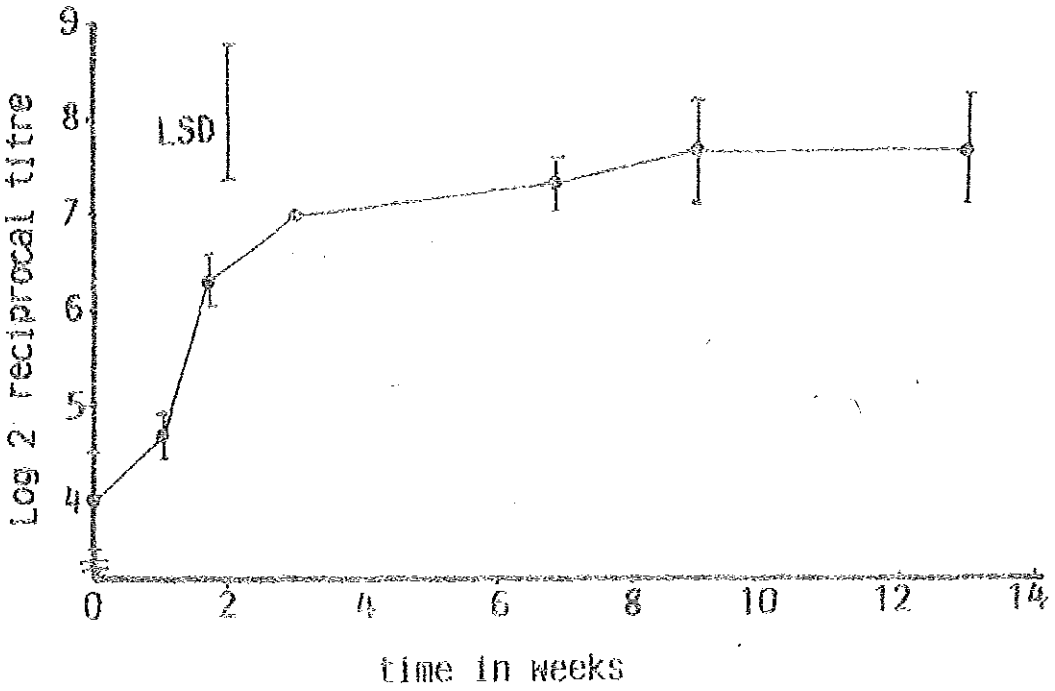


Fig.6 Reciprocal agglutinating antibody titres in artificially immunized hyraxes



including, anaphylaxis, agglutination, complement fixation, lysis, inhibition of parasite growth (Turk and Bryceson, 1971) and enhancement of infection. Temporal suppression of CMI in visceral disease, and the increased severity of cutaneous lesions in guinea pigs preimmunised with antigenic extracts of leishmania (Zuckerman and Lainson, 1977) are attributed to enhancement by circulating antibodies. Whatever the fate of the parasites which were inoculated experimentally into the hyraxes the antibody titre did not decline suggesting that parasite antigens are still present. In addition, these antibodies may play a role in either modulating the immune response against the parasites or even aiding their uptake by macrophages. The absence of cytolytic antibodies in the experimentally infected hyraxes may also be significant in the survival of the parasite in these animals.

Sandfly Biting Experiments

Laboratory reared and wild collected Phlebotomus longipes were allowed to feed on hyraxes. Forced feeding with the use of test tubes (Foster et al., 1970) was unsuccessful. However, sandflies kept in a modified Barraud's cage, fed readily on restrained hyraxes. Feeding took place from the nose, nostrils, lips and eyelids. In retrospect, all of these sites should have been sampled in the wild caught animals.

Sixteen sandflies were fed on inoculated hyraxes and 85% of the flies digested their blood meals within 8 days. Since, captive gravid flies might die after oviposition (Gemetchu, personal communication), all were dissected before or on the 8th day. No infections were seen in the dissected flies. Although this finding may be due to the sandflies feeding at uninoculated sites, 12 of the sandflies fed from the nose and 4 from the lips. A single fly required about 12.4 $\pm$ 7.7 minutes to become engorged. Feeding was continuous in all bites. It was calculated that 6 cm^2 of bare skin could support 7 biting insects.

CHAPTER III

SUMMARY AND CONCLUSIONS

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1) Rock hyraxes Procavia sp. and Heterohyrax sp. live primarily in well protected rocky habitats in which they are safe from predators and external climatic instabilities. The rock holes and caves provide a thermally stable microclimate which is highly suitable for hyraxes which are poor thermoregulators. The location of hyrax colonies in rocky habitats means that trapping with conventional live traps is unsuccessful although local snare traps were relatively efficient in some locations.

2) Maintenance and breeding of hyraxes could be undertaken on a long term basis. Although the ideal conditions for establishing a colony have not been analyzed in this study, one important requirement is the need for thermally regulated housing.

3) Endoparasites of hyraxes found in this study include the ascarid worms (Ascaris and Toxocara), Trichostrongyloides and Cyclophyllidean tapeworms. These helminths and the ectoparasites have not yet been identified to the species level.

4) Experimental inoculation of hyraxes with L. aethiopica did not result in overt clinical lesions. In addition, parasites could not be isolated or observed in lesions except immediately after inoculation. It must be assumed that these parasites are either very rapidly removed from the site of inoculation or are killed by the hyrax immune system.

5) Whatever the reason for the lack of parasites within the inoculated sites it is clear that hyrax macrophages readily take up promastigotes and that these subsequently transform

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