AN IMMUNOLOGICAL MODEL OF TRYPANOSOMA (TRYPANOZOON) BRUCEI BRUCEI IN THE VERVET MONKEY (CERCOPITHECUS AETHIOPS)

by

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This thesis is my original work and has not been presented for a degree in any other university.

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Signed.....

This thesis has been submitted for examination with our approval as supervisors.

Prof. T.R. Bowry

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LIST OF ABBREVIATIONS

AD	Anno Domini
BCG	Bacille Calmette - Guerin
BIIT	Blood incubation infectivity test
CO_2	Carbon dioxide
CMI	Cell-mediated immunity
CML	Cell-mediated lympholysis
^oC	Centigrade (Celsius)
cm	Centimetre
CNS	Central nervous system
CSF	Cerebrospinal fluid
Cr^{51}	Chromium^{51}
Co^{60}	Cobalt^{60}
CFA	Complete Freund's adjuvant
Con A	Concanavallin A
cpm	Counts per minute
^{14}C-TdR	(Methyl-^{14}C) - thymidine
cu	Cubic
Ci	Curie
DH	Delayed hypersensitivity
DNA	Deoxyribose nucleic acid
DDT	Dichlorodiphenyl trichloroethane
DEAE	Diethylaminoethyl
DNCB	Dinitrochlorobenzene (= Chlorodinitrobenzene; 1 - chloro-2, 4 - dinitrobenzene)
DNFB	Dinitrofluorobenzene (-2, 4 - dinitro - 1 - fluorobenzene

DNP	Dinitrophenol
dpm	Disintegrations per minute
DMEM	Dulbecco's Modification of Eagles Medium
E	East
EA	East Africa
EATRO	East African Trypanosomiasis Research Organization
ECF	East Coast Fever
EEG	Encephalography
EVE	Edinburgh Veterinary Expedition
ELISA	Enzyme - linked immunosorbent assay
EBV	Epstein - Barr virus
EAN	Experimental allergic neuritis
^{o}F	Fahrenheit
Fig(s)	Figure(s)
F_1	First filial generation
FUdR	5 - Fluoro - 2'-deoxy(6-^{3}H) uridine
F/P	Fluorescein/Protein ratio
FITC	Fluorescein isothiocyanate
FCS	Foetal calf serum
ft	foot (feet)
FFA	Free fatty acids
GPI	General paralysis of the insane
GvsH	Graft-versus-host
g	Gravitational force
gm	gramme; gram
Hb	Haemoglobin
HLA	Human leucocyte antigen (system)

H-2	Mouse histocompatibility system
hrs	hours
Ig	Immunoglobulin
ICI	Imperial Chemical Industries
in	Inch
IgG, IgM	
IgA, IgD	Immunoglobulin (G, M, A, D, T, X)
IgT, IgX	
ILRAD	International Laboratory for Research on Animal Diseases
i.u.	International Unit
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
K cells	Killer cells
Kg	Kilogram; Kilogramme
Km	Kilometre
lb	Pound
L-15	Leibovitz - 15 (medium)
LD	Lymphocyte defined; Leucocyte determinant
LPS	Lipopolysaccharide(s)
Log_{10}	Logarithm to the base ten
Log_2	Logarithm to the base two
LP	Lumber puncture
LNC	Lymph node cells
MPS	Macrophage phagocytic system
MHA	Major histocompatibility antigens

MHC	Major histocompatibility complex
MCH	Mean Corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean Corpuscular volume
MSE	Measuring and Scientific Equipment
MRC	Medical Research Council (of Great Brtain)
2-ME	2-mercaptoethanol
µ	Micron
µg	micro-gram
µµg	micro-microgram
µl	microlitre
µCi	microcurie
mCi	millicurie
mg	Milligram
ml	Millilitre
mm	Millimetre
mm^2	Cubic millimetre
mmol	Millimole
mM	Millimolar
MLC	Mixed lymphocyte culture
MLR	Mixed lymphocyte reaction
M	Molar
Mls	M locus
$NaHCO_3$	Sodium bicarbonate
NH_4Cl	Ammonium chloride
$(NH_4)_2SO_4$	Ammonium sulphate
nm	Nannometre

NK cells	Natural Killer Cells
NZB	New Zealand Black
NZW	New Zealand White
n.d.	not done
NRS	Normal rabbit serum
NIP	Normal immunosuppressive protein
N	North
PCV	Packed cell volume
PBL	Peripheral blood lymphocytes
PSG	Phosphate buffered saline glucose
PBS	Phosphate buffered saline
PHA	Phytohacmagglutinin
PFC	Plaque forming colony
PDT	Population doubling times
PID	Post-infection day
PIM	Post-infection month
PLT	Prime lymphocyte typing
P	Probability
PPD	Purified protein derivative
RBC	Red blood cells
RNA	Ribonucleic acid
R	Roentgen; Responder
RPMI-1640	Roswell Park Memorial Institute 1640 (medium)
SRBC	Sheep red blood cells
SRID	Single radial immunodiffusion
SS	Sleeping sickness
SRS-A	Slow reacting substance of anaphylaxis
S	South; Stimulator(s)

S.G.	Specific gravity
SPC	Spleen cells
sq	Square
SD	Standard deviation
SE	Standard error of the mean
SI	Stimulation index
SR	Stimulation ratio
SmIg	Surface membrane immunoglobulin
SmIg Cells	Surface membrane immunoglobulin-bearing cells
T-D	Thymus dependent
TNP	Trinitrophenol
TE	Trypanosomal extract
TREU	Centre for Tropical Veterinary Medicine of Edinburgh University
vs	Versus
W/V	Weight by volume
W	West
WBC	White blood cells
V/V	Volume by volume
VATs	Variant antigen types
VSGs	Variant surface glycoproteins
VSSAs	Variant specific surface antigens

SUMMARY

The vervet monkey was susceptible to two stocks and a clone of T. brucei. At a dose of 1×10^5 inoculated parasites the stocks killed the monkeys either in acute or chronic infection. Also, the cloned parasite showed a dose-dependent response. Monkeys died during acute infection when inoculated with 1×10^8 cloned trypanosomes. However, those inoculated with 5×10^5 or 1×10^3 parasites gave variable disease pictures and died either during sub-acute or chronic infection particularly when staphylococcal and streptococcal secondary infections supervened.

To effectively interpret the results in a T. brucei-infected monkey it was first necessary to establish the normal parameters for conventional cell-mediated responses in a normal uninfected monkey. In the peripheral blood of the vervet monkey, T (erythrocyte rosettes) and B (SmIg) formed 70 and of 10% lymphocytes, respectively. Peripheral blood lymphocytes at 2×10^6 per ml, responded optimally to non-specific mitogens, Con A and PHA but not to LPS when cultured for 72 - 120 hours and pulsed with ^{14}C-TdR for 16 hours terminally, as also did lymphocytes contained in ten-fold diluted whole blood.

Meanwhile responder lymphocytes at 3×10^6 per ml were optimally stimulated in an MLC type reaction with an equal concentration of xenogeneic CLA_4 cells when co-cultured for 168 hours and pulsed as above. The stimulation by allogeneic or semi-xenogeneic cells from vervet monkeys or humans was poor.

The relationship between infection, treatment and self-cure with respect to immune responses was also studied. After

immunization with tetanus toxoid, and 2, 4-dinitrochlorobenzene (DNCB) monkeys were inoculated with 1×10^4 trypanosomes. Subsequently, their lymphocytes were tested *in vitro* with Con A, PHA and xenogeneic CLA_4 cells (in MLC); their DTH tested *in vivo* with DNCB and their sera examined for specific (anti-trypanosome and anti-tetanus) and non-specific (heterophile) antibodies. Initially total IgM and heterophile antibodies were highly elevated while the IgG levels were only slightly raised. Meanwhile only the primary IgM (but not IgG) anti-tetanus antibodies and the MLR were enhanced. Subsequently, *in vitro* Con A, PHA and MLC lymphocyte responses, DNCB skin reactivity and both IgM and IgG anti-tetanus antibodies were depressed through the course of infections. Similarly, monkeys that survived the infection without treatment also showed depressed cell-mediated immune parameters.

Berenil treatment restored quickly, within 3 days the anti-tetanus antibody responses to normal. However, *in vitro* Con A, PHA and MLC as well as *in vivo* skin responses reverted to normal slowly after more than 30 days in the previously *T. brucei*-infected monkeys. Berenil had no effect on the immune responses of uninfected control monkeys.

Monkeys inoculated with 1×10^8 trypanosomes died of acute infection within 2 - 3 weeks while showing depressed lymphocyte responses to Con A and PHA. In the monkeys infected with 1×10^4 trypanosomes self-cure occurred and cleared the parasitaemia in the blood. However, self-cure did not abrogate the immunodepression as evidenced by persistently depressed stimulation indices from Con A-and PHA-stimulated lymphocytes

during the period of absence of detectable parasitaemia and 155 days after the start of the experiment. This suggested that the trypanosomes were perhaps still present in some occluded sites e.g. CNS or tissues, from where they probably exerted their persistent immunodepressive effect.

During infection leucocytes and their sub-sets changed numerically and proportionally. In monkeys inoculated with 1×10^8 or 1×10^4 trypanosomes leucopenia occurred accompanied by lymphocytopenia, neutrocytosis and monocytosis. The lymphocytopenia, was associated with low absolute erythrocyte rosette (T) and surface membrane immunoglobulin-bearing (B) cells and initially reduced but subsequently normal absolute "null" cell counts.

The proportions of lymphocytes decreased while those of neutrophils and monocytes increased, respectively. Normocytic and hypochromic anaemia was the most prominent feature of T. brucei infection in the vervets. It persisted in animals that died of the disease but was abrogated by Berenil treatment and self-cure both of which restored to normal the total counts and subpopulations of leucocytes in the chronically infected animals.

CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

The African trypanosomiases constitute a multifaceted medical-veterinary-economic problem of vector-borne human and livestock diseases (157, 416) which pose a great menace to human life and needs. Over 500 million acres of fertile but tsetse-infested land for settlement, agriculture, livestock, (except poultry keeping) and economic development denied to man by trypanosomiases could have supported 114-125 million cattle (416). The total effect has been reduced protein production, malnutrition, increased death rate, shortened life expectation, chronic ill-health and reduced power source for tilling the land and for ferrying goods (388).

Today, almost a century since the discovery of African trypanosomiases, very little is known about every aspect of the human and animal diseases. Similarly no ideal model for the human disease has been developed. Since the purpose of this thesis was to set-up an immunological model of Trypanosoma brucei infection in a non-human primate, Cercopithecus aethiops (the vervet monkey) so as to mimick the human disease, literature review will centre on the human and animal diseases caused by T. gambiense, T. rhodesiense and T. brucei and on the animal models.

Human sleeping sickness is a very complex disease involving the causative parasites T. gambiense and T. rhodesiense, the African-confined Glossina tsetse-fly vector, wild and domestic mammalian-reservoir hosts and the associated ecosystems of lakes, rivers and savannah woodlands either rich or poor in game.

Trypanosoma rhodesiense and T. gambiense classically

and, respectively, cause the acute Rhodesian and chronic Gambian sleeping sickness (153) characterised by parasitaemia, polyadenitis, late meningo-encephalitis and death if untreated.

(1) Distribution of sleeping sickness

Sleeping sickness is distributed over a wide Sub-Saharan African area between 12^{o}N and 20^{o}S of the equator while African trypanosomiases occur in over 36 tropical countries. The causative parasites are distinctly distributed though patients with T. rhodesiense may occur where T. gambiense prevails. Clinically the 2 disease types are not easily separable thereby making their distinguishing characteristics such as, virulence, vectors or drug responses only relatively valuable (29, 153).

An intermediate type between the classical forms of sleeping sickness occurs in the Zambezi basin and Botswana.

(2) Parasites

Trypanosomiasis is caused by flagellated protozoa of the genus Trypanosoma (137), family Trypanomastidae, order Kinetoplastida, phylum Protozoa. The genus Trypanosoma is divided into 2 main sections, the Stercoraria and the Salivaria (154). All important African trypanosomes belong to the Salivaria and include T. rhodesiense, T. gambiense, T. brucei, T. equiperdum and T. evansi which are homogenous, morphologically identical and closely related important African mammalian trypanosomes of the subgenus Trypanozoon (153, 215). The first two are important to man but all are pathogens of his domestic animals. The Salivaria also include the pathogenic Trypanosoma (Nannomonas) congolense and Trypanosoma (Duttonella) vivax and together with T. brucei they cause nagana, the animal disease.

Trypanosoma gambiense, T. rhodesiense and T. brucei are digenetic parasites, distributed in Africa like their tsetse vectors. Their life cycles alternate between the tsetse's gut and vertebrate host's blood (and/or tissues) wherein flagellates develop, and live, respectively.

In the mammalian blood the Trypanozoon are pleomorphic. The tsetse-borne T. rhodesiense, T. gambiense and T. brucei show all the 3 stumpy, intermediate, and slender forms (constant pleomorphism) while T. equiperdum and T. evansi lack the stumpy forms (inconstant pleomorphism) and are transmitted directly and mechanically between hosts by Tabanid flies or by contact including coitus. The slender forms occur in all the 5 subspecies.

Trypanosoma brucei (302) is probably an economically minor and lowly pathogenic veterinary parasite of livestock. It is apparently not infectious to man (154). Most domestic animals are susceptible to T. brucei and nagana, whose course depends on the parasite stock and host, is characterized by fever, anaemia, oedema, cachexia and frequently partial blindness and paralysis.

Trypanosoma brucei is virulent and fatal in equines, canines (153, 157), subacute in sheep and goats and lowly pathogenic in pigs (153, 353). Cattle are relatively resistant and self-cure. However, it is highly pathogenic and can be lethal within days to all laboratory rodents, for virulence increases with passages. Although, pleomorphic it becomes monomorphic (slender) after prolonged passages in laboratory mammals (153).

(i) The relationship between T. gambiense, T. rhodesiense and T. brucei

These 3 parasites are phylogenetically related and morphologically identical in their hosts (153, 353) and vectors. However, strictly they differ. They are separable biologically, physiologically and nosologically in minor serological, clinical or host characteristics which probably intergrade (153). The 2 human parasites produce in man acute and chronic sleeping sickness, respectively, while laboratory rodents are highly susceptible to the virulent T. rhodesiense and T. brucei. Both are zoonoses and share the same vectors and animal reservoir hosts. Laboratory rodents are relatively refractory to T. gambiense, an essentially human parasite with livestock reservoir hosts, e.g. the domestic pig. Yet, the Zambezi T. rhodesiense causes chronic Gambian-like and virulent diseases in man and rodents, respectively. The classical diseases are, therefore, possibly linked by the Zambezi form. The 3 parasites are probably races, stocks or types which clonally segregated from homogeneous parental species with new characteristics and by favourable natural selection adapted themselves to new hosts (153). Such variants are denoted by the term deme with the prefix indicating the nature of the differentiation criterion like nosodeme, plastodeme (153, 215). Whether a virulent stock or race of T. gambiense gave rise to T. rhodesiense (12, 29, 153, 398) or T. gambiense (purely anthroponotic) originated from T. rhodesiense (anthropozoonotic) which in turn came from T. brucei (purely enzootic) 29, 153, 196) is not known.

The variable pathogenicity of stocks and demes even within species probably affect the clinical signs. Hence biological differences have sometimes resulted in the parasites being accorded independent species status. Thus the above three parasites have long been regarded as separate species because of differences in their distribution. However, whether immunologically and biochemically trypanosomes which probably belong to one species deserve their own species status, is not a subject of this thesis. Suffice it to say the three parasites are closely related.

(ii) Life cycle

Trypanosoma rhodesiense, T. gambiense, and T. brucei show an identical cyclic development in the vector's gut and salivary glands and hence the inoculative transmission of infective metacyclic forms into the mammalian host (29, 153, 353).

In the mammal the parasites' development is initiated by infective metacyclics actively inoculated by the vector's bite. In man during the 9 days' prepatency the metacyclics' development is confined to the primary chancre (nodule). Thereafter, the parasites invade the blood stream, and throughout infection, multiply continuously into the long trypanosomes (trypomastigotes) by equal binary fission (153, 208). The frequently dividing slender forms numerically predominate over the rarely dividing intermediate and stumpy forms which are infective to tsetse wherein they develop (153, 409).

The brucei sub-group trypanosomes are characterised by parasitaemic waves of alternating pleomorphic forms (153, 391).

Within the mammal the brucei sub-group are primarily tissue parasites that also circulate in the blood (29, 352).

(3) Pathogensis

The pathogenesis of trypanosomiasis is complex. The minimum infective dose of T. rhodesiense for man was, by probing technique 300 - 400 (100) and by mice titration using Glossina morsitans 0 - 40,000 (mean 3,200) metacyclics (140). The prepatency and the number of parasites inoculated were inversely and logarithmically related (26). A higher dose of the Ugandan T. brucei stock produced a higher parasitaemia and more severe infection than the Botswana and Tanzania stocks which produced identical infections regardless of the challenge doses (288). The metacyclics injected directly into blood lack prepatency unlike those traversing tissues from the blood pool meal (153, 208, 288).

Pleomorphism has been related to the parasite's virulence and course of infection. The parasite's growth rate or its suppression by the host determines its virulence. Laboratory passaged stocks become more virulent probably because rapidly growing ones are selected before immune inhibition occurs.

Thus experimentally the type of infection is a property of both the parasite and the host. For instance, prolonged passaged stocks of T. rhodesiense showing only thin forms with smaller or larger loculae cause acutely progressive diseases in man and rat or chronic disease in man, respectively (288). Also during trypanosomiasis epidemics transmission by Tabanid flies like syringe-passage probably increases the parasite's virulence.

The brucei sub-group trypanosomes cause lesions through their increased metabolism and through the host's aberrant immune responses to successive variant antigens which the host vainly immunodepresses. Except in rodents the parasitaemia in the blood is low and therefore is not an ideal guide to the exact numbers of trypanosomes which may be abundant in the tissues.

Clones and/or stocks markedly differ in virulence. Some trypanosomes kill animals in 7 to 9 days while others induce chronic diseases after 3 parasite waves or self-limit (abort) after 2 months, possibly due to the parasite's pathogenic effects.

However, sometimes virulence depends on trypanosome stocks and not on the host's health because in the blood numerous mild avirulent trypanosomes (288) and also in "virgin" areas very virulent T. rhodesiense-like T. gambiense parasites that involve the CNS within 2 post-infection months occur (13, 92, 288).

High parasitaemia may quickly kill the host but an effective antibody response probably cures the disease, with or without (399) permanent and sterile immunity to local variants. Usually as local and physiological changes are rectified the parasite and the host seemingly reach a precarious compromise easily upset by secondary infections, stresses, such as, famine, malnutrition, other diseases and childbirth (13) for, paradoxically, the host's immune response to heterologous antigens are suppressed.

Tissue damage results from antigen-antibody reactions activating plasmogen, releasing pharmacologically active

substances like kinins, changing the blood-clotting mechanisms and also from immune complex depositions in the kidneys and other tissues. The excessive trypanosome motility damages tissues indirectly through its excessive carbohydrate demand as an energy source (119). However, the pyruvate and parasite levels are directly proportional (66, 119). The lactate and pyruvate levels increase 2 and 5 times, respectively, (119) in tissue fluids and blood. The lactate initiates fatty degeneration in connective tissue and skeletal and possibly heart muscles and also reduces collagen fibre formation (85, 119, 122).

(4) Clinical picture

Clinically typical sleeping sickness cases are rare for the parasites may be distributed in each others area and because an intermediate disease form due to T. rhodesiense exists (15, 153).

The chronically mild insidious and well-tolerated T. gambiense that lasts up to 21 years is sometimes discovered only during routine examination (13) in Africans but is acute in people of European descent, followed in both races by almost asymptomatic and eventless months or years except for increased headaches and occasional pyrexia. The progressive CNS signs of somnolence and extrapyramidal lesion tremors gradually appear. The body pales and wastes while the course and death may be aggravated and hastened to within 1-3 year(s) by secondary infections, such as, pneumonia and by stress.

Trypanosoma rhodesiense characterized by high

parasitaemia may be more or less virulent with toxaemic death occurring in 1 or 2 post-infection week(s) while in the same area concurrently showing a normal course with the patients remaining fairly well. Treatment is usually delayed because correct diagnosis depends on patent parasitaemia. Between 3 - 12 weeks sequentially oedema and cardiac and CNS symptoms appear. Untreated, the disease normally kills between 3 - 9 post-infection months. In a newly introduced unlike in an endemic area T. rhodesiense is initially very virulent and acute but it becomes mild, chronic, asymptomatic and even self-limiting with time and endemicity (13). Healthy asymptomatic carriers with human parasites virulent for laboratory animals and intermediate between T. gambiense and T. rhodesiense were common in Zimbabwe (Rhodesia), Malawi and in Ngamiland but few or none in Tanzania and in Uganda (13, 15, 29, 153). Thus the virulence probably increases from South to North (153). Hence today the disease is more mild in Malawi, than in Tanzania.

The clinical features of human trypanosomiasis include: the transient trypanosomal nodule or chancre (13, 85, 152) due to the tsetse bite; malaise, headache, joint pains and pyrexia coincident with each parasitaemic wave; rash, oedema (13), cachexia, anaemia (418); jaundice and hepatomegaly due to hepatitis (13, 288); lymphadenopathy (Winterbottom's sign) (13); splenomegaly and ocular lesions. Other features are cardiomegaly, bradycardia, tachycardia, pan-carditis (304), conduction defects, cardiac ischaemia (13, 288, 398); sterility, abortions, impotence and orchitis. The late central nervous system symptoms involve

headache, mania, sensory and motor nervous system disorders like polyneuritis, paraesthesia, locomotor ataxia, somnolence, terminal epileptiform convulsions, coma and death.

Trypanosomiasis in children and as a congenital disease with hydrocephaly and parasitaemia due to placental transmission though rare have been reported (13, 288).

In domestic animals T. brucei clinically produces fever, apathy, lethargy, oedema, cachexia, anaemia, ocular lesions, intercurrent infections and terminal locomotor ataxia and death (153, 353).

(5) Pathology

The pathology due to T. rhodesiense, T. gambiense and T. brucei in their respective hosts is similar except for details such as, perivascular cuffing which is minimal in the acute T. rhodesiense but heaviest in the chronic T. gambiense and the Zambezi T. rhodesiense infections.

Dermal, lymphoid tissue, hepatic, cardiac, haematological and cerebral (syphilitic-like but without demyelination and neurone damage) affections in a patient are early sleeping sickness changes (13, 29, 208, 288). The most classical lesions occur in the brain and are associated with the advanced disease. Depending on the type progressive cerebral lesions evidenced by increasing headaches, diurnal drowsiness and nocturnal insomnia probably appear from 3 - 8 post-infection months.

Chronically very severe and extensive meningo - encephalitis with the dura adhering to the arachnoid matter occur at the base of the brain in Trypanosoma gambiense infections

(208, 288). It is due to either lymphatic or haemotological spread of infection into the CSF across the 'brain barrier'. A tap of the lateral cistern unlike a lumbar puncture (L.P.) is more diagnostic (13, 288).

The inflamed, thickened and adherent piarachnoid is infiltrated by 1 - 30 cell thickness of lymphocytic, plasma and morular cells. The last cells are pathognomonic. They occur in perivascular cuffs during a typical chronically advanced disease and are obtained at L.P.

Progressive microgliosis occurs in the subarachnoid space. Perivascular cuffing may be severe and advanced in T. rhodesiense infection that normally has little or no cuffing. The brain damage and mental deterioration (208, 288) are associated with chromatolysis and Wallerian GPI-type degeneration of neurones and their tracts around the perivascular cuffs of T. gambiense but probably not of T. rhodesiense infection. The brain damage is more severe in T. gambiense than in T. rhodesiense infections. Severe cortical tissue destruction, residual dementia even after treatment of T. gambiense is however, equivocal. Probably cerebral degeneration due to syphilis and perivascular cuffing of trypanosomiasis are irreversible from their onset (208, 288).

Except for the greater cerebral degeneration superficially trypanosomiasis resembles syphilis, polymorphonuclear - rich septic meningitis and acute viral encephalitis without morular cells of chronic infection but not cerebral malaria , a non-meningo-encephalitid,characterized by pigmented schizonts in the blocked brain capillaries. However, petechial haemorrhages

due to ruptured capillaries occur in malaria and also in acute trypanosomiasis in which thrombosis is rare.

The brain, skeletal muscle and pituitary gland lesions due to T. brucei in ungulates, equines, canines (208) and monkeys (298) resemble those in humans.

The CSF, pericardial, peritoneal and pleural effusions, aqueous and vitreous humour have numerous trypanosomes.

(1) Immunology of trypanosomiasis

The mammalian immune system can respond to an antigenic challenge in three ways: (a) produce a detectable immune response either humoral, cellular or both, (b) become tolerant or unresponsive, (c) a combination of all these responses. Generally the type of response produced depends on the antigen dose, the degree of antigenicity, the presentation of the antigen and the immunological status of the host. During trypanosomiasis all these features apparently vary. Probably antigenic variation makes the trypanosome more or less immunogenic during infection and; antigens may be presented either on a trypanosomal cell surface or as freely soluble. Physiologically, biochemically and hormonally the infection may alter the host immunologically. Also protozoal antigen challenge may be a primary event in a clean normal host or a secondary one involving immunological memory in a previously exposed host. Obviously then the mammalian host response to trypanosome antigens will not be simple.

In this section the antigenicity of T. brucei is first reviewed.

(a) Antigenicity of Trypanosoma brucei

The antigenic status of T. brucei and indeed of any

trypanosome, protozoon or parasite including a helminth is difficult to define. This is because, firstly, some protozoal antigens are rapidly varying, secondly, protozoal antigens are not strongly immunogenic and thirdly it is possible, that soon after inoculation by tsetse-bite or by the syringe the parasite not only possesses all trypanosomal antigens but also modifies, acquires or exhibits various host antigens. When discussing protozoal antigenicity it is therefore essential to define the antigen under study and to state its location.

As a result of trypanosomal antigenicity a mammalian host may respond by antibodies to the trypanosome-derived antigens which occur in the serum.

(b) Antigens

The common, exo-, heterophile and variant surface antigens are associated with trypanosomiasis (85).

(i) Common antigens

These are homogeneous intracellular and nucleo-protein components (85, 372, 399). They are apparently qualitatively unchanged during infection and elicit complement fixing, agglutinating, fluoresceing and precipitating antibodies (13, 29, 85, 326, 399). Although they are typical proteins they are common to (cross react with) different trypanosome stocks and sometimes species (85, 125).

The common antigens are released during the crisis of each parasitaemic wave and are probably eliminated rapidly by the antibody or by catabolism. Their importance in protective immunity is doubtful but they are useful in immunodiagnosis.

(ii) Exo-antigens

The soluble T. brucei exo-antigens are loosened cell particles covered with surface plasmanemes (filopodia) and react with agglutinating, protecting and precipitating antibodies (85, 372, 391, 398, 399). Clinically they resemble and have been associated only with cell-free variant antigens (65, 85, 372, 391). However, as surface antigens they possibly occur only in vitro where parasites disintegrate when environmental conditions change (253, 372, 399). A new antigenic variant appears simultaneously with the corresponding new variant specific exo-antigens, possibly produced by it. Also exo-antigens probably include the freely circulating common antigens and some antigenic products (85). However, the release of protective variant antigens either on filopodia (253, 372) or in soluble form probably protects the trypanosomes against the host and also causes disease in the host.

(iii) Heterophile antigens

These occur on the surface of tissue cells of various animals (especially horse, sheep, mouse, etc), plants and bacteria. They show extensive interspecies cross-relationship. In man and rabbits, species which lack them, these antigens stimulate heterophile antibodies.

Heterophile agglutinins (148) revealed in 85% of T. gambiense patients are probably due to a Forssman-like antigen (158) but the Forssman antibody detectable by sheep erythrocyte agglutination test may occur as a natural antibody in human serum. Their increased titres following a trypanocidal

treatment suggests that the heterophile antigen is within the trypanosomal cell.

(iv) Variant antigens

These are best considered together with antigenic variation. The variant specific antigens are potent protective immunogenic glycoproteins externally located on living bloodstream trypanosome surface coat (but on malaria - and Babesia - parasitized erythrocytes) which is replaced by shedding. The parasites thereby change antigenically during infection (372, 391).

Each T. brucei variant specific glycoprotein is the predominant parasite surface constituent, that is, antigenically distinct, for a particular clone (72). However, despite the identical size T. brucei glycoproteins (85, 125, 372) from different antigenic variants or stocks are large structural proteins (polypeptide chains) of different amino acid compositions (sequences). However, those from one variant are homogeneously related glycoproteins sharing a common antigenic determinant and neutral sugars, D-mannose and D-galactose (9, 85, 281, 372). Thus in variant specific surface antigens (VSSAs) variable determinants specific to individual and cross-reacting determinants between VSSAs isolated from different species of trypanosomes occur.

Variant antigens stimulate in the host protective antibodies against the homologous variant parasites without cross-protection between heterologous variants of the same stock or species (253).

The variant antigens are probably produced by antigenic variation. This is a phenomenon whereby the sequentially succeeding parasites from single protozoal species on infecting a mammal are recognized by the host's immune response as antigenically distinct. The specific antibodies formed against each population of, say, T. brucei or T. congolense (265) antigenic variants can be shown by agglutination, immunolysis, cell surface protein antigens,neutralization, re-challenge and double diffusion technique (125, 399). The antigenic variants in succeeding parasitaemic waves do not cross-react serologically (412).

Antigenic variation occurs in all pathogenic mammalian African trypanosomiases even when initiated by one organism. The antigenic variants clonally distributed are probably numerically unlimited for each species (85, 104, 372). However, the variation may be limited by certain frequently occurring types through reversion, of the numerous different antigen types arising in mammal hosts during infection with one T. brucei stock, to one serotype (basic, predominant or common antigen variant of the stock) on cyclical passage in one or several tsetse(s) to new normal mammalian hosts regardless of the antigenic type or the origin of the tsetse fly ingested bloodstream trypanosomes, or on rapid syringe-passage in rodents in which the parasites avoid exposure to specific antibody (125, 372). Hence only a few variant antigen types are probably transmitted by the flies.

After ingestion by a tsetse fly the trypanosomes lose

the surface coat and therefore the variant antigen as well as the infectivity to the mammalian host. These properties are re-acquired during the development of the infective metacyclic's stage in the fly's salivary glands (391, 392).

Natural isolates sometimes contain genetically different parasites as a mixture of stocks. When cyclically transmitted such isolates simultaneously revert the stock to their corresponding metacyclic antigen types in a tsetse fly.

Thus structurally and antigenically the variable antigen of the freshly extruded mature metacyclics is different from that of the corresponding tsetse fly ingested bloodstream forms of each of T. congolense and T. brucei (91, 267, 392). However, it is identical antigenically to the metacyclics which arise after cyclical transmission of different variant antigen types of the same stock. Yet, the metacyclics derived from different T. brucei stocks apparently also differ antigenically; that is, they show different variable antigen types (267).

In the reversion of different antigen types of a stock to a common type on cyclical transmission it is not known whether the metacyclics are uniformly identical in surface antigen or how many basic antigens occur within a given parasite species.

During the rising of undulating parasitaemia variant antigens are produced by antigenic variation. Each trypanosome has genetic loci any of which can be switched on, transcribed and be translated by a temporary interlocus repression mechanism and be expressed at an appropriate time as a new

variant antigen molecule into the parasite's surface coat (47, 65, 86, 125, 265, 392).

Antigenic variation has many implications in protozoology. The variant antigens and the antigenic variation in Trypanosoma, Plasmodium and Babesia, Entamoeba, Paramecium and Tetrahymena are probably important for survival (65) and for evading the host's specific protective immunity against the protozoa (91).

The total variant types that arise during pathogenic protozoal infections with clones or stocks; the repertoire of antigenic diversity of a given species in a defined locality or country; the relevance of its (antigenic variation) possible limitation by the appearance of certain basic predominating antigens following cyclical transmission; the relevance of the common variant antigenic type to the epidemiology of the diseases; the mechanisms of antigenic variation, their relevance to the epidemiology and the control of these diseases in mammalian hosts are unknown. Probably through random (genetic) antigenic variation (267) the antibody modulates, selects (266, 267) rather than induces (125) African trypanosomes to synthesize new variants. These antigens protect the parasites from the cytolytic antibodies against the previous serotypes as they invade the blood and tissues from the host's privileged sites.

Biologically a variant antigen would probably never appear twice during an infection (125) though one trypanosome produces similar variants in different sequence in different hosts. However, during T. brucei and T. congolense cloned

infections in cattle variable antigenic types (VATs) re-appeared and self-cure occurred (267). Whether the VATs were due to trypanosomes "running out" of the ability to synthesize new variable antigens and therefore reverted to types already formed or due to those similar but not identical cross-reacting antigenic determinants between VSSAs, is unknown.

(c) Immunity

The immunity against a pathogen may be non-specifically and/or specifically innate and/or acquired while acquired immunity may be weak or strong. A useful immune response against trypanosomes contributes to the host's resistance by reducing the parasitaemia through non-specific (innate) or specific acquired immunity while a detrimental response contributes to the disease pathogenesis.

Originally it was thought immunity to trypanosomiasis probably disappeared concurrently with the eradication of infection and the recovered patients were susceptible to re-infections (17, 100, 399, 410) probably because the immunoglobulin antibodies were thought to be non-specific and memory cells absent. Indeed, clinical evidence for specific and effective immunity against human and sometimes animal trypanosomiasis is disputed because it is difficult to come by. However, experimental animal and human studies have revealed and confirmed that mammalian hosts possess both humoral and cellular immunity to trypanosomal antigens only that the latter is very depressed.

Innate resistance to protozoa such as trypanosomes is

parasite and/or host specific and not dependent on phenotypic pre-exposure of the host to the pathogen. Closely related protozoa may infect different hosts. For example, T. gambiense unlike T. brucei cannot infect readily numerous mammals possibly because of herd immunity arising from contact with the pathogen for generations. This would be like the resistance and susceptibility to, respectively, yellow fever and tuberculosis, "old" and "recent" diseases to Africans.

Whether man's resistance to T. brucei is genetically controlled through mutation or enzymes like the Negroid resistance to P. falciparum through sickle cell haemoglobin (7) is not known.

The complement in host's blood is a non-specific defence mechanism comprised of many enzyme systems. When activated classically or through alternate pathway it produces anaphylotoxin and chemotoxin and induces phagocytosis. All these probably occur in trypanosomiasis. IgM is much more efficient than IgG in binding complement.

Innate resistance is probably responsible for the greater resistance of some indigenous cattle breeds compared with exotic. The N'Dama and Muturu than Zebu cattle tolerate the infection much better due probably to a genetically-endowed innate immunity reflected in a more efficient acquired immune response because hybrids of N'Dama and Zebu are intermediate in resistance (59).

Natural resistance against trypanosomes occurs in calves, indigenous cattle and game. Young calves better than

adult cattle of the same breed resist trypanosomiasis, generally recover after the acute phase (104), survive longer and seldom die because their immune response more efficiently enables them to ultimately control the trypanosomes. This experience of infection in calf-hood when animals can cope better with the trypanosomes is very important because indigenous breeds born where trypanosome challenge is absent show immune responses and resistance like the susceptible Zebu cattle (399). Trypanosoma congolense-infected adult cattle become chronically diseased after the acute phase, gradually waste away and die within 5 - 7 months (104).

Also the apparent innate resistance in the healthy-looking game but not in the diseased domestic animals in the tsetse area seems genetic. It represents several related and unrelated operating factors.

Innate resistance (153, 372) in trypanosome-infected wild but not domestic animals was ascribed to nutritional and toxic factors and acquired protective mechanisms. The active principle of the natural toxic agglutinating anti-lewisi in the refractory mouse's serum is an IgG. However, the anti-vivax in the serum of a refractory rat either are serum proteins, albumin, gammaglobulins, fibrinogen or is a 2-ME resistant (IgG-like) specific macro- β -globulin-associated factor (372). Unlike natural antibodies it is an Ig whose titre is not raised by specific or cross-reacting antigens (162, 163, 372). Also, it resembles a multivalent plant-like lectin and some invertebrate-like protein that reacts with surface antigenic determinants on

T. vivax and thereby prevents infection of cotton rats with the parasite.

Similarly, T. brucei unlike T. rhodesiense and T. gambiense does not infect man because of the active serum trypanocidal IgM-associated macroglobulin and alpha-2 proteins which are more in adult than in the newborn (86, 142, 372). Thus some of the anti-vivax, and -brucei factors in the cotton rat and human sera, respectively, are probably powerful non-immunoglobulin protein agents of innate resistance. They accidentally structurally fit the trypanosome receptors and protect the refractory heterologous host (372).

The innate protective mechanisms of a host refractory to a heterologous parasite can sometimes be abrogated by serum supplements from a susceptible host. For example, T. (D). vivax and Trypanosoma (Herpetosoma) lewisi parasites of sheep and rat, respectively, require normal sera as supplements from these natural hosts to abrogate the innate resistance of, and to infect, the heterologous refractory cotton rat and mouse (372), respectively.

On the basis of innate immunity the blood incubation infectivity test (BIIT) was developed to distinguish T. brucei from T. rhodesiense (315). In the laboratory but not in the field (366) the test is consistent for T. rhodesiense and T. gambiense and not for T. brucei stocks. Probably when parasites are long maintained in the laboratory hosts they resist the serum following incubation and remain infective for rats.

Natural immunity to protozoal infection may also be cell-mediated as against either Babesia in humans or P. vivax, P.falciparum and P. ovale malarias in chimpanzees (65, 374). The "agile" and short-lived polymorphs and, the sluggish but long-lived monocytes and macrophages as phagocytes are attracted to the site of infection by different chemotactic factors, resulting in parasite ingestion, and digestion through enzyme release. Furthermore, since macrophages directly and non-specifically destroy malignant cell membranes possibly this also occurs in trypanosomiasis after the active trypanosome is immobilised.

Acquired immunity may also be non-specific, so that the parasite's survival and proliferation are probably influenced by the host's non-specific immunity factors including physiological changes such as the inability of T. vaginalis to develop in the female genital tract before puberty. Also, concurrent bacterial infections like B. subtilis, C. perfringens or A. aerogens modify the susceptibility to protozoa making commensals like E. histolytica and E. coli infective (271, 299); yet, also inhibiting other protozoal infections. For example, T. brucei in mice was curtailed by spirochaetes while T. rhodesiense, T. congolense, T. cruzi and P. berghei were inhibited by live or dead bacteria such as Borrelia or their endotoxin (65).

Cross-immunization, macrophage activation and induction of interferon synthesis all of which inhibit toxoplasma and pre-ocytic forms of malaria (65) are also probable. operative

protective immune mechanisms in trypanosomiasis.

Specific acquired immunity in African trypanosomiasis probably occurs. However, man does not develop strongly effective and permanent immune responses against T. rhodesiense and T. gambiense (399) and hence the reported healthy carriers (65, 286).

Premunition is a specific immune response to parasites. It results in clinical recovery associated with low persistent parasitaemia and resistance to specific challenge (65). Although not reported in human African trypanosomiasis (despite self-cure), it occurs probably in T. rhodesiense of wild ungulates, and of humans as healthy carriers (65, 286), in P. falciparum and several other mammalian protozooses. In these diseases clinical resistance is associated with periodically detectable parasitaemia (65).

Sterilising specific immunity is associated with clinical cure, complete parasite elimination and life long specific resistance to re-challenge. This occurs in human cutaneous leishmaniasis and trypanosomiasis in N'Dama and Muturu cattle which have been exposed to infection for generations. Similarly, the rapidly lethal and fulminating P. knowlesi in Macaca mulatta produces premunition in M. fascicularis. In contrast, L. tropica is either transient or chronically relapsing in different persons. The degree of acquired immunity to the same pathogen is thus unestablished. However, acquired immunity, be premunition or sterilising, in human and experimental malaria and African trypanosomiasis is generally species and stock and sometimes (in

malaria) even stage specific (65).

(B) Immunopathology

In trypanosomiasis detrimental (harmful) immune responses lead to immunopathology.

Immune complex phenomena, autoimmunity (219) and intravascular coagulation in man (34), monkeys (326) and rabbits (43) probably cause trypanosomiasis pathology. The anaemia, nephrosis, liver degeneration and pulmonary oedema in cattle T. congolense infection suggests a host's hypersensitivity reactions against trypanosomal antigens (85, 104, 119, 208). Trypanosomes themselves probably secrete an LPS-like polyclonal activator, a substance that haemolyses erythrocytes and, antigenically combines with the antibody to produce immune complexes in antigen excess.

Haemolytic anaemia in African trypanosomiasis is an early immunopathology. However, it is rapidly cured by trypanocides which are effective against even late or chronic trypanosome-induced aplastic anaemia. Although direct lysis of erythrocytes by "Max" factor (erythrotoxin) appears early in experimental disease probably autoimmune anaemia also occurs. The erythrocytes adsorb trypanosomal antigen on their surfaces, are thereby sensitized and subsequently lysed by the antibody (150). The erythrocyte-adsorbed antigen in vivo combines with the excess heterophile and autoantibodies and the resultant complexes functionally activate macrophages resulting in erythrophagocytosis by cells of the expanded mononuclear phagocytic system (MPS).

Also the host's immune responses to trypanosomes are

potentially harmful in other ways. The sensitized erythrocytes and platelets aggregate (86, 399) and antigen-antibody reactions anaphylactically release the potent pharmacologically active substances like kinins, histamine, slow reacting substances of anaphylaxis (SRS-A) and possibly 5-hydroxytryptamine (5-HT) and prostaglandins. The kinins are then activated probably by the Hageman factor adsorbed on the surface of antigen-antibody complexes (119, 208) during pathogenic trypanosomiasis (41, 42, 104, 118, 253, 288). They damage tissues, and increase vascular permeability leading to oedema, haemostasis, tissue anoxia and cardiovascular collapse. Pulmonary fibrinoid deposits due to exudation from capillaries (44, 118, 119) and plasmin activation with a resultant drop in plasma plasminogen level and an increase in fibrinogen degradation products occur in T. brucei-infected rabbits. These substances interfere with haemostasis while the blood vessel occluded by platelets and leucocytes disintegrates.

Other autoimmune lesions are probably associated with production in mammals of autologous anti-liver, anti-Wassermann (85, 118, 119, 220, 372) and possibly anti-heart (288) antibodies during T. cruzi infections. Man than other mammals probably produces more antibodies so that severe anaphylaxis follows the continuing destruction of the few available trypanosomes as after a trypanocidal therapy.

The perivascular cuffing and the destruction of brain cells outwardly are non-specific. Therefore the choroid plexus and the kidney pathology due to immune complexes resemble.

The meningo-encephalitis is associated with scanty trypanosomes which make microscopic diagnosis difficult during advanced disease. Whether the observed pathology is of host's immune origin, probably due to altered antigenicity from tissue damage by infection, or due to escape of B cells, programmed for autoantibodies from T cell control (85, 119, 374) and not due to direct toxic or mechanical damage by the parasite is not known.

Immune complex-associated (43, 119) renal insufficiency with proteinuria in T. brucei-infected rabbits (119), glomerulonephritis and decreased plasma complement component levels C3, C4 and properdin in T. rhodesiense-infected rhesus monkeys indicate increased complement binding (264).

Massive variant specific antigens are repeatedly produced by dying trypanosomes during infection. These antigens combine with antibodies to form circulating immune complexes in antigen/or antibody excess which lead to immunopathology. Most likely in disease antibodies and cell-mediated immune reactions involving polymorphs, cytotoxic lymphocytes and K cell produce the inflammatory responses. In T. brucei-infected mice (85, 195, 253) the lesions are due to variant antigen-IgG and-IgM antibody immune complexes in glomeruli, myocardium and choroid plexus as well as due to anti-DNA antibodies in the heart.

Clinico-immunological and histological evidence for the role of immune complexes in trypanosome pathology include urticaria, lymphadenopathy, splenomegaly and perivascular monoculear infiltrates of the heart and, brain (85) resembling experimental allergic encephalitis. The chancre with its

perivascular leucocytes (29, 86, 208, 288) is also an immune reaction because the multiplying trypanosomes in the skin are a continuous antigen source. The antigens combine with the circulating plasma antibodies to form immune complexes, the corresponding hypersensitivity and increased capillary permeability as in Arthus' type of reaction.

Coombs' positivity occurs in trypanosome-infected monkeys but it is unknown whether or not it is due to immune complexes (118). The lymph nodes and spleen are histopathologically massively also altered. Paracortical or T- but not B-dependent areas are depleted and replaced by macrophages and plasma cells. The disrupted architecture makes antibody response through cellular co-operation impossible even with functional cells.

Thus the trypanosomiasis pathology is probably of immune hypersensitivity type mediated by cells, antigen-antibody complexes or possibly by several processes combined. However, the rapid cure of lesions suggests that autoimmunity is probably less important and since the CNS lesions are immunopathological in origin in man the immune responses must be different from those in experimental animals.

(C) Immunodepression

In trypanosomiasis the raised IgM and IgG production is strangely accompanied by immunodepression to heterologous (non-trypanosomal) antigens, reduced complement (261), and lethal bacterial infections (208, 288).

Immunodepression occurs in trypanosome-infected animals (119, 121, 208, 291, 292, 319, 320) and in T. gambiense-infected

man (129). As each antigenic trypanosome variant is decimated by the corresponding antibody production, the trypanosomes interfere with the host's immune response to heterologous antigens like sheep erythrocytes (86, 118, 119, 208, 253), experimental allergic neuritis (10), worms (385), bacteria like Salmonella typhi vaccine and the skin sensitizing reagent dinitrochlorobenzene (DNCB) (130).

The nature and mechanism of immunodepression are uneludicated. In subacute T. brucei infection of rats (257, 258), and of mice (119, 165 , 372) increased phagocytic activity occurs throughout the infection. Early in disease lymph nodes and spleens are heavily populated by lymphoblasts dividing and differentiating into plasma cells. However, late in trypanosomiasis they are depleted of immunological cells, thereby rendering the animals unable to produce antibodies against any antigens. Possibly, too, trypanosomiasis breaks the control link between T and B lymphocytes (85, 119, 374) so that T cells probably prevent B cells from transforming into the antibody producing plasma cells (8). Similarly, the escape from control possibly allows B cells to make IgM antibodies partly as autoantibodies. However,other responses requiring cellular interactions (co-operation) are possibly depressed.

(D) Self-cure

Spontaneous recovery or self-cure of T. gambiense probably occurs in many healthy-looking initially positively diagnosed but untreated patients (14, 288, 330) who apparently become infection-free after 3 to 8 years. It is exceptional in

the acute T.rhodesiense and probably in the mild T. gambiense-like (Zambezi) T.rhodesiense but not in the virulent form (14, 15). Self-cure also occurs in ungulates (253) and non-human primates with African trypanosomiasis (17).

(6) Animal models

(i) Change from human studies to animal models

Ideally human diseases should be studied in man but animals are advantageous in controlled disease studies of pathogenesis, the interaction of genetic and environmental factors, recognition of physiological processes (only made apparent by a pathological deviation), mode of inheritance and aetiological theories or new treatment methods.

Numerous natural diseases occur in many species but have seldom been used as human disease models. This is possibly because communication between human and animal investigators has been lacking and also because animals with a specific disease are too few for studies. Also the few qualified investigators cannot always be located in time and place to utilize the diseased animals. Fortunately researchers interested in natural animal models have been numerically increasing steadily because of communication between them (180).

Models help understand the basis of many problems although they do not necessarily help solve them. The results obtained in animals such as mice when extrapolated to human situations sometimes bring the researcher either close to the solution or into disrepute (56). Yet, he still continues, for "the real objective today is to use the ability of men and women

with these (scientific) qualities to devise ways by which patterns of behaviours laid down in million years can be modified, tricked, twisted, if necessary, to allow a tolerable human existence in a crowded world. In the laboratories scientists have sought or contrived living models of their interest. Given ingenuity and technology almost all aspects of biology relevant to man can be interpreted in terms of simple models. As intellectual concepts the models are forumulated in numerous ways like in terms of organic or electro-chemistry, of thermodynamics, hydrodynamics, genetics or cytology" (56). Properly applied they elucidate the living systems for the purpose of human need and desire.

Why scientists have changed from human studies to animal models is best answered if specific diseases are cited. For instance, the development of effective human antimalarials have relied on avian, simian and rodent malarias as the primary test objects. All the above animal test systems cannot be predicted but the resultant defects can be offset if induced malarial infections in man are readily available and can be manipulated for pre-field evaluation of possible drug agents in experimental malarias. However, the access to "offsets" has been diminished drastically by changes in attitudes towards using human volunteers for induced disease studies and by the need to work on multi-drug resistant parasites which require great caution in non-immune subjects. These restrictions have increased the need for animal models in which plasmodial infections are studied directly. Some similar success in developing animal models has

been achieved in African trypanosomiasis, schistosomiasis and other diseases.

Human experimentation with trypanosomiasis is either unethical, uneconomical or both and the few volunteers have had their infections quickly terminated therapeutically without allowing them to proceed to chronicity and CNS involvement. Thus very little sequential knowledge of the disease processes has been obtained from the equally few human studies. Natural human trypanosomiasis cases seen by doctors are always in advanced pathological stages of the disease and are of unknown duration. Because of this and because once the disease is diagnosed immediate treatment is instituted many questions about various disease aspects have remained unanswered except probably terminally at autopsy which, though important, in many instances cannot, for various reasons, be performed in endemic areas.

The attainment of independence by African states has either limited or almost halted the access to human material of trypanosomiasis because of changes in general attitudes. People question, sometimes with resentment, the reasons for performing autopsies on their dead. Taboos and religious veneration of the sick or dead are often over magnified. Only few countries such as Tanzania and Uganda have legislated autopsy of the dead to advance medical and scientific knowledge.

Also as a result of independence finances have become meagre to spare for and to encourage field research studies. Hence, unless supported by an outside agency, research is a luxury to most governments. The emphasis is therefore on preventive

services based on experimental results and conclusions collected from and reached elsewhere, respectively. In Europe, America and the Far East there are scientists eager to work on tropical diseases including African trypanosomiasis, schistosomiasis and malaria. They have financial supports from their national governments but no access to diseased human material. Scientists have consequently employed various animal-disease models to enquire into some arising medical questions. However, these animal models have their limitations.

(ii) Change from lower animal to non-human primate models

There are several reasons, too, for change from use of lower to non-human primate models in research. Basically it is the need to understand a disease and its pathological processes so as to effect better and proper control and/or cure. Also it is because of the general realization to employ, in tropical disease research programmes, animals which are closer evolutionarily to the human situation that has led to the use of non-human primate models.

Animal models are sometimes either quite interesting or unsatisfactory. For example, the mouse-T. brucei systems do not all uniformly show CNS involvement and chronicity. These are shown by only certain mouse and T. brucei stocks. Meanwhile in man the CNS involvement is the most important, commonest and best documented feature of human T. gambiense and sometimes T. rhodesiense.

The full relevance of the lower animal-trypanosome model systems including the mouse model to human trypanosomiasis is

precisely unknown. Therefore, results obtained from the mouse system can only be partially and gingerly extrapolated to the human situation. Anaemia, for instance, is well documented in human and animal trypanosomiasis. Self-cure of African trypanosomiasis is not documented in the mouse and rabbit and not so well documented in the rat, goat and sheep as in man and cattle. The mouse is small, easy to breed, has much genetic data on mode of inheritance and linkage available and well assembled and has a short life span. However, it precludes repeated sampling of blood, and clinical studies while identification of individual chromosomes is not yet feasible. Furthermore only few biochemical bases for the defects have been determined (180).

Also, guinea-pigs, rabbits and rats are highly resistant to chronic human S. mansoni disease (421). The rats abort the infection after 3 - 5 weeks while the guinea-pigs and rabbits also somehow self-cure though more slowly than the rats. In non-human primates schistosomiasis persists for years and the baboon, the vervet and the chimpanzee do not show the dramatic drop in egg output like the rat. The rhesus monkey aborts S. mansoni suggesting that immunity is complete while the baboon develops an acquired partial immunity whose degree is far less than that of the rhesus monkey (421). In the mouse, also a susceptible host, aberrant infections occur. The S. haematobium worms lodge in the mesenteric and not vesical veins and the eggs are discharged in the stools but not in the urine. The relevance of some of these interesting results to the human situation is unknown.

The chimpanzee is better than the baboon as a *S. haematobium* host but for its availability which hinders its use in studies. The vervet monkey is the third best host for human schistosomiasis because colonic and vesical lesions, respectively, due to *S. mansoni* and *S. haematobium* infections are produced experimentally as evidenced parasitologically and radiologically (421). Because of the above set-backs and aberrations researchers have changed from the mouse and other lower animal to non-human primate models. Meanwhile in Africa there are numerous fairly cheap non-human primates. These can be used for research locally and can also be exported. This has not only led to the establishment of new local research centres supported by external agencies but also to the increased monkey demand for export and local consumption.

It must be pointed out that though many non-human primates are naturally infected with human diseases others are not, even in the laboratory. Thus not all primates are susceptible to human diseases. For example, the baboon was refractory to human trypanosomiasis but lesions and death were produced only by intraspinal (intrathecal) inoculation of parasites (17). Also in *patas* monkey *T. rhodesiense* produces an acute, subacute or chronic disease while *T. gambiense* is difficult to establish and the resultant disease has a protracted course (407).

(iii) The monkey model

The need for an easily manipulated laboratory host for human diseases like sleeping sickness, schistosomiasis, malaria, etc., has long been recognized. Natural test systems have been

primarily used for biological and chemotherapeutical studies, for example, of sleeping sickness parasites. They comprise of acquired infections in people in endemic areas, volunteers and ungulates. A similar situation obtains in malaria in which induced infections in military personnel, neurosyphilitic patients and in prisoner volunteers; simian plasmodia in Old and New World monkeys as well as birds and rodents with their malarias have been used.

The Old World monkeys are non-human primates which have been associated the longest and the closest with scientific study. Aristotle and Pliny described several recognizable species while Galen probably mainly dissected out Macaca sylvana. However, the rhesus monkey, Macaca mulatta, is the modern medical research monkey because historically in the 19th century Indian rhesus were the most easily available monkeys and once entrenched in the literature they have therefore been ordered routinely for research work. Other species have since been introduced into the laboratories because of the sudden increased demand for them for the development of virus culture techniques using monkey tissues as media. Air transport has made it economical to exploit distant and inland monkey species while the growth of medical research facilities in the tropics in established medical schools in,say,Philippines, Thailand, Indo-China, Formosa, Singapore and Kenya together with the ban on exportation of Macaca monkeys by the Indian government, either for religious veneration or conservation, have all encouraged and even forced researchers to exploit and use other monkey species.

An ideal laboratory primate has to be identifiable toxonomically, readily available in quantity, easily maintained in captivity and of a convenient size for laboratory experiments. For medical research it should also respond to disease and surgery like man but should be free from human disease hazards. The normal ranges of its major physical, physiological and anatomical systems should be known.

The Cerocopithecidae are advantageous as experimental animals in medical research because of their intermediate phyletic position between the Hominidae, on one hand, and the Platyrrhini and Prosimii, on the other. Though generally less similar to Homo sapien anatomically and physiologically than to Pongidae and Hylobatidae they are closer than any other animal group. Yet, the monkeys have taken long to develop evolutionarily since the original splitting of the Catarrhine stock. They are not a stage through which human evolution has passed but the result of a separate radiation from a primitive stock. Consequently no one Cercopithecidae is any "closer to man" phylogenetically than any other in spite of the occasional claims (180).

The intermediate position of the Cercopithecoid monkeys has a practical value. Although more expensive, less easily available and more difficult and slower to breed than the usual domestic laboratory animals they are superior in all these respects to the non-human Hominidae (180).

The Macaca, vervet and patas monkeys as well as the chimpanzee are apparently biologically satisfactory hosts for

African trypanosomes while the chimpanzee and the gibbon, Old World monkeys are ideal for malaria. Yet, the achievements obtained, for example, with monkey-malarial models have not been pursued intensively probably because of scarcity, cost and husbandry problems. These set-backs have curtailed the availability of animals so that sometimes it has not been feasible to employ many of these non-human primates (anthropoids) in experiments to meet drug development programmes.

(vi) African trypanosomiasis in monkeys

Natural brucei sub-group infections occur in vervet monkeys (28) while experimentally T. brucei and T. rhodesience produce acute or chronic diseases and/or self-cure (17, 101).

An infected tsetse bite produced a chancre in chimpanzees (117) but baboons were probably trypanosome-resistant except probably by intraspinal infections. Trypanosoma brucei, T. gambiense and T. rhodesiense have produced similar diseases in monkeys (326), and various other animals (208).

Experimentally T. rhodesiense in rhesus monkey has a brief prepatency. The animals lose weight and appetite and become progressively listless. They assume a slumped posture with bowed heads. The mucosa pales and petechiae develop over the chest and extremities while the fur ruffles. Focal neurological signs unlike rigidity and contraction of the lower extremities are rare (326).

The haemogram rapidly decreases associated with reticulocytosis but without consistent significant changes in total and differential leucocyte counts. Meanwhile the platelet

counts also decrease due to active sequestration, disseminated intravascular coagulation or immune complexes (326).

The serum transaminases (probably of parasite or of host tissue origin), creatinine, blood urea, nitrogen and gammaglobulin increase. Meanwhile the total proteins and albumin decrease due to either increased catabolism, decreased synthesis or abnormal loss in the urine. However, carbohydrate demand by either the host and/or the parasite is apparently normal. Biochemical changes are probably due to the effects of trypanosomes or infection like anoxia, haemolysis and severe anaemia (326).

The fluorescent antibody titres are higher in unimmunized animals than in immunized and protected animals suggesting that the antibodies detected are not protective (326). Reticuloendothelial hyperplasia of the spleen, liver, lymph nodes, perivascular mononuclear cell infiltrate in liver, kidneys and probably heart but also proliferative glomerulonephritis occur in rhesus monkeys with trypanosomiasis (326).

The numerous trypanosomes in heart, kidney and suprarenal connective tissues indicate extravascular parasite multiplication.

Epicardial oedema, exudative pericarditis of mononuclear cells, myocarditis with degenerative and necrotic fibres and free and phagocytosed parasites are the inflammatory reactions. Also the myocardium has amastigotes, granulomas and fibrosis.

Polyserositis evidenced by peritoneal, pericardial and scrotal (hydrocele) effusions and mononuclear and trypanosome infiltrations of the serous membranes and connective tissue

feature regularly. The effusions contain epithelial and mononuclear inflammatory exudate while the trypanosomes exceed those in the blood.

Meningoencephalitis with grossly thickened pia mater occurs in severe and chronic T. gambiense cases and occasionally in acute T. rhodesiense and T. brucei while perivascular and diffuse mononuclear cells in the meninges and neuropil, with the most extensive lesions in the medulla oblongata, are the brain lesions. The more chronic the infection the deeper the submeningeal affection. Neuropil lesions are less severe but the choroid plexus is markedly inflamed and infilrated with numerous perivascular trypanosomes. Also the inflamed brain is (27, 117, 208, 399) associated with cerebrospinal fluid changes in trypanosome-infected apes and monkeys.

The spleens are either hyperplastic; haemorrhagic with small follicles or fibrotic with irregularly enlarged Malpighian follicles, thickened trabeculae and capsule with or without trypanosomes. The bone marrow is grossly red and gelatinous and sometimes shows marked erythrocytic hyperplasia, haemorrhages or atrophy while the megakaryocytes are prominent and significantly abundant. Depending on animal species and anatomical location lymph node changes vary but are generally hyperplastic, congested and occasionally haemorrhagic. The follicles are hypertrophied with numerous macrophages in the sinuses, perivascular accumulation of lymphocytes and plasma cells. Clumped or phagocytosed trypanosomes are more readily detected in the lymph nodes than in the spleen (326) and also

in the spleen and bone marrow than in the blood and liver (408).

The liver shows mononuclear cell infiltration of the portal triad while trypanosomes are rare. True trypanosomal nephritis is rare but when present it resembles myocarditis. Occasionally numerous trypanosomes and extensively inflamed adrenals as well as mononuclear infiltrations of the epididymis and oedema of the eyelids, eyebrows and supraorbital spaces occur (208, 326).

Acute and chronic T. brucei infections are, respectively, associated with cycling high and low self-limiting parasitaemia. Leucocytosis, neutropenia, (406), increased sedimentation rate, decreased haemoglobin and leucopenia occur early (117). In T. gambiense infection serum albumin and gammaglobulin decrease and rise, respectively (348).

This study was therefore designed:

(1) to determine the susceptibility of the vervet monkey to T. brucei stocks and clone by needle challenge;

(2) to explore if the vervet monkey can be used as an experimental animal model for studies on immunological responses to T. brucei infection;

(3) to investigate immunological status of the vervet monkey and the effect of treatment or self-cure on the course of T. brucei infection and on the immunological responses of infected and uninfected control vervet monkeys.

The studies reported here were conducted in 3 different broad experiments.

(a) Experiment 1

(i) A study of the susceptibility of the vervet monkey to 2 stocks and one clone of T. brucei, and

(ii) the effect of various parasite doses of Clone 227 on the course of T. brucei infection in the monkey.

(B) Experiment 2

(i) Selection of foetal calf-serum and determination of its optimal concentration, the suitable medium for in vitro micro-culture of vervet monkey peripheral blood lymphocytes.

(ii) Determination of optimal cell and Con A, PHA and/or MLC concentrations; culture and pulse times for monkey peripheral blood lymphocytes.

(iii) Determination of an optimal dilution of whole blood, mitogen concentrations and harvesting time for cultures stimulated with PHA and Con A.

(C) Experiment 3

(i) A study on the effect of infection on haematological parameters and the immune system of the vervet monkey.

(ii) A study on the effect of Berenil treatment or spontaneous self-cure on haematological and immunological changes in T. brucei-infected vervet monkeys.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

GENERAL MATERIALS AND METHODS

Most of the general materials and methods that have been used in the studies reported in this thesis are described in this chapter and will be referred to at will in the text so as to avoid repetition and unnecessary digression. Cross-reactivity between monkey and human reagents (79, 305, 346) has enabled the use in this work of some reagents of human origin and are cited. At the beginning of this thesis, a list of abbreviations is included, many of which are commonly used and will be employed in the text without further elucidation. Also a list of suppliers is given at the end of this volume.

(1) Experimental animals

(a) The monkeys (Cercopithecus aethiops)

The non-human primates used in these experiments were adult male and female vervet monkeys (Cercopithecus aethiops). These were trapped locally and purchased from the Institute of Primate Research Tigoni, of the National Museums of Kenya, Limuru.

(i) General description

Cercopithecus aethiops belong to the Order Primate; Sub-order Anthropoidae which are higher primates; Superfamily Cercopithecoidae of Old World monkeys; Sub-family Cercopithecinae of cheek-pouched monkeys some of which are partly or predominantly terrestrial; Tribe Cercopithicini and Genus Cercopithecus commonly called guenons. The other genus of the tribe Cercopithicini is Erythrocebus of patas monkeys and the two genera are confined to Africa unlike the genera Papio and Macaca of the tribe Cercocebini (sub-family Cercopithecinae) which are also found in Asia (180).

Cercopithecus aethiops species often called green, grivet, vervet, malbrook, etc., are greenish-grey white fringed, dark-faced monkeys found in the sub-saharan savannah habitats from Senegal in the West, Ethiopia and Sudan in the East, and South to the Cape of Good Hope. Cercopithecus sabaeus was introduced into St. Kitts, West Indies and into St. Jago, Cape Verde Islands in the seventeenth century (188).

There are three major types or races of C. aethiops each with sub-divisions or sub-species. The types range over the southern, C.a. pygerythrus with its two sub-divisions, C.a. cyanosurus in Angola and C.a. arenarius in the arid N.E. Africa, the northern C.a. aethiops in Ethiopia and C.a. tantalus as its second westerly division and; and the far western savannah C.a sabaeus.

The vervet monkeys, unlike E. patas dwellers of the drier savannah, inhabit rain, secondary montane, gallery and lake shore forests as well as open and woodland savannah. Although rare in the arid zone C. aethiops have been reported from Air Massif (18^{o}N and 8^{o}E). They are diurnal feeders and secondary only to baboon as crop-raiders. They eat leaves, green shoots, buds, fruits, many cultivated food crops and bark gum. They are also partly insectivorous occasionally feeding on small invertebrates and their eggs.

Genetically, the Cercopithicini have variable chromosome numbers but generally exceed 44 in all species (180), a situation believed to be primitive. The haemoglobin is probably polymorphic and identical with human A while the blood

groups resemble human groups A and B.

There are no breeding seasons but the births may be dispersed in one year and more concentrated in another. The gestation period probably varies from 140 - 160 (188) or from 180 - 213 days (180, 270). Single births are common and twining is rare.

Cercopithecus aethiops is very successful as it is hardy in captivity where it breeds occasionally.

The vervets used in this study were routinely screened either by clinical, parasitological, immunulogical or by haematological examinations for trypanosomes, simian plasmodia, tuberculosis (T.B), intestinal helminths and protozoa.

Animals positive for trypanosomiasis by immunofluorescence technique (see below) were discarded, those positive for malaria were treated with parenteral (i.m.) chloroquine for a week, ones with helminths were de-wormed three times with oral piperazine citrate (Willows Francis) while those positive for T.B. by Mantoux test were destroyed. The animals were generally used for experimental work four or more weeks after the last treatment.

(ii) Housing and Feeding

The vervet monkeys used in this study were conventionally housed in the animal house of the Department of Human Pathology, University of Nairobi Medical School. Each animal was kept individually in a locally made press-back wire-meshed (5 x 5 cm) cage measuring 75 x 75 x 75 cm and firmly suspended about a metre above the floor. In the animal house, the monkeys did

not need acclimatization because the temperature and humidity were almost like in the natural environment.

The diet included ripe bananas, raw sweet potatoes and carrots, green and tender maize and monkey-biscuits supplemented with vitamin C (Proctor and Alan Ltd). Each morning and late afternoon, the food and water were dished out into bowls for the animals to eat at will. When kept on this diet, the vervets remained in good health, gained weight and improved their haematological variables such as the haemoglobin and packed cell volume.

(iii) Experimental variables

At least five vervet monkeys were normally used but in the "Susceptibility and Trypanosome Titration" experiments fewer animals were used. Various parameters including clinical, haematological and immunological changes were evaluated both before and after inoculation of the monkeys with trypanosomes.

(b) The rabbits (Oryctolagus cuniculus)

Out-bred adult male New Zealand White (NZW) rabbits were supplied by the National Public Health Laboratory Services, Nairobi. They were conventionally housed in the animal house in the Medical School.

(c) The Sheep

A local breed of sheep was used in these studies. The animals were conventionally housed in the animal house of the National Public Health Laboratory Services, Nairobi. They were bled from the jugular veins whenever sheep red blood cells (SRBC) were required.

(d) The mice (Mus musculus)

Inbred Balb/C mice were bred and maintained at the International Laboratory for Research on Animal Diseases (ILRAD), Kabete, Nairobi.

(2) Parasites

(i) Trypanosomes

The origins of T. brucei clone 227 and EATRO 2234 seem to be the same, as EATRO 939, a derivative of EATRO 795 which was itself derived from Uhembo 77 isolated from an ox at Uhembo, Central Nyanza, Kenya by K. van Hoeve in 1964. The various cloned and uncloned derivatives are shown (Fig. 2.1). Trypanosoma brucei TREU 667 (EVE 10) was isolated by the Edinburgh Veterinary Expedition (EVE) to the Busoga District, Uganda in 1966 (311). When stabilates were received at the Centre for Tropical Veterinary Medicine, Edinburgh, 2 rats were infected; stabilates produced from these rats were TREU 667 (73). These parasites were kindly supplied to me by Dr. Max Murray, ILRAD. Stock TREU 667 has been used in other animal-trypanosome model studies (177, 257, 258, 352).

(3) Screening of monkeys for natural trypanosome infections.

Some of the monkeys used in these studies originated from tsetse fly belts and bordering areas. Therefore, all monkeys were routinely screened for natural trypanosome infections by haematological (May-Grunwald-Giemsa stained thick films) (74) and by indirect immunofluorescence technique (39).

Blood samples were collected from the femoral veins of monkeys and sera eluted from the clotted blood. Sera from known uninfected and infected monkeys or humans were used as

negative and positive controls, respectively. The sera were diluted into 1/10, 1/20, 1/40, and 1/80 and the indirect immunofluorescence test performed as follows: Trypanosoma brucei parasites were raised in normal Balb/C mice and thin blood smears were made on dry slides pre-cleaned with chromic acid and rinsed thoroughly in distilled water. Using untinted nail varnish or a diamond marker the slides were marked into 10 divisions so that two partitions were opposite each other to make 2 rows of 5 divisions each.

The parasites on the slides were fixed by dipping the latter in pure acetone for 2 seconds. The slides were then washed three times in PBS pH 7.2. Each wash lasted 5 minutes. The test and the control sera were left on the slides to react with the parasites for 30 minutes in a moist chamber and washed as above before adding the anti-human Ig fluorescein conjugate (FITC), F/P molar ratio 3.1 (Wellcome, U.K.) to react again, for 30 minutes in the moist chamber. The excess FITC conjugate was similarly washed off with the buffer. They were finally mounted in glycerol-PBS (9:1) and the edges of the applied clean coverslip were sealed with the untinted nail varnish before reading the slides on the fluorescence microscope (Orthoplan, Leitz). They were read immediately for the best results as fading occurred after storage at -20°C overnight.

Any monkey whose serum showed real or doubtful positivity at 1/20 dilution and below was regarded positive for trypanosomiasis and was not used in the experiments.

(4) Separation of trypanosomes from mouse blood and the infection of monkeys.

Balb/C mice lethally irradiated with 900R per total body surface from "Caesium 137" source at ILRAD were inoculated with T. brucei clone 227 to raise the required number of parasites for infecting monkeys. The trypanosomes were separated (197) at ambient temperature, from the heparinized blood (Heparin Novo, 10i.u/ml final concentration) of infected mice on DEAE - Cellulose (Type DE-52, Whatman) using cold phosphate buffered saline glucose (PSG pH 8.0), a 2.5 cm column (Pharmacia Fine Chemicals), and LKB-Bromma 12 Vario-Perpex peristaltic pump. They were collected on ice, washed three times by spinning at 4°C for 30 minutes at 1200 g in a Minifuge (Heraeus-Christ, GMBH, Osterode), enumerated on a haemocytometer using SM-Lux microscope (x 40 objective, Leitz, Wetzlar), and finally adjusted to the required concentration. The appropriate dose (normally in more than 0.1 ml of PSG pH 8.0) of parasites was inoculated intravenously into the monkeys.

(5) Cryopreservation of trypanosomes

Trypanosomes, either separated or in whole blood, were cryopreserved (216) at-80°C in 7.5% (final concentration) glycerol (Fischer Scientific Company) in heparinized capillary tubes (Matheson Scientific) whose ends were finally sealed with seal-ease Plasticine (Cristaseal, G. Hawksley).

The cryopreserved parasites were used to produce in monkeys trypanosome infections of basically the same origin and antigenic constitution.

(6) Detection of infection and enumeration of trypanosomes in blood.

Parasitaemia was detected by the haematocrit technique (417) whereby blood was put into heparinized capillary tubes (Matheson Scientific), spun for 10 minutes in a micro-haematocrit centrifuge (G. Hawksley) and the buffy coat from a cut tube examined for trypanosomes. Quantitation of parasitaemia was done by the haemocytometer method as described under (4) above. They were finally expressed as parasites per millilitre of blood and were plotted as Log_{10}.

(7) Immunization of monkeys with various antigens

(a) Skin sensitization to 2, 4 - dinitrochlorobenzene, (DNCB)

Monkeys were sensitized (383) with 400 µg of 10% DNCB (kindly supplied by Dr J. Mauel of WHO, Lausanne, Switzerland) solution in acetone and applied over an area of 1 cm diameter on the shaved chest of an anaesthetized animal. The acetone was evaporated immediately. Thereafter, the animals were re-challenged with 20 µg of 1% DNCB solution in acetone applied over a similar area on the lateral aspect of the thighs. Control areas received 2 µl of acetone alone. Two diameters of the reactions were measured after 48 hours and the mean diameter calculated therefrom. Skin tests were undertaken at various times (Fig. 5.i).

(b) Tetanus antigen

Monkeys were also immunized with tetanus vaccine (Adsorbed Tet/Vac/PTAH; The Wellcome Foundation, London, Lot 95563) at 0.5 ml dose (10Lf per 0.5 ml) given intramuscularly (i.m.) in the thighs. Revaccinations were with, 0.5 ml i.m. of the

same lot of tetanus vaccine (Fig. 5.1).

(8) Indirect passive micro-haemagglutination test.

The tetanus antibody levels in serially collected monkey sera were estimated in "V" - shaped microtitre plates (Cooke) by the indirect passive haemagglutination test (149) in the presence or absence of 0.1M 2 - mercaptoethanol (2-ME) (BDH) and using formalinized, tanned (1:10,000 tannic acid in PBS pH 7.2) and tetanus antigen - (purified tetanus toxoid (1.7 Lf MWC 4554/F, kindly supplied by Wellcome U.K.) - coated SRBC. The sera were heat-inactivated (56^{o}C for 30 minutes). By doubling dilution in 1% (in PBS pH 7.2) normal rabbit serum (NRS) from which heterophile antibodies had been absorbed away, the monkey sera were serially distributed in the wells.

A positive tetanus antiserum (also supplied by Wellcome) of known haemagglutination titres, was the positive control and all tests were always performed to attain those titres. Pre-immunization normal monkey serum was the negative control. The tanned SRBC used for absorbing heterophile antibodies from the monkey and the normal rabbit sera (NRS) and, coating with the antigen were from the same batch and animal. The 1% NRS in PBS pH 7.2 was used as diluent and the protein in it acted as a carrier medium to prevent false agglutinations.

(9) Direct haemagglutination assay

The titres of heterophile antibodies to SRBC were assayed by the direct microhaemagglutination test (158, 159) in "V" microtitre plates (Cooke) using equal quantities (25µl) of 1% SRBC, washed three times in PBS pH 7.2, and of unabsorbed

heat-inactivated monkey sera diluted in 1% NRS.

Two - mercaptoethanol (2-ME) at 0.1 M in PBS pH 7.2 was again used to measure the 2-ME-sensitive and-resistant agglutinating antibodies.

(10) Enumeration of erythrocyte rosette (ER) (T) cells

Putative T lymphocytes in vervet monkeys were identified by their receptors for sheep erythrocytes and were assayed by rosetting with neuraminidase-treated SRBC (396).

A mixture of 0.3ml of 2 x 10^6 lymphocytes/ml, 100 µl of 2% fresh SRBC (washed three times in normal saline and reconstituted in L-15 medium), 100 µl neuraminidase (Stock 500 units/ml; Vibrio cholerae, Behringwerke) and 50 µl of heat-inactivated (56^{o}C for 30 minutes) foetal calf serum (FCS) (absorbed with one-fifth of its volume with SRBC for 2 hours at 4^{o}C) was made up in a Kahn tube. It was spun at 80 g for 10 minutes at room temperature, then incubated at 37^{o}C for 30 minutes and finally kept overnight at 4^{o}C before counting the rosettes.

The rosetted cell pellet was resuspended very carefully to avoid disruption of rosettes. Tapping by hand or slow mechanical rotation was more reliable than pipetting. Aliquots of resuspended rosette suspensions were carefully pipetted into a haemocytometer and the cells were observed under phase contrast (x 40 objective). Viable lymphocytes surrounded with 3 or more adherent SRBC were considered to be rosetting cells. The lymphocytes were expressed as a percentage or as an absolute number of total live (rosetted and non-rosetted) lymphocytes.

Initially red blood cells from sheep, goat, cow and camel were screened for their ability to adhere to monkey lymphocytes and make rosettes. The SRBC surrounded the largest number of lymphocytes.

(11) Enumeration of lymphocytes bearing surface membrane immunoglobulins (SmIg) (B Cells)

Putative B lymphocytes in the peripheral bloods of monkeys were enumerated after they were identified by their membrane bound immunoglobulins detectable by the direct immunofluorescence staining technique (112) using a fluorescein (FITC) conjugated (375, 376) rabbit anti-monkey immunoglobulin.

(a) Preparation of immunoglobulin - FITC conjugate

The rabbit anti-monkey immunoglobulin-FITC conjugate for staining and enumerating "B cells" was prepared as follows : Pure monkey gammaglobulin was prepared by using a modification of the method described by Bernier (38). Pooled normal monkey serum was tinged with a drop of bromophenol blue solution (Kallestad Laboratories) as a tracking dye. Twelve millilitres of 1.2% solution of agarose B (Pharmacia, Lot. No. CH 1813) in barbital buffer pH 8.6 was poured onto a pre-cleaned dry 82 x 82 mm glass plate (E. Kodak) and was allowed to set in a humid chamber. Ten 1.5 diameter wells were cut in a line 15 mm from one end of the plate. Each well was carefully filled with the dye-tinged serum which was electrophoresed for 45 minutes at a constant current of 24 mA on a VoKAm SAE (Shandon) electrophoresis apparatus using barbital buffer pH 8.6 in the trough, and paper wicks (LKB 2117-203) to complete the electric circuit.

The albumin moves faster than the gammaglobulins and by this difference in the electrophoretic mobilities of these proteins their separation was effected. The gammaglobulins were easily identified in the agar as a discrete clear area which was carefully removed and homogenized with 1 ml of normal saline. The homogenate was emulsified with an equal volume of complete Freund's adjuvant (Difco) and was injected subcutaneously at four sites on the back of each of the two New Zealand White rabbits. Three weeks later the rabbits were re-immunized with monkey gammaglobulins prepared similarly from the same pooled normal monkey sera and emulsified in incomplete Freund's adjuvant (Difco) and the animals bled ten days later.

Anti-monkey gammaglobulin sera from the two rabbits were pooled and a pure globulin-rich fraction from this was first prepared by ammonium sulphate precipitation at 50% and then at $33\frac{1}{3}$ % concentrations. After each precipitation, lasting one hour, the mixture was spun at 2000 g for 30 minutes at room temperature. The first and the second precipitates were re-dissolved in 1 ml of PBS pH 7.2 and normal saline, respectively. The final precipitate dissolved in saline was dialysed over-night against several changes of saline to remove the sulphate ions (the absence of sulphate ions was checked with 1% barium chloride). The globulin solution in a dialysis tubing was then concentrated using polyethylene glycol (Aquacide III-A, Calbiochem) and loaded onto an ion exchange column made out of a 35 ml plastic syringe containing DEAE-Cellulose equilibrated with phosphate buffer 0.017 M pH 6.3, for fractionation. The IgG was eluted with PBS pH 6.3 and appeared

in the first peak. The samples that gave a line with a single spur in immunoelectrophoresis against rabbit anti-monkey whole serum were pooled.

The IgG fraction was concentrated as described above to give a 10 mg/ml protein solution. The fraction was dialysed overnight at 4°C against 0.025 M carbonate/bicarbonate buffer pH 9.5. The dialysis tubing and its contents were transferred into a measuring cylinder containing fluorescein isothiocyanate (FITC, Baltimore Biological Laboratories) at 10 mg/100 ml buffer (or 10 ug/mg protein). The conjugated immunoglobulins were dialysed against several changes of PBS pH 7.2 at 4°C. The conjugate was then spun at 1500 g at 4°C to remove aggregates. The conjugated immunoglobulins in the supernatant had an F/P ratio of 2.07 between OD 280nm/OD 495nm. They were aliquoted in small portions to avoid damage by repeated freezing and thawing and were stored at - 20°C until use.

(b) Enumeration of B cells

The number of surface membrane immunoglobulin bearing lymphocytes (SmIg, putative B cells) was determined by using the FITC-conjugated rabbit anti-monkey Ig. One millilitre of monkey lymphocytes at 2 x 10^6 /ml was put into a Kahn tube and spun at 90 g for 10 minutes at 4°C. The supernatant was removed and 100 µl of cold L-15 medium and 10 µl of freshly centrifuged (1500 g, 15 minutes) rabbit anti-monkey Ig-FITC conjugate added. The mixture was incubated at 4°C for 30 minutes. The tube was then filled with ice-cold L-15 medium containing 0.01% sodium azide (BDH) to stop capping and the cells were washed three

times by spinning at 70 g for 10 minutes each time. All tests were carried out at 4°C to further minimize redistribution of cell-bound antibodies and consequent loss from the surface. The supernatant was poured off, the lymphocytes resuspended and a drop put on a clean dry slide and covered with a clean coverslip. The wet preparation was either sealed with untinted nail varnish before counting the B cells or was examined without sealing.

(12) Single radial immunodiffusion

The serum concentrations of IgM and IgG were quantified by the single radial immunodifussion technique (226) using thoroughly pre-cleaned and pre-coated (0.2% agar) 82 x 82 mm glass plates (E. Kodak.), goat anti-human IgG (8.54 mg/ml) and IgM (7.47 mg/ml) (Behringwerke) (0.4 ml of the reconstituted suspension added to 12 ml of 1.2% special agar-noble (Difco) (containing 0.1% sodium azide in PBS pH 7.2 to limit bacterial growth and equilibrated at 56°C), a levelling board (kit) (consisting of a horizontal table, three legs-one fixed and two adjustable and a spirit level (LKB 2117 - 404), a humid box (chamber) at 4°C to solidify and equilibrate the gelled plates, sample wells 2 mm diameter (cut out using a locally made plastic template), a gel puncher (LKB O.D 2 mm, 94-92-6058) and suction of a high vacuum pump to remove the agar-plug.

The plates for measuring IgG and IgM were incubated for 4 and 24 hours, respectively, at room temperature in the moist boxes, because in agar IgM diffuses much more slowly than IgG. Thereafter the plates were first washed in 0.1M NaCl for

30 minutes to remove the non-precipitated proteins from the gel and then in distilled water for 24 - 48 hours with at least 3 changes to remove the sodium chloride and any remaining free protein from the gel before drying them slowly at room temperature but under filter paper (Whatman No.1) to avoid cracking of the drying gel. They were subsequently stained for 10 minutes in 0.5% Coomassie brilliant blue - R - 250 stain (E. Merck) made from 100 ml of glacial acetic acid, 450 ml, each, of 96% ethanol and distilled water and 5.0 gm of the Coomassie blue, left overnight and filtered before use. However, the filtered mixture could be stored in a covered plastic box. The plates were destained to differentiate for 10 - 15 minutes with three changes of a destaining solution made from 450 ml of 96% ethanol, 100 ml of glacial acetic acid, 450 ml of distilled water and tri-sodium citrate at 0.51% final concentration (range 0.5-10%) to clear the back-ground. They were finally dried at room temperature and the diameters of the precipitation rings were read using a hand micrometer.

The dilutions (neat, $\frac{1}{2}$, $\frac{1}{4}$, 1/10 and 1/20) of the standardized (local or international) serum samples of known antigen composition were included in each plate.

(13) Reticulocyte Count

Reticulocytes are immature and larger than normal erythrocytes. By a supra-vital staining technique involving basic dyes like brilliant cresyl blue they are precipitated into a blue reticulum mesh work, remnants of basophilic ribonucleoprotein normally found within the cell cytoplasm. The

more mature the cell, the less the reticulum.

Reticulocytes were assayed by using equal volumes of filtered saturated solution of brilliant cresyl blue (a mixture of 0.6 gm sodium chloride in 100 ml distilled water to which 1 gm of brilliant cresyl blue (E. Merck) was added and dissolved) and blood (2 - 4 drops of blood, more, if the animal was anaemic) in a Kahn tube, gently mixed and incubated at 37^{o}C for 15 - 20 minutes (74). Thin films made from the mixture were allowed to dry quickly. The unfixed and uncounterstained films were examined under oil immersion (x 100 objective, SM-Lux microscope) and the number of reticulocytes which appeared as greenish blue cells with deep blue precipitate network was determined by examination of 1000 erythrocytes and their percentages calculated therefrom (173).

Permanent preparations were made by counter-staining the smears with 0.2% Leishman stain in methanol (BDH Chemicals Ltd) (a Romanowsky stain). After fixing for 30 seconds in undiluted Leishman stain (containing methyl alcohol) to decolorise the cresyl blue staining of the RBC while leaving the precipitate in the reticulum, the cells were then treated for a similar period with dilute (equal volumes of 0.2% Leishman stain and methanol) stain which was washed off the slides and dried in the usual way. The reticulocytes were enumerated as in the unfixed and uncounterstained films above.

(14) Tissue culture

(A) Culture media

The following culture media were used :-

(i) Leibovitz L-15 (Modified) medium (199) (Flow, Lot 1222039) was used in the preparation of lymphocyte suspensions and as an incubation medium for CLA_4 cells used in mixed lymphocye cultures (MLC). It was also used to reconstitute mitogens and (methyl-^{14}C) thymidine.

(ii) Dulbecco's Modification of Eagles Medium (DMEM) (93) (Flow, Lot 1207029) is a chemically defined synthetic medium. It was buffered with $NaHCO_3$ at 3.70 gm per litre and used to incubate lymphocytes in mitogen stimulation and MLC.

(iii) Roswell Park Memorial Institute culture medium 1640 (RPMI-1640) (243) (Gibco-Biocult, Batch No. U47406) is a rich general purpose medium basically buffered with $NaHCO_3$ and is also suitable for lymphocyte culture.

(iv) Medium 199 (244) (Flow) is another chemically defined medium containing all amino acids, vitamins, nucleic acid constituents, intermediary metabolities and accessory growth factors. Although, the medium can maintain cell cultures viable for long periods foetal calf serum (FCS) is needed for initiating cellular growth. The medium was made from commercial sachets (Flow), buffered with $NaHCO_3$, prepared and sterilized by the Medical Research Centre of the Royal Tropical Institute Amsterdam, The Netherlands, Nairobi.

The media L-15, DMEM and RPMI 1640 were supplied in sterile 500 ml quantities. However, all the 4 media were always stored at 4^{o}C and just before use they were supplemented with sterile 20 mg glutamine (2mM) (Flow, Lot 1540038), 100 units of penicillin (Gibco-Biocult) and 100 µg of streptomycin

(Gibco-Biocult) per 100 ml of medium and with various concentrations of heat-inactivated FCS (Flow) when used for culture.

(B) Mitogens

A micro-culture method (171) was used in stimulation of separated lymphocytes or whole blood, with a panel of non-specific mitogens dissolved and diluted in sterile L-15 medium without FCS.

(i) Phytohaemagglutinin (PHA) (Bacto-PHA P, Difco Control 63625) was dissolved to provide a 0.1% (1000 µg/ml) PHA stock solution,

(ii) Lipopolysaccharide (LPS) (Bacto-LPS W-E Coli 0.55. B5, Difco Batch No. 51044) was dissolved to give a stock solution of 3000 µg/ml concentration and,

(iii) Concanavalin A (Con A) (Serva Feinbiochemica, Lot No. 3120-25) from Canavalia ensiformis was also dissolved to a stock solution of 3000 µg/ml.

All stock solutions of mitogens were sterilized by millipore filtration (0.45 µm, Millipore Corporation) and together with the appropriate concentrations were stored in liquid nitrogen until required.

(C) (i) Collection of Blood

Venous blood was always obtained aseptically from the femoral veins of vervet monkeys using preservative-free heparin (Heparin Novo, Novo Industri, Batch No. 48+17) as an anticoagulant (10 i.u.per ml blood, final concentration). It was collected in sterile plastic universal containers (Sterilin).

With practice femoral vein punctures were easily and quickly carried out on unanesthetised but restrained animals with minimal trauma to the monkeys and the technicians.

(iii) Separation of lymphocytes from the peripheral blood of monkeys.

Lymphocytes were separated from the peripheral venous blood of animals (46) using equal volumes of sterilized (autoclaved at 15 lb/sq inch for 15 minutes) Ficoll-Triosil S.G. 1.0825 solution prepared from a mixture of 37.5% (w/v) sodium metrizoate solution (Triosil 440, Nyegaard) and 9% Ficoll 400 (Pharmacia) in a ratio, of 1 part Triosil and 2.5 parts. Ficoll (the sterile Ficoll-Triosil solution was always stored at room temperature in the dark). The undiluted heparinized venous blood was slowly and gently layered on top of the Ficoll-Triosil in sterile universal containers which were then centrifuged for 30 minutes at 900 g at room temperature in an MSE centrifuge with the braking system switched off. The plasma was first removed and stored at - 30°C for subsequent analysis and then the lymphocytes at the plasma-Ficoll-Triosil interface were carefully removed and washed twice with L-15 medium. Any excess red blood cells in the lymphoid cell suspension were removed at this stage by resuspending the cell pellet in 10 ml of sterile 0.17 M ammonium chloride (NH_4Cl) solution and by incubating the mixture at 37°C for 5 - 15 minutes. This was gently mixed 2 - 3 times by hand during this period. The lymphocytes were then washed 3 times with cold L-15 medium for 15 minutes at 400 g in a refrigerated centrifuge at 4°C.

After the final wash, the pellet of lymphocytes was resuspended in the required culture medium containing the appropriate concentration of FCS, counted on a haemocytometer and adjusted to the required cell numbers, again, in the right culture medium.

In all cases when sterile lymphocytes were required most of the procedures were done in a sterile laminar air-flow cabinet.

(iii) Enumeration of lymphocytes

All lymphocytes, including stimulators and responders (see MLC below), after the final wash, were resuspended and diluted in the appropriate medium containing the right concentration of heat-inactivated FCS. Cell concentration and viability were determined by phase contrast microscopy (108) (x 40 objective). The intact viable lymphocytes appeared bright whereas dead, dying and damaged cells appeared much darker and somewhat flatter. The trypan blue exclusion technique whereby 100 µl of 1% w/v trypan blue solution (prepared as 5% w/v stock solution in distilled water, filtered and diluted in PBS or L-15 medium) was added to 1.0 ml of appropriate dilution of the cell suspension and allowed to react for 5 minutes, was also attempted. The enumeration was done on a haemocytometer. Total lymphocyte count involving viable, as unstained cells and dead, as stained lymphocytes, was made and the viability of the cells in the suspension was finally calculated. More than 90% of the lymphocytes were viable by phase contrast while by the trypan blue method more than 95% lymphocytes excluded the dye. The cells were subsequently adjusted to the appropriate concentration in the incubation medium containing the FCS at the required

concentration.

(iv) Preparation of lymphocytes from lymph nodes and spleens of vervet monkeys

These were prepared according to the method described by Ford (108). A spleen and brachial, axillary and inguinal lymph nodes were aseptically removed from a monkey deeply anaesthetised by intramuscular injection of Ketamine hydrochloride (Parke-Davis) and ether (May & Baker) inhalation on cotton wool. The organs were transported in sterile containers of L-15 medium supplemented with 10% FCS, antibiotics (penicillin, streptomycin and gentamycin 5000 ug/ml L-15). In a sterile atmosphere of the laminar air-flow cabinet the spleen and the lymph nodes were minced gently with a pair of sterile scissors. The cells were gently teased out from the respective organs using the sterile piston of a 2 ml plastic syringe and a sterile stainless steel tea-strainer with a 60 gauge mesh suspended in fresh and sterile L-15 transport medium (above) contained in a small sterile stainless steel 'bowl'. The contents in the bowl were transferred into a sterile universal container and large pieces of tissue were allowed to settle at the bottom of the tube in 3 - 5 minutes. Then the top lymphocyte-rich layers were pipetted off. The lymphocytes were subsequently washed three times in L-15 medium, each wash lasting 10 minutes, by centrifugation at 300 - 400 g and resuspended in L-15 medium with 10% FCS. The spleen cells were collected separately while the lymph node cells were pooled. These cells were used as stimulators in the mixed lymphocyte cultures. No bacterial

or fungal contamination of these cells was observed during culture.

(a) Cultures of separated lymphocytes

Appropriate dilutions in microgrammes (µg) of Con A, PHA and LPS were added as 20 µl volumes to the appropriate concentration of peripheral blood lymphocytes contained in 180 µl of culture medium. Cultures were then set up in triplicates each replicate of 200 µl per well in sterile flat-bottomed microtitre plates covered with loose plastic covers (Linbro, Tissue culture grade). The outer wells of the micro-culture plates were filled with sterile buffered saline to prevent evaporation from the culture wells. Different culture plates were set up for each time point studied and were always incubated at 37°C in a humidified.5% CO_2 in air atmosphere and blast transformation allowed to take place.

(b) Whole blood cutures

Samples of peripheral venous blood of vervets were diluted either 5-, 10-, or 20- fold in DMEM (without FCS) and the appropriate dilutions of Con A, PHA or LPS were added as for separated lymphocyte cultures described above. The mixture was then dispensed into sterile flat-bottomed microtitre culture plate wells in triplicates of 200 µl per well. These cultures were then subjected to the same culture conditions as the separated lymphocytes above.

(c) Mixed lymphocyte cultures

Another non-specific stimulation method of lymphocytes was by either allogeneic or xenogeneic mitomycin C-treated

lymphocytes. The DNA synthesis was assayed after an *in vitro* one-way micro-culture mixed lymphocyte reaction (MLR) (294).

The responder lymphocytes were obtained from peripheral bloods of vervet monkeys while the stimulator cells were either peripheral blood lymphocytes of vervets (allogeneic), Colobus and Syke's monkeys and a lady technologists (xenogeneic), lymph node and spleen cells from vervets (allogeneic) or CLA_4 cells (xenogeneic) of a continuous cell line, CLA_4, of human lymphocytes originally infected with Epstein-Barr virus and therefore essentially a B cell line (84).

The CLA_4 cell line had been maintained in culture in L-15 medium supplemented with glutamine, penicillin, streptomycin and gentamycin and 5% FCS at $37^{o}C$ in 5% CO_2 humidified air atmosphere at the Kenya Tuberculosis Investigation Centre, (K.T.I.C.) Nairobi for about 18 months at the time of experimentation and was kindly supplied to me by Dr David Humber, of the M.R.C./K.T.I.C., Nairobi. The CLA_4 cells were normally cultured in sterile flat-sided plastic culture flasks (Falcon) for 3 - 5 days each time before use as stimulator cells in the MLC. The cells were counted on a haemocytometer and a volume containing 30 - 40 x 10^6 cells was centrifuged at 400 g for ten minutes and then inactivated with mitomycin C.

(i) Inactivation of stimulator cells with mitomycin C.

An easily measurable one-way (unidirectional) MLR occurs when stimulator cells are first inactivated with mitomycin C to prevent their response to the stimulation of responder cells. Any blast transformation that occurs is

therefore essentially that of responder cells.

The supernatants from all centrifuged stimulator cells, peripheral blood lymphocytes, lymph node, spleen and CLA_4 cells, were discarded. The cells were resuspended in 1 ml of L-15 medium and to this 125 µl of mitomycin C stock solution added at a final concentration of 25 µg/ml. The cells were then incubated for 30 minutes at 37°C and thereafter they were washed three times, 10 minutes for each wash, in L-15 medium at 400 g to remove the mitomycin C. The cells were reconstituted in the appropriate culture medium with FCS, enumerated on a haemocytometer and adjusted to the required concentration in the culture medium with the right dilution of FCS, ready for culture.

(ii) Setting up of the mixed lymphocyte cultures (MLC)

Equal volumes of appropriate concentrations of viable responder and stimulator cells were mixed thoroughly and aliquots of 200 µl of this cell mixture of (sometimes varying) cell concentrations per culture were distributed in each well of the flat-bottomed microtitre plates. The cultures were run in triplicates. The controls received an equal volume of either 100 µl of responder cells or 100 µl of mitomycin C-treated stimulator cells and 100 µl of MLC medium (DMEM). These mixed lymphocyte cultures were then treated like the mitogen-stimulated ones, above.

(iii) Estimation of DNA synthesis

Lymphocyte blast transformation and therefore DNA synthesis was measured by incorporation of (methy-^{14}C) - thymidine (^{14}C - TdR) into a water/methanol insoluble

material. Separated lymphocyte, whole blood and mixed lymphocyte cultures were cultured for varying times depending on the experiment but each culture was always labelled (pulsed) with 0.02 μCi (in 20 μl of L-15 medium) of ^{14}C-TdR (The Radiochemical Centre Ltd., Code CFA 532, Specific activity, 52 μCi/mmol; Radioactive concentration 50 μCi/ml, Batch 17). After the appropriate pulsing time the cultures were terminated either by storing the plates at 4^{o}C before harvesting or by harvesting them immediately.

(iv) Harvesting and counting of cultures

The cultures were harvested onto glass fibre filter mats (Skatron) with a semi-automatic multiple Titertek cell harvester (Skatron). The filters were first pre-wetted for 2 seconds and then the cultures were washed onto them for 20 seconds with de-ionized or mono-distilled water followed by a 15 seconds methanol wash and subsequent air-drying. The procedure rendered the resultant glass fibre discs fairly colourless. The discs were dried thoroughly for 30 minutes in an incubator at 37^{o}C before putting them in the scintillation glass vials and adding 5 ml of toluene—based scintillation fluid containing 0.3% PPO (2, 5 - Diphenyloxazole) (BDH, Chemicals Ltd. and 0.03% of POPOP (1,4 - Bis-(5-phenylloxazol)-2- yl)-benzene (Ernst Merck) scintillation grade. All samples were counted for 100 seconds on a liquid scintillation counter (LKB, Wallac 1210, UltroBeta) to assess the amount of radiolabel, ^{14}C -TdR, incorporated by the DNA in the cells. Machine background substraction was first performed and quench correction was done using an external

standard and the mean disintegrations per minute (dpm) used to calculate stimulation indices (SI) or ratios (SR).

(15) Fluorescence microscopy

This was performed using an Orthoplan, Leitz, fluorescence microscope (Ernst Leitz GmBH D-6330, Wetzlar, Germany, 895018). This was equipped with an HBO 200 W/4 Super pressure mercury lamp as a light source for the incident epi-illumination and for the blue excitation radiation for the ploem fluorescence vertical illuminator; KP 490/2 x KP 490 (2KP 490 - KP 500) as exciting filters, 2 x 3 mm BG 12 filters with dichroic beam splitting mirrors with built in suppression (barrier) filters TK 510/K 515; and periplan GW 8 x MF eyepiece and, modified to permit rapid switching between the fluorescent, phase contrast and plain light.

The lymphocytes were examined and enumerated under oil immersion with x 100 and 100/1.30 (1.70/0.17) fluorescent, phase contrast and plain light objectives while the trypanosomes were examined under oil immersion using only the above fluorescent objective.

(16) Haematology

Haematological variables, particularly the haemogram, were collected on a Coulter Counter Model "S" (Coulter Electronics Inc.) in the Haematology Section, Nairobi University Medical School. The counter was calibrated for the monkey cells.

The staining of thin blood smears for differential white blood cell counts was performed on an automatic staining machine (Shandon) using May-Grunwald-Giemsa's stain (74).

(17) Weighing of animals

Anaesthetised (10 mg/kg body weight Ketamine hydrochloride, Parke-Davis) monkeys were weighed on a balance (Salter Model 502 Thermscale, England)). They were starved overnight to reduce hazards associated with vomiting during anaesthesia.

(18) Treatment of monkeys

Each infected animal that was treated with a trypanocide received only one injection (i.m.) of Berenil (-(4-, amidinophenyl) - triazene- (N-1:3) diaceturate) at 10 mg/kg body weight.

(19) Expression of results and statistical analysis

Unless otherwise specifically stated, (i) the concentrations indicated in the text, materials, methods, results and tables of these experiments were the final ones; (ii) the results are expressed as the mean and its standard error (SE) from 5 or 6 animals; that is, individual points on figures represent the mean values of 5 or 6 determinations and; (iii) sera were inactivated at 56^{o}C for 30 minutes.

Statistical calculations were carried out using a Texas Instrument SR-51-II or Casio Scientific fx - 68 programmable calculator. Means, standard deviations, standard error of the means, Students' t-test and analysis of variance were calculated using the formulae given in Snedcore & Cochrane (349).

The proper application of many statistical tests requires data that have a normal (Gaussian) distribution and homogeneous variances within the groups to be compared. However, biological

phenomena often do not conform to these criteria and mathematical transformation of the raw data may be necessary in order to fulfil these requirements. This is true of data from the lymphocyte transformation assays as the distribution of the disintegrations per minute is skewed and variances increase with the mean (124). The Log_{10} transformation gives good approximation to normality and reduces heterogeneity of variances (124). The transformation used in this study was Log_{10} $(x \pm 1)$ where x is the arithmetic mean of the radioisotope disintegrations per minute (dpm). This allows for zero values (422). Also after the dpm were Log_{10} transformed to normalise their distribution the statistical significance was calculated using the Students' t-test and expressed as probability.

Similar considerations apply to the majority of data presented in this study and all raw data were screened before any statistical analysis was carried out to ensure that the populations under investigation were normally distributed and had almost similar variances.

Various transformations were tried out to normalise data and the final methods chosen are shown in Table 2.1.

Stimulation indices (SI) or ratios (SR) were calculated by dividing stimulated by the corresponding unstimulated or background dpm values.

The dpm counts in lymphocyte transformations were from 3 replicates (per animal) which were averaged; that is, each dpm determination is a mean of 3 replicates.

Animals' weights were analysed by considerations of

Log_{10} animal weight on animal number as shown to be appropriate by Healy and others (144).

Table 2.1. Data transformation and expression

Data	Transformation
Haemagglutination titres	Log_2 titre per sample
Parasitaemia	Log_{10} mean parasites per ml blood
Animal Weight (mg)	Log_{10} mean body weight per animal
Disintegrations per minute (dpm B)	Log_{10} dpm sample; Log_{10} $(x \pm 1)$ mean dpm per sample
Lymphocytes, neutrophils, monocytes, erythrocyte rosettes, SmIg and null cells	Per cent, mean absolute numbers; Log_{10} absolute numbers per sample; Log_{10} $(x \pm 1)$ mean absolute numbers per sample.

CHAPTER THREE

SUSCEPTIBILITY OF THE VERVET MONKEY TO *TRYPANOSOMA BRUCEI* AND TITRATION OF THE CLONED PARASITE

INTRODUCTION AND REVIEW OF THE LITERATURE

Literature abounds with reports on various aspects of human and animal African trypanosomiasis. In this section literature on susceptibility, parasitaemia and anaemia associated with T. brucei T. rhodesiense and T. gambiense in man and the non-human primates is reviewed while bearing in mind (415) the definitions of infectivity, virulence and resistance based on bacteriological models, and the modifications (212) intended to iron out inconsistencies and ambiguities with regard to these terms, which tended to confuse discussions, when used in parasitology.

Infectivity is the capacity of an organism to establish a primary lodgement on the animal at the body's surface and also to spread from one host to another under specified conditions of exposure to risk of infection (415). The term also covers cultures and vertebrate and arthropod hosts. Lodgement is a situation in which the introduced organism probably reproduces at least to some extent.

Virulence is the capacity of an organism to cause disease, the host and the conditions being specified or an average resistance implied. High virulence in protozoology applies to acute situations when organisms multiply to the extent of overwhelming the host, as in trypanosomiasis, and producing a fulminating parasitaemia. Low virulence strains are those which produce long term low intensity infections, and still cause disease (415) though probably secondarily by immunological mechanisms rather than by the direct effects of the organisms.

Wilson and Miles (415) have used degrees of virulence to qualify individual cultures and strains and have reserved pathogenicity to apply to classes of bacteria. However, pathogenicity and virulence have been regarded as synonymous (82) and invasiveness as comprising the capability of organisms to establish themselves intracellularly, to protect themselves from host attack by capsules, or to adapt themselves to the host environment and toxigenicity, compromising their ability to produce exotoxins and endotoxins which attack the host.

Statements on the resistance of a host ideally should either specify the stock of a parasite or imply a parasite's infectivity and/or virulence.

(1) Non-human primates

Reports on natural T. brucei, T. rhodesiense and T. gambiense infections in non-human primates are very rare presumably because Glossina species feed only infrequently on lower primates (29, 107, 398, 400). None of the Cercopithecus aethiops, for instance, used for experiments at Tinde, Tanzania was originally naturally infected though some came from the tsetse-fly belts (17). Similarly at Tororo, Uganda, of the 22 vervets examined for natural brucei-subgroup trypanosomes none were infected (116). However, natural infections of monkeys have been reported from East Africa as T. gambiense, probably in C. aethiops and from Portuguese Guinea as T. brucei in C. aethiops (28).

Experimentally all primates except Papio species seem susceptible to trypanosomes and fatal infections have been readily initiated in C. aethiops (17) and chimpanzee (27, 117).

Monkeys infected experimentally with T. brucei and T. rhodesiense produce either acute or chronic diseases. Typically, T. rhodesiense kills monkeys but self-cure also occurs. For instance, 5 vervets spontaneously recovered from T. rhodesiense and even after 3 of these were re-infected with the same parasite stock between 2½ and 6 years later, one monkey again recovered (100, 410). Also of vervets cyclically and parenterally infected with T. brucei (17) three monkeys recovered spontaneously and one died with high parasitaemia 65 days after re-infection with T. rhodesiense. Thus most T. rhodesiense and T. brucei - infected vervets usually died and only about 1% recovered spontaneously and could still be re-infected with the same or different parasite.

Cyclical transmission of T. rhodesiense by G. morsitans in C. aethiops over 7 years increased the virulence of the parasite which progressively reduced the survival times of the animals from 169.7 $\pm_{SD}$ 28.4 to 33.6 $\pm_{SD}$ 51.0 days (410). In contrast, T. gambiense transmitted parenterally, directly and almost in rotation between vervets, guinea-pigs and rats retained its low pathogenicity in the monkeys and some guinea-pigs and rats. It maintained enhanced virulence for other rats and guinea-pigs but not for monkeys when inoculated in rat blood. A goat had a transient infection on post-infection day (PID) 323 while a sheep was completely refractory to the trypanosomes virulent for guinea-pigs and rats (17, 68).

Experimentally immunized Macaca mulatta were readily infected with T. rhodesiense and survived for 6-49 days but they died earlier as parasites syringe-passages numerically and

and rapidly increased, concomitantly with its virulence (95). After inoculating these Macaca with 1 x 10^6 trypanosomes the prepatency varied from 4 - 8 (mean 5) days. The parasitaemia increased rapidly to 150-200 x 10^6/ml on PID 10, peaked at 700 x 10^6/ml by PID 15 and then fluctuated between 50-500 x 10^6/ml blood until the animals died.

One to two weeks after parasite inoculation, the haemogram dropped progressively to about 10% and 2 x 10^6 erythrocytes/ml blood for the PCV and RBC counts, respectively, while concomittant reticulocytosis also occurred (326).

Marked bone marrow erythrocytic hyperplasia occurred but the total and differential leucocyte count changes were inconsistent. The weight loss and gain for the infected and control animals were 1000 and 450 gm, respectively, in 6 weeks.

Houba and others (159) also successfully infected Macaca parenterally with the 3 brucei-subgroup trypanosomes and studied heterophile antibodies.

Trypanosoma gambiense infection is difficult to establish in monkeys and the resultant disease is chronic. In Erythrocebus patas-patas Wijers (407) cyclically induced acute T. rhodesiense and chronic T. gambiense with occasional low self-limiting parasitaemia, with 128,000 and 104,000 parasites per cu.mm., respectively, as the highest. Yet, cycling and relatively high parasitaemia was reported in T. brucei-infected monkeys. Leucocytosis, lympocytosis and neutropenia (406) also occurred. Trypanosoma brucei, rhodesiense and gambiense infections in monkeys are probably similar (326) and T. rhodesiense invades the cerebro-spinal fluid (CSF) (17, 68).

Attempts to infect baboons with pathogenic trypanosomes succeeded in the case of T. gambiense and T. congolense by intraperitoneal inoculation while a lethal T. gambiense, with a predilection for the CNS, inoculated intrathecally produced no trypanosomes in baboon blood (17). Therefore, baboons have been considered resistant to trypanosome infections except possibly by intrathecal inoculation which, however, has not been successful with T. brucei and T. rhodesiense, either (17).

Since man's susceptibility is the recognized method of differentiating T. brucei, T. rhodesiense and T. gambiense only a few attempts have been made to experimentally infect the anthropoid apes for the purpose. Following successful infection of T. rhodesiense in a chimpanzee, attempts were made to try and differentiate T. brucei from T. rhodesiense by infecting intramuscularly 2 chimpanzees, respectively, with 25×10^6 T. rhodesiense and 15×10^6 T. brucei parasites (27). The former animal produced a parasitaemia between PID 10 and 13 and appeared healthy but chronically infected, showing a fluctuating low grade parasitaemia for 5 months. However, the CSF collected 3 weeks and 4 months after infection showed pathologically raised cell counts and protein concentrations. The prepatency for T. brucei was 8 days but the animal with moderate parasitaemia, died of intercurrent infection on PID 12. Thus, T. rhodesiense also invades the CNS of the chimpanzee like that of man and produces similar CSF changes suggesting that the chimpanzee is experimentally useful for studying T. rhodesiense pathology. However, unlike man it cannot be used to differentiate T. rhodesiense and T. brucei because it is susceptible to both

parasites (28).

The above apes were infected parenterally with parasitized blood. Later, cyclically tsetse-fly-transmitted T. rhodesiense, T. gambiense and T. brucei were compared in the chimpanzee (117). An infected tsetse-fly bite produced a localized nodule (chancre) in the skin. The prepatent periods for T. brucei, T. rhodesiense and T. gambiense were 6, 8 and 9 days, respectively. The T. brucei rapidly multiplied to about 400 trypanosomes per 100 fields of blood film by PID 12 and then decreased to only one trypanosome, per 100 fields of blood film and 20 parasites/cu. mm. CSF by PID 14 when the chimpanzee was moribund. However, the animal was clinically unwell from PID 4-18, lost 1.4 kg and suffered from heavy Strongyloidiasis and, mild anaemia which disappeared after therapeutic cure of the T. brucei.

In contrast, T. rhodesiense relapsed with a parasitaemia of up to 100 trypanosomes per 100 fields of wet blood film. The parasitaemia first peaked on PID 13 (5 days after the first day of patency) and later at 5-10 days intervals until the chimpanzee died on PID 44 with only 2 trypanosomes per cu. mm. CSF and with low Hb and erythrocyte count. However, leucopenia had occurred earlier. On PID 35 the T. rhodesiense-infected chimpanzee developed sulphatriad-resistant sores in the gum and was ill and lethargic by PID 41. It died probably from barbiturate overdosage on the weak animal when being anaesthetized for routine examination. The animal was almost normal initially but lost 1.0 kg between PID 28 and 35.

The Trypanosoma gambiense infection became chronic with a

maximum of 300 trypanosomes per 100 fields of chimpanzee wet blood film. During the first 90 days, the parasitaemia peaked at 6-10 days'intervals and seldom exceeded 20 trypanosomes per 100 fields. Thereafter the parasitaemia was only occasional for 11 months and none for a further 9 months (that is, until the 18th and 27th months, respectively, of infection) when the experiment was stopped. The CSF on PID 65 showed only one T. gambiense parasite per 200 fields (×40 objective). By PID 99 only the microscopically negative CSF was, for the last time, infective to a 12-day weanling rat. Throughout the infection the Hb decreased only slightly but the animal seemed clinically well and for 4 months it maintained the pre-infection 10.5 kg body weight which increased to 12.6 kg by PID 252 (117). In these chimpanzee experiments T. brucei was the most pathogenic parasite. Death of the chimpanzee on day 9 was averted by treatment (117) while the chimpanzee of Baker (27) died on PID 12 but the T. rhodesiense-infected animal lived 3 times as long as the T. brucei-infected ape. The chronically T. gambiense-infected chimpanzee seemed clinically well (117). Intercurrent infections as helminthiasis and gross lung disease probably influenced the outcome of T. brucei and T. rhodesiense, respectively.

(2) Man

The morphologically identical T. brucei, T. rhodesiense and T. gambiense are indistinguishable even on electron micrographs. The main distinguishing characteristic is that T. brucei is not infective for man and the concept of T. rhodesiense and T. gambiense sleeping sickness epidemiology rests partly upon that long-held belief that the infectivity of

T. rhodesiense and T. gambiense and the non-infectivity of T. brucei for man are constant and stable properties (314). The three parasites are here regarded as distinct subspecies in order to simplify arguments and that T. brucei is lysed and killed quickly by human serum and unlike the other 2 parasites is, therefore, neither infective for, nor pathogenic to, man.

Man is highly susceptible to T. rhodesiense which kills him in weeks or months (29). However, the parasite also multiplies prolifically in various non-human reservoir hosts (351), producing high but less acute parasitaemia to ensure its propagation and survival through the tsetse-fly without involving the bed-ridden, sick and dying man (29).

The human seems naturally and experimentally very susceptible to T. gambiense (29) which normally kills him after a few years (29). However, T. gambiense epidemics can be very virulent (57).

In the 4 volunteers cyclically infected with T. rhodesiense (100) the prepatent periods were 35, 27, 45 and 10 days, respectively. The nodule puncture of the second volunteer was positive on PID 12 but negative for trypanosomes by microscopy or by rat inoculation on PID 14 and 15. The third volunteer had an incubation period of PID 8 and on PID 9 he showed malarial parasites in his blood. However, by microscopy and rat inoculations he was trypanosome-free between PID 16 and 19. The fourth patient was treated on PID 15.

Heisch and others (145) inoculated a white volunteer with 25 ml of trypanosome-laden blood (100 parasites per one oil immersion field) from rats sub-inoculated with blood from a

bush-buck. Trypanosomes were seen in the material aspirated from a swelling at the inoculation site on PID 6, and few in the blood by PID 8 associated with moderate pyrexia of 39.4°C and hyperpyrexia of 41°C. Treatment was commenced on PID 9. Rats inoculated on PID 9 with the blister exudate and with blood from the ear-lobe prick became infected.

Also Onyango and others (285) inoculated about 7.4×10^6 trypanosomes (from an isolate from cattle) into an African volunteer who on PID 2 developed a nodule and on PID 8 showed midcervical, axillary and supraclavicular lymphadenopathy and malaria parasites but no trypanosomes in 3 stained thick blood films taken sequentially that day. The patient received chloroquine intramuscularly. On PID 9 he was hyperpyrexic (40.4°C) and in agony, but only one trypanosome per microscope field (x800) of wet blood was seen. The parasites increased numerically on PID 10 and 11 only to disappear on PID 12. On PID 17 hyperpyrexia and 2 trypanosomes per field (x800) were present while obvious right posterior cervical triangle lymphadenopathy appeared on day 19. Mice inoculated with the volunteer's blood on PID 11, 13, 14 and daily from PID 25 and CSF examinations, 5 days and 5 weeks after therapy were negative for trypanosomes and normal for protein and cell contents. The 8 days' incubation period, the infectivity of the trypanosomes and the pyrexia typify and even resemble natural T. rhodesiense infections.

In nature T. rhodesiense from the primary chancre invades the lymph, blood and tissues, increases logarithmically in the blood and then seems to disappear from the circulation.

The intervals between parasitaemic waves in man initially vary from 1-8 days but thereafter they increase while the parasitaemia decreases. Thus early T. rhodesiense is characterized by acute severe symptoms and high cycling parasitaemias which strangely do not ease diagnosis because, except in laboratory rodents the parasitaemias possibly do not reflect the true counts in the host's body. The organisms may be scanty or apparently absent from the blood while teeming in tissues because the brucei-subgroup trypanosomes are connective tissue parasites that circulate in blood (29, 352). For instance, even in a moribund cyclically infected white patient only up to 6 T. rhodesiense parasites per high power field were seen in his blood between PID 7-11 (34). His blood showed poikilocytosis, monocytosis and irregularly contracted erythrocytes while the PCV swung from 34% to 25% between PID 7-17 and back to 34%, fourteen days after chemotherapy. The Hb, WBC and platelet values were 11.3 gm/100ml, 5,200 and 36,000/cu.mm. blood, respectively, associated with coagulation changes. The weight loss was 4.25 kg in one week and axillary and femoral lymphadenopathy was evident.

In a 2½ weeks old natural and probable T. rhodesiense infection in a patient from Zaire the Hb dropped to 10.8 gm/100 ml of blood and a haemolytic anaemia-associated factor was revealed by the shortened life-span and rapid splenic sequestration of the ^{51}Cr - labelled RBC. The infection and anaemia responded well to Mel B but not to suramin (418).

Again, six trypanosome-infected Ugandan patients were compared with others with, strictly, the big spleen disease (BSD) only (419). Both groups of patients had autoimmune anaemia

associated with complement-coated and short-lived ^{51}Cr - labelled erythrocytes. It was, therefore, concluded that radioactivity was accumulated in the spleen by erythrophagocytosis within it and that cytolysis in trypanosomiasis possibly contributed to the anaemia and splenomegaly.

De Raadt (85) has correlated no specific clinical signs with early T. rhodesiense except for pyrexia which always coincides with the parasitaemic waves. As the infection progresses the pyrexia decreases and the more typical signs including anaemia and the CNS manifestations develop normally between 3 - 12 weeks post-infection. Untreated, human T. rhodesiense infection usually kills between 3 - 9 months after infection. Intercurrent bacterial infections of lungs possibly accelerate the disease outcome (85).

Monkeys seem refractory to T. vivax (208) which probably requires adaptation like T. gambiense by either serial passages or serum supplements as in rodents and ungulates (372). However, unlike T. congolense, 2 human cases of T. vivax from Ghana and from Uganda have been reported (142). Again, monkeys are probably not naturally infected with T. congolense, yet experimentally, they show acute, subacute and chronic infections with high parasitaemia, even terminally (17, 208). Chronic anaemia, probably of immune origin, and emaciation also occur (208). Thus in many ways the acute and chronic T. gambiense and T. congolence infections in monkeys resemble. However, like T. brucei and unlike T. rhodesiense and T. gambiense, T. congolense is not infective to man (208).

In summary, in monkeys T. brucei can occur naturally,

and, the parasite can produce different disease pictures in different or in the same species and/or strain of the host, like self-cure and death in monkeys, while different parasites produce almost the same disease in different hosts. Anaemia, for instance, is produced by all African trypanosomes whether they are plasma parasites like T. congolense and T.vivax or tissue parasites like T. brucei, T. rhodesiense and T. gambiense. Probably other unknown factors are also important in the pathogenesis of trypanosomiasis.

After establishing that an animal is susceptible to a parasite previous investigators have tended to ignore determining the effects of the size of the dose of the parasite on the course of the infection. Yet, it is well known that varying the numbers of parasites inoculated, at least, influences the incubation period of the infection so that few trypanosomes are associated with long incubation periods whereas high parasite doses are associated with short incubation periods (26).

Since the object of this thesis was to set up an immunological model of trypanosomiasis in the vervet monkey it seemed reasonable to begin by investigating experimentally the susceptibility of the animal to parenterally inoculated T. brucei, by determining the course of infection, the pathogenicity of the parasite and the effects of different parasite doses on the course of the resultant disease.

MATERIALS AND METHODS

Experimental design

(A) Susceptibility

In the first experiment three monkey groups of two, each, were inoculated with approximately 1 x 10^5 trypanosomes (in heparinized mouse blood diluted in PBS pH 7.4) to determine the susceptibility of the vervet monkey to the parasites. The challenge dose was not very critical, neither was the separation of trypanosomes from the mouse blood before inoculating the parasites into the monkeys (Table 3.1).

As a consequence of initial financial constraints which limited purchase of more animals each group of monkeys has acted as its own control and hence the results have been compared to the pre-infection values (177).

(B) Titration of clone 227

In the second experiment which was consequent upon the above experiment whereby monkeys were susceptible to the lethal stocks of TREU 667 and EATRO 2234 and to the self-limiting Clone 227, monkeys were inoculated with different doses of the clone to determine the resultant infections. The idea was to observe whether the monkeys always self-cured regardless of the size of the inoculated dose of the cloned parasite or whether sometimes they were killed by it.

Eight trypanosome-free male vervet monkeys weighing between 2.0 - 3.5 kg were divided into 4 groups each of 2 animals. One group acted as the control while each of the remaining 3 groups was infected with either 1 x 10^8, 5 x 10^5 or 1 x 10^3 parasites of Clone 227 (Table 3.14). The parasites for

inoculation were raised in lethally irradiated mice, separated on DEAE-Cellulose, carefully enumerated on a haemocytometer and finally adjusted to the appropriate concentrations in PSG pH 8.0 such that the total injected parasites were contained in 2.0 ml.

Daily parasitological and regular haematological variables were recorded as described under "General Materials and Methods".

RESULTS

(A) Susceptibility

(i) Parasitaemia

The changes in the mean parasitaemias are shown (Fig. 3.1., Table 3.2).

The animals became clinically ill and some even died as the infection progressed. This indicated that the monkeys were susceptible to all the two stocks and one clone of T. brucei used. Stocks TREU 667 and EATRO 2234 and Clone 227 produced acute, subacute and chronic infections whose prepatency was 1, 3 and 2 days, respectively. In the acutely infected monkeys the parasitaemia was persistent and relentlessly non-relapsing until the animals died on PID 19, one aparasitaemic and the other with 1.2×10^6 trypanosomes/ml blood. The maximum mean parasitaemia for the two animals was 138.1×10^6 trypanosomes/ml blood on PID 7.

The subacute infection formed a mean plateau-like parasitaemic wave until the animals died on PID 44 and 62 with 15×10^6 and 3.2×10^6 parasites/ml blood, respectively. The highest mean parasite count was 27.1×10^6 trypanosomes/ml blood on PID 39.

The chronically infected animals formed the typical fluctuating parasitaemia with periods of remission during which parasites were undetected in blood. The first mean parasitaemic wave peaked on PID 4, and each wave lasted 6 - 12 days. The maximum mean parasitaemia for the group was 21.1×10^6 trypanosomes/ml blood on PID 35. Thereafter the peaks decreased as the infection progressed to self-cure on PID 37 and 61

because for several weeks thereafter parasites were not detected in the blood either by microscopy or by sub-inoculation into mice and the PCV was back to normal.

(ii) Haematology

The changes in the mean haematological parameters are shown (Figs 3.ii - 3.xii ; Tables 3.3 - 3.13), respectively, and can be summarised as follows:

Anaemia featured prominently in all infected monkeys. In animals infected with stock TREU 667 (acute infection) the anaemia was brisk and severely progressive. By PID 18 the RBC count, the Hb and PCV (Figs. 3.ii, 3.iii, 3.iv; Tables 3.3, 3.4, 3.5) had decreased by 48.9% (5.79 - 2.96 x 10^6/mm^3 blood), 51.8% (13.9 - 6.7 gm/100ml blood) and by 48.1% (39.90 - 26.65%), respectively. There was also reticulocytosis (0.65 - 5.80%) whose maximum value of 9.20% was recorded on PID 15 (Fig. 3.v, Table 3.6). The MCV, MCHC and MCH (Figs. 3.vi, 3.vii, 3.viii, Tables 3.7, 3.8, 3.9) each was terminally the same or changed only slightly from 70.5 - 70.5 μ^3, 34.85 - 32.25% and from 24.05 - 23.20 uug, respectively, while final leucopenia of 70.1% (8.70 - 2.60 x 10^3 WBC/mm^3), lymphocytosis of 57.5 - 84.0%, neutropenia of 40.5 - 11.5% and slight monocytosis of 2.0 - 4.5% were noted (Figs. 3.ix, 3.x, 3.xi, 3.xii; Tables 3.10, 3.11, 3.12, 3.13). The anaemia in this phase of infection was mainly normocytic and moderately hypochromic.

In the subacute infection due to EATRO 2234 the anaemia was moderate and slightly remitting. By PID 43, just before the first animal in the group died, the mean RBC count, the Hb and PCV had decreased by 40.2% (5.77 - 3.45 x 10^6/mm^3 blood), 44.5%

(13.65 - 7.60 gm/100 ml blood) and by 39.7% (40.80 - 24.75%), respectively, (Figs. 3.ii, 3.iii, 3.iv; Tables 3.3, 3.4, 3.5). The mean reticulocyte count, which initially rose from 1.25 - 8.60% by PID 21, was back to 1.20% on PID 43 (Fig. 3.v, Table 3.6). Meanwhile, the MCV wavered between 69.5 - 70.5 μ^3 (Fig. 3.vi; Table 3.7) and the MCHC and the MCH decreased from 31.65 - 30.65% and from 23.60 - 22.15 µµg, respectively, (Figs. 3.vii, 3.viii; Tables 3.8, 3.9). Leucopenia of 43.8% (8.90 - 4.95 x 10^3 WBC/mm^3) and neutrocytosis of 37.5 - 63.0%, like in the acute disease, lymphocytopenia of 64.0 -37.0% (for monkey no.12) and monocytopenia of 2.5 - 0.0%, unlike in the acute disease, were evident in this monkey group when last investigated on PID 49 (Tables 3.10, 3.11, 3.12, 3.13). The anaemia was normocytic and mildly hypochromic.

In the monkeys chronically infected with the clone the anaemia was mild to moderate and remitting (transient). The RBC, and PCV dropped by 40.07% (5.79 - 3.47 x 10^6 cells/mm^3 blood), 42.0% (13.80 - 7.95 gm/100ml blood) and by 41.7% (41.65 - 24.25%), respectively, by PID 18 (Figs. 3.ii, 3.iii, 3.iv; Tables 3.3 , 3.4, 3.5). By PID 21, however, some of the haematological values like RBC count and Hb started to increase and the reticulocyte count reached its highest value of 8.60% and was back to the pre-infection level of 0.9% on PID 73 (Fig. 3.v; Table 3.6). The drop in RBC count, Hb and PCV was 12.6% (5.79 - 5.06 x 10^6 RBC/mm^3 blood), 18.8% (13.80 - 11.20 gm/100ml), and 12.0% (41.7 -36.7%), respectively, by PID 73 as compared to the PID 18 values (Tables 3.3, 3.4, 3.5).

Concomitantly the MCV wavered between 70.5 - 72.0 μ^3 and the MCHC and MCH dropped from 32.70 - 30.75% and from 23.75 - 22.25uug, respectively, (Figs. 3.vi; 3.vii; 3.viii; Tables 3.7; 3.8, 3.9). Leucopenia of 42.4% (8.50 - 4.90) on PID 18 disappeared by the return to 8.50 x 10^3 WBC/mm^3 blood by PID 73, unlike in the acute and subacute infections, was evident, while lymphocytopenia from 60.5 - 48.9%, neutrocytosis from 37.5 - 52.0% by PID 49 and almost normal monocyte count from 2.0 through 4.0% and back to 2.0% on PID 25, were observed (Figs. 3.ix, 3.x , 3.xi , 3.xii; Tables 3.10, 3.11, 3.12, 3.13).

The lymphocytopenia in this chronic phase unlike that of acute infection was similar to that of subacute infection . The neutrocytosis resembled that of acute and subacute infections. The anemia here was normocytic and hypochromic in type.

(iii) Clinical picture

Clinically the infected animals showed progressive emaciation (due to loss of appetite), apathy, irritability, decreased muscle mass, limb rigidity, perineal oedema and slumped postures with bowed heads. Inguinal lymphadenopathy was evident from the second post-infection week (PIW 2). The hair was dispersed.

(B) Titration of Clone 227

(i) Parasitaemia

The changes in the mean parasitaemia are shown (Fig. 3.xiii ; Table 3.15). The two animals inoculated with 1×10^8 trypanosomes lacked prepatency and the parasitaemia increased relentlessly without forming parasitaemic waves, to a maximum mean peak of 232.3×10^6 trypanosomes/ml blood on PID 11. The animals died on PID 12 and 15 with high terminal parasitaemias of 201.0×10^6 and 38.2×10^6/ml blood, respectively.

The prepatency in monkeys inoculated with 5×10^5 parasites was 2 days. Typical parasitaemic waves emerged with remissions of 1-2 day(s) during which the parasites could not be detected in blood by the haematocrit method. The first wave peaked on PID 5 and each wave lasted 7-9 days. However, as the infection progressed the animals acquired secondary staphylococcal and streptococcal infections as multiple pyogenic abscesses and died on PID 26 and 117 with high terminal parasitaemias in their bloods. The maximum mean parasitaemia per ml blood was 51.3×10^6 on PID 20 while the maxima for individual animals in this group were 91.0×10^6 on PID 20 for monkey no. 6 and 54.7×10^6 trypanosomes /ml blood on PID 98 for monkey no. 10.

The incubation periods of infection in the monkeys inoculated with 1×10^3 trypanosomes were 5 and 7 days. Again, the characteristic parasitaemic waves were evident. The first wave appeared on PID 11 and each wave occurred every 9-11 days. The maximum mean parasitaemia was 57.8×10^6 trypanosomes/ml on

PID 20 and monkey no.3 that died on PID 41 had its highest parasite count of 114.75 x 10^6 trypanosomes/ml on PID 20 but, it was 96.2 x 10^6 trypanosomes/ml on PID 40. The second animal (monkey no.1) had its maximum parasitaemia of 136.5 x 10^6 trypanosomes per ml on PID 70. It had 3 distinct parasite waves and the trypanosomes disappeared from the blood on PID 53 only to reappear on PID 60. Then the parasitaemia rose relentlessly to its maximum. In this group, too, intercurrent staphylococcal and streptococcal infections occurred and they were probably responsible for the death of the animals on PID 41 and 79.

(ii) Haematology

The changes in the mean haematological values are shown (Figs. 3.xiv - 3.xxiv; Tables 3.16 - 3.26) and are summarized as follows:-

The control monkeys were not inoculated with T. brucei and therefore, their haematological values remained the same or increased. For instance, the Hb rose from 13.9 - 16.7 gm/100 ml blood (Fig. 3.xv; Table 3.17) and the MCV was finally the same (77.0 - 77.0 μ^3) (Fig. 3.xviii; Table 3.20).

In the animals inoculated with 1 x 10^8 trypanosomes, by PID 10, a few days before monkey no.12 died the mean RBC count, Hb, and PCV had dropped by 36.3% (5.26 - 3.35 x 10^6 RBC/mm^3 blood), 42.3% (12.30 - 7.10 gm/100 ml blood) and by 36.1% (40.40 - 25.75%), respectively, (Figs. 3.xiv, 3.xv, 3.xvi; Tables 3.16, 3.17, 3.18). There was reticulocytosis of 1.15 - 7.00% (Fig. 3.xvii; Table 3.19) while the MCV changed only slightly from 76 - 78 μ^3 but

the MCHC and MCH decreased from 30.00 - 27.90% and from 23.45 - 21.35 uug, respectively, over the same period (Figs. 3.xviii, 3.xix, 3.xx; Tables 3.20, 3.21, 3.22). Leucopenia of 17.1% (8.20 - 6.80 x 10^3 WBC/mm^3), lymphocytopenia of 36.5 - 27.0%, neutrocytosis of 61.5 - 71.0% and the same monocyte count of 2.0 - 2.0% were also noted (Figs. 3.xxi, 3.xxii, 3.xxiii, 3.xxiv, Tables 3.23, 3.24, 3.25, 3.26). The anaemia was normocytic and hypochromic in type.

In the monkeys which were inoculated with 5 x 10^5 parasites, by PID 22, a few days before monkey no. 6 died the RBC count, the Hb and the PCV, had decreased by 35.7% (5.58 - 3.59 x 10^6/mm^3 blood), 40.0% (13.50 - 5.10 gm/100ml blood) and by 32.6% (42.80 - 28.80%), respectively, (Figs. 3.xiv, 3.xv, 3.xvi; Tables 3.16, 3.17, 3.18). The reticulocytosis varied between 0.65 - 10.5% (Fig . 3.xvii; Table 2.19) while the mean MCV remained virtually the same (78.0 - 77.0 μ^3) but the mean MCHC and MCH dropped from 30.85 - 28.20% and from 24.30 - 21.90 uug, respectively, (Figs. 3.xviii, 3.xix, 3.xx; Tables 3.20, 3.21, 3.22). Leucopenia of 24.5% (7.90 - 6.00 x 10^3 WBC/mm^3 blood), lymphocytopenia of 34.5 - 24.5%, neutrocytosis of 64.0 - 73.5% and almost the same monocyte count of 1.5 -2.0% were also observed (Figs. 3.xxi, 3.xxii, 3.xxiii, 3.xxiv; Tables 3.23, 3.24, 3.25, 3.26). The anaemia was normocytic and moderately hypochromic.

In the monkeys that received 1 x 10^3 parasites the RBC count, Hb and the PCV (Figs. 3.xiv, 3.xv, 3.xvi; Tables 3.16; 3.17; 3.18) had decreased by 39.6% (5.55 - 3.33 x 10^6 RBC/mm^3 blood), 45.1% (13.25 - 7.30 gm/100 ml blood) and by 39.2% (42.90 - 26.10%) respectively, by PID 37, just a few days before

monkey no.3 died. There was reticulocytosis of 1.00 - 9.50% (Fig. 3.xvii; Table 3.19) while the MCV was, terminally, the same (78.5 - 78.5 μ^3) but the MCHC and MCH decreased from 31.25 - 27.95% and from 23.80 - 22.10 µµg, respectively, (Figs. 3.xviii, 3.xix, 3.xx; Tables 3.20, 3.21, 3.22). Leucopenia of 46.9 (8.10 - 4.30 x 10^6 WBC/mm^3 blood), lymphocytopenia of 56.5 - 45.5%, neutrocytosis of 41.5 - 51.5% and the same monocyte count of 2.0 - 3.0% were noted (Figs, 3.xxi, 3.xxii, 3.xxiii, 3.xxiv; Tables 3.23, 3.24, 3.25, 3.26). The anaemia in this phase was typically normocytic and hypochromic.

The haematological changes were obvious as from PID 2 (Tables 3.16, 3.17, 3.18). The anaemia was more severe at the peak of, and 1 - 2 day(s) after, a parasitaemic wave than at the base of and 1 - 2 day(s) after a 'trough'. For example, in monkey no.10 inoculated with 5 x 10^5 trypanosomes, on PID 37, 41, 45, 47 and 51 the corresponding parasitaemias per millilitre blood were 0, 15.5 x 10^6, 0, 5.25 x 10^4 and 36 x 10^6 (Table 3.15) while the respective values of Hb in gm/100 ml blood were 8.1, 9.2, 8.2, 9.3, and 8.9 (Table 3.17) suggesting a probable association between the parasitaemia and anaemia.

In summary, the stocks TREU 667 and EATRO 2234 and Clone 227 produced acute, subacute and chronic infections whose prepatent periods were 1, 3 and 2 days, respectively. The parasitaemias were relentlessly unremitting, plateau-like and fluctuating, and remitting for acute, subacute and chronic infections, respectively.

The normocytic hypochromic anaemia was severe, moderate and mild in acute, subacute (intermediate) and chronic infections,

respectively. These changes were progressive and persistent until the animals died of acute and intermediate infections but showed remissions from around PID 19-22 in the chronically infected monkeys which also underwent self-cure. Reticulocytosis was stepwise and continuous in the acute and subacute infections but the reticulocytopenia was stepwise in the latter. The early reticulocytosis and late reticulocytopenia in chronic infection were both stepwise.

The monkeys that received the highest parasite dose of, 1×10^8 of Clone 227 had: no prepatency, the highest and non-relapsing parasitaemias and the least survival times while those that received intermediate (5×10^5) and the lowest (1×10^3) challenge doses had longer prepatent periods, lower and relapsing parasitaemias and longer survival times. Normocytic hypochromic anaemia evident by PID 3 was an outstanding feature in this experiment, regardless of how long the animal survived the infection. The acutely infected animals that received 1×10^8 trypanosomes had the severest anaemia which was progressive and non-relapsing. Paradoxically animals inoculated with 1×10^3 parasites showed a more severe anaemia than those monkeys inoculated with 5×10^5 trypanosomes. Intercurrent bacterial infections probably accelerated the course and outcome of the trypanosome infections. However, the animals that received 5×10^5 and 1×10^3 trypanosomes lived longer and were clinically less ill than those monkeys that received 1×10^8 parasites. Also partial but temporary remissions of the anaemia occurred after each parasitaemic wave in animals that vainly attempted to effect spontaneous recovery and lived longer than those animals that

received 1×10^8 trypanosomes. Of the animals infected with 5×10^5 parasites, one died on PID 26 while the second outlived the monkeys inoculated with 1×10^3 trypanosomes and died on PID 117. As a result of these data three patterns of infection emerged:

(1) Three vervets, two inoculated with 1×10^8 and one with 5×10^5 trypanosomes died of acute infection on PID 12, 15 and 26 respectively.

(2) One animal that received 5×10^5 parasites died of chronic mild infection on PID 117 and,

(3) Two monkeys that were infected with 1×10^3 trypanosomes died of chronic severe disease on PID 44 and 79.

DISCUSSION

This is probably the first detailed report describing the experimental infection of the vervet monkey by T. brucei. The results indicate and confirm that the vervet monkey was susceptible to the needle challenge with bloodstream forms of two strains and a clone of T. brucei. The disease produced varied from acute, subacute to chronic. Acute infection was associated with high non-relapsing progressive parasitaemias and brisk and relentless anaemia due to stock TREU 667, subacute infection with moderate plateau-like parasitaemias and slightly remitting but still persistent anaemia due to stock EATRO 2234 while the chronic form was associated with self-limiting parasitaemias and transient anaemia due to Clone 227.

The results also showed that the host either died or recovered spontaneously from T. brucei infection. Furthermore by varying the size of the challenge dose of the cloned parasite it was possible to influence the course of the infection and produce either acute fatal rhodesiense - like disease, sub-acute fatal or probably chronic fatal gambiense - like disease with corresponding severe, moderate or mild anaemia and clinical conditions.

Thus the vervet monkey like M. mulatta and the chimpanzee was readily susceptible to T. brucei but was unlike man who was refractory to T. brucei sub-group of trypanosomes.

The course of T. brucei in the vervet monkey was either acutely fatal, thus confirming the work of Ashcroft and others (17), like, T. brucei in the chimpanzee (27, 117); T. rhodesiense in M. mulatta (95, 326), vervet monkey (17, 68, 410), the chimpanzee (27, 117), and in man; or chronically fatal like

T. gambiense in man and T. congolense in the vervet (208). In the experiments reported here two of the vervets infected with T. brucei Clone 227 at 1 x 10^5 parasite dose recovered spontaneously as did the T. brucei - and T. rhodesiense - (410) infected monkeys. Thus T. brucei and T. rhodesiense infections in the vervet monkeys are probably similar in all ways but can also be fatal (17, 410). Spontaneous recovery was not reported in T. brucei, T. rhodesiense or T. gambiense infections of the chimpanzee but has been reported in human T. rhodesiense and T. gambiense (15).

The course of the T. brucei infections in the vervet monkey varied with the stock and the size of the inoculated parasite dose. For instance, the prepatency of the less virulent EATRO 2234 and of the lower parasite doses 5 x 10^5, 1 x 10^5 and 1 x 10^3 of Clone 227 were 3, 5, 2-7 and 5-7 days, respectively. Meanwhile, the most lethal T. brucei strain TREU 667 and the highest dose, 1 x 10^8 parasites, of the clone did not show prepatency in most monkeys for the parasite appeared in the blood almost immediately, after inoculation on PID 1. The prepatent periods of the lower parasite doses and of EATRO 2234 closely resembled those produced by cyclical transmission of T. rhodesiense in the vervet monkeys (17, 68) and of T. brucei, T. rhodesiense and T. gambiense in chimpanzees (117) which were 4-7, 4,8 and 9 days, respectively. They were also similar to those produced by needle challenge of M. mulatta with T. rhodesiense (95) and of the chimpanzees with T. brucei and T. rhodesiense (27) which were 4-8, 8 and 10 days, respectively. However, they contrast those of Ashcroft and others (17) and of Willett and Fairbairn (410) who,

respectively, reported prepatencies of 16 days in T. brucei - infected vervets and of 9-28 days in cyclically T. rhodesiense - infected monkeys.

Trypanosoma brucei (TREU 667 and EATRO 2234) at 1 x 10^5 and Clone 227 at a dose of 1 x 10^8 parasites did not produce the typical trypanosomal waves because the parasitaemia was non-relapsing. The animals died of either acute infection with high parasitaemia like M. mulatta infected with T. rhodesiense (95) or of subacute infection (EATRO 2234) like the chimpanzee infected with T. rhodesiense (117). Parasite doses of 5 x 10^5 and less of the Clone 227 produced in the vervet as in man the typical cyclic trypanosomal waves with periods of remission. Most animals died with high parasitaemias though a few died with low or even no parasitaemia just like the chimpanzee infected with T. brucei which had one trypanosome per 100 fields of blood, terminally (117). That, in African trypanosomiasis, animals die with either high or low parasitaemia is a well documented phenomenon.

The association between a high parasite dose on one hand and a short prepatency, high parasitaemia and short survival time on the other and vice versa would tend to confirm the observations of Baker (26) and also of Ashcroft (16) who noted that the smaller the dose of inoculated T. rhodesiense the longer was the life of the infected rats e.g. the mean survival times of the rats were 22.6, 23.6 and 37.2 days when inoculated with 7.5 x 10^6, and 1.5 x 10^6, and 3 x 10^5 trypanosomes, respectively. However, Baker (26) also noticed that smaller rats (50 gm) survived longer than heavier rats (75 gm) when given the same parasite dose.

The 5 x 10^5 parasite dose would seem to be the critical dose

in this study because at that dose it was possible to produce either an acute or a chronic disease, probably also depending on the health of the monkeys.

The parasitaemic patterns changed from cyclic waves to relentless and non-relapsing type when the animals became infected secondarily with Staphylococcus and Streptococcus, strongly suggesting that these intercurrent infections also influenced the course of T. brucei infections at 5×10^5 and 1×10^3 parasite doses. The effect of concurrent infections on other infections has also been reported in other model systems. For instance, the course of the self-limiting P. yoeli was changed by the intercurrent T. rhodesiense (75, 76) or by the self-limiting T. musculi (69). The chimpanzees that were infected with T. brucei and T. rhodesiense had intercurrent helminthiasis and bacterial lung infections (117) and it is possible that their survival times were further reduced by these secondary infections. Another possibility is that trypanosomiasis can unmask other latent diseases. For example, in the volunteers (100, 285) inoculated with T. rhodesiense malaria became patent.

Ideally the number of animals in each group should have been increased to prove or disprove these results statistically but limited funds and animal space did not allow purchase of more monkeys at the start of these experiments. All the same, the results suggest that the lives of the vervets were prolonged by lowering the challenge dose and that the animals with secondary infections reduced the survival times of T. brucei-infected monkeys.

Anaemia was evident in all monkeys inoculated with trypanosomes. It is a common and important feature of African trypanosomiasis of man and animals and it is well documented. In this study the anaemia was evident as early as PID 2 in all phases of infection and tended to be of rapid onset and progressively severe and persistent in animals that died of acute infection within 2-3 weeks. Such rapidly developing severe anaemia has also been reported in acute infections of mice and rats with T. brucei (30, 161, 256) and with T. vivax (370); of guinea-pigs with T. brucei, T. rhodesiense or T. gambiense (208) and; of cattle with T. congolense (208); or rabbits with T. brucei (208), of cats with T. congolense (208); of goats with T. congolense (136) or with T. vivax (389); of sheep with T. congolense (136, 208) or with T. brucei (208); of horses with T. vivax (208) or with T. brucei (208); of the chimpanzee with T. brucei (117); and of Macaca with T. rhodesiense (34, 419, 420).

In some cases the anaemia showed either total, partial or temporary remission, that is, it was transient, particularly in monkeys that aborted the infection or that died of subacute or chronic trypanosomiasis. Transient anaemia has also been reported in subacute and/or chronic infections of sheep and goats with T. congolense (136); of cattle with T. congolense (208) and with T. vivax .(80); and of the chimpanzee with T. gambiense (117). Also, the anaemia that tended to be more severe at the peak of a parasitaemic wave was normally followed by a slight and temporary remission 1-3 day(s) after the parasitaemic waves.

Anaemia of late onset was not observed in this study, thus contrasting the reports on late anaemia in trypanosomiasis of rats, mice, rabbits (207, 208), of dogs (208) with T. congolense; of cattle with T. brucei (208), T. congolense (104, 208) or T.vivax (208); and of the chimpanzee with T. gambiense (117).

The monkeys that died had PCV values of less than 30% with an average of 24.07 ± S.D. 3.3% and a range of 17.8 - 26.2%. Kramptz and others (194) reported that for cattle with PCV of less than 18% death could be predicted with certainty within days. Similarly most sheep and goats that died of T. congolense infections had PCV of less than 25% (136).

The severity of most infectious diseases is probably determined by the number of infective organisms that start the infection. In this study it has been shown that in the monkeys infected with T. brucei Clone 227 the size of the parasite dose inoculated influenced the prognosis of the resultant disease so that a high parasite dose produced a more rapidly severe disease than a low dose. Cyclically transmitted trypanosomiasis is produced when metacyclics are injected into a host by a tsetse-fly. The infection of the host depends on the infection rate of the fly, the number of fly bites and the number of metacyclics which varies between flies and between feeds of the same fly (100, 139, 140, 414). Fairbairn & Burtt (100) concluded that an average man required a dose of 300-450 metacyclics from G. morsitans to be infected by T. rhodesiense but there were men whose infective dose was higher (1067 metacyclics) or lower (170 metacyclics) and even less than 50 bloodstream forms could produce infection in man (24). The above figures are variable and possibly depend on

the virulence and the pathogenicity of the parasite as well as on other conditions.

One trypanosome of T. brucei, T. rhodesiense or T. congolense can infect a rodent in the laboratory and seven T. rhodesiense parasites produced an infection in rats with a prepatency of 7 days (100). In the case of monkeys and the chimpanzees the doses have ranged from 1×10^3 to 25×10^6 parasites of the brucei - sub-group trypanosomes; yet known infective tsetse, fed on a susceptible animal, sometimes have not produced infection (95, 100). One fly bite could infect a man or an animal (100) and as few as 1×10^3 T. brucei parasites would be sufficient to infect a monkey and produce a severe disease meaning that one or a few flies could produce trypanosomiasis. The 1×10^8 parasite dose was associated with the most severe disease in the monkey and this would be like many infected tsetse flies biting and each infecting a different stock of the same parasite or different species. The vervet monkeys are small animals and are therefore usually "taking cover" up the trees and are rarely bitten by tsetse flies except when they are down on the ground in search of food. It is conceivable that in natural conditions vervets may be inoculated with a small dose of antigenically similar trypanosomes while a tsetse is feeding. This may then lead to a spontaneous recovery of the host. This could possibly account for the few reports on natural T. brucei and T. rhodesiense infections in monkeys which spontaneously recover and become resistant to re-challenge or, the monkeys are just never re-challenged.

The phenomenon of self-cure is very interesting because it

operates despite the ability of the trypanosomes to undergo antigenic variation and the monkey, cattle and possibly even men are able to eliminate the brucei - sub-group trypanosomes following needle or cyclical inoculation. Thus apart from producing acute, moderate and chronic trypanosomiasis, self-cure can also be obtained in the laboratory by needle challenge of vervets by varying the size of the infecting dose of Clone 227. This is important because a tsetse bite contains between 10^2 and 10^5 infective metacyclics and the dose that produced spontaneous recovery in this study was 1×10^5 trypanosomes.

The fact that the vervet monkey was susceptible to T. brucei and that different doses of the cloned parasite produced either acute fatal rhodesiense - like disease, subacute fatal or probably chronic fatal gambiense-like disease with corresponding severe, moderate or mild anaemia and clinical conditions (the parasite-dose responses) were the basis for the experiments described in the next chapters, on standardization of the invitro correlates of cell mediated immunity and on the vervet monkey - T. brucei model itself.

CHAPTER FOUR

STANDARDIZATION OF THE *IN VITRO* CELL-MEDIATED IMMUNE CORRELATES OF THE VERVET MONKEY

INTRODUCTION AND THE REVIEW OF THE LITERATURE

Only a few workers (79, 105) have tried to delineate some of the vervet's haematological and immunological baseline indices. In this chapter are first reviewed the *in vitro* correlates of cell-mediated immune responses of animal and human lymphocytes to antigens, mitogens and to allogeneic and xenogeneic stimulator lymphocytes as probes. Subsequently described are the optimization of some of these lymphocyte responses for a normal uninfected vervet monkey.

1. Transformation of separated lymphocytes

Morphological transformation of small lymphocytes into large dividing blast-like cells occurs when known numbers are cultured *in vitro* exposed directly to known quantities of either specific antigens, non-specific mitogens, allogeneic or xenogeneic lymphocytes as stimulants (387, 394). The responses are measured as dividing cells rather than antibodies. Since re-exposure of the cell donor to the antigen is unnecessary the *in vitro* lymphocyte transformation technique has become an ideal clinical and research tool for assessing the cellular immunity of an individual.

Antigens of, or the products of, protozoa, bacteria, fungi, viruses, pollens, purified transplantation antigens, drugs, synthetic amino acids and other proteins, (260, 387, 394) including purified protein derivative (PPD, tuberculin), candidin, lepromin, measles virus, etc., are specific stimulants. They have been used to pre-sensitize specific antigen-sensitive lymphocytes of a host to produce specific cell clones which on re-exposure to

the same pre-sensitizing but not to a non-sensitizing material proliferate and sometimes even produce antibodies (387). Thus, lymphocyte transformation to in vitro antigenic stimulation is a secondary immune response. It is transferable by lymphocytes only.

Several mechanisms can probably act at the cell surface and initiate lymphocyte stimulation in vitro. Lymphocytes from primates, rodents and birds have proliferated in response to antigens (60, 61, 141, 184, 223, 231, 387), to antibody directed against cellular antigens and against lymphocyte-surface immunoglobulins (54, 387) and also to mitogens. Mitogens are substances that non-specifically induce mitosis and transformation in a wide (polyclonal) range of lymphocytes unlike antigens which activate only specific or restricted numbers of clones. Some mitogens are lectins, plant-derived proteins with specific carbohydrate moeities (sugars and oligosaccharides) (151). They probably bind to glycoprotein and glycolipid receptors, the external constituent hydrophilic portion of cell membranes (293). Certain lectins agglutinate cells of specific blood group(s) and bridge the lymphocytes (217); still lymphocyte binding and stimulation by lectins have sometimes been uncorrelated. However, because of their other properties lectins have been extensively used to study cell membrane structures and lymphocyte subpopulations, functions and stimulation (293).

In the experiments reported below, 3 non-specific mitogens phytohaemagglutinin (PHA), concanavalin A (Con A) and lipopolysacharides (LPS) were tested to stimulate the vervet monkey peripheral blood lymphocytes. The phytohaemagglutinins

are lectins extracted from the red kidney bean seeds of Phaseolus vulgaris or P. communis and when partially purified they yield protein-(PHA-P) or mucoprotein-rich (PHA-M) extracts which agglutinate certain erythrocytes by their binding affinity for N-acetyl-B-D-galactosamine residues. The soluble and insoluble PHA are strongly mitogenic for human and murine T and, for B lymphocytes, respectively. It was the use of PHA in blood cultures which first revealed the differentiation and division potentials of lymphocytes (283, 387, 394).

Concanavalin A (Con A), a tetrameric polyvalent ligand and lectin from the jack bean (Canavalia ensiformis), has 4 binding sites for polysaccharides. It binds glycopyranosides, mannopyranosides and fructofuranosides, the common glycoprotein constituents of cell membrane to form clusters thereon and agglutinate cells. It is mitogenic for T lymphocytes (40).

Lipopolysaccharides (LPS) refer to the bacterial polysaccharides of O-antigen (endotoxin) of Gram-negative enterobacilli like E. Coli, Salmonella and Shigella species and, probably Haemophilus and Bordetella pertussis (151). Basically they differ antigenically and structurally in their specific polysaccharides which link to the phospholipid,'lipid A', of the O-antigen but not their physiological effects like shock production, initial leucopenia, late leucocytosis, pyrexia (hence called pyrogens), general and local Shwartzman reactions, potent lipid A-associated adjuvant activity as in triple vaccine and B-lymphocyte mitogenicity.

Other common lectins include soybean agglutinin (from Glycine max), lentil (Lens esculenta), castor bean (Ricinus

communis), potato lectin (Solanum tuberosum), peanut (Arachis hypogoed), wheat germ agglutinin (Triticum vulgaris), pokeweed mitogen (PWW; Phytolacca americana), Helix pomatia agglutinin and Ulex uropeans, all with lectin-specific or cross-reacting (shared) sugar specificity. Some are mitogenic for either B, T or both lymphocytes (151). B lymphocyte polyclonal activators have a high molecular weight and repeating antigenic determinants on a solid matrix. Many are thymus-independent antigens including the LPS, polymerized flagellin, dextrans, levan, polyvinyl-pyrollidone, type III pneumococcal polysaccharide etc. However, some like dextran sulphate and pentosan sulphate have low molecular weight derivatives with a strong net charge.

Concanavalin A and/or PHA stimulate DNA synthesis in rodent and primate T lymphocytes (40, 53, 81, 98, 126, 141, 172, 184, 217, 223, 224, 231, 355, 378, 387) while LPS stimulates murine and bovine but probably also enriched human B lymphocytes (217, 293, 317). Thus Con A, PHA and LPS, each, possibly stimulate into blast mainly one type of small lymphocytes (387), unlike PWM a mitogenic lectin for both T and B lymphocytes (45, 90, 387). However, unlike antigens, they all stimulate, without pre-sensitization, and activate most blood, lymph node and spleen lymphocytes (387).

2. Mixed lymphocyte cultures

Blast-like cells are also formed when leucocytes from two genetically different (allogeneic or xenogeneic) mammals, birds or amphibians are mixed and co-cultured (mixed lymphocyte culture, MLC) in vitro and hence the term mixed leucocyte reaction (MLR) (22, 25, 97, 263, 387).

Mixed lymphocyte cultures (MLC) basically define histocompatibility antigens; the mixed lymphocyte (proliferative) reaction (MLR) and the generation of cytotoxic lymphocytes (cell-mediated lympholysis, CML) are two such assays used for detecting cell surface or lymphocyte defined (LD) antigens.

The mixed lymphocyte reaction (MLR) is an in vitro assay for cell-mediated reactivity to cellular antigens and it measures the degree of proliferation of the stimulated cell population usually by the incorporation of a radiolabel into their DNA.

In the cell-mediated lympholysis (CML) test, the effector cells are derived from several days' old MLC. The ^{51}Cr-labelled target cells which are either transformed or tumour cells carry histocompatibility antigens. The target cells are lysed by the available effector cells and the released isotope is assayed.

Neither the MLR nor the CML reactions are really good in vitro models for graft reactions, nor do they accurately predict allograft survival. The number of blasts directly relates to the degree of histo-incompatibility of the two cell donors (387). Sequentially the events in lymphocyte transformation due to MLC and mitogens resemble (387).

The MLR has been measured either directly by estimating in the cultures the fractions of blasts and/or dividing lymphocytes (22, 25, 263) or indirectly by assaying the uptake, by the cultured cells, of radioactive amino acids (^{3}H-leucine) (263) or DNA precursors like ^{3}H-methyl thymidine (^{3}H-TdR) (22, 23, 263), ^{14}C-methyl thymidine (^{14}C-TdR), ^{125}Iodo-

deoxyuridine (^{125}IUdR) (97, 263) into either acid precipitable material or single cells by autoradiography (25, 263)

Various culture systems, media and lymphocyte sources, preparations and numbers and harvesting techniques have been used (22, 25, 31, 33, 263, 387), depending on the animal species and the test. When untreated genetically dissimilar leucocytes are co-cultured both populations respond giving a bi-directional (two-way) reaction (22, 25, 263, 387) which complicates the interpretation of the results. Therefore MLR are normally made unidirectional (one-way) either by blocking irreversibly DNA synthesis of stimulator lymphocytes (stimulators) with either mitomycin C-treatment (23, 263) or by mixing parental ('responders') with F1 ('stimulators') lymphocytes. The basis of this is that MLR is genetically controlled by loci closely associated with the human leucocyte antigen (HLA) systems, obeys the transplantation laws and is the afferent (recognitive and proliferative) arm of an immune response to the major histocompatibility antigens (MHA) on the stimulator cells (263, 387).

The proliferative MLR of the MLC has several characteristics some of which occur regardless of the mechanism of lymphocyte stimulation.

Lymphocyte proliferation in MLC is essentially a specific and genetically determined (25) primary immune response due to histo-incompatibility.

Greater MLC responses in mice are associated with strain differences at the strong H-2 locus than at the weak non-H-2 locus (387) though strains that differ at 12 or more weak loci

give a positive MLR (312).

Similarly, the human MLR is not triggered off by differences at the loci governing the serologically defined (SD) antigens but by those closely linked to the HLA genes that govern the LD products (antigens), as evidenced by recombinant human family studies (263, 387). A single locus with 30 or more alleles possibly controls the MLR test. The MLR however, cross-reacts with serological typing (387). The MLC-identical siblings also type identically with most antisera detecting the HLA antigens. Yet, in the unidirectional MLC stimulation still occurs between siblings that differ at one or two HLA chromosomes and not between identical siblings (387). The stimulation is twice when siblings differ at 2 HLA chromosomes than at one (312) although some HLA antigen differences stimulate more than others. However, antisera which recognize determinants in the cell-mediated reactions, including some which inhibit the MLR exist. Consequently, the distinction between the two, SD and LD, antigen classes in breaking down.

In the MLC, lymphocytes also respond specifically to xenogeneic cells (234, 235, 263, 344) and against the MHC LD antigen of the sensitizing species (234, 235). Human LD antigens, on target cells, which are recognized by lymphocytes from specifically immunized hosts rather than antibodies against the SD determinants are also present on xenogeneic cells (234, 235). Thus the MLR is a specific response of T lymphocytes to allogeneic and xenogeneic MLC-controlled LD antigens (235).

Remarkable primary structure homologues of MHC-controlled SD antigens conserved in evolution exist between humans and

mice (387). However, the primed lymphocyte typing (PLT), a test also specific for LD antigens of the sensitizing species, has revealed in xenogeneic combinations, extensive sharing of LD antigens between humans and dogs and humans and cattle but none between humans and mice (234, 235). Yet, even in rhesus monkeys the shared specificities are probably not the only means of recognition because on the xenogeneic cells extra LD determinants not shared with humans exist and have been recognized in a primary xenogeneic MLC (235). Also the significant xenogeneic MLC reaction between human and mouse lymphocytes has suggested specific recognition by human lymphocytes of mouse H-2 LD antigens. This trongly indicates that xenogeneic antigens are recognized in MLC even when they are not cross-reactive with human LD antigens. Thus many initially responsive cells in MLC also include specific cells recognizing allogeneic and non-cross-reactive xenogeneic LD antigens (235).

Only about 2% lymphocytes in the MLC, tuberculin and in initial graft-versus-host reactions are recruited into the reactions because the responding cells are derived from few precursors of specific target-cell (antigen) sensitive clones (263, 387). Although pre-sensitization with cross-reacting antigens occurs generally and accelerated graft rejection follows experimental immunization with some bacteria in man the MLC like the graft-versus-host reaction is essentially a primary, without inadvent pre-immunization, and probably not a secondary immune response (387) like the tuberculin reaction. The evidence for a secondary immune reaction is weak and is against the selection theory (55) for too many antigens are involved and cannot explain

the strong MLR and concurrent weak responses to non-histocompatibility antigens by cells of normal non-sensitized donors.

The generation time for human lymphocytes was 18-21 hours while cell division occurred after 42 hours of culture with PPD. However, it is unclear if new cells proliferated for the first time late in reaction as in the MLC of rats (387). Thus lymphocyte stimulation is probably proportional but not equal to the cell numbers per culture initially responding to the antigen. This is because of sequential entry of new cells into the responses following stimulation and proliferation of the few cells which initially react to the stimulant (387).

Lymphocytes initially not responding to the stimulants are recruited into proliferation through mediation of a soluble mitogenic factor (94) which makes even the non-sensitive cells incubated with it respond to additional antigen doses. In MLC the factor is probably a transplantation antigen. Yet, strangely in the rat MLC involving many cells recruitment is insignificant (387).

During antigenic and MLC stimulation lymphocyte proliferation <u>in vitro</u> requires contact between purified lymphocytes and/or autologous non-sensitized macrophage-like adherent cells (387) and a high lymphocyte density on a small area. This probably provides the non-specific cell-cell surface contact phenomena needed for initiating the immunological responses to antigens (387) through macrophages. These cells process and then present the immunogenic antigen on their surfaces (384) to the antigen sensitive lymphocytes in contact. For a little

undegraded antigen on the macrophage surface is probably more effective in stimulating specific antigen-sensitive lymphocytes than a large amount of free antigen in vitro (387). Similarly in the MLC the allogeneic or xenogeneic macrophages probably initiate proliferation and increase the stimulating capacity of the cell surface histocompatibility antigens (387).

Human cord blood unlike adult MLR produces slightly larger blast transformation (387). Also neonatal rat thymocytes (172, 263) but not lymphocytes from neonatally thymectomized animals (387) respond to allogeneic MLC.

In allogeneic MLC the T lymphocyte 'responders' involved are stimulated by B lymphocytes ('stimulators') (263, 387). Probably the situation is the same in xenogeneic MLC.

Lymphocyte stimulation and proliferation also generate killer cells (109) specific for the target SD antigens in cell mediated lympholysis (CML) of MLC (263).

Biochemical studies of membrane sites for various activators and kinetic and inhibition studies of early events indicate that activation occurs at the lymphocyte membrane (394). Physiologically and biochemically the quiescent small lymphocyte is derepressed to become a metabolically active dividing cell.

The rapid (within 1-2 hours of activation) cytochemical events in the PHA-transformed and proliferating lymphocytes involve increased cyclic-AMP and adenylcyclase, membrane phosphorylation, acetylation of histones, net RNA synthesis (especially ribosomal RNA) and its rapid migration out of the nucleus, lysosomal permeability and enzyme movement into the cell sap, protein synthesis, incorporation of thymidine into

DNA, glycogen levels, and DNA synthesis (89, 387).

The MLC has been usefully employed in transplantation immunity to measure genetic disparity between 2 individuals with regard to histocompatibility antigens of man and/or inbred strains of animals as well as in typing leucocytes from an HLA serologically identical recipient and a prospective donor. It is an aspect of *in vitro* allogeneic recognition by lymphocytes (22, 25, 263, 387) and a partial *in vitro* analogue of a homograft rejection because in the triggered MLR are generated effector killer (109) cells that specifically recognize and lyse target (SD) cells in CML (263) sharing MHA with stimulator cells. The resultant cellular differentiation, help correlate, assess and predict, though not very accurately, the prognosis of an organ graft, its survival and rejection, and also the outcome of tolerance induction to transplantation antigens (312, 387).

Lymphocyte transformation in response to antigenic stimulation, *in vitro*, correlates well with positive delayed hypersensitivity (DH) reaction in the cell donor but not with positive antibody response. For instance, it is initiated by proteins but not by polysaccharides while together with DH unlike antibody production, is transferable to previously non-sensitized recipients by the transfer factor. It occurs with lymphocytes from donors with or without positive DH skin test and from animals immunized by methods that do not induce the classical DH reaction. These methods include giving the antigens intravenously, in saline, in complete Freund's adjuvant (CFA) or with alum precipitated diphtheria toxoid associated with immediate hypersensitivity reactions to, such as, penicillin

and pollen, re-stimulated by these pre-sensitizing antigens (387).

Lymphocyte proliferation and carrier-protein specificity have been correlated through the interaction of an antigen with the antigen-sensitive lymphocyte that transforms *in vitro*, and through the stimulation of the secondary antibody response and elicitation of the DH skin test *in vivo*. Carrier specificity is important in DH because *in vitro* lymphocytes proliferate when re-stimulated only by the pre-sensitizing hapten-protein conjugate originally given intravenously or in CFA. However, a hapten conjugated to a different carrier protein induces *in vitro* a vigorous cell proliferation associated with secondary antibody responses detectable by a hapten coupled to either the new or the pre-sensitizing carrier-protein. This is because the antibody specificity is normally directed against the hapten (37, 387).

Only compounds containing 7 (heptamers) or more L-lysines (e.g. in DNP-polysine conjugate system) are immunogenic, elicit delayed hypersensitivity skin reaction *in vivo* and stimulate lymphocyte proliferation *in vitro* while the hapten coupled to smaller oligolysine chains or different proteins react with the antibody (387).

Various sensitizing techniques, the strength of, and the time between immunization and the *in vitro* experiments possibly account for the different transformation results. For instance, spleen lymphocytes transform in the absence of DH in the cell donor whereas blood and lymph node lymphocyte transformation correlates well with the positive delayed hypersensitivity skin test in the cell donor. It is possible that more immunoglobulin-

bearing (B) lymphocytes are contained in the spleen than in the blood while species differences are possibly responsible for different lymphocyte numbers in the humoral and cell-mediated immunities. However, transformation of lymphocytes from donors with negative delayed hypersensitivity skin reactions strongly suggests a greater sensitivity of the *in vitro* test which possibly also correlates numerically with lymphocytes, *in vivo*, that can specifically be induced into proliferation by an antigen (387).

3. Whole blood cultures

The lymphocyte or cell-rich plasma cultures dominated *in vitro* lymphocyte culture. The separation techniques were tedious until whole blood cultures were described for mammals, man and monkeys (98, 184, 217). Mitogen and antigen stimulation of whole blood micro-cultures as a T lymphocyte functional test has been developed and perfected by diluting heparinized whole blood ten-fold and stimulating 0.20ml cultures. The test which can be performed daily on infants or small animals requires minute blood volumes and fewer procedures unlike the laborious and time-consuming cultures of separated lymphocytes (98, 141, 184, 217). Yet, in man and marmosets the results of both whole blood and separated lymphocyte cultures have been comparable in extent, composition and reproducibility (184).

Cells survive, transform and differentiate in both PHA-stimulated whole blood and purified lymphocyte cultures (184, 217, 378, 387, 424). However, in the former cultures a better and prolonged lymphocyte growth, maximal DNA metabolism and high blast cell counts occur (98, 217).

Reproducible results would ideally need whole blood cultures of constant lymphocyte numbers calculated from total and differential leucocyte counts. Yet, whole blood micro-cultures normally depend on fixed blood volumes whose adjustment to fixed T-cell numbers is impracticable because serum concentration changes modify the optimal mitogen dose and excess RBC slightly inhibit the lymphocyte response (217).

Numerous factors including various immunodepressive conditions, autologous serum factors and other cells numerically and selectively influence stimulation ratios and absolute counts which concurrently increase with mitogen responsive and non-responsive T cell subsets in cultures (98, 217).

Whole blood and separated lymphocyte cultures differ because of the numerous erythrocytes and granulocytes in the former cultures. These cells probably influence mitogen-lymphocyte stimulation in separated lymphocyte cultures because optimal facilitation by erythrocytes, granulocytes and non-T mononuclear cells occur at physiological blood levels (184, 217, 387) and sometimes inhibit lymphocyte responses to PHA and other T-cell stimulants (217). In whole blood cultures possibly erythrocytes and non-T mononuclear cells also markedly facilitate PHA responses partly through monocytes. In contrast isologous erythrocytes and serum factors possibly potentiate by providing a suitable micro-environment for optimal lymphocyte survival, free movement, transformation and proliferation such as, on the surface of the erythrocyte pellet (217).

Other factors including optimal culture conditions and soluble products from monocytes and serum factors through

induction also facilitate cells to proliferate fully. The adherent peritoneal cells release potentiating factors because their number is too low to possibly potentiate through direct contact with the stimulated T cells (217).

The PHA-stimulated whole blood cultures labelled with ^{125}IUdR, conveniently assayed DNA synthesis in the lymphocytes and produced stimulation ratios identical to those labelled with thymidine (98), while the FUdR augmented the radiolabel incorporation. The cultures were radiolabelled between 48-64 hours and the incorporated label increased directly with the numerical T-cell increase although ^{125}IUdR is a metabolic inhibitor. The test could be performed routinely during regular working hours while the detection of ^{125}IUdR incorporation by gamma-counters was an extra advantage.

That few variables exist in the whole blood culture test has helped define the normal range of mitogen (e.g. PHA)-lymphocyte responsiveness and has enabled direct comparisons of results from different laboratories when expressed as the ratio of counts per minute (cpm)/T cell in whole blood (217). Thus in spite of the complex cellular events the whole blood test reflects T lymphocyte functional potentials when lymphocytes are stimulated by cell surface ligands binding under conditions appropriate and similar to those for antigen presentation. Powerful mitogens like L-PHA can stimulate even under sub-optimal conditions. For instance, single lymphocytes transformed and proliferated (217), but were stimulated further as the cell densities optimized (171, 217, 378). The less powerful H-PHA, PWM and Con A mitogens possibly depend upon secondary

facilitation (217). Yet, L-PHA unlike H-PHA binds mainly to RBC.

In summary, since Nowell (283) showed and others confirmed that phytohaemagglutinin (PHA) could produce large dividing blast-like cells from the small lymphocytes in human peripheral blood cultures, other activators (lymphocyte stimulators, mitogens) have also been employed.

Lymphocyte mixtures from 2 genetically non-identical individuals can also stimulate each other in vitro (the MLR) and the genetic and immunological analysis of this phenomen has made the MLC a measure of the histocompatibility between persons and an important tool for studying immunobiology and genetics of tissue transplantion.

Lymphocyte activators are either specific or non-specific. Some (e.g. PHA) stimulate a sizeable proportion of the lymphocytes of all normal individuals. They induce detectable biochemical and physiological changes quite soon, within 1 hour, after addition to lymphocytes. However, specific activators (antigens, such as, PPD) stimulate in vitro lymphocytes from only sensitized individuals showing skin hypersensitivity. Stimulation by lymphocytes (the allogeneic or xenogeneic MLC) while possessing many qualities of specific activation does not require presensitization of the donor and is therefore not readily categorized as above. Lymphocytes morphologically showing stimulation by specific activators are proportionally few and detectable changes in these cultures occur about 3 days after those non-specifically stimulated.

The biochemical and physiological changes induced by

specific and non-specific activators are qualitatively similar. However, the time and degree of response quantitatively differ and are relative to the proportions of cells in culture initially responding to the activator. Small lymphocytes are functionally heterogeneous and their responsiveness to activators may be restricted to either of the functionally defined cell, such as, T or B lymphocyte which responds differently to various activators.

In stimulated lymphocytes morphological changes, induction of DNA synthesis and increased RNA and protein synthesis occur. Biochemical changes all occurring within the first hour or two of activation, are followed by derepression of eukaryote genomes. The interactions of activators with the lymphocyte membrane are complex and poorly understood. The degree of reaction of cultures varies with the area of culture surface and with both the concentration of activator and the ratio of activator molecules to cells. The time of peak or detectable response varies with the cell type and also with the activator. Thus the establishment of optimal conditions for an assay while considering these variables is necessarily empirical.

The assays of the in vitro responses are based on the morphological and macromolecular synthesis changes in stimulated lymphocytes. The mouse lymphocyte culture techniques have provided extensive information on the genetics of the H-2 (histocompatibility) locus in inbred and genetically well defined strains.

Several microassays which can determine responses with less than 10^6 lymphocytes per culture exist. Measurements of

of the rate of DNA synthesis is the basis for most assay systems used in quantitating lymphocyte activators.

The measured event is both temporally and physiologically far removed from the initial activation event of the initially responsive cultured lymphocytes when the macro-molecular synthesis rate is to measure the degree of activation. Activated lymphocytes produce factors which potentially modify the response and obscure quantitatively the initial response. In the more than 2 days' old cultures cell division and death also complicate the analysis of the responses. An ideal assay, therefore, should be practical, rapid, precise and based on an early irreversible step in the activation process to estimate better the number of cells responding to an activator and their degree of activity.

Lymphocyte stimulation is clinically and experimentally a cell-mediated immunity parameter. Lymphocyte activation is either by isolating cells and analysing their responses under defined conditions such as, in synthetic serum-free media or as in the more practicable mitogenic (PHA) stimulation and functional assessment of T cells under optimal conditions in the early exponential phase when responses are probably limited only by the performance of stimulated lymphocytes in whole blood cultures.

The in vitro tests alone are ideal for screening purposes but other concurrent techniques like skin testing using antigen (e.g. PPD) or sensitizing reagents (e.g. DNCB) are recommended to correlate cell-mediated immunity.

Since the aim of this thesis was to set up an

immunological model of T. brucei in the vervet monkey it was first imperative to standardize the cellular immunological baseline data of a normal uninfected animal in order to clearly and effectively interpret the results from an infected animal. The experiments described below were designed to standardize some of the in vitro cell-mediated immunity correlates of the vervet monkey using non-specific mitogens in separated lymphocyte and whole blood cultures.

MATERIALS AND METHODS

(i) Source of lymphocytes

(a) Responder lymphocytes

In each experiment, the peripheral blood from 5 adult male vervets provided the responder lymphocytes.

(b) Stimulator lymphocytes

The peripheral blood lymphocytes (PBL), lymph node (LNC) and spleen (SPC) cells from a male and the PBL from a female vervet (Cercopithecus aethiops) monkeys provided allogeneic stimulator cells while the PBL from the male Colobus (Colobus abyssinicus) and Sykes' (Cercopithecus mitis) monkeys and from human as well as the CLA_4 cells were the xenogeneic stimulator lymphocytes.

(ii) Culture conditions

The detailed materials and their sources; the methods and preparations of lymphocytes and whole blood for culture; and the general culture conditions are described under the "General Materials and Methods".

RESULTS

(i) Separation and viability of peripheral blood lymphocytes

Throughout these studies about 1-3 x 10^6 viable lymphocytes/ml of whole blood were obtained routinely on separation. By phase contrast microscopy more than 90% of the lymphocytes recovered at the plasma-Ficoll-Triosil interface were viable although the yield varied between monkeys and even from the same animal on different days. Clumping was minimal with monkey lymphocytes and routine use of heparin in the media was not essential.

(ii) Medium and foetal calf serum batch and concentration

Media and FCS batches were screened for their ability to support PHA stimulation of lymphocytes. Foetal calf serum (FCS) Batch No. 292459 gave high stimulation and low background (control, unstimulated) dpm values (responses, cell turn over) and was subsequently employed in this study.

Lymphocytes at 2 x 10^6/ml were cultured in either Dulbecco's Eagles (DMEM), RPMI-1640 or M 199 medium each containing either 5, 10 or 20% FCS, incubated for 72 hours and stimulated with Con A or PHA. The cells were pulsed with ^{14}C-TdR for 18 hours terminally before harvesting.

When the RPMI-1640 medium was supplemented with 5, 10 or 20% FCS in lymphocyte cultures stimulated with 10 ug/ml of Con A statistically identical dpm values (Log_{10} transformed) were obtained. The highest absolute dpm values and stimulation indices were associated with 5% and 10% FCS, respectively, in RPMI-1640 and 10 ug/ml of Con A. In contrast, with 25 ug/ml of Con A, the highest stimulation indices were obtained with 20%

FCS in RPMI-1640 probably due to the lowest background dpm values. Significant differences occurred between 5 and 10% ($P<0.05$) and between 5 and 20% ($P<0.001$) but not between 10 and 20% FCS (Fig. 4.i, Table 4.1)

Lymphocytes cultured in DMEM with 10% FCS produced the highest responses (dpm values) and SI with 10 µg/ml of Con A. These responses statistically resembled those obtained with 5 or 20% FCS. However, with Con A at 25 µg/ml the highest responses and SI were also associated with 20% FCS. These responses only slightly and statistically ($P<0.01$) exceeded those obtained with 10 and 5% FCS, respectively, (Fig. 4.i, Table 4.2).

When lymphocytes were cultured in M 199 medium supplemented with 5, 10 or 20% FCS and stimulated with 10 or 25 µg/ml of Con A the highest stimulation ratios were obtained with 5% FCS and Con A at 10 µg/ml. However, statistically the responses due to these FCS and/or Con A concentrations were identical (Table 4.3).

The highest to the lowest stimulated absolute dpm values though statistically identical were obtained with lymphocytes cultured in DMEM, RPMI-1640 and M 199 media, respectively.

The mean SI from PHA lymphocyte cultures were highest and most consistent when 5, 10 or 20% FCS supplemented DMEM, RPMI-1640 and M 199 media, respectively, down in that order. The highest SI and most consistent responses (dpm) were obtained with lymphocyte cultures in DMEM supplemented with 5 or 10% FCS and stimulated with PHA at 10 and 15 µg/ml, respectively, (Fig. 4.ii, Tables 4.4, 4.5, 4.6).

Thus the 5, 10 or 20% FCS concentrations were identical in supporting cell stimulations within each medium (intra-media comparisons) but they significantly differed between the 3 media (inter-media comparisons). The corresponding responses associated with the three FCS concentrations in DMEM and RPMI-1640 media were almost similar. However, they were statistically different ($P<0.05$ to $P<0.001$) from those in M 199 medium. Since culture responses fluctuated widely with FCS changes and mitogen concentrations in M 199 medium but were fairly reproducible in DMEM and RPMI-1640 media, DMEM supplemented with 10% FCS was selected for future work. Furthermore, lymphocytes in RPMI-1640 and M 199 media produced the highest SI when the highest FCS (20%) and mitogen (25 µg/ml of Con A) concentrations were employed. Yet, the same SI were obtained with slightly lower FCS (10%) and Con A (10 µg/ml) concentrations in DMEM.

(iii) Mitogen stimulation of separated lymphocytes

(a) Lymphocyte concentration

This experiment was designed to determine the optimal peripheral blood lymphocyte concentration for stimulation with the various mitogens. The results are shown (Figs. 4.iii, 4.iv, 4.v; Table 4.7, 4.8, 4.9).

The maximum SI and the ranges for PBL of the individual animals show the potency of the mitogens used. The highest to the lowest absolute dpm values were obtained with lymphocytes at, respectively, 4-, 2-, and 1 x 10^6/ml with both Con A and PHA. However, because the unstimulated dpm values from 4 x 10^6 cells/ml were also the highest the calculated SI were lower than

those due to cultures at 2 x 10^6 cells/ml which, therefore, had the highest SI. Significant differences occurred between responses due to 1- and 4 x 10^6 lymphocytes per ml at background and at peak mitogen values ($P<0.001$) but not between 2- and 4 x 10^6 cells/ml (Tables 4.7 and 4.8)

The maximum to the minimum stimulated absolute dpm values with LPS were obtained with 4-, 6-, 5-, 2-, 1-, and 8 x 10^6 lymphocytes per ml, respectively, while the highest unstimulated values were recorded with 6 x 10^6 lymphocytes/ml (Fig. 4.v; Table 4.9).

At all the cell concentrations tested PHA and Con A peaked at 15 and 10 µg/ml, respectively. However, the magnitude of stimulations with all the 3 mitogens used varied widely. Concanavalin A and PHA in microcultures produced high SI within a narrow and broad range of mitogen concentrations, respectively. Lipopolysaccharides did not stimulate monkey PBL adequately over a wide range of concentrations. The degree of PBL stimulation with all mitogens varied between animals although the highest was uniformly achieved with almost the same mitogen dose and cell concentration.

The lymphocyte concentration of 2 x 10^6/ml was the optimum for both Con A and PHA and because, responses due to it statistically resembled those due to 4 x 10^6 lymphocytes/ml it was therefore adopted for future work.

(b) Mitogen dose-time response curves

The optimal mitogen concentrations and harvesting time were determined by culturing 2 x 10^6 lymphocytes/ml in DMEM supplemented with 10% FCS and stimulated with various Con A,

PHA and LPS doses for 24 - 192 hours. The cultures were then pulsed with ^{14}C-TdR for 18 hours, terminally.

The typical dose-response curves for the 3 mitogens are shown (Figs. 4.vi, 4.vii, 4.viii; Tables 4.10, 4.11, 4.12). The optimal value for Con A and PHA were 10 and 15 µ/ml, respectively,(Table 4.10, 4,11). Cultures containing LPS, again, gave weak responses even at 192 hours (Figs. 4.viii; Table 4.12). The lipopolysaccharides was, therefore, abandoned in subsequent experiments.

The lymphocyte responses to Con A and PHA at 72, 96 and 120 hours were significantly ($P<0.05$) greater than those at 48 hours of culture although the responses were statistically identical between those times (72 and 96; 72 and 120; 96 and 120 hours). In both Con A- and PHA- cultures the highest stimulated and back-ground dpm were at 96 and 120 hours, respectively. Thus the dpm values increased slowly with prolonged culture time so that the highest to the lowest stimulation indices for Con A and PHA occurred at 72, 96, 120 and 48 hours, respectively. Since the absolute dpm responses were statistically identical for 72, 96 and 120 hours it was decided to adopt 72 hours as the harvesting or culture time to exclude, the possibility of secondary facilitation by activated T cells. Thus the dose-response curves for PHA and Con A sharply peaked whereas LPS produced broad dose-response curves (Figs. 4.vi; 4, vii; 4. viii). The optimal doses of mitogens per culture of 200 µl were 2 and 3 µg of Con A and PHA (i.e. 10 and 15 µg/ml for Con A and PHA), respectively.

(c) Pulse time

In this experiment all cultures were exposed to the same culture conditions. Only that the radiolabel pulse times were varied (Figs. 4.ix, 4.x).

Lymphocytes at 2 x 10^6/ml were cultured with Con A and PHA for a total of 72 hours but were pulsed with ^{14}C-TdR for 48, 24, 16, 8 or 4 hours, terminally. The stimulation indices were highest when Con A cultures were pulsed for 4 hours (due to the lowest background dpm values) and for 16 hours for PHA (Tables 4.13, 4.14). There was an approximately linear increase in ^{14}C-TdR incorporation up to 16 (PHA) and 24 (Con A) hours. Thereafter the rate of radiolabel incorporation plateaued. However, it was decided to adopt 16 hours as the pulse time as this point fell within the linear upward part of the curve and was logistically more acceptable.

(iv) Mixed lymphocyte cultures (MLC)

(a) Cell concentration

Unidirectional MLC between vervet monkey responder lymphocytes and mitogen C-treated allogeneic or xenogeneic stimulator cells were cultured for 168 hours but pulsed with ^{14}C-TdR for 16 hours terminally before harvesting. The optimal cell concentrations were 2 - 3 x 10^6/ml (4 - 6 x 10^5/culture) for the responder lymphocytes and 2 - 3.2 x 10^6/ml (4 - 6.4 x 10^5 cells culture) for the inactivated allogeneic and xenogeneic stimulator lymphocytes (Fig. 4.xi; Tables 4.15, 4.16). Although the highest absolute dpm values were obtained with xenogeneic MLC when equal (at 4 x 10^6 cells/ml) responders and stimulators were used the difference was not significant.

Xenogeneic MLC of equal responders and stimulators at 3- unlike at 2 x 10^6 cells/ml gave consistently well stimulated and reproducible lymphocyte responses. Hence, the former cell mixtures and concentrations were adopted in subsequent experiments. Xenogeneic MLC of 2 x 10^6 responders/ml and 3.2 x 10^6 stimulators/ml also gave high stimulation indices.

(b) Comparison of MLC with lymph node and spleen cells and PBL as stimulators

The cells from lymph nodes (inguinal and axillary), peripheral blood and the spleen of a vervet monkey, PBL from a Colobus and a Sykes' monkey, each, and CLA_4 cells were inactivated with mitomycin C, and at various cell concentrations were the stimulators in the MLC.

The responses were poor with all these stimulators except with CLA_4 cells (Fig. 4.xii; Tables 4.17, 4.18). However, 2- and 3 x 10^6 stimulators/ml from the Sykes' monkey (Cercopithecus mitis) PBL (semi-xenogeneic lymphocytes) elicited the second highest (after CLA_4 cells) dpm and stimulation indices followed by the allogeneic (vervet monkey), spleen cells, PBL, xenogeneic (Colobus) PBL and allogeneic (vervet) lymph node cells, down in that order. Thus xenogeneic CLA_4 and allogeneic cells were apparently the best and poorest (weakest) stimulators, respectively, of vervet monkey responder lymphocytes in the MLC (Tables 4.17; 4.18).

Unstimulated dpm values for the vervet but not for the CLA_4 stimulator cells increased directly with the cell concentrations. The CLA_4 cells as stimulators were, therefore, adopted for future work.

The responses due to allogeneic stimulators from male and female vervets were statistically identical while the stimulation indices were also reasonably high with human (female) xenogeneic stimulators at 3 x 10^6 lymphocytes per/ml (Tables 4.17, 4.18).

(c) The effect of harvesting (culture) times on MLC.

When CLA_4 cells were the stimulators in the MLC for 72 - 216 hours but pulsed with ^{14}C-TdR 16 hours terminally the highest to the lowest stimulation indices were obtained at 168, 120, 72 and 216 hours, respectively, (Fig. 4.xiii; Tables 4.19, 4.20, 4.21) and the responses (dpm) at 120, 168, 72 and 216 hours, respectively. The optimal harvesting (culture) time was therefore taken as 168 hours.

(V) Whole blood cultures

The effects on whole blood cultures of diluting the blood, of mitogens and of varying the culture times were also observed.

Peripheral whole blood from each vervet was diluted five-, ten-, and twenty-fold in DMEM (without FCS), stimulated with various concentrations of Con A, PHA, and LPS for culture times of between 48 and 144 hours and labelled with 0.02 µCi of ^{14}C-TdR in 20 µl (0.02 ml) of L-15 medium in a 16-hour pulse, terminally.

The responses of whole blood cultures resembled those from separated lymphocytes (Figs. 4.xiv, 4.xv, 4.xvi, 4.xvii, 4.xviii).

Moderate stimulation obtained with the 3 blood dilutions was first noticeable at 0.5 µg/ml and peaked between 10 and 50 µg/ml of either Con A or PHA depending on the blood dilution

(Figs. 4.xiv, 4.xv; Tables 4.22, 4.23). Although the 3 blood dilutions were statistically identical the highest dilution (1/20) was more markedly inhibited by increasing the mitogen concentrations of Con A and especially of PHA. The ten-fold dilution produced the most consistent results and was therefore, the optimum for both stimulants and was adopted for future work.

The PHA and Con A time curves were similar for the 1/10 blood dilution when cultured for 72 - 120 hours but prolonged culture times (96 or 120 hours) resulted in a rapidly declining lymphocyte activity. The peak responses (and stimulation indices) for Con A between 72 and 120 hours of culture were economically obtained with 25 µg/ml, (Fig. 4.xvi; Table 4.24). There were significant ($P<0.05$) differences between 48 and 72, 96 and 120 and between 72 and 120 hour cultures. However, the 72 and 96 or 96 and 120 hour cultures were statistically identical. Stimulation indices and dpm at optimal Con A concentration were highest at 120 hours. Therefore, the 96-hour point was taken as the optimal culture time for Con A at 25 µg/ml concentration.

The responses of PHA-stimulated and ten-fold diluted whole blood cultures peaked with 15 µg/ml at 48 and 120 hours and with 25 µg/ml at 72 and 96 hours with significant ($P<0.01$) differences between the responses at 48 and 72, 96 and 120 hours but not between these times (e.g. 72 and 96 hours). However, the stimulation indices peaked with PHA at 25 µg/ml and were highest to lowest at 72 hours 96, 120 and 48 hours, respectively (Fig. 4.xvii; Table 4.25). Seventy-two (72) hours would appear to be the best compromise harvesting time for whole blood

cultures stimulated with PHA at 25 µg/ml.

There were no significant responses to LPS in whole blood cultures (Fig. 4.xviii; Table 4.26).

In summary microculture conditions for vervet monkey lymphocytes stimulated by mitogens and MLC were determined.

The lymphocyte reactivity in vitro was evaluated as DNA synthesis. This was assayed after separated lymphocytes and those in diluted whole blood were stimulated in culture with PHA, Con A, LPS and/or with mitomycin C-treated normal allogeneic and xenogeneic cells in the MLC.

The ability of mitogens to stimulate monkey PBL under different experimental conditions showed that Con A and PHA strongly induced DNA synthesis, while LPS stimulated vervet monkey lymphocytes very weakly.

The lymphocyte concentration and the responder/stimulator cell ratio during the primary contact in a one-way MLC showed that a combination of equal volumes of responders and stimulators both at 3×10^6 per ml gave optimal reproducible responses when cultured for 168 hours. This concentration was therefore used in later experiments.

DISCUSSION

There seems to be no report on stimulation of responder vervet monkey peripheral blood lymphocytes by either mitogens, allogeneic or xenogeneic cells except by autologous and heterologous sera (54). Hence very little appears to be known about the vervet monkey lymphocytes and their immunological functions. Because of this it was decided to test and study the dynamics and extent of vervet monkey lymphocyte stimulation by Con A, PHA, LPS and allogeneic and xenogeneic cells. The conditions for stimulation by mitogens were optimized and compared in a micro-culture system. Several sets of results emerged from the study.

The vervet monkey can be added onto the list of mammals like the mouse, rhesus monkey, marmoset, baboon, cattle and man in which cell-mediated immunity can be assayed by *in vitro* micro-techniques using mitogens to stimulate cultures of separated lymphocytes and/or whole blood. It is also possible to use separated lymphocytes in xenogeneic MLC in the vervet monkey. Since in mice, cattle and man which are evolutionarily very diverse, PHA and Con A predominantly stimulate T cells while LPS activate B lymphocytes it is possible that these mitogens also stimulate proliferation in the same lymphocyte sub-populations in the vervet monkey.

Concanavalin A, PHA and LPS all stimulated DNA synthesis in the monkey PBL under conditions similar to those found optimal for stimulation of human lymphocytes. In this study PHA and Con A produced high levels of vervet monkey PBL activation while

LPS produced extremely low responses. These findings are similar to those observed in man (98, 317).

The relationship between cell concentrations and proliferation in response to mitogens in culture were influenced by availability of nutrients and mitogens, pH changes and cell interactions (202). As these factors affect cultures with each stimulant the differences in the relationship between cell concentration and proliferation for each stimulant probably reflects differences in the population of cells being activated and their modes of activation. Although Con A tended to produce slightly higher responses than PHA when the same lymphocyte concentrations (2×10^6 or 4×10^6/ml) were used, the responses were statistically identical. This suggested marked similarity in the kinetics of lymphocyte responses to Con A and PHA which in turn indicated that these two mitogens activated similar lymphocyte populations by identical modes.

At 2×10^6 and 4×10^6 lymphocytes/ml the mitogen-Log_{10} dose-response curves for Con A and PHA showed approximately linear responses at doses below optimal levels. At higher mitogen doses used in this study the curves tended to plateau suggesting that excess Con A and PHA were probably tolerogenic, a property also observed by Anderson and others (11) who eluted the mitogen from the lymphocytes and enabled recovery of the full response. Lack of either tolerogenicity or toxicity by LPS is shown by the very broad Log_{10} dose-response curves. The lymphocyte responses to mitogens and the background (unstimulated) absolute dpm values tended to increase disproportionately as the cell concentration increased so that the stimulation indices in

turn decreased. The DNA synthesis was usually stimulated optimally by Con A and PHA when monkey lymphocytes were used at a relatively low density (2×10^6 cells/ml) though this concentration exceeded that used in the mouse and bovine systems (1×10^6 lymphocytes/ml) (291, 292, 293).

The RPMI-1640 and DMEM media were statistically identical in their ability to support lymphocyte proliferation and showed consistently reproducible results. They were unlike the M 199 medium which had the lowest background responses and therefore fairly high stimulation indices with Con A and 5% FCS but not with PHA. Also the responses of the mitogen-stimulated cultures in M 199 medium were significantly lower than those due to DMEM and RPMI-1640 media. In fact DMEM tended to be superior to RPMI-1640. The effect of varying the FCS concentration did not seem to alter their efficiency ranks in stimulating lymphocyte responses. Since no comparisons with autologous or other heterologous sera were made (90) no further comment is made on the FCS.

The extent of activation of lymphocytes was discerned from the rate of DNA synthesis as measured by the incorporation of the radiolabel, ^{14}C-TdR. The pulse time affected the correlation between the rates of radiolabel incorporation and DNA synthesis (378). In this study, at constant concentration and specific activity of the ^{14}C-TdR, the incorporation was approximately linear up to 24 hours, a finding also observed by others (378, 387). However, the stimulation indices were highest for Con A when the label was pulsed for only 4 hours while for PHA 16 hours was the optimal time. For logistic

reasons it was decided to adopt the 16-hour pulse period, as this still fell within the linear part of the curve.

The responses of cultures harvested between 72 and 120 hours were statistically identical. Therefore to measure direct T cell activity rather than secondary facilitation by activated T cells it was decided to harvest cultures early, at 72 hours. This also ensured that culture conditions were not limiting (202, 378).

The fact that the lymphocyte responses peaked at different mitogen concentrations on different days would strongly suggest, for practical purposes when studying a particular parameter, the need for using 3 mitogen concentrations, one on either side of the optimal value and also the need for setting up different cultures to be harvested on two consecutive days with the optimal harvesting time probably as the first. This measure would probably unravel any delayed peaking due to, say, a disease, such as trypanosomiasis, under study.

The conditions for optimal stimulation of DNA synthesis in the vervet monkey lymphocytes at 2 x 10^6/ml, PHA and Con A at 15 and 10 µg/ml peak doses, respectively, cultured in DMEM supplemented with 10% FCS, pulsed with ^{14}C-TdR at 0.02 µCi/well for 4-16 hours and harvested after 72 hours of culture, resembled closely those of the mouse and man.

The lipopolysaccharide did not seem to stimulate monkey peripheral blood cultures well, a situation also seen in man (317). However, Pearson and others (291) reported bovine peripheral blood lymphocyte stimulation by LPS at very high cell concentration (5 x 10^6 lymphocytes/ml) while in the vervet

monkey system lymphocyte responses were very weak even at a higher cell concentration than that used by Pearson and his group. The inability of LPS to give reasonable levels of stimulation may be due to either low numbers of B cells found in the peripheral blood as the mitogen predominantly stimulates B lymphocytes or a phenomenon seen with mouse lymphocytes where low density cultures favour only B lymphocyte differentiation and immunoglobulin secretion (187). Very carefully controlled further studies are probably need to determine optimal conditions for the stimulation of vervet monkey PBL with LPS.

In the vervet monkeys PHA and especially Con A produced reproducible levels of activation in diluted whole blood cultures. The peak responses to mitogens of the lowest (1/5) and the highest (1/20) blood dilutions were almost identical particularly with regards to Con A. This finding suggested that culture conditions were limiting and the cultures were not expressing their full proliferating potentials.

The aim of lymphocyte transformation in vitro was to estimate functional changes in lymphocyte activity and as such a choice of conditions that detect both elevated and reduced activity levels would be essential. An elevated lymphocyte activity would be detected only by a supra-optimal mitogen dose provided the culture conditions would support increased activity. In contrast a depressed activity would be detected by optimal or sub-optimal mitogen dose.

The following conditions for the assessment of PHA and/or Con A responsive T cells in the vervet monkey whole blood are suggested - a ten-fold dilution of whole blood

by DMEM supplemented with antibiotics and glutamine but not FCS and stimulation with optimal and supra-optimal doses of PHA and Con A, the final concentrations of which are shown (Figs. 4.xvi ; 4.xvii). Although the cultures harvested between 96 and 120 hours were statistically identical it would be preferred to harvest cultures early as this would more accurately reflect T lymphocyte activity rather than secondary facilitation effects. Also it would ensure that the medium is not exhausted by highly prolonged lymphocyte activity.

Lymphocyte transformation studies of time courses and/or dose response kinetics required large numbers of cultures. The fact that the simple time-saving and economical whole blood microcultures and the use of semi-automatic harvesting were also possible in the vervet monkey model,was very encouraging. It meant experiments on the above scale were practicable using small volumes of blood which would be required from this small primate to set up 50 and 200 culture wells from 1 ml of blood and would provide a simple practical method for estimating PHA and Con A responsive T lymphocyte activity in vitro. However, whole blood cultures have the disadvantage that the number of cells in the cultures that one is dealing with is unknown.

Consistently, low allogeneic MLC responses were found in this study compared with those obtained from other models (291). Even under optimal conditions these reached only 5 to 20% of the xenogeneic (CLA_4) MLC responses. Xenogeneic lymphocytes from Sykes' and Colobus monkeys and humans, and allogeneic spleen cells gave improved MLC responses compared

with lymph node cells, but they were still low at 7 to 20% of the xenogeneic responses, respectively.

The responses in the one-way MLC depended more upon the stimulator than the responder cell concentrations. At constant responder cells per ml in the allogeneic and xenogeneic MLC systems the absolute dpm values increased and decreased, respectively, as the stimulator cell concentrations increased from 2- to 6.4 x 10^6 cells/ml.

The higher cell concentrations were less effective possibly because of crowding while the lower cell combinations produced virtually no stimulations even when the cultures were maintained for 192 hours probably due to lower concentrations of B cells in peripheral blood. Subsequently MLC at the optimal and stimulator cell concentrations of 3 x 10^6/ml, cultured in DMEM medium with 10% FCS and pulsed with ^{14}C-TdR for 168 and 16 hours, respectively, were adopted.

In the allogeneic MLC stimulator lymphocytes from male and female vervets gave identical responses suggesting that sex differences were not influential.

The standardization of the in vitro cell mediated immune correlates of the vervet monkey achieved using non-specific mitogens in separated lymphocytes and whole blood cultures as well as xenogeneic MLC and the susceptibility of the vervet monkey to T. brucei were the basis for setting up an immunological vervet monkey-T.brucei model described in the next experiments.

CHAPTER FIVE

IMMUNOLOGICAL RESPONSIVENESS

OF

T. BRUCEI-INFECTED VERVET MONKEYS

INTRODUCTION AND THE REVIEW OF THE LITERATURE

The aim of this thesis was to investigate the vervet monkey as a possible experimental model for immunological studies of human trypanosomiasis. It is only through a solidly established foundation of reproducible experimental data on the parasitological, pathological, immunological and chemotherapeutical aspects of sleeping sickness of man, that valid conclusions can be drawn about the relevance and use of the monkey as a model for human trypanosomiasis.

The results so far obtained in this study have revealed that the monkey is susceptible to T. brucei and that it shows a parasite dose-responsiveness associated with a spectrum of diseases. The normal immunological baseline data of a vervet monkey are now known but how these values vary during T. brucei infection is not known.

Furthermore, rodent and ungulate trypanosomiasis studies have contributed a lot of information on the immune responses, immunological aberrations and immunodepression. However, very few studies have been undertaken on the immune responses in man suffering from sleeping sickness. Because of this gap in human studies a vervet monkey - T. brucei model was set up to investigate how the immunological baseline data vary during trypanosomiasis. Various immunological tests including in vitro correlates of cell-mediated immunity (CMI) involving lymphocyte transformation in response to mitogens and xenogeneic MLC, enumeration of vervet monkey putative B and T lymphocytes, skin sensitization and antibody estimations against a heterologous

antigen were employed.

In this section immunology of trypanosomiasis, humoral and cell-mediated immunity, immunodepression and self-cure are reviewed.

(1) Immunology of trypanosomiasis

The immunology of trypanosomiasis is complex, complicated by challenges due to the serially and continuously changing trypanosomal variant specific surface antigens and also by aberrant immune responses of heightened antibody production, paradoxically in the face of immunodepression to heterologous antigens. Protective antibodies against T. equiperdum were shown decades ago (85), still little is known about trypanosomiasis immunology and immunity (91, 213). Mononuclear lymphocytic and plasma cells and macrophages probably participate in the trypanosomiasis immune responses. However, the plasma cell series characterize all disease stages.

(2) Immunity

An immunologically competent host like man reacts early to an infectious agent such as T. brucei by immediately expelling it using non-specific mechanisms so that the parasite does not make a foot-hold in it. Alternatively, initially the parasite entrenches itself in the host and later induces either strong fully expressed acquired immune responses which control the infections such as mouse T. musculi (367), rat T. lewisi (365), and N'Dama and Muturu cattle T. congolense and T. brucei (259) or partially expressed (depressed) immune responses so that the parasite evades the effective and destructive immune responses,

multiplies and fatally overwhelms the host as in human *T. rhodesiense* (85) and mouse *T. brucei* (237) and *T. congolense* (246) infections

(a) Humoral immunity

This is mediated by the plasma, lymph and body tissue fluid antibodies which may also become attached to cells (cytophilic antibodies).

Protozoa including trypanosomes, malaria and leishmania stimulate massive production of immunoglobulins. Hence, hypergammaglobulinaemia occurs in human (129) and bovine (193, 210, 262, 280) trypanosomiasis. Originally these immunoglobulins were assumed not be specific to the infecting trypanosomes, that is, directed against antigens unrelated (heterologous) to the infecting trypanosomes (148, 159); weakly correlated with clinical immunity (321) and with little affinity for the pathogen. This is because protozoa including trypanosomes were regarded poorly immunogenic and the antibodies probably produced by polyclonal B cell stimulation (129, 166). *Trypanosoma brucei* infections also show highly raised immunoconglutinins (170).

Cattle infected with *T. congolense* (62, 193, 236, 262) or with *T. brucei* (262, 267) produced both IgM and IgG. In contrast, little or no change in IgG2 levels occurred in cattle infected with *T. vivax* (280). Yet, cattle immunized artifically with isolated VSGs (262) and with heterologous antigens (DNP-ovalbumin) (240) produced both IgG1 and IgG2 after an initial rise in IgM. The reason for this difference

is unknown. However, complement affects antibody production *in vivo* (297). Its activation *in vitro* and *in vivo* through both the classical and alternate pathways results in decomplementation which probably depresses the immune response particularly of IgG (296). Decomplementation in mice and chickens leads to production of mainly IgM and inhibits the switch from IgM leading to repeated cyclic immune responses consisting mainly of IgM (277). Decomplementation leading to hypocomplementaemia probably also constantly occurs in African trypanosomiasis (193, 261, 280) presumably in the presence of IgM antibody. This increases the metabolism of complement (132, 133, 278, 279, 280) leading to formation of large molecular weight antigen - IgM antibody immune complexes. These stimulate B cells through the C3 receptors on the cell surface membrane (284) resulting in dysfunction in the switch mechanisms due to the parasites preventing inductive synthesis of IgG which mainly switches off the IgM responses. Meanwhile where 19S could not completely switch to 7S antibody production this was induced experimentally by decomplementation (277).

It is possible that the switch mechanism from IgM to IgG in trypanosome-infected animals is probably uniquely selective for IgG heavy chains. It probably also depends on the trypanosomes, the nutritional and immunological status of the host and the immunological tests used as the immunoglobulins detected may not be reacting with identical antigens on the parasites. However, in the production of specific antibodies to *T. congolense* and *T. brucei* in cattle polyclonal activation

and apparent B cell stimulation (129, 166) leading to dysfunction in the control of IgM and IgG production, plays insignificant role in bovine trypanosomiasis. That is, it may not be responsible for the high levels of these immunoglobulins in bovine trypanosomiasis (236, 262). Most likely the changes in immunoglobulin synthesis result from a continuous production of specific antibodies against several variable antigen types that arise during chronic infection.

In trypanosomiasis the precipitating, agglutinating (340, 372), antiglobulin, heterophile (170, 372) opsonizing, fluoresceing, complement fixing and immediate hypersensitivity reaction antibodies are diagnostically useful like the pathognomonic raised IgM (119, 220, 372). Its concentration remains high long after cure of the trypanosomiasis and when trypanocidal antibody tests are negative.

The nature and mode of action of the protective trypanosomal antibody have not been proved. However, most likely it is complement dependent and stock specific. In the host the Salivarian trypanosomes stimulate massive antibodies which with or without complement lyse the parasites in vivo and in vitro.

The antibodies against the trypanosome serotypes of early infection, soluble serum antigens or against various metabolites absent from the surface of intact living parasite do not specifically destroy it. However, passive immunization in mouse T. gambiense provides variant specific protection and also prevents relapse and death if the serum dose is large and

the parasite challenge is small. These factors probably inhibit subsequent antigenic variation (85, 125, 253).

Similarly, resistance against or inhibition of the rat T. lewisi is passively transferable by homologous but not heterologous antiserum (immune serum) (372). This contains complement-dependent trypanocidal antibodies, absorbable by living trypanosomes, and also contains the low affinity and unabsorbable gammaglobulin-associated ablastin which inhibits the reproduction but not the viability or the motility of T. lewisi. Whether ablastin and the trypanocidal antibodies are separate or the same entities is still controversial (215, 288).

All parasites probably induce specific antibodies, but, which rarely eliminate them completely. Hence, in the mammal trypanosomes are sometimes acutely or chronically lethal because they evade the acquired immune response they induce and persistently survive in its presence. However, parasites also persist through either re-infection with distinct species, stocks, relapse from tissue forms like persisting pre-erythrocytic stages of some plasmodia, long survival or recrudescence from pre-existing systemic infections. The mechanisms of parasite survival and proliferation in hosts able to mount a strong specific immunity probably partly resemble those occurring in progressive tumour growth in the face of active immunity (65). This strongly implies trypanosomiasis-associated immunological aberrations.

Aberrations in functional responses of the immune

system in the trypanosome-infected mammals occur commonly and probably through antigenic variation-associated prolonged and intense stimulation massive immunoglobulins are produced. Aberrations include production of auto-antibodies to normal tissue antigens (219); immune complex (I.C.) disease (251, 253, 264); IgM rheumatoid factor-like antibodies (159); heightened levels of total IgM (209, 210) and heterophile IgM antibodies (cross-reacting with xenogeneic red blood cells, bacterial LPS, antigens, etc. (159, 288), and C-reactive protein (377). Yet, trypanosomes through immunodepression produce lower levels of specific antibodies to immunizing heterologous antigens such as sheep erythrocytes (SRBC) (65, 85, 118, 250, 257, 258, 372); suppresses experimental allergic neuritis in trypanosome-infected rabbits (10); increases susceptibility to secondary infections in infected humans (130) and animals (155, 222, 253); produces free Ig light chains (131) and depresses both B and T lymphocyte functions (118, 228, 231, 257, 258, 372).

(i) Immunoglobulin M (IgM)

In African trypanosomiasis raised erythrocyte sedimentation rate (ESR) (13, 288) is due possibly to elevated globulin concentration. A fraction of the massive IgM in sleeping sickness (85, 398) has agglutinating, neutralizing, precipitating and lysing antibodies (85) elicited against each new parasite variant specific surface antigenic population arising during infection.

IgM appears early in immune responses and the elevated

levels in man (13, 85, 129), rodents (166, 334), cattle (80, 209) and non-human primates (159) are probably due to sequential production of antibodies to various tissue antigens and the rhematoid factor (158, 159, 189, 191, 220, 339). They are also due to increased synthesis but not impaired catabolism (208, 348) while the anti-trypanosome antibodies (253, 335) are due to rapid succession of antigenic variants each inducing a new specific antibody synthesis. In rhesus monkeys the raised non-trypanosome antigen specific IgM is about 95% (159) and is non-protective against the infection. However, accurate measurement by specific absorption is complicated by antigenic variation.

In trypanosomiasis low 7S IgM containing antigenic determinants and high, 19S, molecular weight IgM are produced (190). The high IgM levels are probably over-estimated by the Mancini technique for in agar 7S diffuses faster than 19S IgM (182). However, in rabbit T. equiperdum infections only about 5% of the IgM is monomeric (113). Whether the 7S IgM is formed by splitting 19S IgM by trypanosomal infuence, is an excretion product or is formed directly by plasma or morular cells, is unknown.

(ii) Immunoglobulin G (IgG)

IgG antibody levels are normally low despite the numerous plasma cells which, however, produce only massive IgM. This is possibly due to the unusual pathogenesis of trypanosomiasis, such as, antigenic variation (125, 399) which allows only enough time for variant antigen specific IgM

but not for delayed IgG whose synthesis is possibly also interfered with directly.

IgG antibodies against common antigens are pathogenetically important during trypanosomiasis because circulating antigen-IgG immune complexes occur repeatedly and cause diseases (264).

The protein concentration in tissue fluids is lower than in plasma because the capillary wall bars high molecular weight proteins. The available useful pentameric IgM cannot therefore cross barriers to reach some sites, such as the testicles (288). Still, probably effective IgG antibody titres are attained and the trypanosomes in the tissue fluids cannot, therefore, completely evade the humoral immunity although their destruction needs high IgG concentrations (253, 361).

IgG is very effective in the agglutination reaction but less effective in causing lysis and requires about 8 times the equivalent amount of IgM concentration to produce the same effect like preventing take of infection (358).

Concurrently given (337) homologous antiserum from even a 2 day infection (359) or specific IgM and IgG antibodies (361, 426) strongly protected rodents against T. gambiense (361) or T. brucei (253) and cattle against T. rhodesiense (404).

Concentrations of 0.03 and 6.5mg/ml of IgM and IgG, respectively, prevent infection of mice with T. gambiense (337, 358) while low IgM concentrations induce new variant

serotypes in mice (358). Whether or not IgM at lower quantities is more protective than IgG (358) or IgG more than IgM (426), is unknown. However, agglutination reaction, variant serotype formation and protection against infection are associated with both IgG and IgM, though to a varying extent.

(b) Cell-mediated immunity (CMI)

Strong evidence for cell-mediated immunity in trypanosomiasis lacks except from heart and nodular histopathological suggestions and from skin tests (4, 230, 231). From the brain and heart pathology, probably CMI and increased permeability due to immune complex mediated hypersensitivity cause perivascular cell infiltrations dominated by small monocuclear cells which favour CMI.

The role of polymorphs is probably minimal in human trypanosomiasis despite the 2-4 times increase (85). Eosinophilia is probably mediated by antigen-antibody complexes (85).

Mononuclear cells fill connective tissue spaces while phagocytes stick to the endothelium and phagocytose and destroy the numerous trypanosomes (118, 120).

In trypanosome-infected mice the absolute B, T and null cells numerically and/or proportionally change (237, 246). In acute infection T cells unlike the fluctuating B cells do not numerically and markedly change while null cells rise relentlessly. In chronic infections, again, T cells do not significantly change while B cells initially undulate but finally decrease gradually. Null cells fluctuate up and down

numerically. They contain IgG intracytoplasmically and are probably memory cells that never become plasma cells. The numerous null cells possibly effectively dilute the splenic antigen-sensitive cells in T. congolense-infected mice (319, 320). Oedema and hypovolumea do not occur or affect proportionally the splenic T cells which are lower in infected than in uninfected animals (319, 320) but the ER (T) cells (21) are numerically almost normal. This implies that immunodepression is not due to a numerical reduction in antigen-sensitive or responding cells. These cells, however, cannot clonally expand from early in trypanosomiasis (292, 319, 320). Also immunodepression is probably due to numerically increased and highly proliferative less mature B lymphocytes (319, 320) in the spleens and bone marrow of infected mice (237, 246).

The categorical nature of null cells (Ig, Thy-1) is unknown (319) but they are probably a mixture of pre- B cells, monocytes, normoblasts and other erythroid precursors stimulated by the concurring anaemia in trypanosome-infected mice or K and NK cells that rapidly divide during trypanosomiasis.

The immunodepression of B or T cells in T. congolense infected mice is probably due to dilution of antigen-sensitive cells by null cells and selective loss and replacement of responding T cells and also probably due to the active depression by one or several suppressor cell type(s) (292) as reflected by generalized non-antigen-specific immunodepression

(319, 320).

Although cell-mediated immunity is equivocal in trypanosomiasis unlike in leishmaniasis in which in vitro lymphokines are produced and lymphocytes are cytotoxic it is possible that trypanosomes after peak parasitaemia in mammals are cleared by complement mediated antibodies, possibly cell-mediated immunity and phagocytosis. Cell-mediated cytotoxicity involves an immune T effector cell with surface Ig receptors able to kill a target cell. However, certain lymphocyte subsets, polymorphs, neutrophils and eosinophils can also kill because they have surface receptors for the Fc of Ig while binding involves specific recognition which in turn requires minute amounts of IgG antibody.

All target cells show susceptibility which, however, differs in degree as some are quite resistant (241). It is possible, however, that immunity to protozoa does not work easily and CMI is probably easily blocked by antigen and/or antigen-antibody complexes utilizing IgG antibody which on getting diminished therefore switches on IgM.

(3) Immunodepression

Immunodepression is a generalized non-antigen-specific unresponsiveness of a host's immune system induced by diseases, drugs, or irradiation and is unlike the antigen-specific immunological tolerance.

In trypanosomiasis circumstantial evidence for immunodepression in humans and animals existed. For instance, pneumonia and streptococci occurred in blood and CSF of

patients killed by trypanosomiasis (153). Death was thus secondary to bacterial purulent meningitis, pneumonia, and dysenteric ulcerative colitis (13). Similarly, frequent secondary piroplasmosis, anaplasmosis and babesiosis complicated cattle T. vivax and T. congolense (104, 208, 328). Also, in T. vivax-infected sheep and cattle Salmonellosis dublin and in goats Pasteurella haemolytica pneumonia occurred (198, 206, 222, 253).

Humoral and/or cell mediated immunodepression occurs in early and late human T. gambiense (129, 130, 135) and in acute and/or chronic experimental mouse, deer mouse, field vole, rat, guinea-pig, rabbit, goat, sheep and cattle trypanosome infections (121, 228, 291, 292, 319, 320).

(i) Human trypanosomiasis-associated immunodepression

In late rather than in early human T. gambiense infections T lymphocyte functions are more severely depressed as evidenced by reduced induction and expression of in vivo skin sensitizing agents such as dinitrochlorobenzene (DNCB), PPD, Candida and streptococcal antigens (130, 135). However, only B lymphocyte functional expression, shown by poor antibody responses to Salmonella typhi antigen was equally depressed in early and late disease. Although non-sequential these studies imply a progressive and persistent immunodepression.

There seems to be no report of immunodepression in T. rhodesiense-infected humans.

(ii) Animal trypanosomiasis-associated immunodepression

Trypanosoma brucei-induced immunodepression to

heterologous antigens in infected animals has been the most investigated (118, 121). Trypanosoma brucei-infected mice and rabbits poorly responded immunologically to injected SRBC. The haemagglutinin IgM antibody titres were lower in the infected than in the uninfected animals. Immunodepression became markedly progressive and was severest during the third post-infection week (PIW 3) (121, 256). In T. brucei-infected than in normal mice fewer Jerne plaque forming cells (PFC) to pneumococcal polysaccharide (SIII) and to SRBC and LPS antigens occurred (258) while the skin reactivity to oxazolone was normal. Histologically lymph node and spleen T-D areas were invaded by plasma cells. The impaired IgM responses to the antigens were due to a B cell defect while the immune complexes probably contributed to the expansion of the macrophage phagocytic system (MPS) and to normal T cell responses (258). They reverted to normal after treatment, meaning that immunodepression was associated with live trypanosomes.

Spleen cells from T. brucei-infected mice were unresponsive in vitro to Esch. coli-LPS (174). The secondary and primary immune responses to heterologous human erythrocytes in T. gambiense-infected field voles (Microtus montanus) were also depressed (1,2). However, previously established antibody responses were unaffected. When the voles were treated twice with suramin and then re-challenged with the same antigen 3 days after treatment they responded like uninfected control voles.

Early in infection IgM- and IgG-producing cells were,

respectively, unaffected and reduced to below 20% of the control count but 10 days later both were reduced to below 2% of the controls (111). Antigen-binding or sensitive cells were present in the spleen. They could respond to an antigen because the trypanosome-infected mice treated with Berenil and concurrently immunized with SRBC responded normally immunologically, thus strongly suggesting that immunodepression is exerted at the induction phase (initiation stage) of the immune response (111).

Immunodepression has also been shown to other antigens such as adult Nippostrongylus brasiliensis worms which could not be expelled normally by a T-D phenomenon (386) from concurrently T. brucei-infected rats. Production of protective (IgG) and reaginic (IgE) antibodies was impaired in T. brucei-infected rodents which showed initially normal T-dependent antibody responses to polysaccharides, less affected IgM but depressed IgG anti-SRBC responses, T-dependent (T-D) expulsion of N. brasiliensis worms and delayed hypersensitivity reaction to oxazolone (386). However, by the erythrocyte rosette (ER) test the primary antigen-sensitive spleen cells were numerically normal and could clonally expand after immunization (111, 386). Thus T-dependent responses were only slightly depressed suggesting no cellular injury but only interfered with and markedly depressed T and B cell co-operative responses.

The presence of cell-mediated immunity in trypanosome-infected hosts has been much debated but little investigated.

Trypanosoma brucei-infected rabbits were protected by immunodepression from developing experimental allergic neuritis (EAN), an autoimmune disease predominantly caused by cell-mediated immunity (10). However, some of the animals did develop EAN. Thus the presence of CMI in trypanosome infected rabbits is probably equivocal. Depressed cell-mediated immune responses to Candida, PPD and DNCB in T. gambiense-infected humans (135) and DNA synthesis by spleen cells stimulated with Con A, PHA, and allogeneic cells (174) have confirmed (67) a 90% depression of T cells to PHA stimulation for thymidine incorporation from a 5 day T. brucei mouse infection. Similarly depressed lymphoblastic transformation to PHA and responses to dinitrofluorobenzene (DNFB) and ovalbumin in T. brucei-infected guinea-pigs (204) as well as to oxazolone in T. gambiense-infected field voles have also been reported (1,2). However, CMI to oxazolone in guinea-pigs (203) and in mice (111, 253, 258), could not be detected until after at least 3 weeks of infection. The discrepancies have been ascribed to differences in trypanosome stocks and hosts used (1, 2).

Cell-mediated immune (delayed hypersensitivity) and primary T cell responses to oxazolone and ^{125}IUdR in T. brucei-infected mice showed normal and subnormal lymph node radiolabel incorporation in early and late infections, respectively. Meanwhile the late subnormal (immunodepressed) incorporation was restored to normal by a trypanocide (176).

In deer mouse (Peromyscus maniculatus) T. equiperdum infection severe immunodepression occurred (248). Spleens and

lymph nodes showed hyperplastic germinal centres and plasma cell infiltration of the red pulp and medullary cords and disrupted B and T dependent areas. The responses of splenic T and B cells to stimulation with PHA, Con A, pokeweed and LPS, as well as humoral responses to SRBC were severely depressed unlike those from animals injected with radio-attenuated trypanosomes that mounted a normal or enhanced immune response.

Immunodepression of humoral and/or cell-mediated immune responses has been shown in T. congolense infections. Primary antibody responses to SRBC and pneumococcal polysaccharide (SIII) were depressed in infected rodents (227). In contrast, these animals had, during most of the infection, elevated primary in vivo splenic PFC responses to T cell independent antigens (e.g. SIII, PVP). However, secondary antibody responses were also depressed in T. congolense-infected mice to SIII and PVP, in sheep to Vibrio foetus antigen (222) and in cattle to polyvalent clostridial antigen (156). Similarly in T. congolense-infected mice a generalized immunodepression was evidenced (291, 292, 319, 320) by unresponsiveness of spleen cells to LPS, Con A, allogeneic spleen cells differing at the H-2 or Mls loci, to generate cytotoxic lymphocytes (291) and to stimulate in MLC (319, 320). The inability to reject allogeneic skin grafts also implied a depressed T-lymphocyte reactivity in vivo (319, 320)

Previously T. congolense-infected mice prolonged subsequently inoculated T. musculi parasitaemia (51). Also T. congolense-infected rabbits had depressed in vivo responses

to PPD, *in vitro* lymphocyte transformation to PPD and PHA and migration inhibition factor (231).

There has been only one report of immunodepression, for at least 25 days to *L. biflexa*, *Br. arbotus* and *M. mycoides* antigens in cattle with *T. vivax* (and *T. congolense*) (323). The immunodepression probably affected IgG more than IgM antibodies.

In humans (130, 135) and animals (1, 2.111, 177, 248, 256, 258) with trypanosomiasis the impaired immune responses and histological changes reverted to normal after a single or double Berenil or suramin treatment. Therefore, immunodepression was associated with live, multiplying, virulent and varying trypanosomes which could also elicit immunological and inflammatory responses and which directly or indirectly affected the host's immune system.

Except for *T. cruzi* which is pathogenic to humans and other mammals, Stercorarian trypanosomes are typically non-pathogenic. However, they do induce immunodepression in their hosts. For instance IgM and IgG antibody formation to burro erythrocytes (64) and cell-mediated immune response to oxazolone and BCG (309) were depressed in *T. cruzi*-infected mice. Otherwise, immunodepression due to Stercorarian trypanosomes has been deduced indirectly. Thus *T. theileri*, the ubiquitous non-pathogenic cattle parasite, has been associated with intercurrent diseases (215, 278). However, as *T. theileri* and bovine leukosis (225) were not correlated it is not known whether the trypanosomes or the secondary infections were immunodepressive.

Mice concurrently infected with T. musculi and a lowly virulent T. gambiense died of infection (114) but it is not known which trypanosome caused death. Again, T. musculi (69) or T. rhodesiense (75, 76) enhanced murine P.b. yoelli infections. Similarly in vivo and in vitro responses to SRBC, PHA, Con A and LPS of spleen cells from T. musculi-infected mice were depressed (5). The immunodepression which correlated with the splenomegaly and parasitaemia was abrogated when the spleen returned to normal size. Saline extracts from freeze-thawed T. musculi and serum from infected mice had an immunodepressive effect on the in vitro humoral antibody response of normal spleen cell cultures to SRBC. The depression is probably due to parasite-derived soluble substance(s) which act(s) directly on B lymphocytes or essential assistant (secondary) cells rather than by activating suppressor T cells or macrophages (6).

IgM and IgG antibody responses to SRBC in T. musculi-infected mice were also depressed when the parasitaemias of at least 50 x 10^6 trypanosomes per ml blood developed (143). The immunodepression vanished gradually with the disappearance of the parasites.

Humoral and/or cell mediated immunodepression to heterologous antigens (130) also occurs in humans and animals with other acute and/or chronic plasmodia, babesia and theileria (71, 130, 239, 300, 303, 308, 393, 423), helminths, bacteria, viruses (282, 295, 329), malnutrition (103, 275, 356), pregnancy (354), graft-versus-host reactions (342, 343) and

malignancy. In measles (238) immunodepression is transient because the virus just like P.b. yoelii (76) can be self-limiting in the absence of secondary infections.

In summary, in Salivarian trypanosome-infected mammals humoral and/or cell mediated immunodepression occurs. In early and late human T. gambiense it affects the induction and expression of cell-mediated immunity and T cell functions to skin sensitizing agents while only the expression of humoral immunity and B lymphocyte functions to bacterial antigens are depressed. It is progressive and persistent but can be abrogated by treatment.

(iii) Mechanisms of immunodepression during trypanosomiasis

The immunodepressive mechanisms due to trypanosomiasis are either unknown, unclear or speculative only and are probably different for different parasite and host strains because the pathogeneses of the diseases also differ. Trypanosoma brucei is a tissue parasite that localises extravascularly (352) while T. congolense and T. vivax are mainly plasma parasites, but suggestively extravascular (52, 211).

Immunodepression which in trypanosome-infected mammals results in their poor response to challenge with heterologous antigens (121, 324) is probably due to various mechanisms (20, 67, 292, 319, 320). These mechanisms include depletion of antigen-reactive lymphocytes of the infected hosts due to a polyclonal LPS-like mitogenic B cell activation which probably drives lymphocytes to memory and plasma cells and

thereby stimulates them to exhaustion while the memory cells divide probably 10-12 times only and the secondary immune responses to the pre-sensitizing antigen are reduced (18, 209, 232, 411); loss of immune regulation (5, 8, 111, 135, 204, 205, 229, 230, 233, 257, 258, 272, 287); generation of suppressor T lymphocytes (96, 115, 175) and suppressor macrophages (174, 401); altered macrophage function (130, 201); increased protein metabolism (348, 390); histological changes in lymphoid tissues (320, 386); inhibitory plasma factors (111, 204, 249); the presence of live trypanosomes (96, 231, 320); changes in T, B or null cell counts (237, 319); free fatty acids (382); hypocomplementaemia (261, 277, 296, 297, 357); antigen, antibody and antigen-antibody complexes as blocking factors of the immune response (146, 147, 372, 380); and antigenic competition (83, 242, 368) of sequential, molar ratio or intramolecular type (138, 164, 268, 313).

Arguments against some of the postulated immunodepressive mechanisms such as antigenic competition when parasitemia is low (289, 319, 320); impaired protein metabolism (2, 4); clonal activation which exhausts lymphocyte potentials and depresses B or T cell responses in trypanosome infected animals (292); impared macrophage function (118, 248, 257); gross histolcgical changes (248, 252, 257, 258); enhanced suppressor T cell functions (233, 258, 292, 319, 320); and the presence of live trypanosomes (2, 3, 6, 49, 232, 291, 320) also exist.

(4) Self-cure

Trypanosomes possibly through immunodepression and antigenic variation evade the immunity responsible for clearing them. Nonetheless even without treatment they are still cleared from the blood possibly by similar *in vitro* mechanism involving variant specific, opsonizing, immunoadherence-and phagocytosis-promoting antibodies and complement which agglutinate and lyse the trypanosomes (253).

Self-cure of trypanosomiasis without a trypanocide or pre-immunization by homologous parasite is well documented in Stercorarian mouse *T. musculi* (367), rat *T. lewisi* (365) and also in Salivarian cattle *T. brucei* or *T. congolense* (253), sheep and goat *T. congolense* (136) and primate *T. brucei* or *T. rhodesiense* (17, 407) infections.

In the mouse the natural parasite *T. musculi* is limited after 20-25 days, by a T-dependent immune response but not by passively transferred immune serum (306). A strong and durable immunity is conferred (367). However, the T cell role in the development of the resultant immunity is unknown.

In mouse *T. musculi* (306) like in rat *T. lewisi* (365) infections clearance of parasitaemia from the blood is probably by a thymus-dependent ablastin (a reproduction-inhibiting factor) and a thymus independent 'first trypanocidal' antibody. The mechanism of accelerated parasite-elimination, though unknown, involves neither immune (sensitized) adherent cells nor immune serum directly (367). In contrast, acquired immunity to *T. lewisi* is passively transferable by only homologous immune

serum (288).

During chronic African trypanosomiasis the protracted parasitaemia fluctuates and the parasites express several or apparently infinitely numerous different variable antigen types (VATs). Yet cattle chronically infected intravenously with cloned T. congolense, T. vivax or T. brucei underwent self-cure after 32, 11, or 16 weeks, respectively. Thereafter all self-cured animals completely resisted challenge with cyclical homologous or with other clones derived from the corresponding but not heterologous stocks (269).

The mechanism responsible for self-cure is also unknown. Individual factors are probably important because the high resistance of N'Dama cattle against T. congolense depends on their ability to limit the height and duration of parasitaemia more efficiently than the Zebu (253). This factor possibly also operates in individuals and breeds of sheep and goats. This is because although more indigenous than exotic breeds effected self-cure some animals of both exotic and indigenous breeds self-cured or quickly succumbed (136). Also, the T. congolence or T. vivax parasitaemia in the gazelle unlike in Zebu cattle disappeared within 3 months to produce an apparently parasite-free healthy animal (318, 399). The ability to control parasitaemia is probably related to the host's very efficient immune response (399) and is inherited as a dominant trait in inbred strains of mice (246).

Probably, no effective immunity exists against human T. gambiense or T. rhodesiense (399). Apparently healthy-looking

but infected people (healthy carriers) have occasionally been reported in endemic areas (14, 15, 221, 286).

Variant specific antigens induce (125) in T. gambiense infections initially mainly IgM and later both IgM and IgG (334, 340, 358). These variant specific antibodies highly protect the host against challenging homologous but not heterologous trypanosome stocks (333, 337, 372, 399). This is unlike immune cells which effectively protect against, say, T. gambiense in the early stages of immunization (253, 358). Lysis is more efficient in the presence of complement and homologous IgM which leads to decomplementation (261). Similarly, immune rat serum lysed irradiated T. rhodesiense (88, 253).

The variant specific pentameric IgM clears infections in the bloodstream where 80% of it is found but not in the tissue (extravascular) spaces where, unlike the smaller molecular weight IgG, it does not easily reach high and effective concentrations against T. brucei, a tissue parasite (288). However, when IgG antibodies are synthesized the trypanosomes in the tissues have varied antigenically so that the trypanosomes are always one step ahead of the effective antibody (85).

Sera from cattle that received single infections of T. congolense (236, 413) or T. brucei (268, 413) clones or metacyclics from different stocks showed 2 or more recurrent peaks of mostly specific IgM, IgG_1 and IgG_2 antibody activity against the original infecting VATs and those arising later in the infection through antigenic variation. This was

probably due to a re-appearance of the infecting VATs or emergence of VATs possessing surface antigenic determinants identical or closely related to those of the infecting organisms including metacyclics (35, 200, 269). The VATs occur in different sequence in different animals (236). In T.congolense-infected cattle they appeared more often than previously (413) reported.

In chronically infected cattle during recovery the infecting organisms probably exhausts their repertoire of VATs which are virulent in the host, including those that closely resemble the metacyclic VATs. This then leads to "self-cure" through re-appearance of the infecting VATs and subsequent complete resistance to challenge by all VATs belonging to homologous but not heterologous serodemes(269).

Cell-mediated immunity has been observed in T. brucei-(379, 380, 382) and T. gambiense-(4) but not in T. congolense-infected rabbits (230). It is probably present but cytotoxic cells specific for trypanosomes and their effect in vivo are enhanced or facilitated by antibodies (127, 273) and by blocking factors, such as antigen-antibody complexes as in malignancy (147).

In mouse trypanosomiasis the absolute numbers of live spleen T cells by erythrocyte rosetting technique are almost normal despite the leucopenia (291, 292, 319, 320). Trypanosomes inhibit clonal proliferation of helper T cells through liberating anti-lymphocyte proliferation factor which competes with lymphocytes for some factor essential to proliferation.

Phagocytes from actively and passively T. lewisi-immunized rats and homologous parasites interact intraperitoneally (364). Immunophagocytosis of T. lewisi and T. gambiense by exudate cells in vitro is enhanced by homologous immune serum (362, 363, 364) containing a phagocyte-enhancing antibody. It probably mainly and rapidly clears parasites inoculated into immune rats.

Serum and "cell-mediated" antibodies initiate specific immune responses against parasites. Their action in vivo and in vitro may be enhanced synergistically by specific antibody, primed lymphocytes and macrophages leading to phagocytosis (48). The cytophilic antibodies in sera from T. brucei- or T. cruzi-infected mice (see 253) or horses, respectively, cause immuno-adherence of trypanosomes to macrophages with an unknown outcome. Also the immune response against T. gambiense in mice probably results from the activities of resistant anti-mouse thymic cell serum and adherent cell subpopulations. When these cells are mixed with anti-mouse thymic cell serum non-adherent sensitive cells possibly co-operate and enhance the immune responses (359). The adherent cells produce and release the agglutinins, IgG and IgM (345).

The antimicrobial activity of macrophages is non-specifically increased in vivo in animals infected with living organisms (218) such as leishmania and probably also trypanosomes (240, 292). This system, acting in concert with specific antibodies could be the major mechanisms of parasite clearance in the infected host (276).

Finally, in mouse T. congolense and T. rhodesiense infections the resistance was increased, the survival times were prolonged and parasitaemic peak was delayed through the adjuvant (immunologic and pharmacologic) effect of E. coli lipopolysaccharide inoculated previously (253) or through its effect on stimulating interferon production or the alternate complement pathway which stimulates the macrophage phagocytic system.

In T. gambiense mouse infection phagocytosis by macrophages involves sequentially attachment and ingestion (363). The parasite attachment is enhanced by specific antiserum to T. gambiense (363). However, macrophage attachment is greatly and relatively less reduced by soluble and insoluble parasite fractions, respectively (364). Similar results have also been reported with Mycoplasma pulmonis and intact mouse macrophages(183).

On the basis that the vervet monkey is susceptible to T. brucei Clone 227 which showed a parasite-dose responsiveness (Chapter 3 of this thesis) and that the cell-mediated immune correlates of the vervets could be standardized and be reproduced (Chapter 4 of this thesis) experiments were designed to study how these immune parameters altered during either untreated acute fatal and chronic self-limiting infections due to 1×10^8 and 1×10^4 trypanosomes, respectively, or chronic Berenil-treated infection due to 1×10^4 parasites. The materials and methods, results and discussion are reported below.

MATERIALS AND METHODS

Experimental design

There were two sets of experiments reported in this chapter.

The first experiment was designed to investigate immunological responses, before and after treating the monkeys with a trypanocidal drug, Berenil, in vervets infected with a low dose of *T. brucei* Clone 227. The design of the first experiment is shown in Table 5.1.

The second experiment was intended to look at the immune responses in animals with either untreated acute fatal or chronic self-limiting *T. brucei* Clone 227 infections.

(A) *Berenil-treated infections*

The first experiment involved 10 parasite-free adult male monkeys weighing between 2650-3750 gms. The monkeys were divided into 2 groups, each of 5 animals, on the basis of matched weights. One group was infected with *T. brucei* while the second formed the uninfected controls (Table 5.2).

A week before infection with *T. brucei* all the monkeys were weighed, and haematological indices and *in vitro* correlates of cell-mediated immunity (using Con A, PHA and MLC) were performed on their separated peripheral blood lymphocytes (PBL). The pre-infected sera were stored for subsequent serological examinations. Two days before infection all monkeys were primed with tetanus toxoid and also with the skin-sensitizing reagent 2, 4 - dinitrochlorobenzene (DNCB). On day "zero" one group of monkeys was inoculated with *T. brucei*

parasites and thereafter bled daily to estimate the levels of parasitaemia. Also both groups of animals were bled sequentially once weekly for assessing haematological indices, cell-mediated immunity and serology until the experiment was stopped.

One millilitre (1.0ml) of the bloodstream form of Clone 227 containing 1×10^4 parasites raised in irradiated Balb/C mice and isolated on DEAE column was inoculated intravenously into each of the 5 monkeys.

(B) Untreated infections

The results of the first experiment (treated infections) in this section whereby immunodepressed monkeys quickly recovered their humoral immune responses but took long to recover their cell-mediated immune correlates prompted another study to look at both immune responses in animals with untreated acute fatal or chronic self-limiting infections.

In this experiment three groups each of 5 male monkeys were used. One group was infected intravenously with 1×10^8 and the other group with 1×10^4 trypanosomes after separation on DEAE from mouse blood (Table 5.27). The same control group of monkeys served both experiments.

The course of infection was followed by daily parasite estimations by the haemacytometer method. Meanwhile haematology; stimulation of peripheral blood lymphocyte cultures by mitogen and unidirectional xenogeneic (CLA_4 cells) MLC; enumerations of ER (T), SmIg (B) and "null" cells in monkey peripheral blood lymphocytes; DNCB skin sensitization;

estimation of anti-tetanus, total IgM and IgG and heterophile antibodies; weighing of animals; and Berenil treatment were performed as described under "General Materials and Methods".

The reasons for using 3 doses of mitogens and for setting up different culture plates of PBL from the same host for harvesting on two different consecutive occasions have also been given in Chapter 4 above.

RESULTS

The pre-infection parameters in the control and infected monkeys were, each, the same. In the uninfected control monkeys most of these values (except for the anti-tetanus antibodies) remained almost unchanged throughout the experiment and were unaffected even by Berenil treatment. However, they sometimes differed significantly from those of the infected animals.

In monkeys inoculated with Clone 227 of T. brucei the parasitaemias resembled those reported in Chapter 3 where the same parasite and almost identical doses were used. The parasitaemia due to 1×10^4 parasites undulated, with remissions, like that in man suffering from trypanosomiasis. It responded to a single Berenil injection and also limited itself. Meanwhile parasitaemia due to 1×10^8 trypanosomes was progressively non-relapsing and lethal.

Clone 227 induced in monkeys anaemia which coincided with parasitaemic patency. The haemograms decreased rapidly during the first 3 weeks of infection particularly in the monkeys which died of acute infection unlike those which survived longer and went to spontaneous recovery or were treated. In the chronically infected monkeys moderately low haemograms were sustained a little longer. Thereafter recovery was evident by the end of the experiment on PID 155. Thus anaemia was only slightly abrogated by Berenil treatment or self-cure.

The infection induced leucopenia, lymphocytopenia, neutrocytosis and monocytosis. It also reduced the erythrocyte

rosettes, SmIg and null cells. The *in vitro* correlates of cell-mediated immunity were initially enhanced. However, subsequently they were depressed, more briskly and severely in the acutely than in the chronically infected monkeys.

The immunodepression to Con A and PHA was severest on PID 20 as evidenced by the least SI and lowest absolute dpm.

Except on PID 5 at other times before treatment there was limited multiplication of PBL from infected monkeys when stimulated with CLA_4 cells for they responded poorly in xenogeneic MLC.

Following Berenil treatment the *in vitro* cell-mediated immune correlates took about 30 days to return to normal. Similarly the depressed responses to DNCB improved slowly but were significantly lower than those in the control animals by the end of the experiment. This indicated that complete recovery after treatment was achieved only with the humoral immune response to tetanus toxoid. In the untreated self-curing animals the *in vitro* cell-mediated immune correlates were still depressed 155 days after infection. This suggested that the parasites disappeared from the blood into some privileged sites from where they exerted immunodepression.

Trypanosoma brucei-infected, unlike the control, vervets immunized intramuscularly with tetanus toxoid 2 days before infection showed, coincident with the first peak of parasitaemia, an initial enhanced primary 2-ME-sensitive (IgM) but not 2-ME-resistant (IgG) anti-tetanus antibody responses. Subsequently, from PID 11 onwards, depressed primary and

secondary humoral (IgG more than IgM) immune responses to tetanus toxoid occurred. Secondary immunization gave an initial depressed but final normal secondary anti-tetanus antibody titres.

Most sera from both animal groups contained 2-ME-resistant (IgG) anti-tetanus antibodies but the titres in the infected than in the control animals were lower statistically on PID 20 and 33.

Following Berenil treatment of monkeys 44 days after infection, that is, 46 and 18 days after primary and secondary (first re-challenge) tetanus toxoid immunizations, respectively, the suppression disappeared. The previously infected monkeys quickly recovered immunologically. They, like the control animals, mounted a similar anamnestic and normal secondary anti-tetanus antibody immune responses for both IgM and IgG when re-immunized 12 days after treatment. This indicated probable recovery of the humoral immune responses and establishment of memory. Memory was also evident during secondary cell-mediated (DNCB) immune responses. Until the end of the experiment all monkeys were apparently producing at the same rate mainly 2-ME-resistant anti-tetanus antibodies, presumably IgG.

The trypanosome infection contributed significantly and only slightly to the increase of total serum IgM and IgG values, respectively. However, Berenil treatment had the reverse effect on the serum Ig values in the infected monkeys.

During infection IgM but not IgG heterophile antibodies

were mainly and massively produced. However, both types were not affected by treatment.

A. Treated infections

(1) Parasitaemia

Within 3 days following intravenous inoculation of 1×10^4 parasites of Clone 227, trypanosomes were detected in wet blood films from the peripheral blood of all 5 monkeys (Fig. 5.i; Table 5.3).

The parasitaemia in individual animals fluctuated tremendously and it undulated like that in humans with trypanosomiasis. However, it did not indicate complete disappearance of parasites from the blood when animals were considered as a group. For instance, in monkey no. 35 the parasite count rose sharply to the first peak on PID 4 which was controlled and subsequently followed by further waves that peaked on PID 12, 24, 28, 31, 38 and 42. Each wave lasted for 2 - 14 days while the trypanosomes disappeared from the blood for 1 - 12 days. The highest parasitaemia for the group was $30.64 \pm 29.59 \times 10^6$ on PID 16 whereas for individual animals it was 32.00, 3.60, 136.40, 35.60 and 72.00×10^6 trypanosomes/ml blood for monkeys no. 22, 28, 33, 34 and 35, respectively, again corresponding to PID 15, 24, 16, 27 and 12.

Although all 5 monkeys received the same 1×10^4 trypanosome inoculum the parasitaemia was heavy in monkeys no. 33 and 34 and fairly light in monkey no. 28. As the infection progressed the peaks became smaller and the intervals between waves increased while the animals appeared to control

the infection. Thus the parasitaemia in these monkeys essentially resembled that reported in Chapter 3 where 1 x 10^5 trypanosomes of the same Clone 227 were inoculated in monkeys.

A single dose of 10 mg/kg body weight of Berenil on PID 44 uneventfully cleared the trypanosomes from the peripheral blood within 24 hours. Until the experiment was stopped 34 days later, on PID 78, no relapse was observed using the haematocrit centrifuge technique (417) or by inoculation of monkey blood into mice. Thus Berenil treatment was effective against T. brucei in the vervet monkeys.

(2) Haematology

(i) Red cell indices

An almost persistent anaemia evidenced by reduction in nearly all the haematological indices featured significantly throughout T. brucei infection in the monkeys. The differences in the red cell indices between the infected and control animals became evident by PID 5 (Figs. 5.ii - 5.vii, Tables 5.4 - 5.9). Some differences involving the erythrocyte (RBC) count ($p<0.01$), the Hb ($p<0.001$), PCV ($p<0.001$), MCV ($p<0.05$) were significant by PID 20 and persisted until treatment on PID 44. The MCH and MCHC values decreased slightly before treatment. The lowest values for the RBC count (4.10 $\pm$ 0.35 x 10^6 cells/cu.mm. blood) ($p<0.01$), Hb (9.52 $\pm$ 0.80 gm/100ml blood) ($p<0.001$), PCV (30.12 $\pm$ 2.59%) ($p<0.001$), MCV (72.0 $\pm$ 1.97 μ^3), ($p<0.05$), MCH (22.48 $\pm$ 0.77 $\mu\mu$g) and MCHC (29.76 $\pm$ 0.59%) were observed on PID 26, 20, 20, and 75, respectively.

When all the monkeys were treated on PID 44, for

sometime thereafter the haemograms in the infected animals remained significantly lower than those in the controls until on PID 54 for RBC ($p<0.05$), on PID 75 for Hb ($p<0.05$) and on PID 61 for PCV ($p<0.05$) (days 10, 31 and 17 post treatment, respectively). However, even at the end of the experiment the RBC counts and PCV were still below those of the controls.

The MCV returned to the control level, within 3 days of treatment, only to drop and persist below it, until on PID 75 (31 days after treatment). The MCH and MCHC values for the infected animals differed only slightly from the controls before treatment. However, on PID 44 and subsequently they became statistically low between PID 47-61 for the MCHC ($p<0.03$) and PID 75 for the MCH ($p<0.05$).

Thus, in the monkeys studied 1×10^4 parasites of Clone 227, induced anaemia which was slightly abrogated by Berenil treatment while the uninfected control animals simultaneously treated sustained their normal haematological indices.

(ii) Total and differential leucocyte counts

In the infected monkeys suggestive and sometimes significant total and/or differential leucocyte count changes were evident within 5 days of infection.

The leucocyte changes from either the pre-infection or control levels were not significant (Fig. 5.viii; Table 5.10) throughout the experiment.

During the slight leucopenia significant per cent lymphocytopenia ($P<0.01$) on PID 26, neutrocytosis ($P<0.05$

at least) on PID 20 and 26, and monocytosis ($P<0.05$) between PID 20 - 54 occurred compared to the control and/or pre-infection levels (Figs. 5.ix, 5.xi, 5.xiii; Tables 5.11, 5.12, 5.13).

The fluctuating absolute lymphocyte counts in infected monkeys statistically ($P<0.05$) decreased gradually and progressively to the lowest value, on PID 20, of 2303 $\pm$ 464 per mm^3 blood compared to the corresponding control value of 5214 $\pm$ 943. Later the counts rose gradually to the highest level, by PID 61, of 5596 $\pm$ 879 which was statistically identical to the control (4799 $\pm$ 894 lymphocytes per mm^3 blood) and the pre-infection (3733 $\pm$ 1000) values (Figs. 5.x, 5.xi, 5.xii, 5.xiv; Table 5.11).

Similarly, in the infected animals the absolute neutrophil and monocyte counts increased gradually from their pre-infection values of 3274 $\pm$ 394 to 4461 $\pm$ 852 neutrophils/mm^3 blood and 92 $\pm$ 14 to 387 $\pm$ 111 monocytes/mm^3 blood, respectively, their highest values and statistically identical to the corresponding control values by PID 40, before treatment. However, the absolute monocyte counts significantly exceeded the pre-infection values on PID 33 ($P<0.01$) and PID 54 ($P<0.001$) (Tables 5.12, 5.13).

Berenil treatment on PID 44 was associated with only a slight leucocytosis to 9.68 $\pm$ 1.45 x 10^3 and then to 8.38 $\pm$ 0.70 x 10^3 WBC/cu.mm. blood by PID 61 and PID 75, respectively. However, 3 days after treatment the per cent lymphocytes and neutrophils were back to their respective pre-infection and control values. Meanwhile the proportions of monocytes dropped

from 4.80 ± 1.08% to 1.20 ± 0.22%, the pre-infection level, only to increase statistically ($P<0.05$) on PID 54 (10 days after treatment) to 8.40 ± 1.75%, above the pre-infection and control levels. They finally decreased to 4.00 ± 1.70% by PID 61.

The absolute lymphocyte, neutrophil and monocyte counts in the infected animals returned to and sometimes even exceeded the pre-infection and/or control values, within 3 days of treatment. However, the absolute monocyte count by PID 54 (day 10 post-treatment) was signficiantly ($P<0.001$) higher than the pre-infection but not the control levels. This was followed 7 days later by an abrupt decrease to the pre-infection and control levels (Tables 5.11, 5.12, 5.13).

(iii) Erythrocyte rosettes (ER), SmIg and "null" cells

(a) Erythrocyte rosettes (ER cells),

Essentially only minor fluctuations in the percentages of ER cells occurred on PID 5 from 65.60 ± 3.91% to 74.80 ± 5.18% which later dropped to a minimum value of 62.20 ± 7.76% by PID 20.

The absolute ER cells significantly ($P<0.05$) decreased gradually from the pre-infection 3030 ± 300 to 1471 ± 370 cells/mm^3 blood, the lowest value by PID 20.

Following treatment on PID 44 the low per cent ER cells slowly increased and equalled the control values. Meanwhile by PID 47 and thereafter the absolute ER cells in the infected but treated animals had returned to the pre-infection and the control values (Figs. 5. xv, 5.xvi; Table 5.14).

In monkey no.33, the absolute ER cells dropped to 13% of the pre-infection level by PID 20.

(b) Surface membrane immunoglobulin-bearing (SmIg) lymphocytes

The proportions of SmIg cells increased significantly ($P<0.01$) from the pre-infection 10.80 $\pm$ 0.96% to 26.40 $\pm$ 4.06% by PID 11. Thereafter they returned, by PID 33, to the control SmIg values which were sustained until after Berenil treatment. The per cent SmIg cells then again, significantly ($P<0.05$ at least) increased above the control and/or pre-infection levels on PID 47 and 54. Subsequently, and until the end of the experiment on PID 75, they were, in the control and infected animals as well as in relation to the pre-infection values, statistically identical (Figs. 5.xvii; Table 5.15).

The absolute SmIg cells increased gradually but significantly ($P<0.05$) on PID 11 above the pre-infection but not the control values. Later they decreased to the lowest non-significant levels on PID 33 (11 days before treatment). However, after treatment, they rose above the pre-infection and control values (Fig. 5.xviii; Table 5.15).

(c) The "null" cells

The per cent "null" cells decreased significantly ($P<0.05$) on PID 5 from the pre-infection 23.60$\pm$3.38% to its lowest significant ($P<0.02$) value of 4.20 $\pm$ 3.40% compared with the controls (22.20 $\pm$ 5.92%) on PID 11. This then increased slightly to 25.20 $\pm$ 9.17% above the control (22.20 $\pm$ 1.87%), by PID 40, and the pre-infection levels (Fig. 5.xix; Table 5.16).

The absolute null cells initially also decreased suddenly and significantly ($P<0.05$) (on PID 5, 11 and 26) from the pre-infection 1114 ± 252 to the lowest value of 136 ± 174 null cells/mm^3 blood on PID 11. Subsequently, by PID 40, they rose to the control and pre-infection levels (Fig. 5.xx; Table 5.16).

After Berenil treatment, the per cent and absolute counts of null cells, that by PID 26 were slightly below the control values, gradually returned to almost the control values by PID 75, and 61, respectively, (Figs. 5.xix, 5.xx; Table 5,16).

(3) Monkey weights

All the monkeys involved in these experiments were weighed when they were aparasitaemic because the effect of Ketamine on the trypanosomes was unknown then. However, recently the drug has been reported to have no effect on the parasites (316).

On PID 50, the control group had gained a mean weight of 50 gm only, possibly due to blood loss and stress resulting from the sequential weekly bleeding (Table 5.17). The infected group however, lost a mean weight of 295 gm compared with the pre-infection value. On PID 78 (34 days after Berenil treatment) the control and infected animals had gained mean weights of 55 and 290 gm, respectively. Thus the infected group was almost back to its pre-infection mean weight. These weight changes indicated overall mean weight gain and loss of 105 and 5 gm, respectively, and corresponding to the control and infected

animals.

The resultant weight changes by PID 50 and 78 for the infected animals were not statistically different from the pre-infection weights and/or from those of the control groups at the corresponding times.

(4) Cell-mediated immunity

The weekly results of cell-mediated immune correlates are illustrated (Figs. 5.xxi - 5.xxiii; Tables 5.18, 5.19).

(i) Stimulation of PBL with Con A, PHA and xenogeneic CLA_4 cells

Micro-cultures of 2×10^6 lymphocytes/ml were stimulated with either sub-optimal, optimal or supra-optimal PHA and Con A doses each for 72 and 96 hours. Also, unidirectional xenogeneic MLC using infected or control monkey PBL responders and CLA_4 cell stimulators all at 3×10^6/ml were set-up and harvested at 120 and 168 hours of culture. The results are shown (Figs. 5.xxi, 5.xxii, 5.xxiii; Tables 5.18, 5.19)

(a) Con A lymphocyte stimulation

Before infection the sub-optimal, optimal and supra-optimal Con A doses of 5,10 and 15 µg/ml used to stimulate PBL from monkeys for 72 hours gave the highest, the second best and the least SI, respectively. From PID 5, through treatment day on PID 44 and until PID 54 and 75, SI were lower in the infected than in the control animals and, the supra-optimal, optimal and sub-optimal doses gave the highest, the second best and the least SI, respectively, when the PBL

were cultured for 72 or 96 hours. Thus before treatment the PBL from infected animals responded slightly more but not significantly to higher Con A concentrations. Yet, the corresponding dpm, dose for dose, of the controls were not attained during infections (Fig. 5.xxi; Tables 5.18, 5.19).

Following Berenil treatment on PID 44 responses identical to pre-infection and control situations were first seen in the infected group by PID 61. That is, it took about 2 weeks for the PBL responses to Con A to show some recovery.

The 96-hour lymphocyte cultures from infected animals had lower SI than those at 72 hours due to increased background dpm which were sustained until, but not after, PID 47. Thereafter the background dpm were almost equal except on PID 75 when they were lower than the pre-infection values. In the 96-hour cultures the supra-optimal, optimal and sub-optimal Con A doses gave the highest to the least SI, respectively, from PID 5, through treatment on PID 44 and until the end of the experiment by PID 75 (31-days after treatment). This suggested that recovery was really not complete as return to the pre-infection and control situations had not occurred.

Similarly, in the 72-hour cultures both the optimal and supra-optimal doses of Con A gave fairly close responses while the sub-optimal dose continued giving very weak responses, also suggesting incomplete recovery by PID 75. This indicated that recovery of CMI responses when measured by Con A was also very slow after trypanocidal treatment of monkeys. The immunodepression to Con A, like to PHA, was severest on PID 20

as evidenced by the least SI and absolute dpm. However, these values were only slightly lower than the pre-infection or the control values.

Significant ($P<0.05$, at least) immunodepression occurred as shown (Tables 5.18, 5.19). Before treatment, neither increasing the Con A dose nor culturing for longer times, say, 24 hours more, improved stimulation indicating that immunodepression was genuine and not artifactual. Again treatment did not abrogate soon (within 48-144 hours) the immunodepression which lingered on for 4-5 weeks thereafter.

(b) PHA lymphocyte stimulation

The final sub-optimal, optimal and supra-optimal concentrations of PHA used were 10, 15 and 20 µg/ml, respectively, for total culture times of either 72 or 96 hours. When PBL from infected monkeys were stimulated *in vitro* with the above 3 doses of PHA and cultured for 72 hours, the pre-infection or control order of the optimal, supra-optimal, and sub-optimal doses being associated with the highest, the second best, and the least stimulation indices (SI), respectively, changed as from PID 5. The highest, the second best and the least SI were by then due to the supra-optimal, optimal, and sub-optimal doses, respectively, down in that order except on PID 75 when stimulations resembled those of control animals. By PID 20 the three PHA doses were eliciting low SI because of great variation in stimulated dpm. Thereafter the stimulation indices improved towards the pre-infection and control values and were associated with significant differences

(Fig. 5.xxii; Table 5.18, 5.19) due to reduced variation.

Berenil treatment on PID 44 shifted the responses towards normal over PID 47-61 and the recovery was partially complete by PID 75. However, the highest to the lowest SI were still due to supra-optimal, optimal and sub-optimal doses, respectively.

When cultured for 96 hours the PBL from infected animals before, except on PID 11, and after treatment gave mostly lower SI than those terminated at 72 hours because the background and stimulated dpm increased disproportionately. Complete recovery of cell-mediated immunity evidenced by the return to the pre-infection and control situations occurred on PID 75 (31 days after treatment). That is, it took more than 4 weeks for Berenil to abrogate the T. brucei immunodepression on CMI correlates. The stimulated absolute dpm from the control and the infected animals, in relation to doses and culture times differed statistically as indicated (Fig. 5.xxii; Tables 5.18, 5.19).

(c) Mixed lymphocyte reaction (MLR)

The ability of PBL to respond in MLC to xenogeneic (CLA_4) stimulator cells are shown (Fig. 5.xxiii; Tables 5.18, 5.19).

The pre-infection stimulation indices and the absolute dpm each due to cultured PBL from infected and control animals were similar.

Before infection of the monkeys the background mean absolute dpm were lower and hence the higher stimulation

indices (SI) for the 120 - than for the 168-hour MLC. After infection, by PID 5, both the background and the stimulated dpm were lower for the MLC harvested at 120 than at 168 hours; yet the SI were higher than the pre-infection and those values due to cultures terminated at 168 hours. Thereafter the background and the stimulated dpm fluctuated as either high or low values giving, respectively, low or high SI (Fig. 5.xxiii, Tables 5.18, 5.19).

Some MLC involving PBL from the infected but not from the control monkeys, from PID 11 on, rapidly deteriorated because the stimulated dpm of individual animals decreased abruptly when harvested at 168 instead of at 120 hours. This occurred before and after Berenil treatment in monkey no. 28 on PID 11 when the stimulated dpm dropped by almost 80% from 9903 to 2166 dpm and in monkey no. 22 on PID 47, three (3) days after treatment, by 61% from 7504 to 4603 dpm, respectively, and corresponding to 120 and 168 hours of culture; that is, by prolonging the cultures for 48 hours. The background dpm changed from 761 to 154 dpm on PID 11 and from 117 to 128 dpm on PID 47 for the corresponding 120 and 168 hours of culture. During those times, the MLC responses for monkey no. 33 were almost completely suppressed.

During the experiment, the lowest SI with PBL infected monkeys pertained to PID 26 and 20, respectively, which were 4.18 and 3.03 while the SI corresponding to the controls were 60.34 and 43.14, respectively, for the 120- and 168-hour cultures. The low SI were due to high background and low stimulated absolute dpm. These latter values were

due to real depression of MLC responses during trypanosomiasis. During infection and before treatment the background dpm from the 120-hour cultures fluctuated widely from 164 to 1138. However, thereafter they gradually dropped as the MLC responses in the infected monkeys showed recovery by PID 75 (the end of the experiment).

The stimulated responses decreased gradually from the pre-infection 7426 $\pm$ 1268 to the lowest 1682 $\pm$ 581 dpm, a value 22.7% lower than the control value by PID 26 for the 120-hour MLC. Significant ($P<0.05$) depression occurred on PID 33 when harvesting was done at 168 hours. Subsequently, the dpm increased to and above the pre-infection values by PID 40 and 47 (4 and 3 days before and after treatment, respectively). Yet, the background responses were, respectively, 5 and 3 times higher than the pre-infection dpm and hence the still low SI. However, from PID 40 and especially after Berenil treatment, the SI rapidly rose due to decreased background and increased stimulated responses. By PID 61, the SI from the MLC of infected monkeys for the 120- but not the 168-hour cultures had exceeded the pre-infection but not the control levels due to "residual" depression and slightly higher background absolute dpm.

The control than the infected monkeys had higher SI and statistically significant stimulated dpm ($P<0.05$) on PID 11 and 33, for the 120- but not for 168-hour MLC whose background responses fluctuated between 115 and 145 and between 140 and 160 dpm, respectively.

(ii) DNCB skin sensitization

During infection and before treatment on PID 40 the infected animals responded to DNCB skin sensitization significantly ($P<0.001$) less (0.83 ± 0.05 mm. mean dia.) compared to the control monkeys (1.43 ± 0.03). Four weeks after treatment, that is, on PID 68 the skin reactivity to DNCB re-challenge in the previously infected animals had improved though it was still slightly weaker (1.58 ± 0.03) than in the control animals (1.65 ± 0.04 mm. mean dia.) (Table 5.20). Thus it took about 4 weeks for the skin reactivity and, therefore, the delayed hypersensitivity to recover from the depressive effect of the T. brucei.

(5) Humoral immunity

(i) Immunoglobulins, M and G (IgM and IgG)

Total IgM and IgG concentrations in sera from infected monkeys increased significantly during the infection but not in the controls (Figs. 5.xxiv, 5.xxv; Tables 5.21, 5.22).

(a) Immunoglobulin M (IgM)

IgM concentration progressively increased from the pre-infection level of 28.00 ± 2.24 mm.mean dia. to the highest peak of 45.25 ± 2.48 mm.mean. dia. on PID 33 (Fig. 5.xxiv; Table 5.21) when measured by the radial immunodiffusion test. Thereafter it decreased to 43.80 ± 2.68 mm.mean dia. on PID 40, four days before treatment. In the controls the mean serum IgM levels varied between 27.00 and 31.50 mm.mean dia. (the peak on PID 11) and were significantly ($P<0.02$) lower than in the infected monkeys.

Following Berenil treatment on PID 44 the serum IgM levels in the infected monkeys decreased gradually through 44.75 ± 3.84 to 34.0 ± 0.82 mm mean dia., by PID 47 and 75 (3rd and 31st post-treatment days), still statistically ($P<0.02$) above the serum levels in the control animals which were virtually unaffected by treatment.

(b) Immunoglobulin G (IgG)

The serum IgG concentrations in the control monkeys fluctuated slightly, between 7.86 ± 0.19 and 8.38 ± 0.27 mm. mean dia., throughout the experiment (Fig. 5.xxv; Table 5.22).

In the infected monkeys the pre-infection serum IgG level of 7.85 ± 0.33 increased progressively to a peak of 8.98 ± 0.25 mm mean dia. on PID 26. Thereafter, it dropped to 8.68 ± 0.17 and 7.93 ± 0.12 mm mean dia. by PID 40 and 75, before and after treatment, respectively. However, the serum IgG concentration in the infected group was statistically ($P<0.05$, at least) high on PID 11 and 20 (before treatment) and on PID 47 (3 days after therapy) (Table 5.22).

(ii) Anti-tetanus antibodies

The characteristics of the primary and secondary anti-tetanus antibody responses estimated by the indirect passive microhaemagglutination titration test are shown (Figs. 5.xxvi, 5.xxvii; Tables 5.23, 5.24).

There were no natural haemagglutination anti-tetanus antibodies titrable in the sera of all the monkeys before inoculating them with tetanus toxoid but they appeared thereafter and the titres rose.

(a) Primary anti-tetanus antibodies

Following primary immunization the total titres of anti-tetanus antibodies in the control animals rose and peaked as Log_2 7.40 $\pm$ 1.64, just below Log_2 9.00 $\pm$ 1.73 for the infected monkeys on PID 20 (Figs. 5.xxvi, 5.xxvii; Tables 5.23, 5.24). Immunizing monkeys with the tetanus toxoid 2 days before experimental infection produced an enhanced 2-ME sensitive (IgM) but not 2-ME resistant (IgG) antibody response in the infected animals. On PID 11 during the second parasitaemic peak, the response of the infected monkeys to tetanus toxoid was statistically ($P<0.02$) less than that of the controls. Although, on PID 20, the parasitaemia was low in infected animals the anti-tetanus antibody response was high probaly due to enhancement resulting from the tryapanosomal adjuvant effects. Thus the titres of primary anti-tetanus antibodies peaked on PID 20 in both the infected and control animal groups. Thereafter, by PID 26, the anti-tetanus antibody titres in the infected group dropped dramatically below those in the control animals.

Most sera up to PID 26, contained predominantly 2-ME-sensitive (IgM) antibodies. However, 2-ME-resistant (IgG) antibodies were also produced early and peaked as Log_2 5.00 $\pm$ 0.94 on PID 20 in the control unlike in the infected animals in which they were depressed as Log_2 1.00 $\pm$ 1.12. Thereafter on PID 26 the 2-ME-resistant (IgG) titres in the infected animals were still a mere Log_2 1.40 $\pm$ 1.56, only slightly lower than Log_2 3.80 $\pm$ 0.65 in the control animals.

(b) Secondary anti-tetanus antibodies

(b1) First re-challenge

The secondary immune responses in the 2 monkey groups differed qualitatively at the peak titres on PID 33, five days (on PID 28) after re-challenge with tetanus toxoid (Figs. 5.xxvi, 5.xxvii; Tables 5.23, 5.24).

The main characteristic feature of the secondary anti-tetanus immune responses to tetanus antigen in the control but not in the infected monkeys was a rapid switch in the class of antibody produced from 2-ME-sensitive (IgM) to 2-ME-resistant (IgG) antibodies. The IgG antibodies increased from Log_2 3.80 $\pm$ 0.65 to 9.40 $\pm$ 1.10 and the IgM antibodies from Log_2 5.40 $\pm$ 1.40 to 8.80 $\pm$ 0.74 during the 7 days. In contrast, in the infected monkeys, the corresponding titres increased from Log_2 1.40 $\pm$ 1.56 to 3.40 $\pm$ 2.39 for IgG and from Log_2 2.40 $\pm$ 1.64 to 4.60 $\pm$ 2.22 for IgM, respectively.

However, on PID 40, four days before Berenil treatment, the mean 2-ME-resistant (IgG) and 2-ME-sensitive (IgM) anti-tetanus antibody titres were Log_2 10.00 $\pm$ 0.94 and 9.80 $\pm$ 0.74 for the controls and Log_2 8.80 $\pm$ 1.45 and 9.00 $\pm$ 1.06 for the infected animals, respectively, statistically identical within and between the 2 groups. Five (5) days after secondary immunization only in one monkey, no. 4, did the total tetanus 2-ME-sensitive (IgM) and 2-ME-resistant (IgG) anti-tetanus antibody titres differ by greater than one dilution. And from day 12 post-rechallenge until the end of the experiment all monkeys were apparently producing mainly

2-ME-resistant antibodies, presumably IgG.

The control monkeys gave good secondary anti-tetanus antibody responses which in infected monkeys were depressed by T. brucei. Although the antibody titres in the infected animals were lower than in the controls, between primary and secondary inoculations, the difference was significant ($P<0.05$) for 2-ME-resistant (IgG) anti-tetanus antibodies only on PID 20 and 33.

Following Berenil treatment of monkeys on PID 44, that is, 18 days after secondary immunization the 2-ME-sensitive (IgM) anti-tetanus antibody titres for the control monkeys dropped from Log_2 9.80 $\pm$ 0.74 to 9.00 $\pm$ 0.61 (PID 47) and 8.20 $\pm$ 0.42 (PID 61). Meanwhile the 2-ME-resistant (IgG) anti-tetanus antibodies continued rising to a peak of Log_2 10.40 $\pm$ 1.15 by PID 47 but dropped to Log_2 8.60 $\pm$ 0.04 by PID 61. In contrast, in the infected animals both the 2-ME-sensitive (IgM) and-resistant (IgG) anti-tetanus antibody titres rose to their peaks of Log_2 9.20 $\pm$ 0.82 and 11.60 $\pm$ 1.04, respectively, on PID 47 and then dramatically dropped to Log_2 8.20 $\pm$ 0.65 and 10.00 $\pm$ 1.00 by PID 54, respectively. Thus in the infected animals the suppression of anti-tetanus antibody responses apparently disappeared within 3 days (by PID 47) of treatment. Thereafter these animals behaved like the control monkeys, producing at almost the same rate, if not greater, titres of mostly 2-ME-resistant (IgG) anti-tetanus antibodies.

(b2) Second re-challenge

A third challenge with tetanus toxoid on PID 57 (day 13 post-treatment) briskly produced in both animal groups identical anti-tetanus antibody titres predominantly 2-ME- resistant (IgG) of Log_2 12.00 on PID 75 (days 31 and 18 post-treatment and post-"tertiary" immunization, respectively) (Figs. 5.xxvi, 5.xxvii; Tables 5.23, 5.24).

(iii) Heterophile antibodies

Only low pre-infection titres (Log_2 4.0) of 2-ME-sensitive (IgM) and 2-ME-resistant (IgG) heterophile antibodies in the control and infected monkeys occurred. The values in the control animals did not alter significantly throughout the experiment (Figs. 5.xxviii, 5.xxix; Tables 5.25, 5.26)

(a) 2-Mercaptoethanol sensitive (total, IgM) heterophile antibodies

In the infected monkeys the pre-infection total heterophile (2-ME-sensitive) antibody titres of Log_2 0.80 ± 0.42 dramatically rose to 7.80 ± 1.14 on PID 40, four days before treatment (Table 5.25). They statistically ($P<0.05$, at least) exceeded those in the control monkeys from PID 11-75.

After Berenil therapy on PID 44 the already rising titres of 2-ME-sensitive (total IgM) heterophile antibodies continued to Log_2 8.60 ± 1.25 by PID 47 (day 3 post-treatment). Subsequently, they gradually dropped to Log_2 5.80 ± 0.82 by PID 75, still significantly ($P<0.01$) above the controls.

(b) 2-Mercaptoethanol resistant (IgG) heterophile antibodies

The sera of control animals were also negative for the 2-ME-resistant (IgG) heterophile antibodies before treatment (Table 5.26). However, in infected animals gradually the titres increased from the pre-infection Log_2 0.0 ± 0.0 through a mere Log_2 2.00 ± 1.37 on PID 40, before treatment, to the highest value of Log_2 2.60 ± 1.30 by PID 47 (day 3 post-treatment). Later, the titres gradually dropped to Log_2 1.60 ± 1.03 on PID 75 (day 31 post-treatment).

Again, monkey no. 28 had the highest 2-ME-resistant (IgG) heterophile antibody titres of Log_2 6.0 by PID 47 (day 3 after treatment). Meanwhile monkeys no. 33 and 35 had, throughout infection, the lowest titres, with Log_2 3.0 on PID 61 (day 17 post-treatment) and 2.0 on PID 26 (18 days before treatment) as the highest, respectively.

The differences in titres of 2-ME-resistant (IgG) heterophile antibodies between the control and infected animals, before and after treatment, were not significant. However, the 2-ME-sensitive (IgM) titres were significantly higher ($P<0.02$, at least) than the 2-ME-resistant IgG antibodies. This suggested that most of the heterophile antibodies were 2-ME-sensitive, possibly IgM, and not 2-ME-resistant (IgG).

(B) Untreated infections

(1) Parasitaemia

All monkeys inoculated with 1 x 10^8 trypanosomes lacked prepatency for the infection was evident (i.e. levels above

1.0 x 10^4 parasites per ml) the next day in blood and the animals developed acute diseases (Fig. 5.xxx; Table 5.28).

The mean trypanosome counts increased and peaked at 50.0 x 10^6 by PID 6 and persistently fluctuated between 1 - 30 x 10^6 until the animals died between PID 12 - 23. The highest parasitaemias recorded in these monkeys were respectively, 110 -, 55 -, 40 -, 179.5-, and 90 x 10^6/ml blood on PID 9, 17, 10, 5 and 6 for monkeys no. 29, 38, 40, 41 and 44 which survived for 12, 22, 11, 11, and 13 (mean of 13.80 $\pm$ 2.33) days. Two to four days before death a high parasitaemia was sustained in each monkey though it decreased slightly a day before death (Table 5.28).

In contrast, in monkeys inoculated with 1 x 10^4 trypanosomes the prepatency was 2-5 days and each animal developed a chronic self-limiting infection. Throughout the infections typical parasitaemias, resembling those reported in Chapter 3 developed in each animal.

Basically the fluctuations of parasitaemia in this group were similar, but differed from those of animals inoculated with 1 x 10^8 parasites. The parasitaemia varied daily within and between animals and was characterized by transient peaks (up to 20 - 25 x 10^6 parasites per/ml blood). The highest counts reached in individual animals were, respectively, 15.8-, 13.4-, 3.4-, 22.8- and 23.6 x 10^6/ml blood on PID 10, 26, 32, 23, and 17 for monkeys no. 37, 39, 42, 43 and 45 (Fig. 5.xxx; Table 5.28). The daily parasitaemias in acutely (1 x 10^8) diseased monkeys were up to 23 times

greater on PID 6 and also significantly ($P<0.02$) higher between PID 6 - 10 than in those with chronic (1×10^4) infections whose levels subsequently decreased temporally as the animals attempted self-cure. The parasites were detected only occassionally by the haematocrit method during chronic stage of infection and were last detected on PID 54, 46, 86 and 74 in monkeys no. 39, 42, 43, and 45, respectively but on PID 121 in no. 37 which had a relapse on PID 150.

(2) Haematology

(i) Red cell indices

The haematological indices in the control monkeys bled simultaneously and sequentially once weekly with infected animals, fluctuated persistently within the normal ranges throughout the experiment. For instance, the PCV value non-statistically changed from the pre-infection 50.76 $\pm$ 3.60% through 55.42 $\pm$ 3.70 and 46.55 $\pm$ 3.13% on PID 8 and 134, respectively. It then gradually returned to its original value, sustained until the last results were recorded on PID 155 (Table 5.31).

As shown (Figs. 5.xxxi - 5.xxxvi; Table 5.29 - 5.34) T. brucei in the vervets was followed, within on week, by an abrupt marked and progressively severe normocytic and hypochromic anaemia evidenced by significant ($P<0.05$, at least) decreases from the pre-infection levels to the lowest mean levels of RBC counts, Hb and PCV, 5.61 $\pm$ 0.65 to

3.04 ±0.53 x 10^6 cells/cu. mm blood, 14.44 ± 1.71 to 7.20 ± 1.27 gm/100 ml blood and 42.84 ±4.76% to 23.06 ± 3.65%, respectively, on PID 8 in the acutely infected animals. Thus the haemograms were severely reduced by more than half in the acutely infected animals that died within 3 weeks (5.xxxi, 5.xxxii, 5.xxxiii; Tables 5.29, 5.30, 5.31) than in the chronically infected self-curing animals.

The chronically infected monkeys also became moderately anaemic during the infection. The RBC counts, Hb and PCV decreased significantly ($P<0.01$) though gradually from 5.54 ± 0.39 to 3.87 ± 0.15 x 10^6 cells/mm^3 13.70 ± 1.18 to 9.20 ± 0.41 gm/100 ml blood and 40.16 ±3.08% to 29.08 ± 1.37% , respectively, by PID 22. Thereafter the values increased slowly, back to the pre-infection values e.g. 41.08 ± 1.90% by PID 155 for PCV. The anaemia occurred between PID 8 and 78 and the erythrocyte counts, Hb and PCV were low, respectively, in 16, 18 and 14 out of 21 determinations. The peak parasitaemia and the lowest PCV temporally corresponded while the recovery coincided with decreasing parasitaemias. Normal RBC, Hb and PCV values were restored on termination of the infection or slightly thereafter (Figs. 5.xxxi, 5.xxxiii; Tables 5.29, 5.30; 5.31).

The MCV values for both infected monkey groups temporally and seemingly but not significantly increased rapidly to their respective peaks on PID 8 and 29 when compared with the pre-infection and control values (Fig. 5.xxxiv,, Table 5.32). The MCH and MCHC did not decrease significantly

in the acutely infected animals. However, they were reduced significantly ($P<0.05$; at least) on PID 15.57, 78, 85, and 92, for MCH, and on PID 15, 64, 85, and 99 for MCHC in the chronically infected animals (Figs. 5.xxxv, 5.xxxvi; Tables 5.33, 5.34).

(ii) Total and differential leucocyte counts

The total and differential leucocyte values did not change significantly throughout the experiment in the control monkeys. However, in both groups of trypanosome-infected animals significant leucopenia coincided with the patent parasitaemia (Figs. 5.xxxvii; Table 5.35). The changes persisted until all the acutely infected animals died of trypanosomiasis. The rapid leucopenia to $3.54 \pm 0.85 \times 10^3$ cells/mm^3 blood, was significantly below the pre-infection ($9.52 \pm 0.94 \times 10^3$ cells per/cu.mm. ($P<0.01$) and the control $8.62 \pm 1.36 \times 10^3$ cells per/cu.mm ($P<0.02$) values by PID 8. The last animal that died in this group had, 2 days before its death, a leucopenia of 3.0×10^3 cells per/cu. mm. blood by PID 22.

In the chronically infected animals the pre-infection total WBC count of $7.56 \pm 0.51 \times 10^3$ cells/cu.mm blood had, by PID 8, dropped precipitously to $4.64 \pm 0.70 \times 10^3$/cu. mm. blood, the lowest significant ($P<0.02$) value from the pre-infection and control counts. Although thereafter the animals attempted recovery, significant ($P<0.05$, at least) leucopenia was still evident on PID 15, 22, 71, 78 and 85.

The leucopenia, which coincided with high parasitaemia

between PID 8 - 85 but not thereafter, was associated with lymphocytopenia and neutrocytosis in both infected animal groups. significant ($P<0.05$) per cent and/or the absolute lymphocytopenia occurred sometime between PID 8-134, compared with the pre-infection and/or control values. However, at other times the per cent and/or absolute lymphocytopenia were only slight (Fig. 5.xxxtx; Table 5.36).

In the infected animal groups and throughout the experiment the significant ($P<0.05$, at least) sequential percentages of neutrophils on PID 8, 22, 29, 50, 78 and 85 were above the pre-infection but not the corresponding control values (Fig. 5.xl, 5.37).

The absolute neutrophils in both infected groups were slightly below the control or the pre-infection levels but occasionally exceeded the corresponding controls (Fig. 5.xli; Table 5.37).

The per cent but not the absolute monocytosis rose significantly ($P<0.05$) above the control values in both infected monkey groups (Figs. 5.xlii; 5.xliii; Table 5.38).

(iii) Erythrocyte rosettes (ER), surface membrane immunoglobulin bearing (SmIg) and "null" cells

There were some significant changes in the proportions and/or absolute numbers of the above cells in the infected but not in the control monkeys (Figs. 5.xliv - 5.xlix; Tables 5.39, 5.40, 5.41).

(a) Erythrocyte rosettes (ER cells)

In the acutely infected animals the proportions of the

ER, by PID 8, slightly rose to 76.20 ± 3.86%, above the pre-infection and the corresponding control levels of 68.00 ±4.74%. and 67.00 ± 2.03%, respectively. Thereafter, on PID 22, just before the last animal in the acute group died its proportions of ER were reduced to 52% (Fig. 5.xliv; Table 5.39).

In contrast, the absolute ER cells were significantly reduced from the pre-infection 5306 ± 505 ($P<0.01$) or the control 4819 ± 849 ($P<0.02$) values to 2136 ± 323 ER/cu. mm. blood by PID 8. The last animal to die in the acutely infected group showed only 952 ER/cu.mm. blood by PID 22, just before its death (Fig. 5.xlv; Table 5.39).

In the chronically infected monkeys the proportions of the ER cell were by PID 8 slightly raised to 74.20 ± 2.00%, above the corresponding pre-infection and control values of 65.0 ±4.68% and 67.00 ± 2.03%, respectively. Thereafter the per cent ER that had decreased gradually to the lowest level of 57.20 ± 6.25% on PID 43 suddenly increased to 73.00 ± 3.26% by PID 50, only, to fluctuate between this latter value and 61.80 ± 3.65% on PID 148 as the animals struggled to control the infection. Meanwhile the absolute ER, however, decreased significantly ($P<0.05$) between PID 8 - 29 and 71 - 85 to below the pre-infection but not the control values (see Table 5.39)

Thereafter, as the animals attempted self-cure and until the end of the experiment the absolute ER cells fluctuated only slightly below the control and the pre-infection values.

(b) Surface membrane immunoglobulin-bearing (SmIg) lymphocytes

The proportions of lymphocytes bearing immunoglobulins on their surface membranes (SmIg) by PID 8 had increased significantly from the pre-infection and the control values of 12.40 ± 1.90% to 20.8 ± 2.80% and from 11.40 ± 1.04 to 24.00 ± 1.70% for the acutely ($P<0.05$) and chronically ($P<0.001$) infected animals, respectively, (Fig. 5.xlviii; Table 5.42). Subsequently, the proportions of these cells rapidly declined to 3% by PID 22, in the last animal that died of acute infection. Meanwhile in the chronically infected animals the per cent SmIg cells, almost throughout the experiment, fluctuated slightly around the control values (Fig. 5.xlvi; Table 5.40).

In contrast, during the entire experiment, the absolute SmIg cells, in both the acutely and chronically infected groups, did not alter significantly from either the pre-infection or the control values (Fig. 5.xlvii; Table 5.40)

(c) The "null" cells

The percentages of the null cells in both infected groups of monkeys decreased suddenly and significantly ($P<0.001$) from 21.80 ± 2.33% to 2.80 ± 1.24% for the acutely and from 23.60 ± 5.20% to 1.80 ± 0.42% for the chronically infected animals when compared to the control (22.20 ± 1.75%) and/or the pre-infection (21.80 ± 2.33%) values by PID 8 (Fig. 5.xlviii; Table 5.41). Thereafter the proportion of null cells for the last animal to die in the acutely infected

group rose abruptly to 40% by PID 22. In the chronically infected group the increase to the first and the highest peaks of 24.20 ±6.28% and 32.50 ± 3.86% on PID 43 and 148, respectively, when compared with the control or pre-infection values was associated with significantly ($P<0.05$) raised proportions above the almost constant control values, such as, on PID 134.

The absolute null cell numbers in the infected groups also decreased suddenly and significantly ($P<0.001$) from the pre-infection 1793 ± 199 to 170 ± 117 cells/mm^3 blood by PID 8. However, in the animal that died last in the group values changed from 696 to 99 null cells per cu. mm.on PID 12 and 22, respectively. Meanwhile in the chronically infected animals they numerically and significantly decreased to below the control and/or pre-infection values on PID 22, 29, 50, 57, 64, 71, 78 and 85. However, from PID 113 until the end of the experiment,the counts fluctuated slightly around the control values (Fig. 5.xlix; Table 5.41).

(3) Cell-mediated immunity

(i) Lymphocyte stimulation with Con A and PHA

Before infecting monkeys with T. brucei the stimulation indices and absolute dpm values from lymphocytes stimulated with various PHA and Con A concentrations for either 72 or 96 hours were similar in the 3 monkeys groups (Figs. 5.1; 5.1i, Tables 5.42 - 5.44).

(a) Concanavalin A lymphocyte stimulation

The stimulation indices of the monkey lymphocytes from

both infected groups by PID 8 had dropped dramatically and statistically ($P<0.05$, at least) to 9%and 6%and, to 49%and 39% of the control values for 72-and 96-hour cultures, respectively(Fig. 5.1; Tables 5.42, 5.43, 5.44). The drop in values was less in the chronically than in the acutely infected animals. However,the absolute dpm were statistically identical for the 72 and 96 hour cultures.

By post-infection day 22 the lymphocytes, from the acutely infected animal still alive, produced stimulation indices of 20 and 30 which were lower than those of 90 and 70 from the controls for the 72-and 96-hour cultures, respectively. Meanwhile the stimulation indices for the chronically infected monkeys were about the same, 20, for the 72- and 96-hour cultures. However, thereafter and until the end of the experiment on PID 155 they fluctuated lowly without recovering to the control value. The lowest stimulation indices of 10 and 7 were obtained on PID 71 from the 72-and 96-hour cultures, respectively (Tables 5.43, 5.44).

The absolute dpm for Con A were statistically ($P<0.05$, at least) lower than those of the controls for the 72-hour cultures between PID 8-72, 113 and 134-155 and for the 96-hour cultures between PID 8-155 (Tables 5.42, 5.43, 5.44).

(b) Phytohaemagglutinin lymphocyte stimulation

The trend of lymphocyte stimulation with PHA resembled that due to Con A which stimulated slightly more than the former mitogen (Fig. 5. 1i; Tables 5.42, 5.43, 5.44).

The stimulation indices of lymphocytes from acutely

infected monkeys decreased dramatically. Also a significant ($P<0.05$, at least) reduction occurred in the absolute dpm values by PID 8 compared to those from chronically infected monkeys. These were moderately and significantly ($P<0.05$) reduced compared to the controls, for the 96-hour cultures. The lowest stimulation indices were obtained on PID 134 followed by those on PID 148 and 71, whether the cultures were terminated at 72 or 96 hours.

The responses of lymphocytes from chronically infected animals were significantly ($P<0.05$), reduced on PID 8 - 92, 113 and 127-155 and on PID 8-64, 92 and 127 - 155 for the 72-and 96-hour cultures,respectively. The lymphocyte responses to various mitogen concentrations within and between 72- and 96-hour cultures for the acutely and chronically infected animals were, each, statistically identical.

Similarly, the recovery of the PHA stimulation indices to the control level did not occur despite the disappearance of the parasitaemia from the blood. These results strongly suggest that the apparent cure or the absence of parasites from the blood was not associated with recovery of the *in vitro* immunological responses. This is because although the trypanosomes were absent from the blood of 4 out of 5 animals at the end of the experiment the *in vitro* immunological responses to Con A and PHA were still depressed.

(4) Humoral immunity

(a) Immunoglobulins M (IgM) and G (IgG)

The sequential changes of serum IgM and IgG

concentrations in the infected and control monkeys are shown (Figs. 5.1ii. 5.1iii; Table 5.45, 5.46).

In the control vervets the immunoglobulin concentrations undulated within the normal values (79) of around 1 and 10 mg per ml for IgM and IgG, respectively. Meanwhile in the acutely infected animals the above values changed from 1.71 ± 0.65 to 3.23 ± 1.12 and 14.66 ± 2.71 to 14.22 ± 3.22 mg/ml, respectively, by PID 8. In contrast,in the chronically infected monkeys the increase in immunoglobulin concentrations could still be related to the course of infection in each individual animal (Tables 5.45; 5.46). The degree of response varied between animals. For instance, in monkey no. 39 the IgM increased progressively to a maximum of 34 times the pre-infection levels of PID 57 compared to a mean of 12 times in the chronically infected monkey group on PID 26. Also in the chronically infected animals, the pre-infection serum IgG rose 4-fold from 13.78 ± 1.42 to 59.69 ± 28.97 mg/ml by PID 57, and returned to its original concentration by PID 85 which did not increase further subsequently.

DISCUSSION

In the vervet monkey-*T. brucei* model in this experiment several observations have been made. Many of these, including the susceptibility of the vervet monkey and the characteristic parasite-dose responses associated with the acutely overwhelming lethal 1×10^8 trypanosomes and chronically self-limiting 1×10^4 parasites of Clone 227, anaemia and numerical cell changes, statistically confirm earlier results in Chapter 3. The results of the *in vitro* cell-mediated immune correlate tests involving mitogenic and xenogeneic (CLA_4 cells) mixed lymphocyte culture stimulation of lymphocytes from uninfected control monkeys confirm their reproducibility, as reported in Chapter 4 above, while those from infected animals indicate that these parameters alter during infection.

In *T. brucei*-infected unlike in the normal uninfected monkeys the baseline cell-mediated immune correlates changed as the infection progressed. Hence, immune responses, death, immunodepression, immunological aberrations, the trypanocidal drug effect and self-cure became evident during infection and are discussed.

In this experiment early enhanced and subsequently depressed T cell responses occurred and were supported by *in vitro* analysis of peripheral blood lymphocytes and their subsets from, and *in vivo* skin test of, monkeys infected with 1×10^8 or 1×10^4 *T. brucei* parasites. Early, by PID 5 but not PID 8, in infection an enhanced or a normal cell-mediated immunity occurred and was associated with slightly raised stimulation

indices from mixed lymphocyte cultures and Con A, but fairly normal PHA stimulation indices. The highest stimulation indices were obtained with the cells from the treated chronic but not from the acutely fatal T. brucei-infected or the uninfected control monkeys. Thereafter from PID 8 the T cell in vitro xenogeneic (CLA_4 cells) MLC, Con A and PHA correlates of cell-mediated immunity (58) in infected monkeys were depressed. Similar early, before PID 5, enhanced humoral and cellular immunity occurred in T. brucei- (166, 167) and T. congolense-infected mice (291), respectively. Also identical subsequent cell-mediated immunodepression was reported after PID 7.5 in T. congolense-infected mice (319, 320).

In the infected monkeys early, severe and progressive leucopenia, lymphocytopenia, neutrocytosis and monocytosis coincided with patent parasitaemia and persisted until the monkeys died of 1×10^8 trypanosome challenge. However, these cell changes were transient in the animals chronically infected with 1×10^4 parasites and either treated with Berenil or self-curing. Thus they confirm results in Chapter 3 above when almost similar doses of the same Clone 227 were inoculated into monkeys. This is unlike in T. brucei-infected cattle (Zebu) (247) in which fairly normal leucocyte counts, lymphocytosis, neutropenia and slight monocytosis, and in those with T. theileri in which leucocytosis and lymphocytosis occurred. However, they closely resemble those in T. congolense-infected Boran cattle in which leucopenia, lymphocytopenia and almost normal neutrophils and monocytes (350) were associated. Yet, in

T. rhodesiense-infected *Macaca mulatta* leucocyte changes were equivocal (326).

During the lymphocytopenia the early slightly elevated erythrocyte rosette per cent in both infected animal groups decreased slightly during chronic infection. Meanwhile the absolute erythrocyte rosette numbers decreased significantly and persisted thus until the animals died of acute infection. However, proportionally and numerically the erythrocyte rosettes returned to normal after treatment and during self-cure of chronic infections. This is unlike in the mouse situation in which the spleen erythrocyte rosettes were numerically normal but diluted by increased null cells during *T. congolense* infection (320).

Berenil treatment killed trypanosomes and released lots of trypanosome antigens. The increase in SmIg cell levels early in infection and soon after treatment suggests that the sessile B lymphocytes were initially massively produced, in response to either the trypanosome antigens or the trypanosomes' adjuvant effect, and recruited from the primary lymphoid organs. They appeared raised in the blood as they migrated to the secondary lymphoid organs.

The percentages and absolute numbers of null cells initially significantly decreased but later slightly increased until the animals died of acute trypanosomiasis. In the treated and self-cured animals the numbers but not the percentages decreased significantly. This contrasts the raised null cell numbers in the spleens of mice infected with

T. brucei (237, 246) or T. congolense (319, 320)

In this experiment all monkeys in the 2 groups responded to DNCB although to a lesser degree in the infected than in the control animals. Thus the skin responses to DNCB were not completely depressed but could still be elicited to a certain extent. This has been shown and confirmed by some (4, 130, 374, 379, 380) but not by other (230, 231) workers using all sorts of African trypanosomiasis models and skin sensitizing agents including oxazolone, DNCB and trypanosome antigens to elicit skin reactivity. Still these results have been regarded equivocal even for tissue parasites like the human T. gambiense because secondary immune reactions to DNCB were depressed in some infected individuals and not in others (130). Also in rodent T. brucei models responses to oxazolone were not depressed implying that skin reactivity is probably determined by the parasite, the host and the phase of infection as well as by the sensitizing agent.

All monkeys in the 2 groups responded to tetanus toxoid given 2 days before infection but the mean levels of anti-tetanus antibodies were higher in the controls than in the infected monkeys. The latter animals initially showed enhancement and elevation of IgM antibodies (SmIg, B cell responses and counts) while IgG antibodies (T-B lymphocyte co-operative functions) were depressed. Yet, early leucocytosis or lymphocytosis was not evident. Similar findings were also reported (166, 167) when sheep erythrocytes were given around the time of T. brucei inoculation into mice.

Immunological enhancement occurred in the infected monkeys in this experiment. The early enhanced or fairly normal cell-mediated immunity which occurred with the mixed lymphocyte cultures, Con A-and PHA-lymphocyte, *in vitro*, stimulation was probably due to unaltered or slightly reduced absolute background dpm in lymphocytes from infected monkeys. It was associated with the early slightly increased erythrocyte rosettes and humoral immune responses during the first week of infection. It is possible that enhancement by PID 5 but not PID 8 was a normal cell mediated immunity response against, and to expel, the parasites. It was probably also due to a slight adjuvant effect of killed trypanosomes on the host's humoral immune responses. However, the term enhancement has been used to describe such a situation probably because the trypanosomes are expected from the very moment of inoculation in animals to induce immunodepression and not normal immune responses.

In monkeys infected with 1×10^4 *T. brucei* parasites total, and heterophile IgM and IgG antibodies were, respectively, significantly and slightly raised. Pronounced IgM and IgG hyper-immunoglobulinaemia are the major serological features of mammalian African trypanosomiasis (278, 372, 382) including of man (128, 129) and cattle (193, 210, 262, 280). Polyclonal hyper-immunoglobulinaemia probably occurs after antigenic stimulation of B cells (129, 166) but the nature, quantity and specificity (340, 372) of the elevated IgM and IgG antibodies

produced for the parasite moieties are unknown and this was not determined in this experiment.

During the estimation of anti-tetanus antibodies in this experiment non-sensitized sheep erythrocyte agglutinins (heterophile antibodies) in sera from infected vervets were absorbed using tanned sheep erythrocytes while from infected cattle (159, 262) trypanosomes were used for absorption. This indicates that trypanosomes can induce heterophile antibody responses due to non-specific stimulation of B cell heterophile antibody clones. Also the multiplicity of antibodies to other unrelated antigens in African trypanosomiasis (192) suggests an extensive cross-reactivity of the many parasite antigens released by dying organisms. Moreover the production of massive antibodies against various determinants such as heterophile, antiglobulin (rheumatoid-like factors) normal tissue (autoantibodies), antigens/haptens and vaccines (159, 191, 229, 381, 382) unrelated to the eliciting trypanosome could result from an autoimmune component of the response. Probably, hyper-immunoglobulinaemia during infection is due to low affinity and cross-reacting antibodies to T. brucei.

Similar high non-trypanosome -specific IgM antibody levels were reported in monkeys (159). In contrast, the elevated levels of IgM and IgG antibodies produced particularly during the first 3 weeks of cattle trypanosome infection were parasite specific. This specificity was evidenced by increased neutralizing capacity of IgG1 antibodies from the second peak which resulted from an anamnestic response following a

re-appearance of trypanosomes with variable surface glycoproteins (VSGs) similar to those of the infecting clones (267). The difference is probably due to the fact that monkey hosts and also a few instead of multiple variable antigen types (VATs) for absorbing the monkey sera (159) were used. Moreover, the cattle sera for absorption were selected for as the infection progressed animals responded to internal antigens making complete absorption of the antibodies more difficult.

The significance of the raised heterophile antibodies in trypanosomiais is unknown apart from strongly suggesting that immune responses during infection are selectively and not completely depressed. No studies were undertaken to determine whether the heterophile antibodies raised throughout infection were produced repeatedly during the course (166, 167) or only at the beginning, of infection. However, the production of IgM but not IgG heterophile antibodies which were abrogated by Berenil strongly suggests repeated production during the infection. This also strongly implies an association between heterophile antibodies and variant specific surface trypanosome antigens, only that they are probably presented at different angles to the various Ig receptors on the antigen-sensitive cells (267). Probably IgM heterophile antibodies were associated with self-cure seen in the T. brucei-infected monkeys and other model systems in which the two phenomena occur. This is because heterophile antibodies have not been reported in mice and rats which rarely undergo self-cure against African trypanosomiasis. Yet, antibodies to other

unseen (not inoculated), including TNP, antigens have been reported in T. brucei infected mice (166, 167) and in primate trypanosomiasis (159). Cross reactivity between one antibody and several other unrelated antigens but having similar antigenic determinants is documented and might be involved in self-cure in T. brucei-infected monkeys. There is a definite need to research more on the dynamics, specificity and significance of heterophile antibodies in trypanosomiasis and other diseases in which they are produced.

Generalized non-specific cellular (T cell) and humoral (B cell) immunodepression occurred in T. brucei-infected monkeys when immune functions were measured by in vitro DNA synthesis to stimulation with Con A, PHA and xenogeneic CLA_4 cells, by the skin responses to DNCB and by antibody responses to tetanus toxoid. Immunodepression was incomplete (partial) as residual cell-mediated immunity was evident; it was real and not an artifact and was associated with the presence of parasitaemia as it was abrogated by Berenil therapy which also cleared the parasitaemia.

The depression of T and B lymphocyte functions was established by PID 11. It paralleled detectable parasitaemia (representing 1×10^4 parasites per ml blood) which coincided with numerical lymphocyte subpopulation changes. Early in infection it affected the expression but not the induction of humoral and cellular immunities because the testing heterologous tetanus toxoid and the DNCB were given 2 - 4 days before infection so that when the trypanosomes were inoculated the

immune responses were fairly well established. Similar results of depressed T and B lymphocyte functions and expression but not induction were also reported in humans with T. gambiense infections (130) and in T. brucei -(252, 257, 258) and T. congolense - infected (291, 292, 319, 320) mice.

In this study T. brucei- infected monkeys showed cellular immunodepression which was progressive and relentlessly severe in acute infections, moderately severe and persistent in self-curing chronic diseases and transient in chronic Berenil-treated infections.

Depressed total serum IgM or IgG concentrations were neither seen in the monkey-T. brucei model nor have they been reported in other models. In this model, whether following treatment or self-cure, the high serum IgM levels were sustained for sometime above the control values, an observation also made by Fink (106). The raised IgM levels are probably due to trypanosomiasis-associated massively produced and persistent antigenic serum 7S IgM. This probably exaggerates the total IgM levels when measured by the Mancini technique if a normal serum standard containing only 19S IgM is used because the 7S diffuses faster than the 19S IgM.

Depression of the primary and secondary immune responses to tetanus toxoid occurred in T. brucei-infected monkeys, re-challenged 30 days after primary immunization (that is, on PID 28). The severest immunodepression in the T. brucei-infected group occurred on PID 20 (22 days after primary immunization). The immunoglobulin classes involved

were IgM and IgG and a T. brucei infection due to 1 x 10^4 parasite had a slight immunodepressive effect on the primary and secondary IgM responses of infected monkeys to tetanus toxoid. The responses were statistically identical in both animal groups on, say, PID 26 and 40, (28 and 12 days after primary and secondary immunizations, respectively.

Since T. brucei significantly ($P<0.05$) depressed the primary and the secondary IgG response in infected monkeys from PID 11 (second week) through PID 20 and 33, respectively, and later following re-challege, it indicated that the trypanosomes were probably more depressive for IgG and T-B co-operative lymphocyte functions than for IgM as in T. congolense-infected mice (143).

Significant anti-tetanus IgG antibody depression occurred on PID 20 and 33 but not on PID 26 and 40 indicating that the depression fluctuated as the infection progressed. For example, serum antibody levels rose in both phases of the immune response when immunodepression was more pronounced. Immunodepression was minimal on PID 26 (28 days after primary challenge) when serum antibodies were lowest between the 2 phases of the immune response and probably representing a time when most cells were producing little antibody.

On pre-treatment immunity, delayed hypersensitivity to DNCB showed significantly ($P<0.001$) decreased mean diameter of skin induration in the infected monkeys 42 days after primary challenge. This suggested a depressed functional T cell expression before (during infection) and after treatment for

DNCB responses were still depressed though treatment had controlled the infection.

A similar type of immunodepression is well established in acute, mild and severe progressive chronic trypanosomiasis of humans and animals in which reduced humoral responses to heterologous Salmonella typhi vaccine and, delayed hypersensitivity reactions to DNCB, oxazolone, PPD (tuberculin) (130) and to in vitro stimulation of lymphocytes with Con A, PHA, LPS and allogeneic MLC (291, 292, 319, 320) occurred.

In this experiment mechanisms of immunodepression were not studied. However, the immune responses can be inhibited either peripherally if the effector functions are interfered with or centrally if the distribution or the numbers of any of the cell types involved in immune responses are altered or if the reaction of the cells to antigens during their interaction with other cells is defective.

Trypanosomes were associated with immunodepression which was abrogated when parasites were killed by the trypanocide, Berenil. However, immunodepression was not eliminated by self-cure. Higher parasitaemia was associated with low stimulation indices and depressed anti-tetanus antibodies, and therefore severe immunodepression. How the trypanosomes caused immunodepression of tetanus toxoid and DNCB given intramuscularly and topically, respectively, is not known. The antigens were dead and hence they could not replicate to provide, continuously, antigens and stimuli to overcome antigenic dilution which possibly causes

immunodepression (252, 257, 258). Although the depression against tetanus toxoid and DNCB was significant this experiment has not shown that antigen dilution did not cause immunodepression.

The immunodepression of potentially effective humoral and cell-mediated immune responses against the trypanosomes were probably a direct result of the organisms' depressive properties because where such responses existed and were fully effective the infection was slowly controlled. Animals inoculated with 1×10^4 trypanosomes apparently controlled the resultant chronic infection. However, the cell-mediated immunity parameters were not restored to normal due to persistent immunodepression and relapsing parasitaemia, at least in one of the "self-cured" animals. In contrast, in the Berenil treated monkeys immunodepression was abrogated by treatment and was therefore temporary and transient. Although restoration of cell-mediated immunity parameters to normal took a long time, the parasitaemia did not relapse.

The stimulation indices remained low after the monkeys had purpotedly undergone self-cure. This strongly suggests that the so-called self-cure was not real but that parasites went into hiding in extravascular spaces, privileged sites or tissues and could not be reached by IgM and/or IgG antibodies for destruction. The parasites were probably released into the bloodstream from the hiding places from where they still exerted strong immunodepressive effects. This is suggested in this experiment by events in monkey no. 37 whose blood was aparasitaemic for 29 days but suddenly low grade parasitaemia,

leucopenia and depressed lymphocyte responses to mitogens occurred. A similar persistent immunodepression was seen in three vervets that had undergone self-cure one and half-years previously (77).

Depressed absolute erythrocyte rosette and null cells but normal SmIg cell numbers occurred in the infected monkeys. These results contrast those in T. congolense-infected mice (248) in which Ig^+ splenic lymphocytes decreased and "null cells" increased.

The Ig^+ lymphocytes are apparently the antigen-reactive cells (290) and were decreased probably by being pushed to plasma cell stage. A mitogen stimulates B cells whose division is probably limited to 10-12 times as they differentiate polyclonally into exhaustion. Also, probably a few antigen reactive cells are distributed throughout the body and particularly lymphoid tissues so that an antigen given once than repeatedly less likely meets the reactive cells. However, a more tangible explanation is required because the recovery rate (numerically and functionally) of Ig^+ cells from peripheral blood examination after Berenil treatment showed that they quickly recovered in the T. brucei-infected group. Recovery changes after Berenil are probably determined by the trypanosome species, its pathogenicity and tissue distribution (323).

In this experiment the vervets' lymphoid organs were not examined histologically. Based on other model systems the lymphocytopenia and reduction of other cell subsets during leucopenia suggests involution and cellular derangement of the

primary and secondary lymphoid organs (50, 257, 320). This further suggests that immunodepression probably partly relates to altered T cell numbers and functions. Also B cell mitogens like the bacterial LPS cause involution in mice (427) The presence of B cell LPS-like mitogens (5, 19, 99, 222, 233, 252, 257, 258) in trypanosome homogenates and autolysates possibly subscribe to the thymus involution.

The stimulation indices of lymphocyte cultures from infected but not from uninfected monkeys after *in vitro* stimulation with PHA or Con A initially showed enhanced immune responses but subsequently, from PID 11, were strongly depressed. For example, by PID 20, 26, 40, 47, 54, and 75 the stimulation indices in the PBL from the infected than from the control monkeys were lower although the absolute dpm values were the same. This was not simply due to the background dpm counts being unequal between the control and infected monkey lymphocytes (that is, higher background dpm and identical but not reduced dpm from the infected animal lymphocytes). It reflected a true depressed response. By PID 26, 33, 40, and 61, the PHA and Con A responses were significantly ($P<0.05$) lower in the infected than in control animals while the background dpm counts were almost identical indicating that infected monkeys were immunodepressed.

Similarly the cells from infected monkeys stimulated in xenogeneic MLC showed limited or poor multiplication (responses) compared with normal control lymphocytes. This resembles a situation when the H-2 identical and Mls different allogeneic spleen cells from *T. congolense*-infected mice in 2 different MLC

systems were used (291). In the MLC, the responders are normally a T lymphocyte sub-population stimulated by certain Ia antigens on the B lymphocyte surface (263). The reduced responsive capacity of lymphocytes from infected monkeys during severe depression of MLR is not due to the absence of stimulator B lymphocytes assuming that the stimulator CLA_4 cells carry the relevant Ia antigens and at a constant concentration. The reduced responsiveness, however, could be due to lower numbers of potentially responsive T cells in the MLC as only about 50% T lymphocytes were present in blood from the infected monkeys.

Although cell-mediated immune responses were more depressed in the acutely than in the chronically infected monkeys the mean absolute dpm were statistically identical. Low stimulation of lymphocytes from infected monkeys was obtained with each of the three PHA and Con A, dilutions from PID 8 and for several days after treatment and throughout self-cure. Meanwhile increasing the mitogen concentration or culture time did not break the immunodepression nor represent a shift in the optimal mitogen concentration or culture time required for lymphocyte stimulation. However, possibly lymphocyte dynamics in the cultures changed probably due to a reduced T cell density by increased death rate in vitro.

These results resemble those from other animal-trypanosome models in which lymphocyte unresponsiveness was not due to a shift of mitogen doses, inability to respond in lymphocyte transformation or length of time required for stimulation (291, 292, 319, 320). However, they contrast those

in which immunodepression was not due to reduced T and B cell numbers (319, 320) despite the changes in the spleen cell populations in the T. congolense- and in the T. brucei-infected mice (237, 246). Also in these other models immunodepression was not antigen-specific, not H-2 restricted to respond in MLC, not due to an intrinsic defect of B or T lymphocytes but was actively caused by suppressor cells because Berenil treatment of infected mice rapidly cleared the parasitaemia, reduced the suppressor cell activity and restored to normal the immune reactivity (258, 320). Other possible mechanisms of immunodepression have been mentioned elsewhere. However, it is unlikely that the trypanosomes like schistosomes (421) covered themselves with host antigens to avoid recognition.

Skin reactions are cellular in type. In Africans and monkeys multiple infections occur compounded by malaria and particularly malnutrition which also depress cell-mediated immunity (103, 275, 356). In this experiment infected but not control monkeys lost weight. Since loss of weight is a sign of malnutrition it is possible that this contributed to the cell mediated immunodepression in monkeys. Hence, skin reactions are depressed and sometimes not seen in indigenous people and possibly also in monkeys that are malnourished.

During active humoral immunodepression animals re-immunized before and after Berenil treatment responded anamnestically to tetanus toxoid suggesting the presence of memory cells. A weak anamnestic (memory) response to re-immunization with tetanus toxoid occurred before and after

Berenil treatment. However, the IgG response was significantly low in the infected animals on PID 33 (5 days after secondary tetanus toxoid challenge). Thus possibly the immunological memories were equal in both animal groups for IgM but not IgG antibodies. However, secondary immune responses from each monkey and on their own showed that the previously infected animals had a rapid and fairly high IgM and slow and low IgG secondary responses. Also infected than control monkeys treated with Berenil and immunized with tetanus toxoid 12 days later had seemingly normal or higher "tertiary" immune responses to the toxoid because the treatment abrogated the immuno-depression. These results suggest an adjuvant effect in previously infected animals, normal induction, poor expression of secondary immune responses and normal "tertiary" immune responses. This has profound implication in immunization campaigns against other diseases during trypanosomiasis epidemics.

Monkeys died of acute high 1×10^8 but not of chronic low 1×10^4 T. brucei-parasite challenge. Death was associated with trypanosomiasis but it was of unknown aetiology in the absence of secondary infections. In contrast, in cattle with African trypanosomiasis death has been associated with anaemia and severe myocarditis due to mononuclear cell infiltration of the heart muscle. In rodents too, a virulent T. brucei stock kills within 5 days with terminal parasitaemias of up to 1×10^9 trypanosomes per ml blood. Speculatively the rapid death of these animals must be due to the immediate effects of

trypanosomiasis, probably associated metabolic disturbances and comparable with exhaustion of a nutrient medium, as in the early stages of T. rhodesiense infections of man. Hence in man pronounced clinical symptoms without important histopathological lesions occur. This contrasts the complicated interactions resulting from the many different antigens that during infections intermittently enter the circulation and cause the important long term effects of the disease.

African trypanosomiasis is commonly associated with immunological aberrations (372) which also occurred in the T. brucei-infected monkeys. This was evidenced by massively increased total IgM and IgG and IgM (but no IgG) heterophile antibodies; depressed generalized non-specific humoral immunity as reduced IgM and IgG anti-tetanus antibodies and cell-mediated immune functions as reduced responses to in vivo DNCB skin sensitization and in vitro cell-mediated immune correlates by stimulation of PBL by T cell mitogens Con A, PHA and xenogeneic MLC. Similar immunological aberrations were reported in T. congolense-infected mice (291), in T. rhodesiense - infected Macaca mulatta (159) and T. brucei - infected rabbits (10, 219) The significance of immunological aberrations is unknown

Previous studies on the effect of trypanosomiasis on the immune responses of mammalian hosts were sometimes inconclusive because of inadequate controls or lack of uniformity in assessing immunodepression. Also interpretation of the results was difficult as often serial control studies were not performed and patients and animals were not allocated at random for

infection. Therefore possibly the apparent immunodepression was due to temporal technical variation compounded by subjective allocation of animals with severe immunodepression.

Most studies on cellular immunodepression during trypanosomiasis need cautious interpretation because cells from infected animals are always contaminated with trypanosomes and their products, none of which can be removed satisfactorily. Enumerations of B and T cells are always problematic. In this study lymphocyte subpopulations were identified by surface membrane immunoglobulins and sheep erythrocytes which in the vervets probably serve as makers for some T and B cells, respectively, and are putative antigen-sensitive T and B lymphocytes. However, no convenient T and/or B lymphocyte markers have been described for the vervet as have been for the mouse (292), the human (181) and for the bovine (293).

Lymphocyte receptors for sheep erythrocytes are easy to work with but they are difficult to standardize. Similarly specific T cell antisera are difficult to prepare and standardize and the results from different laboratories cannot be compared with easily. Receptors for autologous erythrocytes exist but heighten cell values while receptors for in vitro work with measles also exist; however, in Africa virus laboratories are very few to act as supply houses.

Similarly, enumeration of viable B lymphocytes by immunofluorescence staining technique for the surface membrane Ig have set-backs associated with binding of IgG due to Fc receptors, binding aggregated Ig independent of Fc receptors and

non-specific staining of non-viable cells with antibodies like antilymphocyte antibodies directed against membrane antigens. Also monocytes have attached Ig on their surface receptors for IgG binds onto monocytes and in this experiment the monocytes were significantly raised. Similarly, activated T cells sometimes have cytophilic Ig attached on their surfaces and it is possible that they also immunofluoresce during the test for SmIg cells. Some of these set-backs can be avoided by washing the cells several times and by staining cells with pepsin-treated antisera that lack the Fc piece. In the experiment reported here cells were washed several times before staining them ordinarily with a conjugate that was centrifuged fast before use. Finally, erythrocyte rosette and surface membrane immunoglobulin tests are static and not functional.

Expression of results is equally confusing because the same parameter, for instance, the erythrocyte numbers but not per cent was depressed in this experiment.

Most of the studies on cellular immunology of rodents have involved spleen cells and only very few have employed isolated peripheral blood lymphocytes as in this study. Comparison of such results sometimes only creates confusion.

Immunodepression has been intimately associated with several human and animal protozoal, helminthic, viral and bacterial infections (178, 320). Its nature in these diseases is ill-defined but probably involves, partly humoral and/or cell-mediated immunodeficiency. Further, it will be interesting to see if common mechanisms function in the immunodepression seen

in these and trypanosome infections.

Immunodepression due to African trypanosomiasis has profound implications. The control of trypanosomiasis which causes massive economic loss could open up large areas of land for grazing livestock and agriculture. Immunodepression in human (129), bovine (155, 323) and ovine (136, 222) trypanosomiasis is very important in Africa where vaccination campaigns to other diseases are essential for human survival and successful development of livestock industry.

Multiple infections are very common and severe in tropical children and adults. Immunodepression is clinically important because infection with one parasite increases susceptibility to others. In the mammalian host trypanosomiasis causes immunodepression which enhances trypanosomiasis itself and also unresponsiveness to foreign antigens (130, 156, 256). In this way economically and medically trypanosomiasis interferes with, and depresses the response to, immunisation such as, in Trypanosoma gambiense - infected humans (130) and T. congolense - infected cattle (156) with Salmonella typhi and clostridial vaccines, respectively. Therefore, it is recommended to avoid conducting vaccination compaigns in patients during trypanosomiasis epidemics (1, 2, 3). Similar conclusions were reached in children with malaria (239) and T. gambiense (130).

Immunodepression tends to increase the susceptibility of both humans (135) and animals (104, 252, 257, 258, 387) to secondary bacterial (130, 156, 186, 208), viral (1) and

protozoal infections (114), interferes with resistance to and reduces the expulsion of Nippostrongylus brasiliensis (110, 386) and Trichuris muris (301) from rodents. It also prolongs the normally self-limiting murine P.b. yoelii (76) as well as activates latent malaria and tuberculosis (135). Meanwhile through immunodepression streptococcal infections in humans (135, 298) and sheep (168, 222) hasten the prognosis of the trypanosomiasis. The predisposition to secondary infections is thus another way by which the trypanosome exerts its pathogenicity (156).

Also the incidence of neoplasia might be raised in trypanosome-infected (1) just like in animals (327, 397) and humans (78) with viral lymphomas in which malaria facilitates the proliferation and metastasis of malignant cells.

Yet, autoimmune diseases have low prevalence and are rare in the tropics where multiple parasitaemias are widespread (128, 134). This is probably because the depressive effects of parasitic infections such as malaria and also possibly trypanosomiasis on the immune responses of local populations interfere with abnormal immunological processes involved in autoimmune disease production (128) as in experimental NZB/NZW F_1 mice (134).

Also although many features of immunodepression remain similar the degree varies. For example, it is too slight to impair vaccination (323, 331, 350). The clinical or veterinary importance will therefore vary with the host-parasite combination (63).

Although the humoral immune response to some antigens is impaired during trypanosomiasis antibodies active but less protective functionally against the parasites can still be produced. This selective form of immunodepression in protozoal infections possibly reflects how the parasite manipulates the immune response of the host so as to prevent the formation of antibodies with a deleterious effect on its growth and reproduction (130). In malaria the immunodepression is selective so that certain cellular immune responses remain normal (130).

Altered immune responses to numerous heterologous antigens due to repeated exposure of the hosts (10, 231, 257, 258) such as, cattle (156) and sheep (222) results in persistently immunodepressed animals. These are possibly not affected by the immunologically mediated anaemia and myocarditis (195, 252) and thus survive despite the high parasitaemia. The antibody is effective against the intravascular T.congolense and T. vivax and the extravascular T. gambiense parasites.

Restoration of the immune responses after trypanocidal therapy is probably important in areas endemic for trypanomiasis and the immunological memory temporarily suspended by trypanosomiasis is re-established after therapy (252, 257, 258).

Compared with other infections African trypanosomiasis affects many aspects of the immune response, so results obtained in studying this disease could probably indicate the various immunodepressive mechanisms possibly operating in others.

Berenil treatment on PID 44 (16 days after secondary tetanus toxoid immunization) eliminated the trypanosomes within 24 hours and the anaemia after sometime. It also restored the humoral immunity fast, within 3 days, and the cell-mediated immunity slowly, over 30 days.

The removal of parasites by the trypanocide and the subsequent normal humoral immune response suggested specific association of immunodepression with the available live trypanosomes (111). Similar relationship between fluctuating parasitaemias and immunodepression has been shown in goat T. congolense (136), cattle T. congolense or T. vivax (405) and in rodent T. brucei (252, 257, 258) infections. Concurrent trypanocidal therapy and immunization led to considerable recovery of the immune competence of the affected animals. Like in vervet monkeys T. brucei-induced immunodepression in mice persisted for the duration of infections as the parasitaemia cyclically altered (67, 96, 179, 257). However, the suppression of experimental allergic neuritis in rabbits was apparently not related to peak parasitaemias (10). Yet, parasitaemia and immunodepression could not be correlated (174) while the role of antigenic competition in immunodepression was doubtful when trypanosome antigen load was very low. In monkeys parasite elimination by Berenil did not immediately abrogate suppression in vitro or in vivo. This agrees with the mouse situation in which spleen cells from infected mice but also treated with a trypanocide did not respond well in vitro (96). In contrast, Berenil treatment rapidly cleared the

parasitaemia, concurrently abrogated the immunodepression and suppressor cell activity, restored to normal the cellular composition and allowed repopulation of the lymphoid organs (319, 320). This indicates that immunodepression in monkeys like in mice requires, continuously present high parasitaemia of live, virulent, multiplying, and probably antigenically varying trypanosomes as well as, immunological and inflammatory responses (248). These cannot produce normal humoral and *in vivo* skin tests or *in vitro* correlates of cell-mediated immunity to stimulants (231).

In the infected monkeys a single Berenil treatment numerically shifted the leucocytes and the various lymphocyte subpopulations towards normality. The SmIg cells, after a slight increase, and the reduced pre-treatment ER (T) and null cells numerically returned to normal, all within 10 days of therapy. Also in most cases the treatment improved the depressed *in vitro* lymphocyte-mitogen transformation responses to Con A, PHA and xenogeneic MLC and the *in vivo* skin DNCB responsiveness slightly in previously infected animals. These recovered, about thirty days after treatment, just like the responses in all functional assays and the lymphocyte subpopulations. The PHA responses recovered early followed by MLC and outlasted by Con A cultures indicating stimulation of probably slightly different lymphocyte subpopulations by each mitogen.

Treatment had neither adverse effects on the total serum Igs, anti-tetanus antibody responses after re-challenge

and cellular immunity parameters nor did it alter dramatically the levels of IgM and IgG heterophile antibodies. However, it improved the responses to tetanus toxoid and of the lymphocytes to transformation in the previously infected monkeys. The levels of anti-tetanus IgM and IgG antibodies suddenly, within 3 days of treatment, increased substantially.

The anti-tetanus IgM antibody levels in the two animal groups all re-immunized 16 and 12 days before and after treatment, respectively, increased or became statistically identical. However, in the previously T.brucei-infected animals the anti-tetanus IgG antibody levels were even higher than in the control monkeys after treatment.

The cause of the increase in humoral immune responses after Berenil treatment is unclear. It could be because the immunodepression was abrogated by the trypanocide. Also the enhanced IgG anti-tetanus antibody response following Berenil treatment suggests that B cell clonal exhaustion in the infected monkeys as in mice did not occur for these cells numerically increased. However, it is possible that the suspended memory was re-established or that the cells were released from the suppressive effect of T. brucei. Also probably Berenil therapy releases previously hidden trypanosomal antigens and B-cell LPS-like mitogenic factors which have an adjuvant effect and/or that the increase in B lymphocytes is a response to common or to cross-reacting antigens.

The origin of the adjuvant effect was not investigated in this experiment. However, trypanosomal autolysates (19)

and homogenates (99) activate polyclonally mouse B cells *in vitro*. The B cell mitogens like PPD, *E. coli*-LPS, dextran sulphate and polyacrylic acid have an adjuvant effect on the immune response to SRBC depending on the relative time of administration of the mitogen and the antigen. An antigen administered 30 minutes or 4 days after LPS injection was associated with an adjuvant or an immunodepressive effect, respectively.

The association between humoral immunodepression, rapidly restored by Berenil, and living trypanosomes could be via substances secreted by these parasites. It could also be by the parasites themselves *per se* or an essential nutrient for trypanosomes and for the antibody production which these parasites and the immunocompetent cells compete for. The substance would be short-lived, unstable and continuously produced by the trypanosomes which when removed it also vanishes. However, this was not so in mice because such components would have circulated to all lymphoid organs while immunodepression was much more severe with the spleen than with lymph node lymphocytes (320). Yet, lymphocyte sub-populations quantitatively differ in different lymphoid organs and hence possibly the different responses.

The rapid recovery of humoral immunity in monkeys following a trypanocidal therapy resembles that seen in *T. brucei*-infected mice (258). However, the slow recovery of cell-mediated immunity correlates in the *T. brucei*-infected monkeys contrasts the immediate and rapid recovery of cell-

mediated immunity in T. congolense-infected mice (320).

T and B cell functions were much more easily induced into immunodepression from which the T cells took longer to recover following a single Berenil treatment. The delayed induction and the rapid recovery of B cells from immunodepression after treatment resembles immunological tolerance. The slow recovery of cell-mediated immunity has been ascribed to polyclonal activation of suppressor cells (67, 96, 174). These induce non-specific generalized immunodepression and take long to die off. This resembles low zone tolerance. There is need to re-examine immunodepression in the context of tolerance.

Six weeks after infection self-cure, in the absence of drug therapy, occurred in the monkeys in this experiment although in trypanosomes apparently infinitely numerous different VATs exist. It was probably associated with clinical monkey cure but not with complete parasite elimination and sterilizing immunity as in infections of T. congolense in N'Dama cattle, T. lewisi or P. berghei in rats, P.b.yoelii in mice and cutaneous leishmaniasis in man. In these systems resistance is species and stock specific. Self-cure has also been reported in parenterally and/or cyclically infected cattle with T. congolense, T.vivax, T. brucei (254, 255, 267, 403) or T. rhodesiense (404); goats and sheep with T. congolense (136) and vervet monkeys with T. brucei or T. rhodesiense (17). It also occurs in T. gambiense-infected human patients (14, 15, 288) although in T. rhodesiense-infected humans (286) only healthy carriers have been reported.

The mechanisms of self-cure in the absence of a trypanocide or previous immunization by homologous organisms are unknown. It has been suggested that a few potential or more common antigenic variants than others probably exist and certain hosts probably meet, through re-appearance (267), the same variant twice, undergo self-cure and survive like T. congolense-infected cattle (35, 200, 267, 269). Through re-appearance of homologous (the same or identical) VATs (35, 200, 262, 267, 269, 336) or through the VATs-specific anamnestic response the neutralizing capacity of IgG1 antibodies against homologous serodeme improved as the infection progressed.

In the T. brucei-infected monkeys IgM and IgG antibodies were raised. Since self-cure occurred in this model system a special mechanism probably involving the raised IgM and IgG antibodies able to lyse the trypanosomes occurred and must be unique in the monkeys with its "non-specific" (heterophile) Igs. In cattle IgM more than IgG antibodies efficiently kill trypanosomes through lysis particularly in the presence of complement (261) and prevent formation of serotypes (358). In contrast IgG1 more than IgM is probably efficient in the agglutination reaction and in protecting the host from homologous but not heterologous parasites. In cattle infection where trypanosomes do not re-appear IgM mainly eliminates the organisms in each parasitaemic wave (267, 258). However, where the infecting organisms re-appear (267) IgG antibodies probably eliminate the parasites. This is because only 4-8 and

200 µg of IgG antibody from the second and first peaks, respectively, kill 1 x 10^4 trypanosomes, for the second peak of antibody activity results from an anamnestic response (262). It is possible that this also occurred in T. brucei-infected monkeys.

The resurfacing of trypanosomes in the blood after self-cure would be against the attainment of effective and specific IgG antibody concentration in tissue (extravascular) spaces. IgG diffuses into extravascular spaces because of its low molecular weight unlike the large pentameric IgM which therefore cannot effectively reach the tissue T. brucei parasites lodging therein.

It is also possible that in the monkey unlike in the lower animal trypanosome models probably complement is neither quite depleted nor necessary for trypanosome lysis and elimination in the presence of immunoglobulins.

Cross-protection (253) possibly due to common or basic antigens conditions and allows more brisk secondary responses to subsequent variant (or hapten) antigens as in malaria (49). This is another possible self-cure mechanisms. In this model as well as in other primate-trypanosomiasis systems (166) large quantities of non-parasite-specific, including heterophile antibodies, were produced. It is possible that cross-reactions between trypanosome antigens and heterophile antibodies occurred. This is unlike in T. congolense- and T. brucei-infected cattle in which cross-reacting antibodies against heterologous trypanosome antigens

were insignificant (236, 262). Therefore cross-reactivity was an unlikely self-cure mechanism because the bovine IgM and IgG1 antibodies were specific to the infecting parasites (262). Yet, in the mouse T. brucei infections (341) the antibodies are non-specific and self-cure apparently does not occur. In man heterophile antibodies occur but their role is unknown. It is possible that self-cure depends on the host and the parasite.

In this vervet monkey model cell-mediated immunity occurred early although later in infection it was depressed. The existence of cell-mediated immunity is controversial in lower animal trypanosome models. It has been reported absent (230) or present (231) but depressed in infected rodents, as evidenced by in vitro and in vivo cell-mediated immune response, particularly when immunodepression is severest during acute infection. Cell-mediated immunity probably protected the host against T. brucei and T. rhodesiense (extravascular parasites) but not T. congolense (intravascular parasite) in infected rabbits (379) which developed only an immediate and not delayed hypersensitivity reaction (230). Yet, in this experiment during and after self-cure unlike after treatment, cell-mediated immunity did not seem to recover from depression. It is possible that the parasites could still exert their depressive effect from their hiding places.

Cells are probably effectors in protection against trypanosomes. The antibody-producing cells are important effectors of the protective immune response while thymic cells are responsible for protection and agglutinin production (360)

and for induction of the response (359).

Monocytosis occurred during monkey infection in this study. Macrophages engulfed trypanosomes (118) and their phagocytic homologous antigen was not impared in T. brucei - (118, 257) and T. equiperdum-infected (248) rodents but it was deranged for heterologous antigens. The peripheral blood monocytes from T. brucei- and T. congolense-infected cattle have very high phagocytic activity due to the in vivo activation of the mononuclear phagocytic system. This occurs during infection (276) and leads to emergence of receptor sites for IgG1 and possibly IgM on macrophages. The mononuclear phagocytic system acting in concert with VSG - specific IgM and IgG1 antibodies which induce adherence and subsequent phagocytosis of homologous trypanosomes by macrophages is probably the major mechanism of parasite elimination in the infected host (240, 276). Similarly, trypanosomes are phagocytosed and cytolysed by normal and irradiated macrophages in the presence of specific homologous T. gambiense (360, 362, 363) and agglutinating T. brucei antiserum (but not normal serum) (214), by a heat stable and non-complement dependent reaction involving macrophages, cytophilic trypanosomal antibody and T. congolense (253, 425) in a buffalo.

Cytotoxicity is another effector mechanism. It involves normal lymphoid cells and specific antibody against, say, T. dionisii (241), and is effective against extravascular (T. brucei-subgroup) but not intravascular (T. congolense and T. vivax) parasites. This is because extravascularly a wide range of potential effector cell types like lymphoid cells and

macrophages exist (252, 257, 258).

Cross-reacting antibodies and a primed and expanded macrophage phagocytic system just as in ovine and bovine trypanosomiasis probaly rapidly cope with new variants (252) while environmental factors, the age of the animal, husbandry, colostral antibody intake and genetic make-up of the animal are also important factors in self-cure. In the field (255) self-cure enables cattle to acquire complete immunity against blood-stream and metacyclic VATs of all the serodemes from different areas (169). Passively acquired immunity probably also helps certain cattle breeds to thrive in trypanosome endemic areas (255) without any trypanocidal treatment. N'Dama are more resistant to trypanosomiasis than Zebu cattle though this resistance is not absolute because some Zebu cattle too are resistant.

What happens in self-cure cannot be said from this study except for speculating that IgM and IgG antibodies some of which were specific anti-trypanosome were raised while monocytes (precursors of tissue macrophages) were also increased numerically. These together with the adjuvant effect of trypanosomes, probably operative during infection, and innate and/or acquired immunity must have acted in concert to eliminate the parasites, at least, from the blood-stream.

These experiments have, however, shown that the vervet monkey is a suitable experimental model for Trypanosoma brucei infections. Hence further studies should be extended to T. rhodesiense and T. gambiense infections.

CHAPTER 6

C O N C L U S I O N S

CONCLUSIONS

The aim of the investigations reported in this dissertation was to set up a model of *T. brucei* in the vervet monkey so as to mimick the situation in man infected with the closely related and morphologically identical *T. rhodesiense* or *T. gambiense* and then to compare the immunological responses in the infected and uninfected control animals. The susceptibility of the vervet monkey, the parasite-dose responses, the reproducibility of the optimized *in vitro* cell-mediated immune correlates and the changes of these correlates during *T. brucei* infections clearly indicate that the vervet monkey is a good model for *T. brucei*.

In this exercise the monkey was first tested for its susceptibility to needle challenge with *T. brucei* parasites and then its response to different parasite doses, that is the dose-response (titration) of the selected parasite. In order to effectively and critically interpret the immune responses of an infected monkey, *in vitro* cell-mediated immune correlates of lymphocyte stimulation, DNA synthesis and transformation employing various non-specific mitogens and allogeneic or xenogeneic MLC of a normal uninfected vervet were also optimized. The final objective was to look at the immunological changes during *T. brucei* infection.

The results show that the vervet monkey was susceptible (17) to two stocks and a clone of *T. brucei*, all at an empirical 1×10^5 parasite dose. The stocks produced acute and subacute infections fatal within 2 and 6 weeks respectively

while the cloned parasite produced a chronic infection, self-limiting after six weeks. All the infected monkeys developed normocytic hypochromic anaemia evidenced by reticulocytosis and depressed haemograms typifying African trypanosomiasis in man, rodents, ungulates and non-human primates (27, 117, 288, 326).

The anaemia was severest, progressive and persistent in monkeys dying of the acute T. brucei infection. However, in those dying of subacute disease the moderate anaemia was severest during the third week of infection and slightly remitting thereafter. These animals never recovered fully their haemograms which even terminally were still below the pre-infection values. In contrast, in the animals that self-cured the anaemia was mild and transient although the haemograms were also lowest during the third week of infection. Thereafter the haemograms rose as the anaemia remitted, quickly to partial recovery. This remission of anaemia was just like that seen in T. brucei-infected N'Dama cattle (259).

Leucopenia, and per cent lymphocytopenia, neutrocytosis and monocytosis also occurred.

Since the Clone 227 was self-limiting in the vervet monkey it was decided to see the effect of different parasite challenge doses. The high, intermediate and low parasite doses of 1×10^8, 5×10^5 and 1×10^3 trypanosomes produced, respectively, fatal acute (within 3 weeks), acute and chronic and subacute and chronic infections. Thus depending on the challenge dose acute T. rhodesiense-like and chronic

T. gambiense - like infections could be produced and be mimicked by T. brucei in the vervet monkey.

Some of the animals inoculated with 5×10^5 and 1×10^3 parasites acquired severe intercurrent streptococcal and staphylococcal infections which probably accelerated their courses and contributed to their deaths. This is very important in Africa where polyparasitism is very common and deaths from secondary bacterial infections have been documented in animals and humans suffering from trypanosomiasis. A mild self-limiting disease in the presence of another mild infection can become fatally severe (76).

In these experiments the parasitaemia lacked prepatency in acute infections in which it was relentlessly progressive and unremitting. However, it was cyclically undulating and the parasites disappeared from the blood in subacute and chronic infections except when their courses were modified by secondary bacterial infections acquired by the monkeys.

Severe, moderate and mild anaemia occurred in acutely, subacutely and chronically T. brucei-infected animals, respectively. It was abrupt, progressive and persistent in the acutely and subacutely infected monkeys but was chronically remitting.

The susceptibility of the vervet monkey to T. brucei suggests that, like Macaca mulatta, E. patas patas, the chimpanzee, common rodents and ungulates but unlike the baboon, the vervet has no innate resistance either as anti-brucei trypanosomal serum protein factors (372) or as cells which

would immediately kill the parasite and prevent the infection from "taking" or making a foothold in it. Since in the dose-response experiments no other animal (rodent, cattle, etc.,) serum supplements were given to monkeys either before, during or after inoculation of trypanosomes which were washed 3 times and resuspended in serum - free phosphate buffered saline glucose (PSG) pH 8.0 (the eluate used in trypanosome isolation on DEAE from rodent blood) indicates that serum supplements are not essential to help T. brucei infection take in the vervet monkey. This is unlike in other heterologous systems of cotton rat - T. (D) vivax (an ungulate parasite) or mouse - T. (H). lewisi (a rat parasite) (372) in which they are essential. Finally, because the trypanosomes were infective it confirmed that the passage of trypanosomes through the DEAE-cellulose did not alter their infectivity (371). However, it could not be ruled out that raising the T. brucei parasitaemia in mice before inoculation into monkeys was not like passaging the parasites through mice and therefore necessary for the infection of vervets. The implication of this is that innate susceptibility is probably associated with the parasite species and stock, challenge dose, passage through other trypanosome hosts as well as the breed, species, age, sex and serum components of the host.

Before inoculation the monkeys, some of which came from tsetse belts, were screened for infection by immunofluorescence test. Although they become infected when inoculated with T. brucei parasites this did not rule out their probable

previous contact with or exposure to T. brucei in the wild because immunity against trypanosomes is probably weak, short-lived and lacks memory (399). Also immunity against trypanosomes is probably not heterologous but homologous parasite -, species - and stock - specific, lacking cross-reactivity (253).

The immunological techniques and parameters for use in the vervet monkey - T. brucei model were standardized. The monkey like the human mononuclear cells can be separated from peripheral blood by the method of Boyum (46) and also be stimulated in vitro by non-specific Con A and PHA mitogens and by xenogeneic CLA_4 cells in the MLC. In addition, Con A and PHA can strongly and optimally stimulate lymphocytes in ten-fold dilute vervet monkey whole blood as in the other primate (human, marmoset and rhesus monkey) situations. However, LPS, a B cell mitogen, and allogeneic vervet monkey lymphocytes cannot stimulate significantly peripheral blood lymphocytes because B cells are few in the peripheral blood. This is unlike with the B cell-rich mouse spleen cells in which therefore significant stimulation with LPS and allogeneic cells occurs.

The optimal cell, medium, and foetal calf serum concentrations and the pulse and harvesting times were all determined for mitogen-stimulated cultures and for MLC. These parameters closely resembled those from the human and it is possible that these non-specific mitogens (stimulants) stimulate

the same sub-populations of vervet monkey lymphocytes as in man and the mouse. Because of the reproducibility and the closeness of these results to the human situation and the susceptibility of the vervet monkey to T. brucei, a vervet monkey - T. brucei model using some of the cross-reacting human reagents was set up.

The vervet - T. brucei model re-confirmed the monkey's susceptibility to needle challenge, the parasite dose-responses, the characteristic trypanosomal patterns of parasitaemia, anaemia, death and self-cure.

Monkeys inoculated with 1×10^4 T. brucei parasites showed fluctuating parasitaemic waves with periods when trypanosomes were absent from the blood just like in humans suffering from sleeping sickness. In the self-curing animals the parasitaemic waves became smaller and infrequent as the infection progressed into the chronic stage while in the acutely (1×10^8 trypanosomes) and fatally infected animals the parasitaemia was relentlessly high and unremitting. The normocytic hypochromic anaemia, in severity and course, resembled that previously described above, parasite dose for dose.

The model also revealed aberrant responses of the immune system in the infected monkeys. These were evidenced by greatly raised IgM anti-tetanus antibodies, non-specific IgM but mildly raised IgG antibodies; initially enhanced in vitro cell-mediated immune correlates to MLC and Con A and IgM, but not IgG, anti-tetanus antibodies. This is like in humans who showed depressed anti-typhoid antibodies (130), in vitro cell-mediated

immune correlates and skin responses to DNCB but raised total Igs during chronic infection. Also evident was leucopenia associated with: proportional and numerical lymphocytopenia; proportionally fluctuating and numerically significantly reduced ER; initially raised proportions and numbers of lymphocytes bearing surface membrane immunoglobulins (Smlg), presumably B cells, which numerically fluctuated around the control values during chronic infections; initially significantly decreased proportions and absolute numbers of null cells which fluctuated up and down after treatment and during self-cure and; depressed skin reactivity to secondary DNCB challenge.

The initial enhanced immune responses were probably due to the animals fighting off infection. However, by the third week the responses were mostly significantly depressed. In the dying animals the cell mediated immune responses, just like the haemograms, were briskly depressed, early, during the acute phase of the infections. Since in these studies the low doses of PHA and Con A used to stimulate lymphocytes were depressed a helper T cell defect must have occurred. Also the antibody responses to tetanus toxoid, a T-dependent antigen were depressed. It is possible, therefore, that the monkeys that died of secondary streptococcal and staphylococcal infections did so as a result of a defective humoral T-B cell co-operation.

In the self-curing animals the immunodepression was severest during the first 5 weeks while the self-recovery during chronic infection was so slow that several days after the

parasites were last seen in monkeys' blood and even at the end of the experiment by PID 155 these animals were still immunodepressed, suggesting residual infections in probably privileged sites. Indeed, one monkey had a relapse of parasitaemia and leucopenia after the so-called self-cure. There is a definite need to research on self-cure using the ideal monkey-brucei model.

A single Berenil treatment of PID 44 killed the trypanosomes in the animals' blood, abrogated the immunodepression and restored to normal the humoral and B cell functional responses quickly, within 3 days, and the cell-mediated immune and T cell functional responses (Con A, PHA, MLR and skin reactivity to DNCB) and haemograms, slowly after about 4 weeks.

Thus T lymphocytes and their functions were immunodepressed faster and much more than B cells and also took longer to recover. The T. brucei clone 227 in the monkey was associated with immunological and haematological changes and was sensitive to Berenil while the animals could curtail, without treatment, a low parasite dose but not the immunodepression.

This is probably the first detailed report of immunological studies in experimental T. brucei - infected vervet monkeys. From the data the infections were apparently easily established in the unaltered vervets and were either acute fatal, subacute fatal or chronic self-limiting infection.

In discussing immunological changes in animal

trypanosomiasis extrapolations are sometimes made between different trypanosome species and between different hosts infected with the same parasite. It is most sensible to consider an infection with each trypanosome in each mammalian species as a separate entity until enough information has accumulated to decide which aspects of the pathogenic mechanisms are common to all of the infections (319, 320).

Common laboratory models of African trypanosomiasis involve rodents which are often more severely affected than man and domestic animals. In rodents and other animals virulent and lethal T. brucei stocks can rapidly kill, sometimes within 5 days, with terminal parasitaemia of up to 10^9 trypanosomes/ml blood without CNS or heart involvement or secondary infections but probably by metabolic disturbances comparable with the exhaustion of a nutrient medium.

However, lesions produced by parasites in man cannot be reproduced consistently in experimental animals. For example, meningoencephalitis, the most important aspect of human sleeping sickness and the heart lesions, can occur in monkeys but its development cannot be guaranteed either by selecting a particular stabilate or by using the same host species each time. They are produced by some but not all trypanosomes. Since the CNS lesions are immunopathological in origin in man the immune responses must be diferent from those in experimental animals.

Bar ethics and the fact that the vervet monkey, a primate, is evolutionarily closer to man than the mouse or the goat is,

the real evidence for the animal being a good and ideal model system for studying T. brucei infections came from the results of the experiments in this study.

The clinical picture and the pattern of T. brucei clone 227 parasitaemia in the vervet consistently resembled those of the human disease particularly with 1×10^3 - 5×10^5 parasite dose. In other animal models T. brucei stocks produce inconsistent infections very different from human ones. Unlike with inbred stocks of mice which are associated with parasite - dose unresponsiveness, monkeys showed parasite-dose responsiveness. A probable spectrum of human diseases from the acute rhodesiense - type to the chronic gambiense-type was produced and seen in monkeys depending on the size of the parasite dose of Clone 227. Of the high (1×10^8), medium (5×10^5) and low (1×10^3) parasite doses used the last 2 were around the 1×10^2 to 1×10^5 metacyclics, a range inoculated by a tsetse fly when it bites a host once. The implications of all these doses have been discussed in relation to the number of bites one or several tsetse(s) inflict(s) and to the epidemiology of the disease.

Anaemia in humans and animals during early and chronic infections and CNS involvement in chronic human trypanosomiasis are well documented. In this study all experiments revealed anaemia evidenced by reticulocytosis and decreased haemograms in early, intermediate and late trypanosomiasis of monkeys. The limb rigidity and posture changes in some animals with intermediate and chronic infections strongly suggest some

breeding of vervets in captivity under suitable surroundings and on a balanced diet, preferably in their native countries, in cages large anough for possible natural sex and age structure to produce physically and psychologically normal animals and thereby also reduce expenses to supply some of the over 250,000 monkeys required annually by laboratories for routine use; (vi) would be dependent on the present economic status of the Cercepithecidae as wild animals - like the legally protected crop-raiders (because of their scarcity) or less important forest confined, regular crop-raiders and agricultural pests, including the vervet monkeys, that are often used in research laboratories; (vii) would avoid animal death and wastage by combining dealers' efforts of trapping for export and authorities responsible for vermin control in agricultural areas; (viii) would lead to "game ranching" on land that is unsuitable for agriculture and cropping of monkeys just like cropping ungulates for meat, in keeping with stock-rearing; and (ix) that would call for basic research in ecology, breeding structure and natural population studies while the control mechanisms of wild monkeys and techniques of trapping, handling and transportation would completely raise the official economic position of wild monkeys from pests to valuable assets.

Thus the development of the vervet-human trypanosome model readily manipulated in the laboratory has much to offer investigators of human sleeping sickness, chemotherapy, the basic biology of parasites and infections. The only undermining factor is the continuing supply of vervet monkeys numerically

The model is particularly attractive because of the probable association of sleeping sickness with CNS involvement and because trypanosomes appear rapidly in all animals after inoculation. Many studies of these problems have been pursued using rodent, ungulate and few non-human primate parasites. They have yielded important and stimulating information. Comparable work with the human trypanosomes probably would yield information more relevant to the disease in man and would enrich the understanding of some aspects of drug resistance. Readily available sizeable numbers of putatively human T. brucei parasites in the infected vervet monkey will make such investigations feasible.

The possible mechanisms of anaemia, immunopathology, immunodepression and self-cure were not undertaken in this study but were mentioned (reviewed). The vervet monkey-brucei subgroup model offers opportunities for exploiting other as yet unresolved chemotherapeutical, pathological and immunological problems and for studying more basic biological phenomena including (1) the search and testing for new and more effective and better tolerated prophylactic and radical curative trypanocides or even vaccine, with the mouse and the vervet as the first and second line research animals, respectively; (2) the exploration of the action of trypanocides, the mechanisms of drug resistance and the biochemistry of the trypanosome parasites; (3) the pathogenesis of trypanosomiasis; (4) the trypanosome immunity due to human trypanosome stocks of diverse geographical origin and manipulation of the infections

to ensure common parasite and antigen burdens; the results presented here should serve as an important beginning but more qualitative and quantitative studies can be met by carefully planned multi-institutional collaborative investigations; (5) the sequential kinetics of humoral and cell-mediated immunities during brucei subgroup trypanosomiasis; (6) the presence, significance and mechanisms of cell-mediated immunity during African trypanosomiasis; (7) the evasion of immune mechanisms by the parasite and the dissection of the possible mechanisms involved in self-cure and immunodepression; (8) the repetition of the parasitological and immunological studies reported here to confirm or disprove these results using multiple antigens; (9) the sequential micro-(histo-) pathological studies involving the use of light, electron-microscopy and immunofluorescence techniques on, particularly, the brain (and its choroid plexus), the heart, the kidney, the liver and the lymphoid tissues so as to demonstrate and appreciate the role of immune complexes in the pathogenesis of the human disease.

In pathophysiology the parasite stocks probably mainly determine various morbidities and tissue reactions (pathology). If these observations are correlated then the vervet monkey affords a unique opportunity for appraising the disease-producing features of different stocks of T. brucei, T. rhodesiense and T. gambiense. Limited explorations to single stocks were undertaken by Peruzzi (298). More detailed investigations at multi-stock levels are needed.

The vervet monkey, however, has the disadvantages of harbouring potentially lethal simian parasites. This is the most serious drawback to the use of monkeys in laboratories to bar them from all non-essential primate work because of the hazard they constitute to human health. The dangerous parasites include the deadly green monkey disease (Marburg viral agent), the herpes virus simiae (B-virus), Q-fever, Psittacosis in Papio, and monkey pox (180).

The monkeys are susceptible to and can maintain human bacteria, helminths and protozoa (180, 274) including Schistosoma mansoni, Watsonia watsoni, Strongyloides fullerborni, Salmonella and Shigella species. These are hazardous to human health in the presence of normal invertebrate vectors or under poor conditions of animal house hygiene particularly if numerous monkeys are kept under semi-wild tropical conditions. Therefore, preventive measures including handling precautions, protective clothing and hygiene are a must in all laboratories where Cercopithecidae are kept.

The precise ages and the exposure histories of the wild unlike the small laboratory-bred animals are usually unknown. Also, inbred monkeys are a distant dream because monkeys are slow breeders; probably with the breeding primate centres in Kenya, the United States and the Far East this will some day be solved.

Other objections to the non-human primate models include secondary infections which are fairly common in animals. Monkeys are fairly expensive; a vervet costs about K.Shs.1000.

(US $ 100.00) locally, excluding its maintenance. Also the supply and conservation of monkeys for experimental medicine are problematical due to a numerical increase of the wild slow breeding Cercopithecoidae that are used in laboratory sacrificial animal experiments.

Monkeys are necessary for research but rational exploitation in the laboratory and in the field is essential to keep the supply flowing. Unfortunately the rhesus monkey used in trypanosomiasis studies (95, 159, 326) is not found in Africa and its populations in Asia has been severely reduced to threaten shortages while the chimpanzee (27, 117) is very expensive. Reports on the use of Cercopithecus aethiops in trypanosomiasis (17, 298), and immunological studies are few and almost nil, respectively.

A more economical use of laboratory monkeys needs a clear and adhered to policy that (i) would determine the choice of the right animal for a particular experiment; (ii) encourage multiple use of research monkeys through liaison between scientists in different research fields; (iii) intensify the search for alternative culture media like human allantois in the important polio vaccine production in which monkeys, currently are essential; (iv) encourage voluntary limitation on the exploitation of wild animals by persuading scientists to appreciate the value and the economical use of research monkeys by raising the price of wild-caught animals so that they compete with captive-bred primates, further conserving the wild populations; (v) promote the

breeding of vervets in captivity under suitable surroundings and on a balanced diet, preferably in their native countries, in cages large anough for possible natural sex and age structure to produce physically and psychologically normal animals and thereby also reduce expenses to supply some of the over 250,000 monkeys required annually by laboratories for routine use; (vi) would be dependent on the present economic status of the Cercepithecidae as wild animals - like the legally protected crop-raiders (because of their scarcity) or less important forest confined, regular crop-raiders and agricultural pests, including the vervet monkeys, that are often used in research laboratories; (vii) would avoid animal death and wastage by combining dealers' efforts of trapping for export and authorities responsible for vermin control in agricultural areas; (viii) would lead to "game ranching" on land that is unsuitable for agriculture and cropping of monkeys just like cropping ungulates for meat, in keeping with stock-rearing; and (ix) that would call for basic research in ecology, breeding structure and natural population studies while the control mechanisms of wild monkeys and techniques of trapping, handling and transportation would completely raise the official economic position of wild monkeys from pests to valuable assets.

Thus the development of the vervet-human trypanosome model readily manipulated in the laboratory has much to offer investigators of human sleeping sickness, chemotherapy, the basic biology of parasites and infections. The only undermining factor is the continuing supply of vervet monkeys numerically

adequate for sleeping sickness research, for work in trachoma (ophthalmology), carcinogenic studies, parasitology, herpes viruses and especially viral oncology and therapy of lymphomas and leukaemias and for kidney cell culture in which the vervet monkey has already served (180). Servicing these research activities probably would stress the reservoirs of the vervet monkey in nature and thereafter eliminate the species. Solid data on existing reservoirs are important and if such are too limited for requirements steps should be taken to secure independence from natural resources supplies. Since the potential of the vervet monkey to contribute to solutions of important biomedical problems is so great, development of information on natural availability and institutions of steps to complement supplies from such sources are currently very urgent.

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