STUDIES ON THE LOCAL SKIN REACTION AGAINST AFRICAN TRYPANOSOMES

by

Alan John Scott, B.Sc. (Glasgow)

Department of Zoology

University of Glasgow

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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"..... there can have been few solitary achievements which were more dependent on others."
Sir Edmund Hilary, talking about the conquest of Everest, 1953.

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Statement of submission

All the work reported in this thesis is my own, and has not been published previously. Some materials, however, were produced or provided by colleagues.

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Summary

This thesis contains the results of an investigation into the immunological events involved in the production of a local skin reaction by rabbits in response to intradermal challenge with Trypanosoma brucei rhodesiense.

An antigenically defined model system is described which utilises cloned, monomorphic lines of trypanosomes stably expressing a single VAT. Intradermal injection of such trypanosomes into rabbits induced the formation of chancres morphologically indistinguishable from those induced by cyclically transmitted trypanosomes. The injection of 10^5 monomorphic trypanosomes was found to be the closest approximation to the bite of a heavily infected fly. All subsequent experiments used the intradermal injection of 10^5 monomorphic bloodstream form trypanosomes expressing a single, defined VAT to elicit the formation of a local skin reaction.

The antigenic definition of the trypanosome population used to elicit chancre formation allowed the VAT-specific serological response to be accurately assessed. The results of these experiments suggest that a high titre lytic antibody response against the challenge VAT precludes the formation of a chancre. Immunity to challenge with one VAT had no effect upon the induction of a chancre to an heterologous VAT, or to the humoral response against it. Some diminution of responses (both chancre formation and antibody production) was observed in late infections.

The lymphocyte composition of the chancre infiltrate has been examined using a panel of monoclonal antibodies which recognise rabbit T-lymphocytes, and anti-immunoglobulin antiserum. These experiments demonstrated that the initial response to trypanosome

challenge was an infiltration into the skin by T-lymphocytes, which preceded a parallel infiltration by B-lymphocytes by 1.5 days. Late reactions were characterised by the presence in the infiltrate of cells which may be cytotoxic/suppressor T-lymphocytes. A method for the rapid separation of mononuclear cells from rabbit blood is described. Analysis of cells isolated by this method demonstrated that the lymphocyte composition of peripheral blood bears little relation to the composition in the chancre.

A method is described for the isolation and culture of viable mononuclear cells from local skin reactions. Cells from local reactions were cultured in the presence of antigenic material derived from the homologous or an heterologous VAT. Cell cultures were depleted of subpopulations of cells in order to more clearly define the responding cell type. These studies suggest that the initial immune response in the chancre is a VAT-specific T-cell response.

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ABBREVIATIONS

DTH	Delayed type hypersensitivity
EATRO	East African Trypanosomiasis Research Organisation
$F(ab')_2$	Divalent antigen binding fragment of immunoglobulin obtained by pepsin digestion.
Fc	Crystallizable fragment obtained after papain hydrolysis of immunoglobulin
FITC	Fluorescein isothiocyanate
GUP	Glasgow University Protozoology
GUPM	Glasgow University Protozoology Monoclonal (antibody)
GUTat	Glasgow University Trypanozoon antigen type
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
ILTat	International Laboratory for research into animal diseases Trypanozoon antigen type
McAb	Monoclonal antibody
PBS	0.017M phosphate buffered saline pH 7.2
PS	Buffered phosphate saline solution used at 6:4 with distilled water, as described by Lanham and Godfrey (1970).
PSG	PS (above) with 10g/l D-glucose added
RPMI 1640	Rothwell Park Memorial Institute, medium number 1640
sd.	Standard deviation
S.L.E.E.	South London Electrical Equipment Co. Ltd.
T_a-cell	Alloreactive T-lymphocyte
T_c/T_s	Cytotoxic/suppressor T-lymphocyte
VAT	Variable Antigen Type
VSG	Variant Surface Glycoprotein.

DEFINITIONS

Monomorphic - populations of bloodstream trypanosomes which do not generate short stumpy form trypanosomes during an infection. The derivations of the monomorphic trypanosome lines used in this study are shown in the appendix.

Monotypic - a population of trypanosomes in which all organisms express the same, defined, VAT. This characteristic was assessed by immunofluorescence on heavily infected blood smears as described in section 2.2.9.

1. GENERAL INTRODUCTION

1.1 The Scale of the Problem

On a global scale, around sixty-five thousand human deaths per annum are directly attributable to flagellated protozoa belonging to the genus Trypanosoma. Some eighty percent of these deaths occur in S. America and are due to Chagas' disease caused by Trypanosoma cruzi. The remaining deaths are due to African sleeping sickness caused by subspecies of Trypanosoma brucei. It is the African disease which is under study in this thesis.

In addition to the human mortality and an associated and greater morbidity, African trypanosomes diminish the quality of life in the sub-saharan regions by killing approximately three million domestic livestock annually (W.H.O., 1979). In the context of the nutritional status of the peoples of these regions the direct effect of the parasites upon humans and their domestic animals is probably not so serious as the trypanosome-forced abandonment of huge areas of Africa which would otherwise be agriculturally viable. It has been estimated that up to one hundred and ten million extra cattle could be raised in Africa in the absence of trypanosomiasis. (Wilson et al. 1963).

1.2 The Parasites which cause the African Trypanosomiases.

The organisms responsible for the African trypanosomiases belong to the Trypanozoon subgenus and can be classified according to the diseases they cause.

The human disease falls into two categories. The chronic form, which is confined to West Africa, is caused by T. brucei gambiense, which originally was thought not to have an animal reservoir. The more acute form is caused by T. brucei rhodesiense and is more prevalent in the central and eastern areas of sub-

saharan Africa. Trypanosoma brucei brucei is a major parasite of domestic and wild animals in these areas but does not infect man. On the basis of their infectivity to humans (or resistance to human serum) T. b. brucei and T. b. rhodesiense used to be considered as separate subspecies, despite being morphologically identical. Nucleic acid analysis, however, could not demonstrate this speciation and it now seems likely that T. b. brucei and T. b. rhodesiense are host range variants of the same subspecies (Tait et al 1985). Similar evidence has shown that T. b. gambiense is a separate subspecies, and also that there is an animal reservoir for this organism (Tait et al 1985, Paindavoine et al 1986).

Several other species of trypanosome are economically important as causes of 'nagana' in African domestic livestock. The most important of these are T.(Nannomonas) congolense and T.(Duttonella) vivax.

1.3 Life Cycles of African Trypanosomes.

Most of the pathogenic species of African trypanosome undergo cyclical development in their tsetse-fly vector, and this discussion will be confined to these.

The ultrastructure and life cycles of the brucei-group trypanosomes, including the two human-infective species, are essentially identical (reviewed by Vickerman 1985).

The trypanosomes found in the mammalian host, the so-called bloodstream forms, are pleomorphic. The extremes of these differentiated forms are represented by the long slender (16-42 um long, free flagellum, rapidly dividing) and short stumpy (12-25um, no free flagellum, non-dividing) trypomastigotes, but a complete range of forms exists between these extremes. All the bloodstream forms possess glycosomes active in glycolysis. The glycosome

contains most of the glycolytic enzymes and appears to be unique to the kinetoplast-bearing flagellates (Opperdoes and Borst 1977). All the bloodstream forms are covered in a dense layer of a single glycoprotein, the surface coat, which appears to protect the organisms from the non-specific immune mechanisms of the mammal. When infected blood, containing the range of pleomorphic forms, is ingested by the fly the stumpy trypanosomes transform into uncoated procyclic forms. Slender forms die or undergo transformation into stumpy forms in the tsetse midgut as only stumpy forms have a partially activated mitochondrion which is essential for the amino-acid based energy metabolism used by the trypanosomes in the fly gut (reviewed by Vickerman 1985).

Four days after infection the procyclics in the gut begin to invade the ectoperitrophic space by penetrating the peritrophic membrane (Evans & Ellis 1983). Subsequent division over the week following invasion leaves the ectoperitrophic space packed with dividing trypanosomes.

The migration route of the procyclics to the salivary glands is an unresolved issue. Robertson (1913) proposed a route from the ectoperitrophic space, through the proventriculus into the endoperitrophic space and then via the oesophagus, mouthparts and salivary ducts to the salivary glands. This view has recently been challenged and an alternative route involving direct penetration of the gut wall into the haemocoele with subsequent invasion of the salivary gland through the gland epithelium into the lumen. (Evans & Ellis 1983). Although neither hypothesis has been proven the presence of trypanocidal factors in haemolymph (Croft *et al* 1983) suggest that the former is more realistic. Further support for this argument is the common ancestry of *T. brucei* and *T. congolense*

(Hoare 1972) as the latter parasite does migrate to the tsetse proboscis without infecting the salivary glands.

Irrespective of the route of migration, differentiation of the procyclic eventually yields a population of epimastigotes attached to the gland epithelium by their flagella. The epimastigotes transform *in situ* into metacyclic trypanosomes which have an unbranched mitochondrion and fully formed glycosomes. These changes, which metabolically pre-adapt the metacyclic trypanosome for life in the mammalian host are accompanied by the synthesis of the variant surface glycoprotein and its assembly as the surface coat (Tetley & Vickerman, 1985). The development of *T. congolense* in the tsetse fly resembles that of *T. brucei* up to the point of migration to the tsetse mouthparts. At this point the epimastigotes are found attached to the labrum wall and it is at this site that *T. congolense* differentiates into mammal infective coated metacyclic trypanosomes (Fraser & Duke 1912). The entire developmental cycle of *T. vivax* in the tsetse fly is confined to the proboscis. Ingested trypanosomes attach to the walls of the food canal and transform into elongated trypomastigotes. Further differentiation to the epimastigote form precedes the formation of coated metacyclics (Gardiner *et al* 1986).

The tsetse flies, which transmit the major pathogenic African trypanosomes, are of the genus *Glossina*. Although over twenty species exist they can be resolved into two groups; the morsitans group which most commonly transmit East African sleeping sickness and the palpalis group responsible for most transmission of *T. gambiense*. This distinction is an ecological consequence of the geography of the regions and is not absolute. The riverine flies of the palpalis group inhabit areas of low mammalian fauna density and as such transmission of *T. gambiense* is essentially from human

to human although infections of domestic animals do occur (Duke 1928, Van Hoof 1947). Flies of the morsitans group however inhabit the animal rich savanna in which there exists a huge animal reservoir for T. brucei, T. b. rhodesiense. It is however possible to transmit experimentally T. b. rhodesiense through palpalis group tsetse flies (Duke 1933) and T. gambiense through morsitans group flies (Corson 1938). Examples of "natural experiments" showing such occurrences have also been documented (Lester 1933, Southon and Robertson 1961)

1.4 Antigenic Variation

In terms of the antigenic stimulus they present to the mammalian host, African trypanosomes are composed of two groups of antigens; common antigens and variable antigens (reviewed by Barry 1986a)

Common antigens comprise structural and metabolic components of trypanosomes, and, with the exception of stage specific antigens, are antigenically invariant throughout the life cycle.

These antigens can also be found in other stocks of the same species and a subset can be found in other species. Some of these antigens persist throughout the life cycle of the trypanosome (Le Ray 1975).

The variable antigens change both between populations and, in a single population with time. They are responsible for the serological variants or variable antigen types (VATs) of the parasite.

As previously mentioned (Section 1.3) all mammalian forms of the trypanosome and the mature metacyclic trypanosomes in the tsetse fly possess a surface coat. The antigenic identity of a trypanosome is localised in this 12-15nm thick, electron dense

layer (Vickerman & Luckins 1969) which is composed of a single glycoprotein, the variant surface glycoprotein (VSG) (Cross 1975).

During a trypanosome infection, successive peaks of parasitaemia occur (Ross and Thomson 1910). Within each peak, the trypanosome population is composed of a mixture of VATs (van Meirvenne 1975), each of which stimulates a VAT-specific antibody response. Each remission in parasitaemia is due to the elimination of a population of trypanosomes by VAT-specific antibody; each recrudescence is due to the growth of antigenic types which have arisen de novo within the trypanosome population. The duration of an infection is limited by at least two factors. Firstly, the survival of the host and secondly, the number of different VATs which can be expressed by a given stock of trypanosomes. Host survival depends on many factors eg. the virulence of the infecting stock and the immune competence of the host. The number of VATs expressed has been demonstrated (in a T. equiperdum infection) to be at least 101 (Capbern et al 1977). In addition, analysis of DNA sequences associated with the variant antigen genes of T. brucei has shown that up to 1000 variant antigen genes may be present (van der Ploeg et al 1982).

If bloodstream trypanosomes are cloned , however, it is possible to grow up a population of trypanosomes of which the majority (>90%) express the same VAT (homotype). Such populations do, however, contain trypanosomes expressing other (heterotype) VATs, and these VATs are seen in greater proportions in relapse populations when the original homotype has been eliminated (van Meirvenne 1975). Relapse populations from clone infections are also composed of a mixture of VATs. The expression of different VATs within these populations however, displays a variable but non-

random frequency distribution within which there may be a statistically definable probability for each VAT switching to any other VAT (van Meirvenne 1975, Miller and Turner 1981). Analysis of the frequency of appearance of new VATs also gave an estimate of the rate of antigenic switching. This rate was calculated as 10^{-4}-10^{-5} switches per trypanosome division.

The sequence of anti-VAT agglutinating antibodies produced in an infected animal suggests that individual VATs in a repertoire appear in a non-random sequence (Gray 1965, Capbern 1977). Agglutinins were produced against certain "predominant" VATs early in an infection and the sequence of these predominant VATs was more predictable than VATs occurring later in the infection. Gray (1965) also recognised a "basic antigen", antibodies against which were the first to appear after cyclical transmission. In a mechanically transmitted infection the "basic antigen" arose immediately after the host eliminated the transmitted VAT. This idea was extended by Capbern (1977) who assigned VATS into three groups , "early", "semi-late" or "late". No conserved sequence within each group was discernible.

In common with bloodstream populations of trypanosomes, the VAT composition of the metacyclic trypanosomes in the tsetse fly salivary gland is heterogenous (Le Ray *et al* 1978, Barry *et al* 1979). This heterogeneity arises with *de novo* synthesis of the surface coat as the attached epimastigotes transform into nascent metacyclic trypanosomes *in situ* (Tetley and Vickerman 1985). Only a limited subset of bloodstream VATs is expressed in the tsetse fly as metacyclic VATs (M-VATs) and these VATs are subsequently the first to appear in the bloodstream (Hajduk *et al* 1981, Hajduk and Vickerman 1981). For a given stock of trypanosomes the different M-VATs are present in the saliva of different flies in

approximately constant proportions. These proportions are unaffected by the VAT ingested by the fly (Hajduk et al 1981). As stated previously, the first VATs detectable in the bloodstream following cyclical transmission are the M-VATs, and these are followed by the group of "predominant" VATs. If flies are infected with a homotype population the ingested VAT (I-VAT) can be expressed as an early member of this "predominant" group of VATs. If the I-VAT is not normally a member of the M-VAT group it is not expressed in the metacyclic population but arises de novo in the first parasitaemia (Hajduk et al 1981).

The use of monospecific and subsequently monoclonal antibody preparations has demonstrated that the "basic" antigen described by Gray (1965) is, in fact, a heterogenous mixture of M-VATs (Le Ray et al 1978, Barry et al 1979, Hajduk and Vickerman 1981).

Trypanosomes cloned from metacyclic or first patent parasitaemia populations are very unstable with respect to VAT. Such clones generate heterotypes with greater frequency than those derived from stable syringe-passaged lines (Le Ray et al 1977). Indeed, after only a few 3-day syringe passages in mice heterotype VATS may almost completely replace the original VAT. This evidence, coupled with the fact that trypanosomes can change their expressed VAT in culture (Doyle 1980) in the absence of any selective pressure from the mammalian immune system shows that VAT switching is an inherent property of the trypanosome and not a reflection of induction by the immune system.

In contrast to cloned metacyclic trypanosomes, repeated syringe passage of bloodstream trypanosomes can lead to the establishment of trypanosome lines stably expressing VATs. Such lines, having selected trypanosomes from the logarithmic growth

phase of a parasitaemic peak, are composed almost entirely of slender form trypanosomes. As a consequence of their monomorphic nature such lines will not infect tsetse flies. The VAT stability they display has however enabled large quantities of pure antigens and monospecific antisera to be produced.

The restricted size of the metacyclic repertoire is an obvious target for vaccination. Knowledge of the size and stability of such repertoires is therefore of great practical importance. Only one metacyclic repertoire has been completely characterised. This work has shown that a single serodeme of T. congolense expresses twelve M-VATs (Crowe et al 1983). Work on T. brucei indicates that it has a larger repertoire (Hall and Esser 1984, CMR Turner unpublished observations). Of greater consequence for vaccination hopes is the finding that the metacyclic repertoire of one serodeme of T. b. rhodesiense is unstable during repeated fly transmissions (Barry et al 1983).

1.5 Variant Surface Glycoprotein

The variant surface glycoprotein (VSG) is the molecule which comprises the electron dense surface coat seen on metacyclic and bloodstream forms of African trypanosomes (Cross 1975). Each trypanosome expresses a single VSG and the surface coat is composed of 1-2 X 10^7 molecules of VSG (Cross 1975, Jackson & Voorheis 1985). Recently, exceptions to the general occurrence of one VSG per trypanosome have been observed (Baltz et al 1986), but these have been found only in culture. Such "double expressors" reverted to the expression of a single VSG upon injection into rodents.

1.5.1 VSG structure

VSGs form a group of glycoproteins of apparent molecular weight 53000-63000D (reviewed by Borst and Cross 1982). When

sequenced they are found to have some homology over the C-terminal 50-100 amino acids but the N-terminal 300-400 amino acids show remarkably little sequence homology, although cysteine residues are conserved (reviewed by Cross 1984). The molecule appears to be conformationally arranged into a C-terminal and an N-terminal domain corresponding to these sequences (Johnson and Cross 1979; Freymann et al 1984). Variant glycoproteins can be divided into two subclasses on the basis of their C-terminal amino-acid sequences (Rice-Ficht et al 1981). Subclass 1 VSGs terminate in an aspartic acid residue whereas serine terminates subclass 2 VSGs. Within each subclass regions of homology extend well into the C-terminal domain. One VSG has been isolated which terminates in an asparagine residue (Majumder et al 1981).

Much of the molecular weight heterogeneity of VSGs is undoubtedly due to variation in the amount of glycosylation of VSGs (Johnson and Cross 1977). There are two types of glycosylation of VSGs. The majority of the carbohydrate coupled to VSG is linked through N-glycosylation (Strickler and Patton 1980, Rovis and Dube 1981). The number of potential glycosylation sites on VSGs is variable, although each VSG carries at least one N-linked carbohydrate. The size and composition of the oligosaccharides attached to VSG, both at individual sites and overall, is also variable (reviewed by Holder 1985).

The C-terminal amino acid of soluble VSG is always glycosylated. The glycosyl chain is linked to the hydroxyl group ethanolamine which is in amide linkage with the alpha-carboxyl group of the terminal amino acid (Holder 1983). The composition of the C-terminal carbohydrate has not been completely elucidated but appears to contain glucosamine, mannose and galactose in the ratio 1:2:4-8 (Holder 1985). One C-terminal glycosyl chain has been

found to contain no galactose (Holder 1985).

Soluble VSGs have been shown to possess an immunologically cross reactive determinant (CRD) (Barbet and McGuire 1978) and this epitope resides in the C-terminal carbohydrate (Holder and Cross 1981). Interestingly, the C-terminal glycosyl chain which does not contain galactose does not react significantly with anti-CRD antisera. As shown by Cardoso de Almeida and Turner (1983), such antisera also fail to react with that form of VSG present on the intact trypanosome, i.e., membrane form VSG (mfVSG). This is thought to be due to steric hindrance by the complex C-terminal glycolipid moiety which anchors mfVSG in the trypanosome plasma membrane (Turner et al 1985).

The polypeptide sequence of released or soluble VSG (sVSG) differed from that inferred from the mRNA sequence (Matthyssens et al 1981), in that it contained coding sequences at both ends which did not appear as amino acids in purified mature VSG. In common with other glycoproteins which are exported to the cell surface the VSG amino terminal has a hydrophobic leader sequence which, before export, is removed in a manner analagous to other signal peptides (McConnell et al 1981, 1982). The length and sequence of these peptides is variable, although they do contain characteristic hydrophobic sequences (Cross 1984).

In direct contrast to the rest of the molecule, the carboxy terminal tail shows great homology between VSGs (Cross 1984). All VSGs which have a C-terminal serine have a 17 amino acid tail while those terminating in aspartic acid or asparagine possess a 23 amino acid tail. The amino acid composition of the tail is very hydrophobic but, unlike membrane-binding sequences in other systems it is an intra-membrane sequence and not a trans-membrane sequence.

One obvious postulate after the discovery of the tail of VSG was that release of VSG was mediated by proteolytic cleavage of the hydrophobic tail (Cross 1984, Turner 1985). The demonstration by Holder (1983) that ethanolamine is in amide linkage with the carboxy-terminal amino acid precludes such a mechanism (see below). The hydrophobic peptide tail appears to be a temporary anchor structure which is replaced during synthesis by a glycolipid moiety and it is this glycolipid which anchors the native membrane form VSG (mfVSG) in the trypanosome membrane (Ferguson and Cross 1984). The sensitivity of the myristic acid component(s) to various chemical treatments suggests that they are in ester linkage with the rest of the molecular complex. There are conflicting reports of the molar ratios of phosphate, glycerol and non-myristyl fatty acyl groups in both mfVSG and sVSG of *T. brucei* and *T. equiperdum* (reviewed by Turner *et al* 1985).

1.5.2 *Release of VSG*

The conversion to sVSG from mfVSG is an enzyme mediated reaction (Cardoso de Almeida and Turner 1983). Initially all the VSG in the surface coat is mfVSG and in this form recognition of the CRD by antibody is impaired. Only after enzymatic release of sVSG can CRD by recognised by such anti-CRD antibodies. It has been assumed that this is due to steric hindrance or epitope masking by the membrane binding complex but these studies do not exclude the possibility that the CRD is linked to sVSG at the time of its release from the membrane bound form.

The enzyme which catalyses the release of sVSG is a membrane bound enzyme present in bloodstream form but not procyclic trypanosomes (Cardoso de Almeida *et al* 1984a, b). The enzyme has a pH optimum of 8.0 and is probably calcium dependent (Turner *et al*

1985, Voorheis et al 1982). Given the phospholipid-like nature of the membrane anchoring structure, phospholipases and lipases have been considered as candidates for this activity. Some activity was noted in a phospholipase C preparation (Jackson 1983) and more recent evidence suggests that the native trypanosome enzyme may have this activity (J. Ward personal communication). Much more information on the stoichiometry of the phosphate, carbohydrate and lipid moieties will be required before the exact nature of the enzymatic conversion is understood.

1.6 The Immune Response to African Trypanosomes.

The immune response to African trypanosomes consists of both specific (involving specific recognition of antigen) and non-specific (not involving specific antigen recognition) mechanisms.

1.6.1 Specific Immunity

1. Humoral Immunity

The systemic immune response against pathogenic trypanosomes is mediated almost entirely by VAT-specific antibodies. In contrast to the typical mammalian primary immune response, in which initial IgM synthesis is replaced by the synthesis of IgG antibodies, trypanosome infected hosts show prolonged and enhanced levels of IgM. Elevated serum IgM levels are a consistent finding in human sleeping sickness and IgM in the cerebro-spinal fluid is diagnostic for the disease (see Mattern et al 1961; Mattern 1962, 1964). Infection of laboratory animals (Seed et al 1969; Hudson et al 1976) and both experimental and natural infections of cattle (Luckins 1976) with T. brucei subspecies also lead to raised levels of IgM. Similar results have been reported for T. congolense and T. vivax (Luckins 1976).

The specificity of the IgM found in infected mammals is a

matter of some debate. Several workers have reported the production of heterophile antibodies (Houba and Allison 1966; Houba et al 1969), and have considered the macroglobulinaemia to be the result of non- specific polyclonal B-cell activation. At least part of the total IgM fraction is likely to be parasite specific, being generated in response to successive trypanosome VATs (Seed et al 1969). More recently, it has been demonstrated that exhaustive absorptions using an undefined range of trypanosome VATs can absorb most of the IgM from the sera of both sleeping sickness patients (Herbert et al 1980) and T. brucei infected cattle (Musoke et al 1981) lending support to the idea that the high titres of IgM seen during trypanosome infections are produced in response to parasite antigens and not induced by mitogens. The temporary drop in IgM levels associated with parasitaemic remission, and also the decline in IgM levels following drug treatment (Luckins 1976), favour neither a mitogenic nor a polyantigenic stimulus for this antibody production, but do, however, serve to reinforce the association between trypanosome infection and IgM production which is the most striking immunological aspect of the disease.

Although VAT specific IgG is produced during infection it is of limited efficacy. In cattle infected with T. brucei IgG is produced concomitantly with IgM but this IgG is of low avidity (Musoke et al 1981). Functionally, IgM is more effective than this early IgG at agglutinating, lysing and neutralising trypanosomes in vitro and also in providing protection in vivo (Seed 1972; Takayanagi and Enriquez 1973; Luckins 1976; Musoke et al 1981). Total IgG levels in experimental infections of cattle with T. congolense and T. vivax are essentially unchanged although in natural infections of cattle and bushbuck IgG levels up to twice normal have been reported (Luckins 1975, 1976). In human sleeping

sickness IgG levels are raised while the levels of IgA and IgG remain within the normal range (Herbert et al 1980).

Despite their absence in natural infections high titres of specific IgG antibodies can be induced by various immunisation protocols. Whole trypanosomes either chemically fixed or incorporated into adjuvants (Herbert and Lumsden 1968) or attenuated by gamma-irradiation (Wellde et al 1975) have been effective. Purified VSG from T. equiperdum is sufficiently immunogenic to confer protection without the use of adjuvants (Baltz et al 1977). All of these immunisation schedules have elicited VAT-specific protective immunity. As discussed previously (see section 1.4, Antigenic Variation) the metacyclic trypanosomes express a limited mixture of VATs, and these present an obvious target for a cocktail vaccine. With this in mind, Van Meirvenne et al (1975) demonstrated that rabbits could be immunised against at least 9 VATs simultaneously. If the metacyclic repertoire had been shown to remain stable upon repeated transmission (Barry et al 1983) a limited cocktail vaccine may have proved possible.

Infectivity of trypanosomes to fresh hosts can be neutralised by antibody although the efficacy of different isotypes varies with host species, IgM being more efficient than IgG in the rabbit (Seed 1977), while in cattle, late IgG is more effective than IgM (Musoke et al 1981). IgM alone is, however, sufficient to control parasitaemia as evidenced by the fact that athymic nu/nu mice (Campbell et al 1978) and irradiated thymectomised adult mice (Askonas et al 1979) survive T. brucei infections better than fully immunocompetent mice.

The effector mechanisms which mediate the destruction of trypanosomes have been studied both in vitro and in vivo. In in

vitro tests VAT-specific antibodies have been shown to agglutinate (Cunningham and Vickerman 1962), lyse (Lourie and O'Connor 1936; Van Meirvenne 1975) and opsonise (Lumsden and Herbert 1967, Takayanagi *et al* 1974) bloodstream trypanosomes. In addition, the binding of specific Ab has an inhibitory effect on aspects of trypanosome metabolism (Thurston, 1958; Diggs *et al* 1976).

Immune lysis of trypanosomes *in vitro* occurs rapidly in the presence of a complement source, most usually guinea-pig serum. An intriguing finding is that stumpy forms of *T. brucei* are rather more resistant to lysis than long slender forms expressing the same VAT (Barry and Vickerman 1977), which may enable the insect-infective form to persist for longer in conditions of developing immunity. Complement appears to be necessary for lysis *in vitro* (Diggs *et al* 1976) although this may not be true at high antibody concentration (Balber *et al* 1979, Crowe *et al* 1984). It is not clear whether the classical or alternative complement pathway is involved in this reaction. It has been reported that EDTA (which blocks only the classical pathway) inhibits lysis of *T. brucei* and *T. congolense* (Balber *et al* 1979), yet *T.b. rhodesiense* is lysed in C4 deficient serum or in the presence of EGTA, both of which suggest that the alternative pathway may operate (Flemmings and Diggs 1978). As all reports stress an absolute requirement for specific antibody the classical pathway is likely to be of greater importance. Although lysis of trypanosomes undoubtedly does occur *in vivo* (Capbern *et al* 1977) the survival time of rodents is not altered by deficiency of C5 (Jones and Hancock 1983). Genetic deficiency of C5 does however extend the time required for clearance of labelled trypanosomes immune mice (MacAskill *et al* 1980).

Opsonisation of trypanosomes is probably the major mechanism

of removal of the organisms from the bloodstream. In vitro murine macrophages attach to, and subsequently ingest, bloodstream forms of T. brucei subspecies, but only in the presence of immune serum (Lumsden and Herbert 1967). Whole antibody, but not $F(ab')_2$, mediates attachment (Takayanagi and Nakatake 1974). Decomplementation has no apparent effect upon ingestion (Takayanagi et al 1974). Despite these findings it seems likely that some opsonisation in vivo will be mediated by the macrophage receptors for complement components. This view is supported by the fact that phagocytes do not have Fc receptors for IgM (Wing and Remington 1980), and indeed, such a site would be unavailable to pentameric IgM. Athymic nu/nu mice, which do not produce IgG are capable of controlling parasitaemia (Campbell et al 1978), and it is likely that lysis mediates this effect.

Extravascular trypanosomes may also come under control by antibody. In this context Goodwin and Guy (1973) demonstrated trypanosome specific antibody in the tissue fluid of rabbits infected with T. brucei. The titre of antibody was one fifth of that in the serum and antibody activity was first detected extravascularly two days after its detection in serum. The antibody detected was initially IgG, although IgM was subsequently found; this was attributed to an increase in vascular permeability which allowed the larger IgM molecule to leave the vascular compartment.

In conclusion, antibody is the most important trypanocidal factor in specific immunity. Although antibody alone can, under certain circumstances, disrupt trypanosomes, most of its lethal effects are dependent on intact complement and reticulo-endothelial systems.

Both trypanolysis and phagocytic uptake of opsonised organisms probably occur *in vivo*. As opsonisation occurs at lower antibody titres than does lysis it is probable that opsonisation occurs earlier in an infection than lysis. Only when opsonic clearance was insufficient (e.g. when the phagocytic system was saturated) to clear an infection would lytic titres be realised.

1. *Cell-mediated immunity*

Cell mediated immune responses appear to play no effector role in immunity to trypanosomes. Indeed, both athymic *nu/nu* mice (Campbell *et al* 1978, Clayton *et al* 1979) and irradiated, thymectomised adult mice (Clayton *et al* 1979) survive *T. brucei* infections longer than their immuno-competent counterparts.

Immunity was not transferred with B-cell depleted spleen cell populations (Campbell and Phillips 1976), but could be transferred with serum or spleen cells treated with anti-thymocyte serum (Campbell and Phillips 1976, Takayanagi and Nakatake 1975). One report that immunity could be transferred by murine thymocytes (Takayanagi and Nakatake 1975) required large numbers of cells which was perhaps due to the presence of B-cell contaminants in the transferred population. It has been reported that human T-cells were cytolytic for antibody-coated *T. (Schizotrypanum) dionisii*, a parasite of pipistrelle bats (Mkwananzi *et al* 1976). The lymphoid populations used, however, were only partially purified, and phagocytosis of the trypanosomes can not be ruled out, especially as *T. dionisii* can be an intracellular parasite (Thorne *et al* 1979). The author knows of no reports of direct cell mediated cytolytic responses against African trypanosomes.

Despite their lack of effector capability T-lymphocytes do respond to trypanosome antigens (Jayawardena *et al* 1978, Campbell

et al 1982, Diffley 1983). Splenic T-cells have also been shown to proliferate transiently in vivo during infection with T. congolense (Morrison et al 1978) and T. brucei (Mayor-Withey et al 1978). No function has been ascribed to these proliferating cells, although it seems likely that the IgG response against trypanosome common antigens (Le Ray 1975) is T-cell dependent. It is also possible that proliferating T-lymphocytes are in part responsible for the generalised immunosuppression which occurs in trypanosome infections (see below).

Delayed-type hypersensitivity (DTH) reactions have been reported in T. rhodesiense infected rabbits (Tizard and Soltys 1971). Cell transfer experiments performed by these authors used very small numbers of cells transferred between outbred animals. As serum was not reported to transfer the Arthus type reaction also observed, the cell mediated nature of these hypersensitivity reactions remains equivocal. Similar work on hypersensitivity reactions to antigens of T. congolense demonstrated only an antibody mediated Arthus-type reaction (Mansfield and Kreier 1972). Hypersensitivity against antigens of both trypanosome species were only observed in animals harbouring long-standing infection.

In conclusion, cell mediated immune responses are not effective against free trypanosomes. Those responses which can be demonstrated occur late in infection and are not protective.

1.6.2 Non-specific immunity

The evolution of the mechanism of antigenic variation argues against the possibility that non-specific immune mechanisms might be effective against bloodstream form trypanosomes.

Complement, however, was activated by bloodstream form trypanosomes, causing depletion of complement activity in serum

even in the absence of specific immunity. Fixed organisms had similar effects (Musoke and Barbet 1977). Purified VSG was found to deplete to a limited extent C1, C2, C4 and C3 indicating activation of the classical pathway. The mechanism of complement activation remains unclear. Despite this complement activation trypanosomes are not lysed in non-immune hosts and it is possible that the pathway is not completed (Neilsen *et al* 1978). An alternative explanation may be that C3b, with its vital membrane attachment site, is too large (M_r = 181000D) to penetrate the surface coat. Uncoated developmental forms of brucei-group trypanosomes lyse in non-immune sera from many mammalian species normally susceptible to infection. As this activity is heat sensitive (56^{o}C) it is probably the result of alternative pathway activation (Rifkin 1978a). The complement pathways therefore, despite their important role in specific immunity (see section 1.6.1(1), appear to play no part in non-specific immunity to African trypanosomes.

Under non-immune conditions phagocytic cells (macrophages, monocytes and polymorphonuclear leukocytes) do not appear to be trypanocidal effector cells. Indeed, in the absence of specific antibody, peritoneal macrophages neither attach to nor phagocytose bloodstream trypanosomes *in vitro*. Stimulants of the mononuclear phagocyte system (BCG and *Propionibacterium acnes*) have, however, been shown to increase survival times in susceptible mice (Murray and Morrison 1979). In wildebeest, which are resistant to the pathogenic effects of trypanosome infection (trypanotolerant), a superior phagocytic capability was reported to be one factor which possibly contributed to their reduced susceptibility (Rurangirwa *et al* 1986). Mice with a genetic defect in macrophage function (C3H/HeJ) survived *T. rhodesiense* infection less well than "normal"

C3H/HeNCrLBR mice which have a similar genetic background and normal macrophage function. The parasitaemias in the two mouse strains were similar (Jones and Hancock 1983). Thus, while ultimate control of parasitaemia is mediated by antibody, an effective phagocytic system appears to reduce the pathogenic effects of parasitosis. Trypanolysis *in vivo*, with its consequential release of trypanosome enzymes, may be more damaging to the mammalian host than an effective opsonic response (see section 1.7.3).

1.6.3 *Trypanocidal effect of normal serum*

Mice infected with *T. brucei brucei* may be cured by the injection of normal human serum (Laveran 1902). Such serum lysed bloodstream forms of *T. brucei*, but not *T. b. rhodesiense*, *in vitro* (Yorke *et al* 1930, Rickman and Robson 1970a). Serum from rodents has no such trypanocidal effect. The activity is heat sensitive (64^{o}C for one hour) but is not restored by complement (reviewed by Hawking 1979). Absorption of serum with high numbers of trypanosomes does not deplete the activity (van Meirvenne *et al* 1973). In human serum high density lipoprotein was suggested to mediate the trypanocidal action as an HDL enriched fraction of serum retained the same activity as normal serum (Rifkin 1978b). No activity was found in the sera of patients with Tangier disease; such sera are deficient in high density lipoprotein. The activity is calcium dependent and may involve the removal of cholesterol from the trypanosome membrane (D'Hondt *et al* 1979, D'Hondt and Kondo 1980).

The biochemical basis of this difference between *T. b. brucei* and *T. b. rhodesiense* remains unknown. Cloned *T. b. rhodesiense* can, however, produce resistant and sensitive forms in a single

infection. Within one serodeme the acquisition of resistance to human serum was always associated with a particular VAT; resistant clones expressing other VATs could then arise from this. As some antigen types have been isolated in both sensitive and resistant forms the gross antigenic specificity does not itself confer resistance (van Meirvenne *et al* 1976).

Resistance to human serum is the basis of the blood incubation infectivity test (BIIT), devised by Rickman and Robson (1970a,b). In this test, trypanosomes are incubated in fresh human blood for 5 hours at 37^{o}C and subsequently injected into rats. If the rats become infected the trypanosomes are probably human infective. In general, *T. b. gambiense* is always resistant while *T. b. brucei* and *T. b. rhodesiense* are variable in their sensitivity.

1.7 Pathogenesis and Immunopathology

A discussion of the plethora of pathogenic and pathological conditions involved in trypanosomiasis is beyond the scope of this work. In the context of the local skin reaction however, some mention of the trypanosome induced changes in the immune system and their effect on the host, is necessary.

The major pathological features of trypanosomiasis are anaemia, lymphoid proliferation, widespread infiltrative tissue lesions and a severe, general immunodepression. All of these features are probably a consequence, at least in part, of successive waves of lymphocytic responses against each peak of parasitaemia.

1.7.1 Lymphoid architecture

The classical symptoms of trypanosomiasis include pronounced splenomegaly and lymphadenopathy. Spleen and lymph nodes are the

site of leukocytic proliferation, although in the spleen the red pulp is also greatly expanded.

Early in infection B, T and null cells are all stimulated to divide but the T-cell response has been reported to be short lived (Mayor-Withey et al 1978, Morrison and Murray 1979). B-cell follicles are found to be greatly increased in size and germinal centres are well developed (Murray et al 1974a, Mansfield 1978). The T-cell areas of the lymph nodes (paracortices) and spleen (periarteriolar lymphoid sheaths) are depleted of small lymphocytes and infiltrated with plasma cells and macrophages (Murray et al 1974 a, b, c; Mansfield and Bagasra 1978). The cortex and medulla of the thymus is also found to be cell depleted and the thymus is reduced in size (Mansfield 1978). As the infection progresses the cellular depletion extends to follicular (B-cell) areas. In the spleen, where the red pulp is expanded, this depletion leads to complete disruption of the normal lymphoid architecture. Similar changes occur in cattle infections (Morrison and Murray 1979). Another cellular abnormality during trypanosome infection is the appearance of large numbers of Mott cells in the spleen. These cells, which manufacture but cannot secrete immunoglobulin, are found in higher numbers in mice which are susceptible to trypanosome infection (Black et al 1985) than in more resistant strains.

In vitro studies on cultured spleen cells have shown that cells from infected mice show increased proliferation. The proportion of cells bearing surface immunoglobulin was also increased, with a concomitant rise in the production of IgM and IgG (Corsini et al 1977).

The major question concerning the changes in the cellular composition of the lymphoid organs is whether they result from

specific responses against the multitude of trypanosome antigen types or from a mitogen released by the parasites (see section 1.8.3.1). A mitogenic action has been ascribed to purified VSG (Diffley 1983), although these experiments did not control for the additional epitopes visible on soluble as compared with membrane incorporated VSG. Studies on trypanosome derived mitogens are hampered, however, by the fact that trypanosomes induce a predominantly IgM response. Given that IgM antibodies are generally of low avidity the probability that antibodies made against trypanosomes will spuriously cross-react with an unrelated antigen is high. Although many of the available data are compatible with the occurrence of a trypanosome-derived mitogen, until such a moiety is characterised this must remain a hypothesis without substance.

1.7.2 Autoimmunity

That autoimmunity may play a part in the pathology of trypanosome infections has been suggested by Seed and Gam (1967). Autoantibodies reacting with brain and cardiac antigens have been detected in *T. brucei* infections of monkeys (Houba *et al* 1969) and rabbits (Mackenzie and Boreham 1974) and also in rabbits infected with *T. congolense* (Mansfield and Kreier 1972). These antibodies however appeared in advance of detectable tissue pathology and chemotherapy of the infection did not lead to an autoimmune pathological condition. The stimulus for this autoantibody formation is unknown; they possibly resulted from tissue injury, polyclonal activation, or could simply be a fortuitous cross-reaction. It is interesting to note that all the autoantibodies characterised have been IgM, which makes cross reaction with trypanosome antigens difficult to disprove experimentally as IgM

antibodies generally are of low avidity.

1.7.3 Immune complex mediated pathology

As mentioned previously, anaemia is a serious pathological effect of trypanosome infections, especially in cattle. The cause of the anaemia is probably multifactorial although it is primarily haemolytic. Haemolysis by trypanosome lysates of all the major species pathogenic in cattle and humans has been observed *in vitro* (Murray *et al* 1979). Free fatty acids have been suggested as possible haemolytic effectors, as trypanosomes release large amounts of phospholipases upon lysis (Tizard *et al* 1978). Haemolytic proteins of M_r 10000D (Huan 1975) and 12000D (Murray *et al* 1978) have been characterised in trypanosome lysates but the mechanism of their lytic action remains unclear. The *in vivo* significance of these observations remains unclear. Haemolytic anaemia has been shown to operate in *T. brucei* infected rats and to parallel parasitaemia (Murray 1978). This activity was generated mainly by dying trypanosomes. In cattle, where parasitaemia is very much lower the significance of these results is questionable.

One mechanism of haemolysis which has been observed *in vivo* is the phagocytosis of red blood cells coated with immune complexes. This mechanism, in which soluble complexes of antigen and anti-trypanosome antibody were deposited on red blood cells and subsequently phagocytosed, has been observed in rabbits infected with *T. brucei* (Kobayashi *et al* 1976) and calves infected with *T. congolense* (Jennings 1976). The expansion of the macrophage population may further enhance the immune complex mediated destruction of red blood cells (Murray *et al* 1974b, Corsini *et al* 1977).

Some tissue damage may be caused solely by the oedema which is

seen in trypanosome infections and this may be at least partly caused by immune complexes. Trypanosome infection decreases the serum albumin concentration (Edington and Gilles 1976) and this leads to a decrease in the vascular osmotic pressure. Immune complex deposition causes an increased vascular permeability and this, in conjunction with the osmotic irregularity, results in considerable oedema (reviewed by Goodwin 1974).

Tissue damage by cellular infiltration is widespread in sleeping sickness patients. Such infiltrates are commonly composed of lymphocytes, plasma cells and macrophages, with polymorphonuclear leukocytes few in number. This infiltration may be the result of chemotaxis following immune complex deposition and, as such, the low numbers of neutrophils found is surprising. The most important of these lesions are the infiltrations of the myocardium and brain which may give rise to the observed changes in ECG pattern and psychological disorders respectively (Greenwood & Whittle 1980). Interestingly, such infiltrative lesions are absent in nu/nu mice (Galvao-Castro et al 1978) and this might imply a function for T-lymphocytes either in control of isotype switching to produce the antibodies responsible for complex formation, or in direct recruitment of cells into inflammatory sites presumably mediated by the lymphokine secretion.

Caution must be exercised in the extrapolation of the results from animal, and in particular rodent, infections to the disease process in humans. Indeed, the paucity of neutrophils in the infiltrative lesions and the absence from human disease of any serum sickness-like condition has led to speculation on the relevance of such findings to human disease (Greenwood and Whittle 1980).

The pathogenesis of brucei-group trypanosomiasis is obviously a multifactorial process. In the context of the immunopathology however there is abundant evidence that such pathology is a result of the immunoproliferative and hyperinflammatory response of the host to successive waves of tissue-invasive organisms.

1.8 Immunosuppression

Infection with African trypanosomes has been demonstrated to cause a marked suppression of the hosts' capability to respond to non-parasite antigens (reviewed by Mansfield 1981, Vickerman and Barry 1982, Roelants and Pinder 1983). It has also been suggested that the response to the trypanosomes themselves is depressed (Gasbarre et al 1981, Luckins and Gray 1983, Dempsey and Mansfield 1983). The mechanism (which is probably multifactorial) of the immunodepression remains unclear, and caution must be exercised in the extrapolation of murine data to human and bovine disease.

1.8.1 Depression of humoral responses

Early work on immunosuppression showed that laboratory animals infected with T. brucei made an impaired antibody response to sheep erythrocytes (Goodwin 1970, Goodwin et al 1972). Immunization 3 days after infection elicited IgG and IgM responses lower than in controls. Immediately after infection (1-4 days) a transient increase in the anti-sheep RBC response was observed (Hudson et al 1976). Depressed antibody responses to other T-cell dependent and also to T-independent antigens have also been noted (Ackerman and Seed 1976, Murray et al 1974b), although normal antibody responses to T-independent antigens have also been reported (Freeman et al 1974). Suppression of IgG responses occurred more rapidly than did suppression of IgM antibody production (Hudson et al 1976). Suppression of VAT-specific anti-trypanosome responses has also

been observed (Dempsey and Mansfield 1983) but these authors ommitted important controls for dosage, and route of administration, of trypanosomes. As in responses to exogenous antigen IgG responses were found to be affected earlier in the infection, although suppression was not apparent until the later stages of protracted infection. Similar observations have been made by Luckins and Gray (1983) in rabbits infected with T. congolense. Infected animals were challenged with an antigenically dissimilar stock and the humoral response against the challenge stock was found to be both delayed and diminished in its neutralising capability.

Interestingly, if immune priming took place before infection of rodents with T. b. gambiense (Ackerman and Seed 1976) or T. musculi (Brooks et al 1983), no significant effect on the secondary response to non-parasite antigens was observed. These findings contrast with those of Askonas et al (1979) who reported that mice infected with T. b. brucei displayed upsets of immunological memory in both the B and T-cell populations.

Immunosuppression in human and cattle infections is not as pronounced as in experimental infections of rodents. Despite this, infected hosts are more susceptible to secondary infections, which are often the cause of death. Patients infected with T. gambiense make lower antibody responses to vaccines and also display lowered skin reactivity to a variety of antigens (Greenwood 1974a). Cattle infected with T. congolense display diminished humoral responses to foot and mouth disease, clostridial and louping - ill vaccines (Holmes et al 1974, Scott et al 1977, Whitelaw et al 1979), although the reduction in neutralising antibody titre was variable. Similar infections made no detectable difference to the response

made by cattle to rinderpest vaccine (Rurangirwa et al 1979). Cattle infected with T. vivax exhibited responses to Brucella abortus vaccine which were proportional to the detectable parasitaemia and this may explain the variable responses observed by others (Rurangirwa et al 1983). Infection with T. vivax only slightly depressed antibody responses to bovine pleuropneumonia vaccine but when challenged with live pleuropneumonia only half the animals were protected (Ilemobade et al 1982). This study highlights the difference between functional, and in vitro detectable, suppression of humoral responses.

1.8.2 Depression of cell mediated responses

Delayed-type hypersensitivity reactions to oxazolone were reduced in animals infected with T. brucei (Urquhart et al 1973) or T. gambiense (Ackerman and Seed 1976). Granuloma formation in response to BCG was also diminished in rabbits infected with T. congolense (Mansfield and Wallace 1974). Conflicting results were presented by Freeman et al (1974) who failed to detect any depression in cell mediated immunity in mice infected with T. brucei. Proliferative T-cell responses against trypanosome antigens have also been reported to be suppressed in mice infected with T. brucei (Gasbarre et al 1980). These authors showed that T. brucei specific T-cells could be demonstrated in lymph nodes for only 2-3 weeks after infection. It was not demonstrated however that lack of responsiveness was due to active suppression or loss of memory. Subsequent work by these authors has demonstrated that the ability of both peritoneal macrophages and lymph node T-cells to mount a trypanosome specific response was severely impaired by infection (Gasbarre et al 1981).

Natural killer (NK) cells do not appear to have any activity

against trypanosomes, as demonstrated by the finding that beige mice, which are defective in NK cell function (Roder and Duwe 1979), show no significant difference in survival time in T. brucei infections (Jones and Hancock 1983).

1.8.3 Effect of drug treatment on immunosuppression

A general finding in work on immunosuppression in trypanosome infections is that cure of the infection with trypanocidal drugs leads to a rapid restoration of the immunocompetent state. An extension of this finding is that the severity of immunosuppression appears to correlate with the parasitaemia, although a slight lag in suppression may be observed (e.g. Askonas and Bancroft 1984). The relationship between parasitaemia and immunosuppression may explain the variation in results found in similar studies because if accurate observations of parasitaemia are not made meaningful interpretation of results is difficult. The central role of the spleen in the immune response to blood borne parasites must also be considered. The spleen is the organ most affected by trypanosome infection, both in terms of cellular disruption and suppressed responses. The response of lymph node cells to exogenous antigen may remain normal for some weeks after the onset of splenic immunosuppression in trypanosome infected mice (Wellhausen and Mansfield 1980, Kar et al 1981). As the majority of workers have concentrated on splenic responses great care must be taken before extrapolating such data to encompass general immunosuppressive mechanisms.

1.8.4 Mechanisms of immunodepression

1. B-cell mitogenesis. It has been proposed that trypanosomes produce a B-cell mitogen and that unregulated division and IgM production subsequently lead to clonal exhaustion and the observed

suppression (Urquhart et al 1973, Greenwood 1974b). This suggestion is supported by histopathological evidence and also from in vitro cultivation of spleen cells from infected mice. Cells cultured from late infections produce much less IgM and IgG, and this suggests exhaustion of B-lymphocyte potential (Corsini et al 1977). Mitogenic properties have been attributed to extracts of T. brucei (Esuruoso 1976) and T. congolense (Assoku and Tizard 1978). Purified VSG from T. rhodesiense has also been reported to be mitogenic (Diffley 1983). Further evidence for non-specific activation of B-cells come from the finding of high levels of heterophile and auto-antibodies in the sera of infected animals (Houba et al 1969). It has also been reported that most of the IgM from such sera could not be absorbed out by trypanosomes (Hudson et al 1976, Terry et al 1980).

In direct contrast to the above results Musoke et al (1981) showed that all of the excess IgM in the first two weeks of infection could be absorbed out using trypanosomes collected sequentially from the same animals, and that 85% could be similarly removed from third week sera. These authors suggest that previous attempts to absorb sera used an insufficient variety of VATs. In a case of human sleeping sickness all the IgM activity could be absorbed out by the infecting organism (Herbert et al 1980).

While B-cell exhaustion does appear to occur in trypanosomiasis, the case for a mitogen remains unproven. Indeed, given the great diversity of VATs which appear very early in an infection (Barry 1986b), it does not seem unlikely that B-cell exhaustion could be the result of specific lymphoid proliferation against the plethora of trypanosome antigens.

2. Suppressor cell generation. Spleen cells from trypanosome

infected mice suppress the normal response of murine spleen cells to mitogens *in vitro* (Eardley and Jayawardena 1977, Wellhausen and Mansfield 1979). Such suppression has been attributed to cells of both the T-lymphocyte and macrophage lineages (Corsini *et al* 1977, Wellhausen and Mansfield 1979). True suppression is very difficult to demonstrate in trypanosome infections due to the transfer of infective organisms with immune cells but Gasbarre *et al* (1981) demonstrated that neither T-cells nor macrophages from infected mice could fulfill their accessory role in the generation of an immune response. The T-cells involved were isolated from lymph nodes. Cells from the same source were found not to develop suppressor activity by Wellhausen and Mansfield (1980). Macrophages which have phagocytosed opsonised trypanosomes can mediate suppression *in vivo* if returned to syngeneic hosts (Grosskinsky and Askonas 1981). Such macrophages displayed many of the characteristics of activated macrophages; there was a reduction in the cell surface expression of Fc and complement receptors, mannose and the F4/80 marker while reactive oxygen intermediates and plasminogen activator were secreted in greater amounts (Grosskinsky *et al* 1983). The expression of Ia antigen was increased in mice infected with *T. b. brucei* (Grosskinsky *et al* 1983) but has been reported to be reduced in mice infected with *T. b. rhodesiense* (Bagasra *et al* 1981). Macrophages isolated from trypanosome infected mice were also found to secrete interleukin-1 (Askonas and Bancroft 1984). As cells with such an 'activated' phenotype are considered to be adequate antigen presenting cells (Unanue 1984) the suppressive activity is difficult to explain. One possibility is that the known suppressive effects of reactive oxygen intermediates and prostaglandin E_2 (Stenson and Parker 1980) outweigh the positive effects of interleukin-1.

Although the evidence for macrophages as target cells for the immunosuppressive action of trypanosomes is accumulating the precise identification of such an action remains obscure.

3.Antigenic competition. The nature of antigenic competition ensures that, in the polyantigenic system of a trypanosome infection, such a mechanism is very difficult to prove or to disprove. Given the nature of the antigenic stimulus in a trypanosome infection, namely recurrent challenge with large amounts of common antigens interspersed with challenge by large groups of VATs with each VAT present at a different level and individual VATs rarely or never repeated, antigenic competition is likely to occur at some point in the infection. By definition, antigenic competition can only operate in a receptor non-specific context. The target for such competition is therefore most likely to be at the macrophage surface. In this respect the reported loss of this cell population from the spleen of infected mice and subsequently the lymph nodes and peritoneal cavity is noteworthy (Bagasra et al 1981). Such depletion would obviously increase the probability of competition. Other workers have however described a marked increase in the macrophage content of lymphoid organs and the peritoneum of infected animals (Murray et al 1974b, Corsini et al 1977).

Whether a defect in processing of trypanosome antigens occurs remains uncertain. Macrophages from T. rhodesiense infected mice could not initiate an antibody response in syngeneic recipients after being primed in vitro to a non-parasite particulate antigen (Mansfield and Bagasra 1978); handling of soluble antigen was not demonstrably affected, which may be attributable to the soluble antigen presenting capability of dendritic cells (Kapsenberg et al

1986).

Although other mechanisms have been proposed to account for the immunosuppression seen in trypanosomiasis (e.g. hypocomplementaemia, loss of T-cell function, increased catabolism of immunoglobulins) the available evidence suggests that they could not account for the observed defect(s) (reviewed by Vickerman and Barry 1982). Such defects may however affect the pathogenesis of trypanosomiasis and could also have a bearing on the increased susceptibility to secondary infection which accompanies trypanosome infection.

1.9 The Chancre

An early account of the appearance of a local skin reaction at the site of a tsetse fly bite, with subsequent progression to sleeping sickness, was given by Graf (1929 a, b). The reaction, known as the chancre because of morphological similarities with the pathognomic lesion of primary syphilis, is the result of dense cellular infiltration into an extravascular focus of trypanosome division.

1.9.1 Gross morphology of the chancre

The course of the local skin reaction is broadly similar, irrespective of the species of trypanosome which induces it. Following the bite of a trypanosome infected tsetse fly there is usually no immediate reaction, although transitory urticarial wheals may be produced in reaction to insect saliva (Lester and Lloyd 1928). Under conditions of repeated challenge (e.g. field conditions) a progression of hypersensitivity responses may occur against insect saliva. After an induction period during which no reaction is made to the bite the animal develops a DTH to insect saliva. Upon repeated challenge DTH responses give way to

immediate hypersensitivity, although a transition period, marked by immediate reactions at new bite sites with eruptions of delayed reactions at older sites, often occurs (Benjamini et al 1960). Desensitisation of both types of response follows further challenge, with the DTH response being lost first (Mellanby 1946, Heilesen 1948, Benjamini et al 1961). None of the reactions against insect saliva resembled the trypanosome chancre in either morphology or kinetics.

Some days after a trypanosome infected bite (typically 4-14 days depending on the species of trypanosome and inoculum size; see below) a discrete nodule (5-10 mm) appears in the skin.

The nodule subsequently thickens and spreads until, 6-10 days later, it forms a painful, indurated plaque. The chancre then continues to spread, with thinning, until it is no longer visible. The appearance of the chancre, and the detection of large numbers of trypanosomes in fluid expressed from the lesion, precedes the detection of trypanosomes in the bloodstream (Willett and Gordon 1957).

Under field (and laboratory) conditions, metacyclic trypanosomes inoculated by the tsetse fly give rise to a chancre (Willett and Gordon 1957). The bloodstream forms of brucei-group trypanosomes (Corson 1932, Fairbairn 1933) and in vitro cultivated metacyclic forms of T. congolense (Luckins et al 1981) will also give rise to a chancre following intradermal or subcutaneous inoculation. Bloodstream forms of T. congolense do not elicit chancres (Awad et al 1969, Emery et al 1980a, Akol and Murray 1982). The use of bloodstream forms of T. brucei has shown that as the dose of trypanosomes is increased the latent period before the appearance of the reaction shortens and the maximal size of the reaction is greater (Willett and Gordon 1957). The observation of

such a dose response may explain why, in fly transmitted infections, T. brucei gives rise to larger chancres than T. congolense and T. vivax; greater numbers of metacyclic trypanosomes are extruded by T. brucei infected flies than by tsetse infected with T. congolense or T. vivax (Otieno and Darji 1979).

Interestingly rodents do not produce chancres in response to cyclically transmitted T. rhodesiense (Willett and Gordon 1957) or T. congolense (Emery and Moloo 1980). Similarly, bloodstream forms of these parasites inoculated intradermally elicit no local skin reactions (Willett and Gordon 1957, Emery et al 1980a, Akol and Murray 1982). All inoculation methods prove infective in rodents despite the absence of local skin reactions. The failure of rodents to make a skin response has been attributed to the skin thickness in these animals (Emery and Moloo 1980, Akol and Murray 1982).

1.9.2 Trypanosomes within local skin reactions

The trypanosomes within the chancre are found in large numbers before they are microscopically detectable in the bloodstream. Subinoculation of blood from infected rabbits into rats however, demonstrates that viable trypanosomes are present in the bloodstream within hours of an infective fly bite. In guinea-pigs, subinoculation proved positive only 5 minutes after the infective bite (Willett and Gordon 1957). Fly-derived infections therefore result in the deposition of two trypanosome populations; some trypanosomes entering the bloodstream directly while others persist in the skin. In goats which had been cyclically infected with T. vivax, cannulation of the efferent duct of the lymph node draining the chancre produced a marked reduction in the first patent

parasitaemia (Barry and Emery 1984). This suggests that most of the trypanosomes inoculated do not enter the bloodstream directly, and also that the major migratory route of the extravascular trypanosomes to the blood is via the lymphatics.

The trypanosomes observed within the chancre induced by T. rhodesiense are morphologically identical to bloodstream form trypanosomes (Willett and Gordon 1957). In contrast, organisms observed in T. congolense chancres were found to be approximately 2 um longer than bloodstream forms from the same animal (Roberts et al 1969). Also, organisms which resembled non-infective insect forms have been observed. These findings, together with the inability of bloodstream forms of T. congolense to elicit local skin reactions may suggest the presence of a distinct developmental and/or morphological form of T. congolense, intermediate in the transformation from metacyclic to bloodstream trypomastigotes. Studies of the morphology of trypanosomes found in the lymph nodes or thoracic duct of infected rats have shown only slender bloodstream trypanosomes to be present following intravenous or cyclical challenge with T. brucei (Tanner et al 1980) or intravenous infection with T. congolense (Ssenyonga and Adam 1975). Pleomorphic populations of T. brucei were reported in the lymph draining from local skin reactions in goats (Barry and Emery 1984). The reason for the observed difference may be that the population examined in the goat was the result of proliferation by the resident population in the chancre, while the lymphatic populations in rats are a result of active migration from the bloodstream.

The distribution of trypanosomes within local skin reactions has been reported in detail for T. rhodesiense in humans (Fairbairn & Godfrey 1957) and for T. congolense in rabbits and cattle (Gray and Luckins 1980, Akol and Murray 1982). Similar findings were

reported in all systems studied. Trypanosomes were essentially absent from the epidermis and were scarce in the dermis, although clusters of trypanosomes were noted around hair follicles and pilosebaceous units. Trypanosomes were most commonly found in association with collagen fibres, especially in and around the junction of the dermis and the subcutaneous fat. The lymphatic vessels draining the chancre were found to harbour many trypanosomes although trypanosomes persisted in dermal collagen when none could be found in the lymphatics (Gray and Luckins 1980). Vacuolated and degenerating trypanosomes were seen in reactions from day 11 onwards, which may be an indication of antibody mediated lysis in the extravascular compartment of the skin (Gray and Luckins 1980).

Antigenic variation in populations of trypanosomes within the chancre has not been studied in detail. The trypanosomes in lymph draining from the chancre however, which are representative of the chancre population, do show extensive antigenic variation. The same sequence of VATs appeared in both the lymph and bloodstream of goats infected with *T. brucei*, although the sequence appeared 2-3 days earlier in lymph (Barry and Emery 1984).

1.9.3 *The cellular infiltrate into the chancre*

Detailed histological accounts of the cellular composition of the chancre have been published for skin reactions induced in humans by *T. rhodesiense* (Fairbairn and Burtt 1946) and rabbits, goats and cattle infected by *T. brucei* and *T. congolense* (Gray and Luckins 1980; Emery *et al* 1980a,b; Akol and Murray 1982). All studies reported a transient influx of neutrophils early in the reaction (day 2-8) with this component being greater in *T. brucei* infected animals than in those infected with *T. congolense* or *T.*

vivax (Akol and Murray 1982).

The majority of the cells in the reaction are lymphocytes which account for about 90% of the infiltrating cells at the peak of the reaction (Emery et al 1980a). Many of these lymphocytes are B-cells (cytoplasmic Ig^+) and these have been shown to comprise up to 37% of the blast cells in T. brucei chancres in goats (Barry and Emery 1984). No further definition of the lymphocytes in the chancre has been recorded. Plasma cell numbers are maximal shortly after the observed peak of the reaction when up to 30% of the infiltrating lymphocytes may be of this phenotype (Emery et al 1980a).

Macrophages steadily accrue within the reaction and can make up 20% of the infiltrate by day 50 (in goats infected with T. congolense), when the reaction is no longer visible macroscopically (Emery et al 1980a).

No functional studies have been performed on any of the cell populations within the chancre or isolated from it. Thus, the antigen which elicits the chancre, its mode of presentation to the local immune system and the control processes involved in the chancre remain undefined.

1.9.4 Local skin reactions to secondary challenge with trypanosomes

Secondary challenge (by infected tsetse flies) of animals infected with the homologous stock of trypanosomes gives rise to an immediate type of skin reaction or no reaction at all (Emery et al 1980a; Luckins et al 1981); this is probably the clearest evidence that the chancre is not a DTH reaction. There appear to be two states of non-responsiveness for chancre formation. Animals which are still infected with the original stock do not form a chancre in response to either homologous or heterologous challenge; animals

which have been drug cured soon after the peak of the skin reaction fail to respond only to homologous challenge (Emery et al 1980a, Luckins et al 1981). In both cases, although no chancre was produced to heterologous challenge, superinfection does occur, as shown by the appearance of antibodies against the M-VATs of the challenge stock (Luckins and Gray 1983). Interestingly, in cattle which were drug cured before the peak of the chancre, immunity to homologous challenge was not total and a small local reaction was sometimes observed. This has been interpreted as a failure of some of the minor M-VATs to reach immunogenic numbers, or as an indication that systemic infection is necessary for the production of a sterile immunity (Emery et al 1980a). The failure to produce a local skin reaction in response to cyclical challenge has been attributed to immunosuppressive mechanisms (Luckins et al 1981, Luckins and Gray 1983). Antibody responses against challenge stocks were found to be both diminished and delayed.

The local skin reaction appears to be species specific, in that rabbits infected with T. congolense for periods which would preclude the formation of further chancres produce a normal local reaction following challenge with T. b. rhodesiense (Luckins et al 1981).

The chancre is, therefore, an acute inflammatory response made specifically against cutaneous challenge with trypanosomes. It is of intrinsic immunological interest because of the remarkable dominance of lymphocytes in the inflammatory infiltrate and also as the first manifestation of the mammalian host response to trypanosome infection.

2. THE DEVELOPMENT OF A MODEL SYSTEM OF CHANCRE FORMATION USING ANTIGENICALLY DEFINED TRYPANOSOME POPULATIONS

2.1 Introduction

The appearance of a local skin reaction to *T. b. rhodesiense*, has been shown to be host species dependent (Willett & Gordon 1957). Although many species of laboratory animals show a cellular infiltrate in response to intradermal challenge with trypanosomes, only rabbits exhibit what is macroscopically obvious as a chancre. Higher mammals including cattle and humans also show chancre formation in response to intradermal challenge with trypanosomes.

Of the species which display chancre formation only rabbits can be considered to be laboratory animals. Given that the rabbit immune system is very poorly defined in comparison with those of the mouse and rat and also in view of the experimental convenience of the smaller species it seemed reasonable to spend some time and effort developing a murine model of the local skin reaction.

The work of Willett & Gordon (1957) showed that the difference between local skin reactions in rabbits and those in small rodents is a phase of persistence and multiplication of the parasite in the skin. In small rodents the injected organisms do not persist in the skin. The possibility emerged, therefore, that any procedure which held the parasites in the skin may lead to a more pronounced lesion.

Another possible reason for the non-appearance of a chancre in small rodents is rapid systemic priming of the immune response leading to eradication of trypanosomes in the skin before the lesion becomes macroscopically visible.

It has been suggested by Akol & Murray (1982) and Emery *et al* (1980a) that the lack of chancres in small rodents is due to the

feeding tsetse not depositing metacyclic trypanosomes in the correct layer of the skin, yet rodents do not exhibit chancres when challenged intradermally with bloodstream forms of T. brucei; under the same conditions rabbits display a local skin reaction indistinguishable from that resulting from the bite of an infected tsetse fly (Willett and Gordon 1957). The nature of this host species difference remains unknown.

Willett and Gordon (1957) stressed that a depot of dividing trypanosomes must be present before chancre formation ensued. In this context any procedure which holds trypanosomes in rodent skin may help to promote chancre induction. To this end, adrenalin treatment, sloppy agar, and oil in water emulsion were employed as procedures which formed depots for variable lengths of time.

Adrenalin treatment of soft tissues (including skin) can restrict local blood flow for 2-8 hours (Howe and Whitehead 1972). Inoculation of trypanosomes into a site injected with adrenalin a few minutes earlier might therefore ensure that trypanosomes are held in the skin for at least one division cycle. Trypanosomes suspended in agar persist subcutaneously for 24-48 hours and intradermally for at least 96 hours (A. Scott, unpublished observations). Antigens in oil-in-water emulsions have been shown to persist at the site of inoculation for several weeks (Warren et al 1986).

It has been suggested that the chancre is, at least in part, a delayed-type hypersensitivity reaction. Work in rodent systems has demonstrated that some reactions of this type can be visualised only after the removal of T-suppressor cells (Askenase et al 1975, Schwartz et al 1978). With this in mind, cyclophosphamide treatment of rodents was carried out before inoculation of trypanosomes.

Suppressor T-cells are highly sensitive to the action of cyclophosphamide and their effects can be abrogated while leaving the humoral immune system intact (Shand and Liew 1980).

One advantage of the use of bloodstream forms, rather than metacyclic trypanosomes, to elicit local skin reactions is that it presents an opportunity to standardise the eliciting stimulus in terms of numbers of trypanosomes and of their VAT composition.

Rapid passage of bloodstream trypanosomes selects for the dividing, long slender forms. After twenty or more passages such lines often display almost complete monomorphism. A further consequence of rapid passaging is the persistent selection of the predominant VAT being expressed in the rodent host. This can lead to the stable expression of a single VAT by virtually all (> 99.9%) the trypanosomes in such monomorphic lines. Antigenic stability appears to be a property of the trypanosomes and not of the particular VAT as antibody induced relapses of such populations stably express the new VAT (Le Ray et al 1977). By using the intradermal injection of such lines it is possible to control accurately both the dose and antigenic composition of a challenge infection.

2.2 Materials and methods

2.2.1 Adrenalin treatment - A solution of adrenaline (20 ug/ml) in PBS was injected intradermally (50-100 ul/site) into animals. Twenty minutes later, trypanosomes in PSG were injected into the centre of the blanched plaque in the skin (see Figure 2.1).

2.2.2 Agar treatment - Trypanosomes were suspended in 0.015% (w/v) agarose in PSG at 37°C. Various concentrations of trypanosomes in 50 ul of agarose were injected intradermally into animals. Viable trypanosomes (having full flagellar mobility) could be isolated

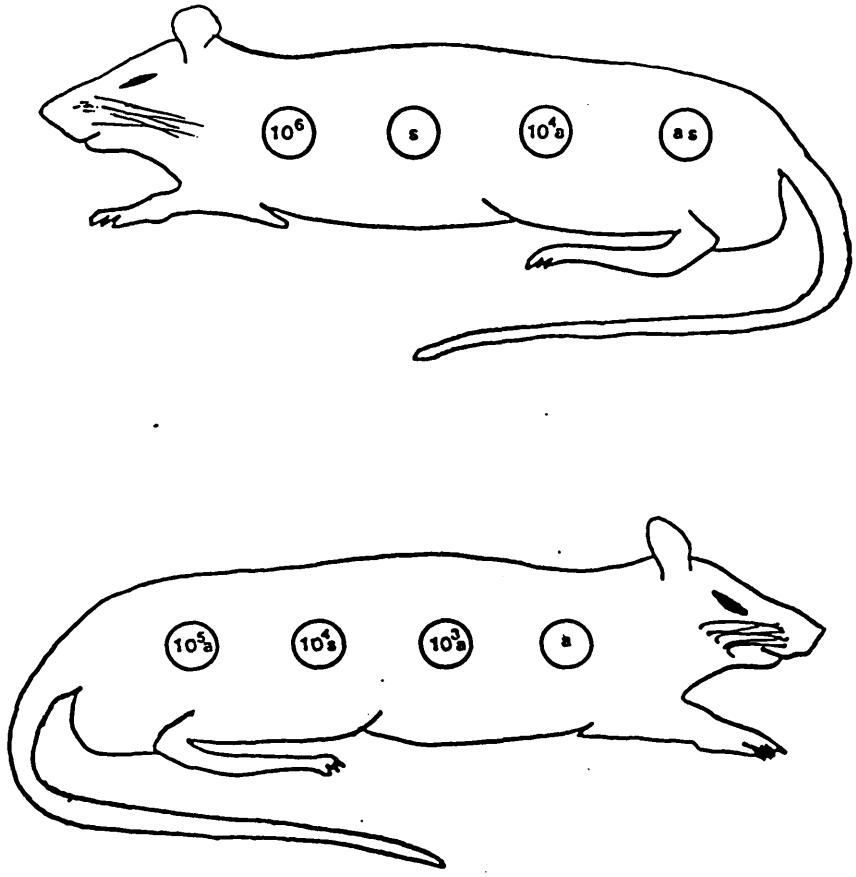


Figure 2.1

Figure 2.1 Diagram of injection sites used in experiments utilising adrenalin, agar or Freunds incomplete adjuvant to confine trypanosomes in rodent skin.

Animals were intradermally injected, at the sites shown, with adrenalin, agar or Freunds incomplete adjuvant (a), PSG (s) or various numbers of trypanosomes as shown. Combinations of treatments were also used, eg 10^5a denotes the inoculation of 10^5 trypanosomes after adrenalin treatment (or suspended in agar or Freunds incomplete adjuvant). Trypanosomes expressing GUTat 7.1, GUTat 7.2, and ILTat 1.3 were used throughout the experiments and each animal was inoculated with at least two different VATs.

from subcutaneous sites up to 72 hours after injection, and from intradermal sites up to 96 hours after injection (see Figure 2.1).

2.2.3 Cyclophosphamide treatment - Animals were pretreated with doses of 250, 100, 50, 10, 5, 1, 0.5 and 0.1 mg/kg cyclophosphamide (Sigma, Poole). Forty-eight hours later, various concentrations of trypanosomes in 50 ul PSG were injected intradermally into animals (see Figure 2.2).

2.2.4 Freunds incomplete adjuvant treatment - Various concentrations of trypanosomes in PSG were emulsified with Freunds incomplete adjuvant (DIFCO) and 50 ul of the emulsion was injected intradermally into animals (see Figure 2.1).

2.2.5 Trypanosomes - The cyclically transmitted trypanosomes used in this study were cloned stocks of EATRO 795. All flies used to elicit chancres had received their infections from a mouse which had been mechanically infected with stabilate GUP 1471. The full history of this stabilate is shown in the appendix. Flies were subsequently maintained essentially as described by Le Ray et al (1977) except that flies were fed on citrated sheep blood through a silicone rubber membrane. Infected flies were detected by examination of saliva probed onto warmed (37^{o}C) microscope slides. In these experiments only flies which probed large numbers of metacyclic trypanosomes were used to infect anaesthetised rodents by placing the individual flies (which were kept in gauze-bottomed cages) on the shaven abdomen of the animal concerned. Rabbits were infected without anaesthesia by allowing individually caged flies to feed on shaven areas of flank.

Three monomorphic, monotypic, bloodstream trypanosome lines were also used in these experiments. The histories of these lines, which are designated by the VAT which they stably express (i.e. GUTat 7.1, GUTat 7.2 and ILTat 1.3), are also shown in the

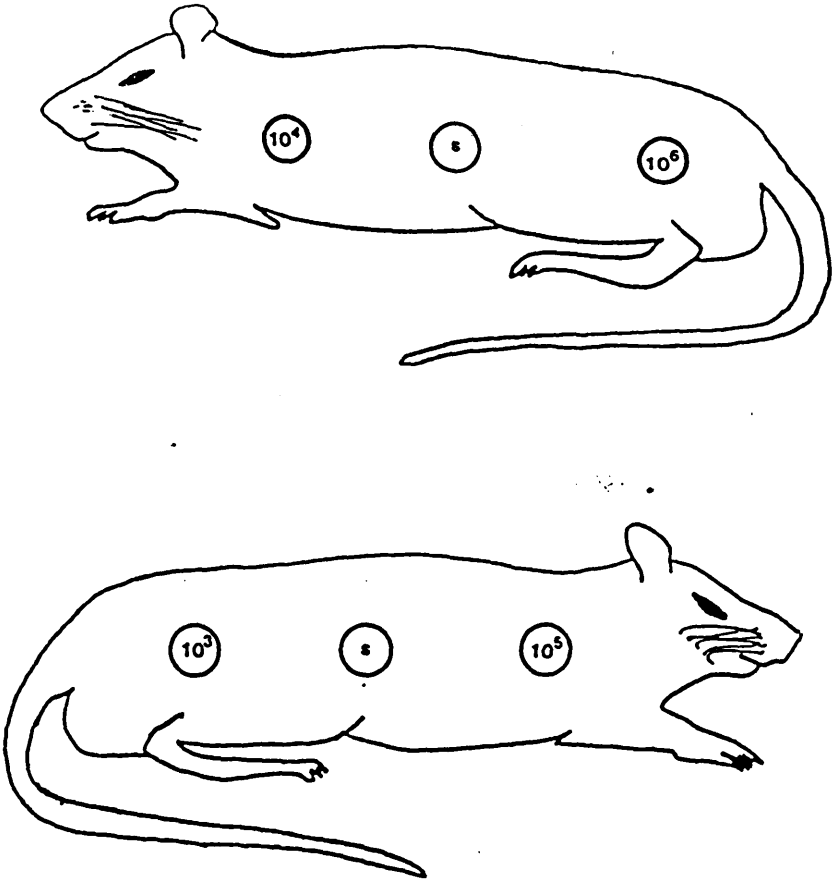


Figure 2.2

Figure 2.2 Diagram of injection sites used to intradermally inoculate rodents pretreated with cyclophosphamide.

Animals were inoculated with various numbers of trypanosomes as shown, or PSG (s). Trypanosomes expressing GUTat 7.1, GUTat 7.2 and ILTat 1.3 were used throughout the experiments and each animal was inoculated with at least two different VATs.

appendix.

2.2.6 Animals

Mice: Male BALB/c (20-25g) and male CBA (20-25g), from the breeding colony, Zoology Department, University of Glasgow.

Female CFLP (25-30g), (Interfauna, Huntingdon, Cambs.)

Male C57Bl/6 (20-25g), from the breeding colony, Department of Zoology, University of Edinburgh.

Rats: Adult female Sprague-Dawley (200-250g) from the breeding colony, Zoology Department, University of Glasgow.

Adult male Wistar (300-400g) rats from the breeding colony, Zoology Department, University of Glasgow.

Guinea-pigs: Adult Dunkin-Hartley guinea-pigs from the breeding colony, Pharmacology Department, University of Glasgow.

Rabbits: Adult female NZW-half lop rabbits (2.5-3kg), (Ranch Rabbits, Crawley Down, Sussex).

All animals had access to commercial laboratory animal diets and water ad libitum.

2.2.7 Monitoring skin reactions - Skin thickness was measured using vernier callipers (Mitutoyo, Japan). Reaction diameter was assessed by measuring two perpendicular diameters of the reaction area and taking an arithmetic average.

2.2.8 Purification of trypanosomes from blood - Bloodstream tryp-

anosomes were purified from infected rat or mouse blood according to the method of Lanham and Godfrey (1970). Briefly, columns were poured in 10ml syringes plugged with absorbent cotton wool using pre-swollen DE-52 (Whatman Ltd, Maidstone) which had been pre-equilibrated in PSG and washed with at least three column volumes of PSG (pH 8.05). The volume of cellulose used was at least five times the volume of infected blood to be treated. When the column was fully equilibrated, infected blood was carefully applied to the top of the column and allowed to penetrate into the cellulose before eluting with PSG (pH 8.05). Trypanosomes were eluted from the column while blood cells were retained.

After elution of trypanosomes the cellulose was recycled by washing in 0.5M NaCl until it was completely free of red blood cell contamination. The recycled cellulose was stored in PS containing 0.02% NaN_3. Cellulose was recycled in this way up to ten times before being discarded when binding efficiency decreased noticeably.

2.2.9 Immunofluorescence on trypanosome infected blood smears - Thin blood films were prepared from a drop of trypanosome infected tail blood, air dried and fixed in acetone for 15 minutes. When dried the slides could be stored desiccated at -40°C.

Reaction zones were drawn onto such antigen slides using plastic ink (Texpen; Mark-Tex Corporation, Englewood, N.J.). Once the ink had dried the slides were rehydrated in PBS for 5 minutes and the area between the reaction zones was dried with absorbent tissue.

An appropriately diluted antibody preparation was then applied to individual reaction zones. Antibodies were prepared as antisera (from rabbits, rats and mice) or monoclonal antibody (McAb) culture

VAT-specific antibody preparations used for immunofluorescence

VAT	Day 6 rabbit antiserum.	Monoclonal antibody	McAb preparation and working dilution	
	Working dilution	(McAb)	Culture supnt.	Ascites fluid
GUTat 7.1	1/600	GUPM 27.1	Neat	1/1000
GUTat 7.2	1/200	GUPM 18.7	Neat	1/800
GUTat 7.13	1/400	GUPM 17.1	Neat	1/300
ILTat 1.3	-	GUPM 23.2	Neat	1/1000
ILTat 1.61	-	GUPM 23.1	Neat	1/500

Table 2.1

FITC-conjugated second antibody preparations
used for immunofluorescence

Antiserum prepared in	Specificity	Source	Working dilution
Sheep	anti-mouse IgG (whole molecule)	Sigma, Poole, England	1/50
Goat	anti-mouse IgG (whole molecule) $(Fab')_2$ fragment	Cappel, Westchester, PA., USA.	1/100
Sheep	anti-rabbit IgG (whole molecule)	Institute Pasteur Production, Paris	1/200

Table 2.2

supernatants or ascites fluids. The method of production and characterisation of the McAbs used has been described by Crowe (1983). The antibody preparations available against each of the VATs used are detailed in Table 2.1. Incubations with the primary antibody preparation were carried out in a humid box at room temperature. After incubation for 30 minutes the slides were washed three times by dipping in Coplin jars filled with PBS, and excess PBS was removed from outside the reaction zones using an absorbent tissue.

The appropriate dilution of a suitable FITC - conjugated second antibody was then applied to the reaction zones. The antibody preparations used are detailed in Table 2.2. All second antibody preparations were diluted in PBS containing either 0.02% (w/v) Evans blue (Merck, Germany) or 0.001% (w/v) propidium iodide (Sigma, Poole) as a counterstain. All second antibodies were titrated for optimal fluorescent activity using a standard concentration of primary antibody directed against a known VAT. Slides were incubated with the second antibody for 30 minutes at room temperature in a humid box, and were subsequently washed twice in PBS before being mounted in PBS/glycerol (50:50, v/v).

Prepared slides were viewed using a Leitz Ortholux II microscope with incident light fluorescence, an HB050 high pressure mercury vapour lamp, a TK 510 dichroic mirror, 2 x KP 490 (exciting) filter and a K515 (suppressing) filter.

2.3 RESULTS

2.3.1 Intradermal inoculation of trypanosomes into rodents pre-treated with adrenalin

Rats (2 Sprague-Dawley and 2 Wistar), mice (3 Balb/C, 3 CFLP) and two outbred guinea-pigs were inoculated intradermally with

adrenalin or saline and ten minutes later with trypanosomes according to the scheme in Fig 2.1.

No skin reactions were seen at any of the inoculation sites. All the animals became blood positive for trypanosomes; mice by day 3, rats and guinea-pigs by day 5 after inoculation.

2.3.2 Intradermal inoculation into rodents of trypanosomes suspended in agar

Rats (2 Sprague-Dawley and 2 Wistar), mice (4 BALB/c, 4 CFLP) and one outbred guinea-pig were inoculated intradermally with trypanosomes suspended in 0.015% agarose in PSG (pH 8.05) according to the scheme in Fig.2.1.

No skin reactions were seen at any of the inoculation sites. All animals did however become blood positive for trypanosomes, mice by day 5 and the larger rodents (with one exception) by day 7. One rat (Sprague-Dawley, male) did not become blood positive until day 13.

2.3.3 Intradermal inoculation of trypanosomes into rodents pre-treated with cyclophosphamide

Groups of rodents (1 Sprague-Dawley rat, 1 Wistar rat, 2 BALB/c mice and 2 CFLP mice) were treated with cyclophosphamide and forty-eight hours later were inoculated intradermally with trypanosomes according to the scheme in Fig. 2.2.

Separate groups of rodents were pretreated with cyclophosphamide at doses of 300, 100, 50, 10, 1 and 0.05 mg/kg).

No skin reactions were observed at any of the inoculation sites. All animals did, however, become blood positive for trypanosomes; mice by day 2 or day 3 (1 CFLP mouse, 0.05mg/kg), rats by day 4.

2.3.4 Intradermal inoculation into rodents of trypanosomes suspended in Freunds incomplete adjuvant

Rats (2 Sprague-Dawley and 2 Wistar), mice (4 BALB/c and 4 CFLP) and one guinea-pig, were intradermally inoculated with trypanosomes suspended in Freunds incomplete adjuvant/PSG emulsion, as shown in Figure 2.1.

All sites injected with adjuvant developed erythematous reactions; sites inoculated with saline only, or trypanosomes without adjuvant showed no cutaneous reactivity.

All mice became parasitaemic by day 3, with rats and guinea-pigs becoming blood positive by day 6.

2.3.5 Experiments in which trypanosomes were injected intradermally into rabbits

As it has been known for many years that bloodstream form trypanosomes inoculated intradermally into rabbits will elicit a chancre, experiments were undertaken to determine if a model chancre, comparable to a tsetse fly bite elicited reaction, could be induced with monomorphic bloodstream form trypanosomes expressing a single VAT (monotypic).

Rabbits were inoculated intradermally with 10^3, 10^4, 10^5 or 10^6 cloned monomorphic trypanosomes expressing VAT GUTat 7.1, GUTat 7.2 or ILTat 1.3. In addition, eight infected tsetse flies were allowed to feed on the shaven flanks of rabbits. In total, six rabbits were inoculated. There was no restriction of one VAT to one rabbit or of one inoculation method (i.e. syringe or tsetse) to a single rabbit. Up to six sites were used on each rabbit as it had been established previously that, providing reactions did not merge, there was no significant effect upon the growth of local reactions between animals bearing six chancres and those with only

a single lesion.

The site of each inoculation was monitored for skin thickness and reaction diameter. The results are presented in figures 2.3 and 2.4.

The results clearly demonstrate that chancre formation is a dose dependent phenomenon. Chancres induced by infected tsetse fly bites correspond most closely to a dose of 10^5 monomorphic trypanosomes. This figure agrees well with the maximum number of trypanosomes which can be probed *in vitro* from infected tsetse flies in this laboratory (Barry *et al* 1983).

Following intradermal injection of trypanosomes, parasites were rarely seen in the blood, by either direct microscopic examination of wet blood films or by the buffy coat method (Woo 1970). Sub-inoculation of 0.5 ml of blood from these animals into mice proved positive (2/5) on day 4 and all mice inoculated after day 5 became infected.

2.4 Discussion

The results presented here demonstrate the dose dependency of chancre formation more clearly than previous work (Willett and Gordon 1957).

The results also suggest that an infective tsetse fly bite may contain up to 10^5 trypanosomes. As metacyclic trypanosomes do not divide (Vickerman 1985), the transformation to dividing bloodstream form organisms must occur very soon after deposition. Willett and Gordon (1957) found bloodstream form parasites in a guinea-pig only five minutes after the infective bite. This finding suggests that transformation occurs before, and not concomitantly with, cell division and supports the view that the infective fly bites, which

Figure 2.3 Graph of incremental skin thickness versus time for skin lesion elicited in rabbits by 10^3 ◆——◆, 10^4 ■——■, 10^5 ●——● , or 10^6 ▲——▲ monomorphic, monotypic bloodstream form T. b. rhodesiense.

Each point is the mean of three estimations (±1sd), and represents data obtained from lesions elicited by trypanosomes expressing at least two, and for most points three, of the VATs GUTat 7.1, GUTat 7.2 and ILTat 1.3. The data for reactions elicited by cyclically transmitted EATRO 795 are shown for comparison O---O. The data for fly bite induced reactions represents the mean of eight lesions (±1sd).

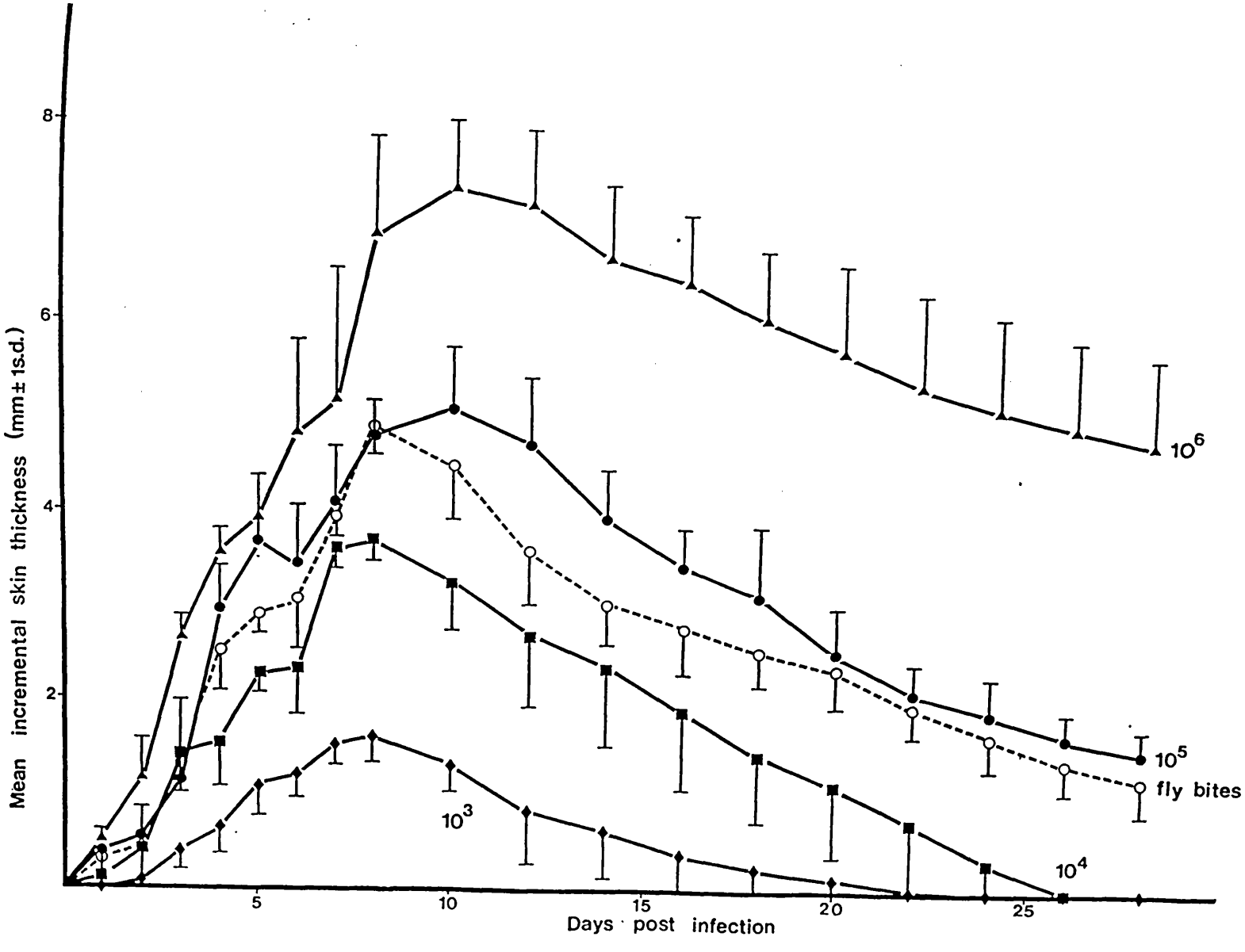

Mean incremental skin thickness (mm ± 1s.d.)
8
6
4
2
5
10
15
20
25
Days post infection
10^6
10^5
fly bites
10^3
10^4

Figure 2.3

Figure 2.4 Graph of reaction diameter versus time for skin lesion elicited in rabbits by 10^3 ◆——◆, 10^4 ■——■, 10^5 ●——● , or 10^6 ▲——▲ monomorphic, monotypic bloodstream form T. b. rhodesiense.

Each point is the mean of three estimations (±1sd), and represents data obtained from lesions elicited by trypanosomes expressing at least two, and for most points three, of the VATs GUTat 7.1, GUTat 7.2 and ILTat 1.3. The data for reactions elicited by cyclically transmitted EATRO 795 are shown for comparison O---O. The data for fly bite induced reactions represents the mean of eight lesions (±1sd).

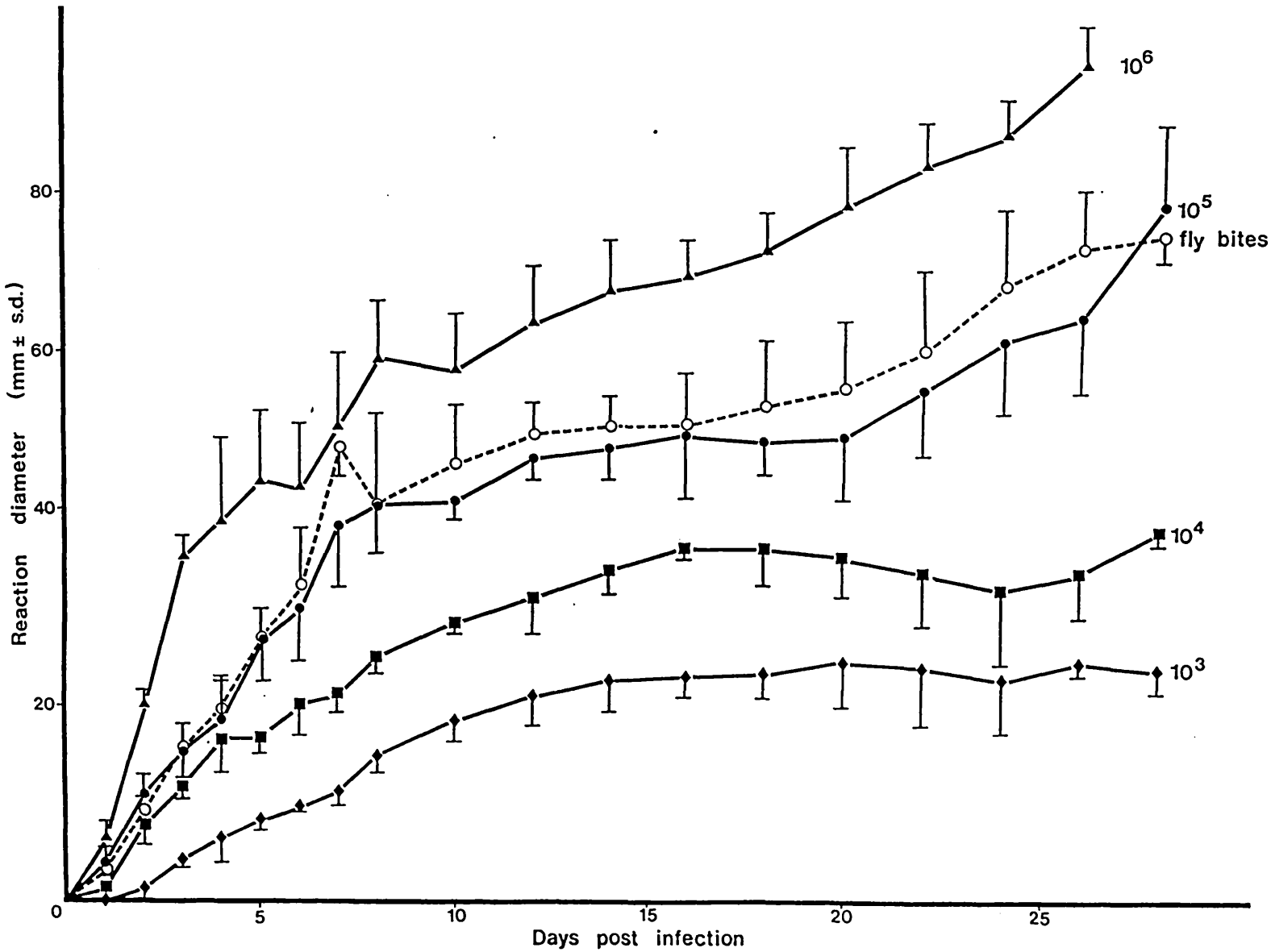

Reaction diameter (mm ± s.d.)
80
60
40
20
0
5
10
15
20
25
Days post infection
10^6
10^5
fly bites
10^4
10^3

Figure 2.4

the rabbits in this study received, contained approximately 10^5 trypanosomes. This figure is slightly higher than most observations of the number of trypanosomes in a tsetse fly salivary probe (Fairbairn and Burtt 1946, Barry et al 1983) but it is possible that much more saliva is extruded during feeding than probing.

The experiments described here also confirm that rodents do not exhibit chancre formation after cutaneous inoculation of bloodstream form trypanosomes (Willett and Gordon 1957). As intradermal injections were used these results do not agree with the suggestion (Emery et al 1980a, Akol and Murray 1982) that rodents make no dermal response because tsetse flies deposit metacyclic trypanosomes in the subcutaneous tissues. The reason for this peculiar unresponsiveness remains unclear, although the work reported here suggests that it is not due to lack of persistence of trypanosomes in the cutaneous tissues. The present work also precludes the suppression of a cutaneous response by suppressor cells in the immune system.

The model for chancre formation outlined in this work aimed at defining certain experimental parameters in order to simplify a highly complex system and thereby facilitate further investigation. The use of intradermal injections of trypanosomes served two purposes. Firstly, it provided complete control over the number of trypanosomes inoculated. As chancre formation has been shown to be dose dependent, only by standardising the dose can kinetic studies on the response be performed on a meaningful basis. Secondly, the removal of tsetse flies from the system prevents any immune response against insect saliva (Benjamini et al 1961) from obscuring the response to trypanosomes.

The use of monomorphic trypanosome populations expressing a single defined VAT facilitates the investigation of immune

responses to trypanosomes by providing material which is not only homogenous, but which is reproducible in an identical form. Antigenic material of this sort removes the problem of assaying for immune responses against a large collection of VATs, each of which is at a different prevalence.

The use of monomorphic populations may detract slightly from the suitability of this system as a model of infection as the rate of antigenic switching is also reduced (Lamont et al 1986). This aspect clearly differs from the natural situation where antigenic variation and responses to new variants can be detected in the lymph draining the site of the chancre (Barry and Emery 1984). Monomorphic populations, however, do undergo antigenic variation (Lamont et al 1986) and so the model system described may only simplify this aspect, rather than removing it totally. The paucity of stumpy form trypanosomes in the populations used may have a marked effect on the immune response to the parasites. It has been reported that only stumpy form trypanosomes induce a VAT-specific serological response (Black et al 1982), although conflicting results have been obtained by others (Zahalsky et al 1976).

The system described in this work provided an antigenically defined model of chancre production which produced chancres which, on morphological criteria, resemble those produced in cyclically transmitted infections. All subsequent experiments in the present study used the intradermal injection of 10^5 monomorphic monotypic bloodstream trypanosomes to elicit local skin reactions in rabbits.

3. THE KINETICS OF CHANCRE FORMATION AND THE ASSOCIATED SEROLOGICAL RESPONSE

3.1 Introduction

The kinetics of the appearance of a chancre following intradermal challenge with trypanosomes are variable. Following the bite of an infected tsetse, a chancre, which develops in about 90% of fly derived infections (Fairbairn and Burtt 1946, Gray and Luckins 1980), appears most commonly between days 4 and 12 but may not be visible until day 36 (Fairbairn and Burtt 1946). Such variability in onset is probably due to the number of metacyclics inoculated (Willett and Gordon 1957). A lesser degree of variability is found in the course of the reaction which almost always peaks 8-10 days after appearance and declines until it is no longer visible 30-40 days after the infective bite. The kinetics of the reaction suggest that a developing systemic immune response may initiate the decline in the chancre.

The local skin response to T. congolense has been found to be considerably altered in animals already harbouring a T. congolense infection (Gray and Luckins 1980, Luckins and Gray 1983). Antigenically heterologous challenge did not elicit a chancre in rabbits which had already been infected for 14 days or more. As antibody responses against the challenge stock were delayed and diminished this has been attributed to an immunosuppressive mechanism (Luckins and Gray 1983, Luckins et al. 1983).

The experiments presented here address the questions of when immunity to homologous challenge develops and how this correlates with the specific serological response. Experiments are also performed to examine the putative immunosuppressive properties (with

respect to chancre formation) of T. b. rhodesiense in a VAT-specific infection and challenge situation.

3.2 Materials and Methods

3.2.1 Production of local skin reactions - All chancres were elicited by the intradermal injection of 10^5 monomorphic, monotypic trypanosomes into rabbits (see Figures 3.1-3.3). The skin thickness at the injection site was monitored using vernier callipers as described previously (see section 2.2.7).

3.2.2 Preparation of sera - Samples of blood (2 ml) were removed from the peripheral ear vein of rabbits every second day. The blood was allowed to clot at room temperature and subsequently stored at 4°C overnight. The expressed serum was removed and spun down at 12,000 X g for two minutes. The supernatant was withdrawn and stored at 4°C for a maximum of 2 days. For long term storage, aliquots of sera were frozen at -40°C.

3.2.3 Immune lysis of trypanosomes - Specific immune lysis of trypanosomes was performed by a modification of the method of Van Meirvenne et al (1975). Monomorphic lines of trypanosomes expressing a single reference VAT were raised in mice and purified as previously described (see section 2.2.8). The VAT composition of the trypanosome population was checked by immunofluorescence on a fixed bloodsmear as described previously (see seection 2.2.9). Monotypic trypanosomes were resuspended at approximately 2 x 10^6/ml in fresh guinea-pig serum.

Serial $\log_3$ dilutions (volume 25ul) of experimental sera were prepared in flat-bottomed 96-well microtitre plates (Nunc, Denmark). To each well was added 50ul of the trypanosome suspension and the plate was incubated with constant agitation (60cycles/s at

28°C; Orbital incubator, Gallenkamp, Loughborough, England.) for 30 minutes. The plate was assayed for lysis by examination on a Leitz inverted microscope (x 32 objective with x 12.5 eyepieces). The lytic titre was taken as the last well in a series in which no motile organisms were observed. On three separate occasions this well was flooded with guinea-pig serum and the contents withdrawn and injected into a mouse. On none of these occasions did the mouse become parasitaemic.

3.3 RESULTS

3.3.1 Repeated inoculation of a single VAT

Rabbits were inoculated intradermally at 2 day intervals with trypanosomes expressing a single VAT. On days 8 and 10 they were also inoculated with an heterologous VAT. Control rabbits were inoculated on these days with 10^5 monomorphic trypanosomes of both the homologous and heterologous VATs. The course of the local skin reactions and the VAT-specific lytic antibody titres against the infecting VAT are shown in Figure 3.1.

The results show that repeated inoculation diminished chancre formation induced by the homologous VAT by day 8 and abolished it by day 10. When the homologous VAT was injected on day 10 it did elicit a local skin reaction, but this was an immediate type reaction, reaching maximum size in 8-12 hours after injection and still being just visible 48 hours after inoculation. The kinetics and appearance of these reactions suggested that they may be Arthus-type reactions.

When the heterologous VAT was injected it gave a lesion with the appearance of a primary chancre on both days 8 and 10. The VAT specific lytic antibody response against the original VAT reached a peak on day 14 and remained high for the remainder of the experiment. A similar response was made against the heterologous VAT.

Figure 3.1 Graph showing the development of a VAT-specific serological response to repeated inoculation of the same VAT.

The graphs display mean incremental skin thickness on the y1 axis and $\log_3$ VAT-specific lytic antibody titre on the y2 axis.

Two experimental groups of three rabbits were intradermally inoculated at two day intervals with 10^5 trypanosomes expressing GUTat 7.1 and ILTat 1.3 respectively. On days eight and ten, the two groups were challenged additionally with 10^5 trypanosomes expressing GUTat 7.2. Graphs A-F display the mean incremental skin thickness (± lsd) of the lesions elicited by the infecting VATs (●——●) and the heterologous challenge VAT (▲——▲). The mean $\log_3$ VAT-specific antibody titre against the infecting VAT is also shown (○——○).

Graphs G and H show the incremental skin thickness of lesions elicited when both the infecting and challenge VATs were simultaneously inoculated into two naive rabbits. Two inocula of 10^5 trypanosomes of each VAT were performed on each control rabbit, and the data therefore represent the mean incremental skin thickness (± lsd) of four lesions. The inocula in Graph G utilised the same trypanosome suspensions used in graph E. Similarly, Graphs H and F represent lesions induced by identical trypanosome preparations.

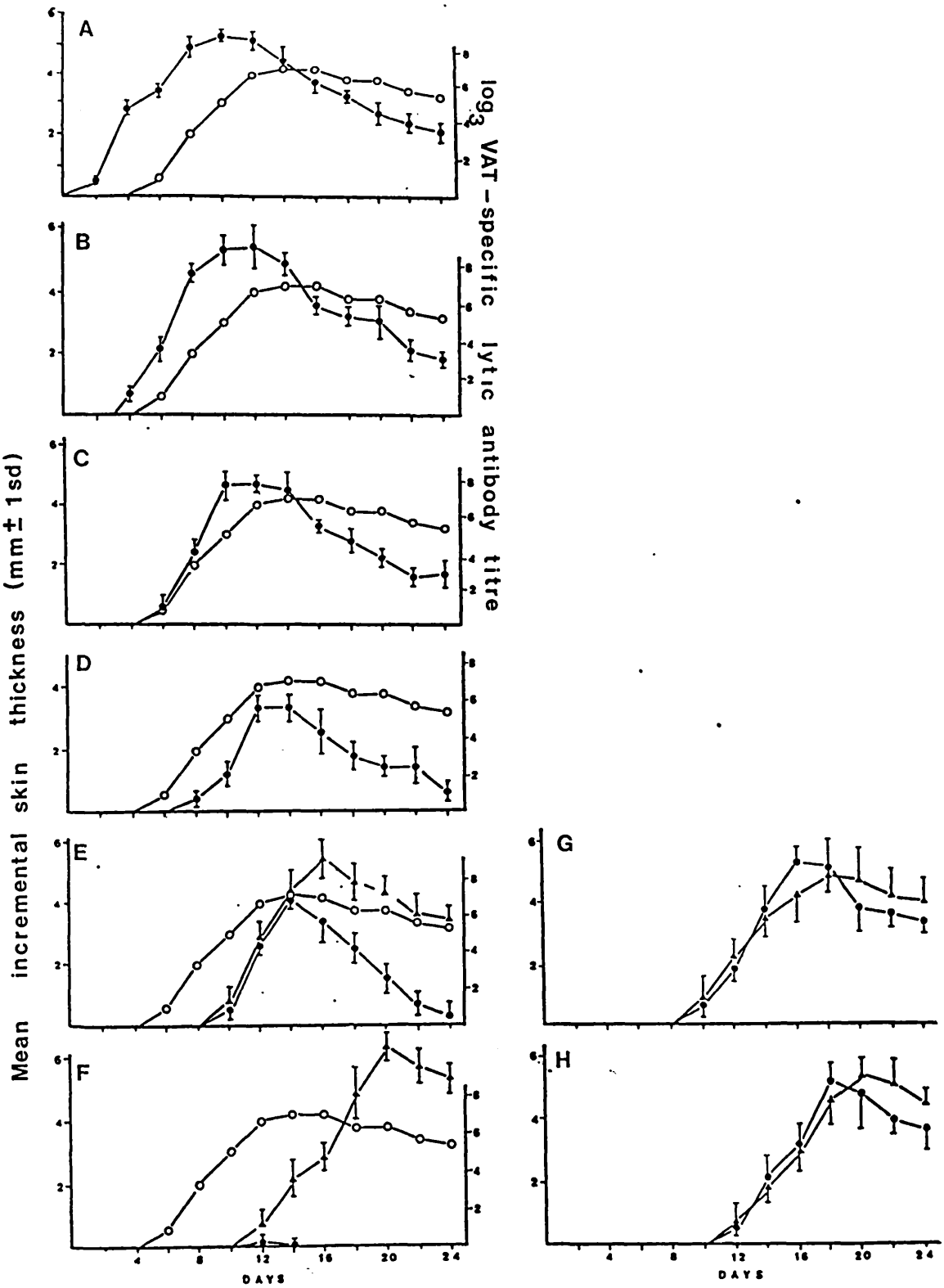

A
B
C
D
E
F
G
H
Mean incremental skin thickness (mm ± 1sd)
log3 VAT-specific lytic antibody titre
DAYS
DAYS

Figure 3.1

These results show that an effective serological response against an intradermally inoculated trypanosome population was made by day 10. Also, challenge with trypanosomes expressing a heterologous VAT on day 10 resulted in the formation of a normal primary chancre. This demonstrates that the immune response against the original VAT did not interfere with the response against the heterologous VAT. In addition, these results suggest that, at least by day 8-10, there is no suppression of the immune response which gives rise to the chancre.

3.3.2 Experiments in which primed animals were challenged with a heterologous VAT on day 10

As the previous experiment had shown that immunity to homologous challenge was present by day 10 experiments were undertaken to determine if the dermal response to heterologous challenge on day 10 was affected by an infection initiated by a single intradermal inoculation.

Rabbits were inoculated intradermally with 10^5 monomorphic trypanosomes expressing a single reference VAT (GUTat 7.1, GUTat 7.2 or ILTat 1.3). Local skin reactions developed at the site of all injections. On day 10 of infection each animal was challenged with 10^5 monomorphic trypanosomes of both the homologous and heterologous VAT. Local skin reactions occurred only at the site of the inoculation of the heterologous VAT. At the injection site of the homologous VAT an immediate type reaction occurred.

Sera were taken from all animals at two-day intervals throughout the experiment. The VAT-specific lytic antibody titres, together with the changes in skin thickness at the injection sites, are shown in figure 3.2. These results support the view that local skin reactions only occur in animals which are immunologically naive with

Figure 3.2 Graph of incremental skin thickness and serological responses of rabbits infected with 10^5 monomorphic trypanosomes expressing a single VAT, and challenged on day 10 with 10^5 monomorphic trypanosomes of the homologous and also an heterologous VAT.

Three rabbits were infected intradermally with GUTat 7.1 and challenged intradermally on day 10 with both GUTat 7.1 and GUTat 7.2. Similarly, two rabbits were infected with GUTat 7.2 and challenged with GUTat 7.2 and ILTat 1.3, while two further rabbits were infected with ILTat 1.3 and challenged with ILTat 1.3 and GUTat 7.2.

The results represent pooled data from all seven rabbits, and are shown as the mean incremental skin thickness for lesions elicited by the infecting VAT (VAT 1)●——●, and the challenge VAT (VAT 2)O——O. The $\log_3$ VAT-specific lytic antibody titre against both the infecting VAT ▲ , and the challenge VAT △ , are also shown.

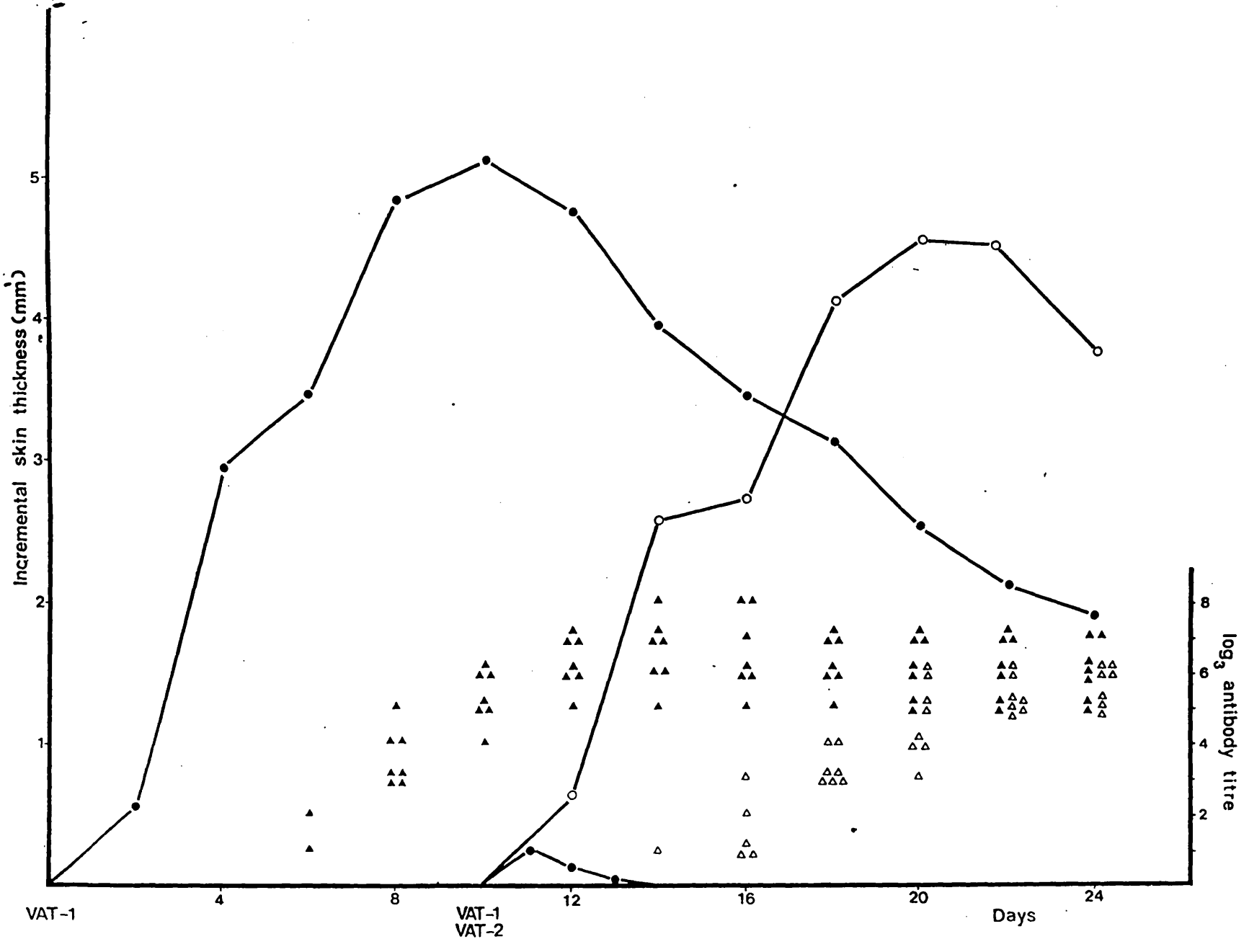

Incremental skin thickness (mm)
1
2
3
4
5
log2 antibody titre
2
4
6
8
VAT-1
4
8
VAT-1
VAT-2
12
16
20
24
Days

Figure 3.2

respect to the inoculated VAT. Immunity to a single VAT therefore has no influence on the behaviour of a heterologous VAT in terms of its capability to induce a local skin reaction.

3.3.3 Repeated inoculation of trypanosomes expressing different VATs

In order to extend the observations reported above rabbits were inoculated intradermally with 10^5 monomorphic trypanosomes expressing a single VAT at four day intervals. Each inoculum expressed a different VAT (in order, GUTat 7.1, ILTat 1.3, ILTat 1.61, GUTat 7.2 and GUTat 7.13). As before, sera were taken at two day intervals and assayed for their VAT-specific lytic antibody titre against each VAT separately. The results of these assays and the changes in skin thickness which occurred at the injection sites are shown in Figure 3.3.

Two rabbits were used as controls. One rabbit was inoculated intradermally with 10^5 ILTat 1.3 and ILTat 1.61 at the same time as the experimental group (i.e. at four day intervals). Similarly, one rabbit was inoculated with GUTat 7.2 and GUTat 7.13. All control inocula gave rise to a lesion with the appearance of a primary chancre.

3.4 DISCUSSION

The experiments reported here have demonstrated that in early T. b. rhodesiense infections chancre induction is a VAT-specific phenomenon. Repeated inoculation of a single VAT produced a local reaction at each injection site until a high titre serological response developed against the injected VAT. Once an effective and specific humoral response had developed an immediate-type reaction occurred at subsequent inoculation sites. At this stage in the infection inoculation with a different VAT produced a normal chancre. The effect of repeated inoculation with the same VAT was to cause

Figure 3.3 Graphs of incremental skin thickness and serological responses of rabbits injected sequentially, at four day intervals, with 10^5 monomorphic trypanosomes, where each inoculum expressed a different VAT.

An experimental group of three rabbits was inoculated at four day intervals with 10^5 monomorphic, monotypic trypanosomes. Each inoculum expressed a different VAT in the order GUTat 7.1, ILTat 1.3, ILTat 1.61, GUTat 7.2 and GUTat 7.13.

Graphs A-E show the mean incremental skin thickness (± lsd), (solid symbols eg ■) and the VAT-specific $\log_3$ lytic antibody titre (open symbols eg □) for each VAT.

Graph F shows the mean incremental skin thickness (± lsd) for three inocula (in a single rabbit) of 10^5 trypanosomes expressing ILTat 1.3 (●). The $\log_3$ VAT-specific lytic antibody titre is also shown (○). Graph G shows similar data for three lesions elicited (in the same rabbit as for graph F) by three intradermal inocula, four days later, of 10^5 trypanosomes expressing ILTat 1.61. The results of similar control inocula (in a further rabbit) of VATs GUTat 7.2 and GUTat 7.13 are shown in Graphs H and I.

Key to symbols:-

GUTat 7.1	▲	△
ILTat 1.3	●	○
ILTat 1.61	◆	◇
GUTat 7.2	■	□
GUTat 7.13	★	☆

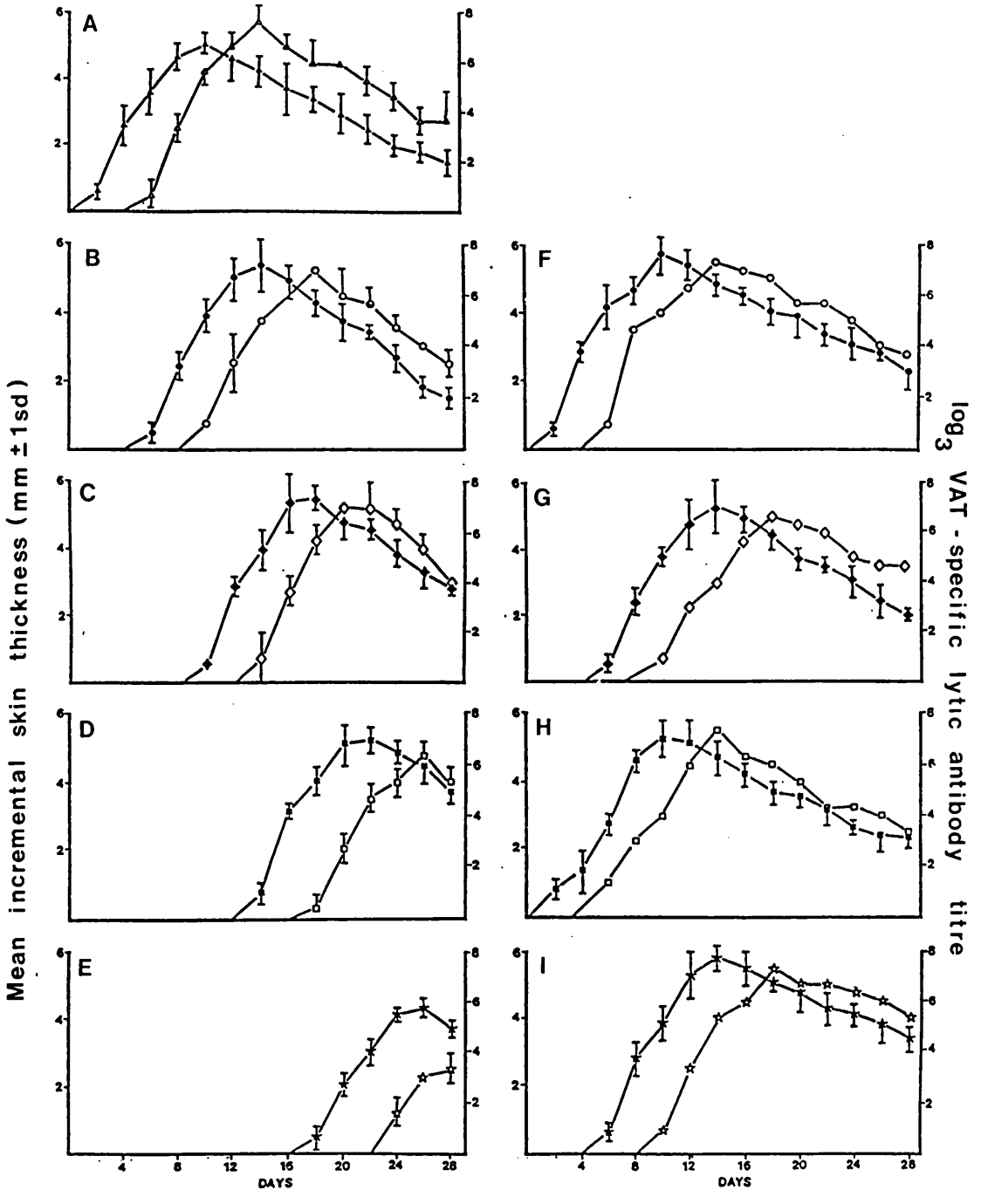

A
B
C
D
E
F
G
H
I
Mean incremental skin thickness (mm ± 1sd)
log3 VAT - specific lytic antibody titre
DAYS
DAYS

Figure 3.3

reactions to decline at an earlier stage in development than would a primary reaction in a naive animal. This led to a reduction in the maximum skin thickness attained by the reaction, and may be indicative of the effects of humoral effector mechanisms.

The development of a cutaneous Arthus-type reaction, with concomitant failure to induce a chancre was coincident with the generation of a high titre lytic antibody response against the infecting VAT. The declining phase of the skin reaction to a single inoculum was also coincident with such a response. Lysis of trypanosomes has been observed within the chancre (Gray and Luckins 1980) and this may be indicative of such an antibody mediated response. Trypanosome-specific (but not VAT-specific) IgG has been demonstrated in the extravascular compartment of the skin (Goodwin and Guy 1973), and in the intense, inflammatory environment of the chancre, where vascular permeability is raised, it seems likely that IgM will also gain access to this site.

Inoculation with a series of different VATs produced a normal skin reaction each time, except the inoculation of GUTat 7.13 on day 16. A VAT-specific lytic antibody response developed against each VAT in turn although the response against GUTat 7.13 was noticeably lower. The lower response against this VAT, both in the skin and serologically, suggest that in late infections some form of immunosuppressive mechanism is in operation. This view is supported by the fact that a control inoculation of this VAT into a normal rabbit produced both a "normal" chancre and a good serological response.

These results differ in some respects from those reported for chancres induced by tsetse fly transmitted T. congolense (Gray and Luckins 1980, Luckins and Gray 1983). Rabbits which had been

infected with T. congolense for as little as fourteen days showed no local skin reactions when challenged with an antigenically different stock of T. congolense (Luckins and Gray 1983). These authors attributed the lack of dermal responsiveness to interference with responses against the parasite during the infection. In support of this idea, it was demonstrated that the neutralising antibody response against challenge stock was both delayed (from 28 to 49 days) and reduced. These results are open to question, however, due to the use of antigenically undefined and mixed populations.

In vitro neutralisation of trypanosomes expressing a mixture of VATs is a measure of the response against the least immunogenic or least prevalent VAT in the mixture as organisms expressing VAT against which the antibody titre is lowest or latest are those most likely to survive when injected into rodents. One problem with neutralisation experiments is that, by necessity the same trypanosome population used for infection cannot be used for antibody testing. The closest such experiments can approach antigenic definition is the use of the same trypanosome stabilate passaged through a different mouse. Any antigenic variation which takes place within such a mouse will therefore reduce the suitability of the trypanosome population as antigenic targets in an assay of humoral antibody against the original stabilate. Any new VATs which arise while growing up trypanosomes for assay purposes will therefore appear as a reduction in the neutralisation capability of the antiserum. The use of an ELISA system to measure the antibody response against metacyclic antigens (Luckins et al 1983) also suffers from the drawback that the antigenic composition of the infecting and assay populations of trypanosomes may be different. The use of homogeneous antigenically defined populations overcomes this deficiency.

One explanation for the observed difference in results could be

the use of monomorphic lines of trypanosomes in the work presented here. Black et al (1985) have reported that such lines do not stimulate the production of either trypanosome specific or non-specific antibody. Indeed, monomorphic lines may not activate B-lymphocytes at all, as no VSG-specific hybrids could be made from the spleens of animals infected with such trypanosomes (Sendashonga and Black 1982). These lines have also been reported as having no immunosuppressive properties (Sendashonga and Black 1982). The work presented here clearly demonstrates that these lines are highly immunogenic in rabbits. Studies on the immunosuppressive properties of different cloned trypanosome lines have correlated virulence with ability to suppress (Sacks et al 1980). Virulence was associated with the population doubling time, which was maximised in populations containing no, or very few, stumpy form trypanosomes (BA Askonas, personal communication). Also, Zahalsky and Weinberg (1976) demonstrated that virulent, monomorphic lines do induce primary antibody responses.

Luckins and Gray (1983) suggested, on the basis of their observations on antibody responses that immunosuppression of responses against the parasite was responsible for the failure of infected animals to produce a chancre in response to heterologous cyclical challenge. No possible defect in any other immune response was however investigated. The results presented here are not entirely consistent with this suggestion, although the diminished response induced by inoculation on day 16 may indicate that such immunosuppressive mechanisms are perhaps operating in late infections.

4. THE SEQUENTIAL CHANGES IN THE CELLULAR INFILTRATE INTO THE CHANCRE

4.1. Introduction

The cellular composition of the leukocytic infiltrate into the chancre was first described in detail by Fairbairn and Godfrey (1957). Their detailed histological findings provided only a qualitative analysis. The first quantitative work in this area was performed by Emery et al (1980a), who, from histological data, formed a sequential analysis of the cellular composition within local skin reactions to T. congolense, thus providing a dynamic account of the infiltrate. After an initial influx of polymorphonuclear leukocytes the majority of the infiltrating cells were found to be lymphocytes. Despite the obvious importance of this cell population it was not possible to characterise them further, except to quantitate the proportion of plasma cells on morphological criteria.

An obvious extension of this work would be to characterise immunologically the lymphocytes involved in this reaction. The use of monoclonal antibodies to identify phenotypic markers on lymphocytes has become a powerful technique in the study of murine and human immune responses (for reviews see Ledbetter et al 1980, Reinherz and Schlossman 1980). Neither of these systems are available for study of the chancre, however, as rodents do not exhibit these reactions (see Chapter 2) and pathological samples of human material cannot easily be obtained. The use of human material would also preclude experimental manipulation of the system. Recently, however, monoclonal antibodies against phenotypic determinants on rabbit lymphocytes have been described (de Smet et al 1983; Watkins et al 1984). Using such monoclonal antibodies, the

sequential cellular changes within the local skin reaction are described in greater detail than previous accounts (Emery et al 1980a).

The McAbs used in this study are : 4B9 - which binds to all peripheral T-lymphocytes.

10B3 - which binds to a major subpopulation of peripheral T-lymphocytes and may include the alloreactive T-cell population.

12C7 - which binds to a minority of peripheral T-cells and may correspond to the OKT5/8 McAb which defines the cytotoxic/suppressor subset of human T-lymphocytes.

The populations of cells recognised by these antibodies will be referred to as T-cells, T_a-cells and T_c/T_s cells respectively although it is stressed that this is not a definitive classification (de Smet et al 1983) but rather a device to help the reader.

Using this panel of monoclonal antibodies (de Smet et al 1983), which were a kind gift (as ascites fluids) of Dr R Hamers, the sequential cellular changes during local skin reactions against T. b. rhodesiense are here described in greater detail.

A similar analysis is described on peripheral blood lymphocytes from rabbits infected intradermally. To facilitate the labelling of lymphocytes from the peripheral blood of several rabbits simultaneously it was necessary to develop a rapid method for the purification of mononuclear cells from small volumes of blood; this is also described.

4.2 Materials and Methods

4.2.1 Media and solutions

Stock Percoll solution - All density gradient centrifugation procedures were carried out on Percoll gradients (Pharmacia, Uppsala,

Sweden). A stock solution was prepared as outlined below:

Percoll	90 ml
Hanks' balanced salts solution (10X),(Sigma, Poole)	8.965ml
HEPES (1M), (Sigma, Poole)	1.00 ml
HCl (1M), (BDH, Poole)	370 ul

This stock solution was prepared aseptically from sterile components (Percoll is supplied in sterile form and the other solutions were filter sterilised with 0.22 um pore size filter) and could be stored for up to six months at 4°C.

RPMI incomplete medium - This medium was used to wash cells during isolation procedures and as a stock solution for culture medium. It consisted of RPMI 1640 (Gibco-Europe, Paisley, Scotland) with 25 mM HEPES at pH 7.2. Five litre batches of this medium were prepared from powder and filter sterilised (0.22 um pore size). The prepared medium was stored for up to six months at 4°C. For cell isolation procedures 100 U/ml penicillin (Glaxo, Greenford, England) and 100 ug/ml streptomycin sulphate (Evans, Greenford, England) were added. These antibiotics were omitted when RPMI 1640 was used as a stock solution for culture medium (Chapter 5).

4.2.2 Histology - Areas of skin were removed from freshly killed rabbits and placed in moulds containing O.C.T. Embedding Compound (Tissue-Tek; Miles, Naperville, NJ., U.S.A.). The tissue was then covered with the embedding compound and the mould was suspended in vapour phase above liquid nitrogen. When the blocks had hardened they were stored at -40°C in evacuated airtight containers.

Sections of skin (7 um) were cut from blocks at -18°C on a S.L.E.E. cryostat (model HRL). Sections were collected on glass microscope slides which had been pre-coated with 0.5% (w/v)

gelatin. Sections were air dried and fixed in methanol before staining with Mayer's haemalum and eosin by the method of Kiernan (1981). Stained sections were dehydrated in graded alcohols before being cleared in Histoclear (National Diagnostics, Aston Clinton, Bucks) and mounted under glass coverslips in Histomount (National Diagnostics).

4.2.3 Cell preparation from skin - An area of skin, including the subcutis, of approximately 4 cm diameter was removed from the site of the local skin reaction using dissecting scissors. The skin was cut into 1mm cubes and incubated in 2.0 mg/ml collagenase (Type IV; Sigma, Poole) in incomplete RPMI 1640 for 2 hours at 37°C. The skin was then disrupted through a nylon mesh by repeated percussion with the barrel of a 10ml syringe to yield a cell suspension. After washing twice in RPMI 1640, with spinning at 550 X g for 1 minute at room temperature, the cells were resuspended in 0.85% NH_4Cl/0.1 M Tris-HCl pH 8.0 (9:1, v/v) to lyse any red blood cells. After incubation (10 minutes at 20°C) the cell suspension was underlaid with FCS and spun down (1000 X g for 1 minute at room temperature) to remove cell ghosts. The cells were washed in RPMI 1640 before being applied onto a Hanks Percoll/incomplete RPMI 1640 (25:17, v/v) gradient and spun at 450 X g for 40 minutes at 4°C. The cell layer which formed at the interface was removed, washed as described above and applied to a 2 ml Sephadex G-10 column at 37°C. The cells eluted from this column were washed in RPMI 1640 before staining. Such cell populations were found to be composed of 90-95% lymphocytes as judged by morphology on Giemsa's stained smears. White blood cell counts performed on stained sections of local reactions and stained smears of cells sampled immediately after red cell lysis during isolation, showed that no gross selection of a particular cell type

occurred during isolation (see Table 4.1).

4.2.4 Cell preparation from peripheral blood - Approximately 750ul of heparinised whole blood was layered above an equal volume of stock Percoll/incomplete RPMI 1640 (13:12, v/v) and spun at approximately 3,000 X g (low setting on MSE 'Microcentaur') for 2 minutes. Cells from the interface were removed and washed twice in PBS before labelling.

4.2.5 Immunofluorescent labelling of lymphocytes - About 2 x 10^6 washed cells were resuspended in 100 ul of appropriately diluted monoclonal antibody in RPMI 1640 and incubated at room temperature for 1 hour. After washing twice in RPMI 1640, as described above, the cells were incubated with appropriately diluted (see Table 2.2) FITC-conjugated goat anti-mouse IgG (whole molecule), $F(ab')_2$ fragment (Cappel Laboratories, Westchester, PA., U.S.A.) on ice for 1 hour. After three washes in ice-cold PBS the cells were fixed in 1% formaldehyde in PBS overnight. The cells were subsequently washed four times in PBS before being resuspended in PBS/glycerol (50:50, v/v) and examined using a Leitz Ortholux II microscope with incident light fluorescence, an HBO 50 high pressure mercury vapour lamp, a TK 510 dichroic mirror, 2 X KP 490 (exciting) filter and K515 (suppressing) filter.

Staining of lymphocytes for surface immunoglobulin was performed by incubating washed cells in appropriately diluted (see Table 2.2) FITC - sheep anti-rabbit IgG (whole molecule) (Institut Pasteur Production, Paris, France) for 1 hour at room temperature. Cells were subsequently washed, fixed and examined as before.

The FITC-conjugated antisera were titrated for optimal fluorescent activity in an indirect fluorescent antibody test on acetone fixed trypanosomes (see section 2.2.8). The optimal

Differential leukocyte counts (percent ± 1sd) of chancre infiltrate cells stained *in situ* or following isolation

Cell type	Day 10 chancre		Day 22 chancre	
	in situ	isolated	*in situ*	isolated
Neutrophils	5 ± 4	8 ± 3	2 ± 1	2 ± 1
Lymphocytes	80 ± 8	86 ± 7	72 ± 11	82 ± 9
Macrophages	8 ± 4	6 ± 3	21 ± 8	17 ± 6
Eosinophils	2 ± 2	1 ± 1	3 ± 2	1 ± 1
Others	2 ± 2	5 ± 3	8 ± 5	6 ± 4

Table 4.1

concentration of each of the anti-lymphocyte monoclonal antibodies was obtained by titrating the monoclonal antibody on samples of peripheral blood lymphocytes. The bound monoclonal antibody was detected with the previously determined optimal concentration of FITC-goat anti-mouse IgG (whole molecule), $F(ab')_2$ fragment.

4.2.6 Production of local skin reactions - Local skin reactions were elicited by injecting 10^5 monomorphic trypanosomes intradermally into rabbits. Trypanosomes expressing VATs GUTat 7.1, GUTat 7.2, GUTat 7.13, ILTat 1.3 and ILTat 1.61 were used. Trypanosomes were grown up and purified as previously described (section 2.2.7). Up to four different VATs were injected into each rabbit (two inoculations on each shaved flank), with two day intervals between the inoculation of each VAT. The order of injection of VATs was varied on each animal and had no apparent effect upon the appearance of the chancres elicited. At least three local skin reactions were studied for each time point. Skin from areas unaffected by local reactions was used in control preparations, in addition to skin from a single uninfected rabbit.

4.3 RESULTS

4.3.1 Changes in the proportions of lymphocyte subpopulations in rabbit peripheral blood following intradermal challenge with 10^5 monomorphic trypanosomes

Rabbit peripheral blood mononuclear cells, collected every second day following the intradermal injection of 10^5 GUTat 7.1 or GUTat 7.2 trypanosomes were stained with monoclonal antibodies and a polyclonal anti-rabbit Ig. The results are presented in Figure 4.1.

The proportion of $^{4B9+}$ T-lymphocytes remained fairly constant throughout the infection. Following a slight, but insignificant

Figure 4.1 Graph of the lymphocyte subpopulation composition of rabbit peripheral blood following the intradermal injection of 10^5 monomorphic trypanosomes.

Cells were stained with McAbs 4B9 (T-cells) ■——■, 10B3 (T_a-cells) ●——● and 12C7 (T_c/T_s-cells) ▲——▲ or with polyclonal anti-rabbit immunoglobulin (B-cells) ◆——◆.

Three rabbits were used and were infected by the intradermal inoculation of 10^5 GUTat 7.1, GUTat7.2 and ILTat 1.3 trypanosomes respectively.

Each point represents the mean determination (±1sd) from the three rabbits.

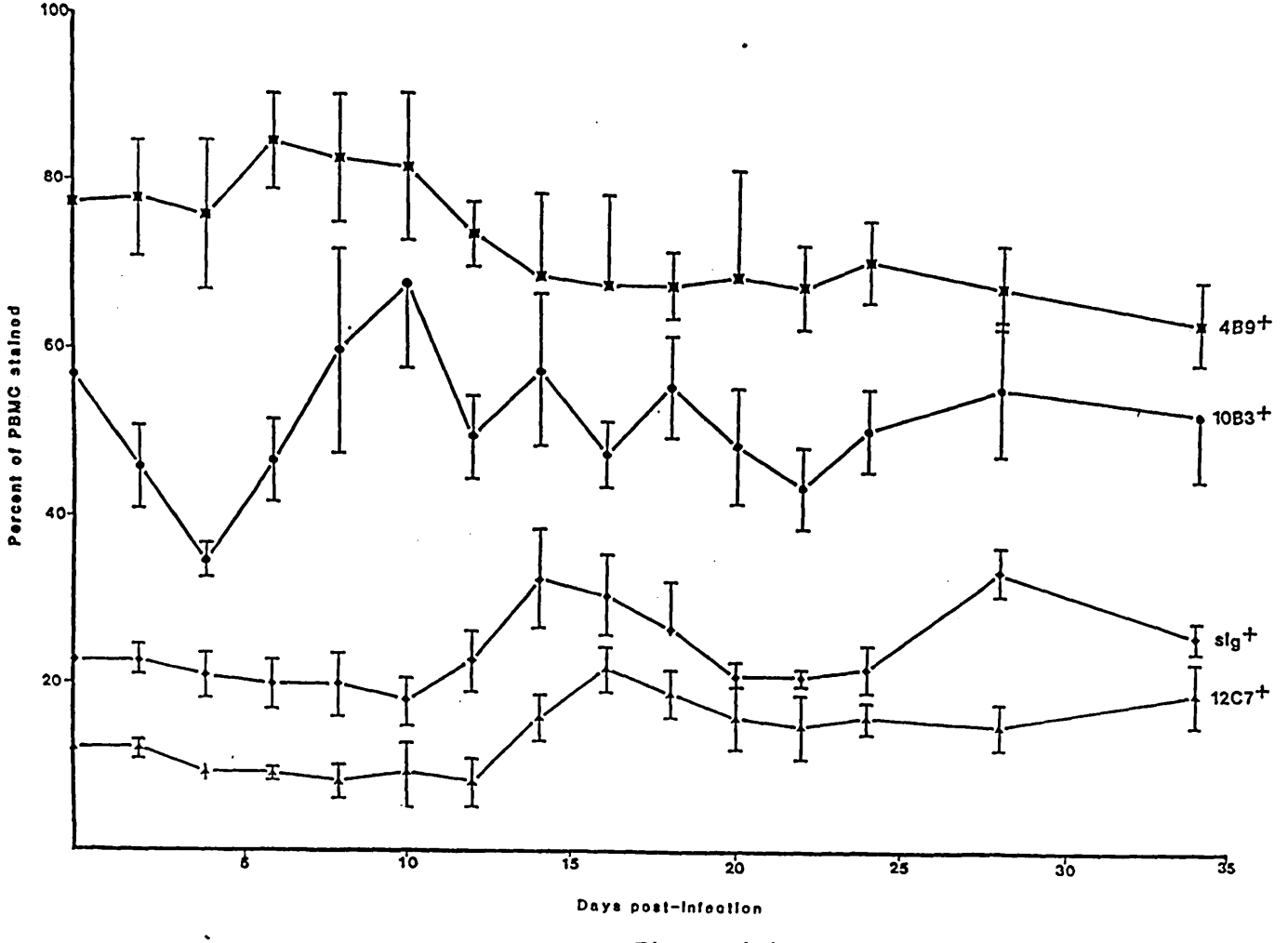

100
80
60
40
20
Percent of PBMC stained
5
10
15
20
25
30
35
Days post-infection
4B9+
10B3+
sIg+
12C7+

Figure 4.1

(Student's t-test, day 6/day 10; $0.1<p<0.2$) peak in the proportion of these cells between days 6 and 10 they declined as B-cell numbers began to rise.

From day 14 onwards they formed about 70% of the peripheral blood mononuclear cell population.

Peripheral blood B-lymphocytes (sIg^+) comprised around 20% of the peripheral blood mononuclear cell population throughout the infection with the exception of two peaks between days 10-20 and 24-34, when they rose to around 30%.

The $12C7^+$ T_C/T_S-cell population comprised 8-12% of the total until day 12. Thereafter the proportion of these cells rose to a peak of 22% on day 16 and persisted at a level of 16-19% for the remainder of the infection.

The proportion of $10B3^+$ T_a-cells in the peripheral blood mononuclear cell population was highly variable, especially early in the infection. Over the first four days the proportion fell from 57% to 35% and subsequently rose to 68% on day 10. Thereafter, although still variable, the proportion remained more constant at around 50% of PBMC. As there was no compensating fluctuation in the proportion of $4B9^+$ T- or $12C7^+$ T_C/T_S-cells, these results imply the presence of at least one other subpopulation of T-lymphocytes against which no McAb is available.

4.3.2 Changes in the proportions of lymphocyte populations infiltrating the chancre

Following intradermal challenge with 10^5 GUTat 7.1, GUTat 7.2, GUTat 7.13, ILTat 1.3 or ILTat 1.61 trypanosomes, skin from the injection site was removed. The mononuclear cells were extracted from the skin and stained with antibodies against surface antigens of T-cell subsets or immunoglobulin. The results presented represent

chancres elicited by at least two, and in most cases three, different VATs for each time point. The results of this analysis are shown in Figures 4.2 and 4.3.

Normal rabbit skin contained very few lymphocytes and the vast majority of these were $4B9^+$ T-cells. Most of the T-lymphocytes found in normal skin were also $10B3^+$ T_a-cells. No $12C7^+$ T_c/T_s-cells were ever observed from normal skin, leaving around 18% of the T-cells untyped.

The proportions of $4B9^+$ T-cells and sIg^+ cells appeared to be inversely related. As the local skin reaction progressed the proportion of B-lymphocytes (sIg^+) increased until day 16, when these cells comprised 54%. During this period there was a corresponding decrease in the proportion of $4B9^+$ T-cells. As the reaction declined the proportion of sIg^+ cells decreased to 26% on day 24. The proportion of $4B9^+$ T-cells rose slightly over this period.

The proportion of $10B3^+$ T_a-cells closely followed the pattern of $4B9^+$ T-cells over the first 12 days of the reaction. After this time however, while the proportion of $4B9^+$ T-cells stabilised and then increased, the proportion of $10B3^+$ T_a-cells continued to fall until by day 24 these cells composed only 10% of the infiltrate.

Cells bearing the 12C7 antigen were first seen in the infiltrate on day 6. These cells steadily increased as a proportion of the infiltrate until by day 18 they composed 30% of the infiltrating cells. These cells persisted at this level until the end of the experiment on day 24.

Trypanosomes were rarely seen in reactions later than day 6 or day 8.

4.3.3 Changes in the absolute numbers of different cell types in the chancre infiltrate

Figure 4.2 Graph of the proportions of T-lymphocytes and B-lymphocytes isolated from chancres induced by 10^5 monomorphic trypanosomes at various times after infection.

Cells were stained with McAb 4B9 (T-cells) ■——■ or polyclonal anti-rabbit immunoglobulin (B-cells) ◆——◆ .

Chancres were elicited by trypanosomes expressing GUTat 7.1, GUTat 7.2, GUTat 7.13, ILTat 1.3 and ILTat 1.61. The results represent the pooled data from twelve infected rabbits each of which received four inocula.

Each point is the mean of three determinations (±1sd) from chancres elicited by at least two different trypanosome lines.

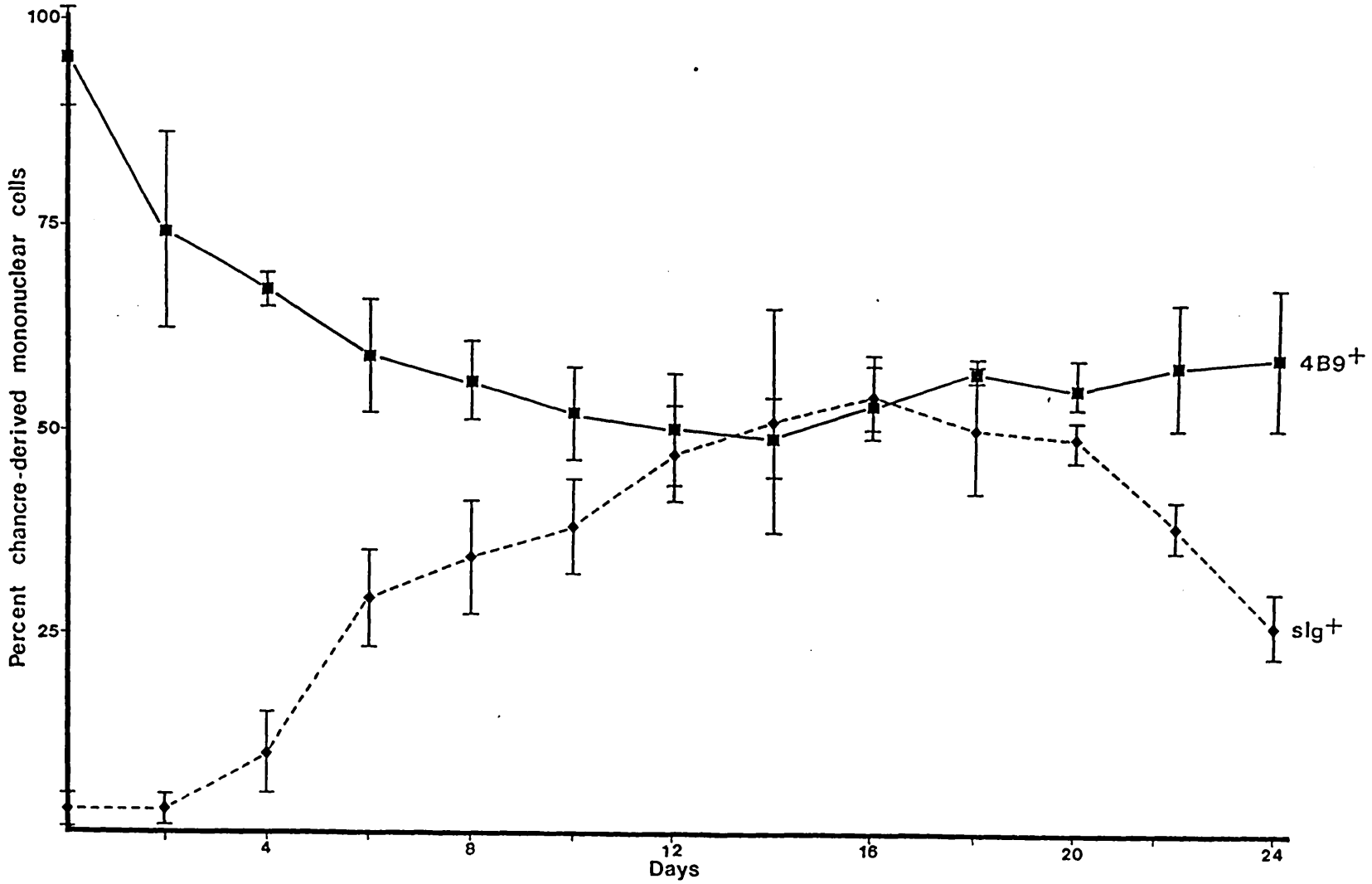

Percent chancre-derived mononuclear cells
100
75
50
25
4
8
12
16
20
24
Days
4B9+
sIg+

Figure 4.2

Figure 4.3 Histogram of the T-lymphocyte subpopulations isolated from chancres at various times following the intradermal inoculation of 10^5 monomorphic trypanosomes.

Cells were stained with McAbs 4B9 (T-cells) ▲——▲, 10B3 (T_a-cells) □ and 12C7 (T_c/T_s-cells) ⊡ .

Chancres were elicited by trypanosomes expressing GUTat 7.1, GUTat 7.2, GUTat 7.13, ILTat 1.3 and ILTat 1.61. The results represent the pooled data from fifteen infected rabbits each of which received four inocula.

Each value is the mean of three determinations from chancres elicited by at least two different trypanosome lines.

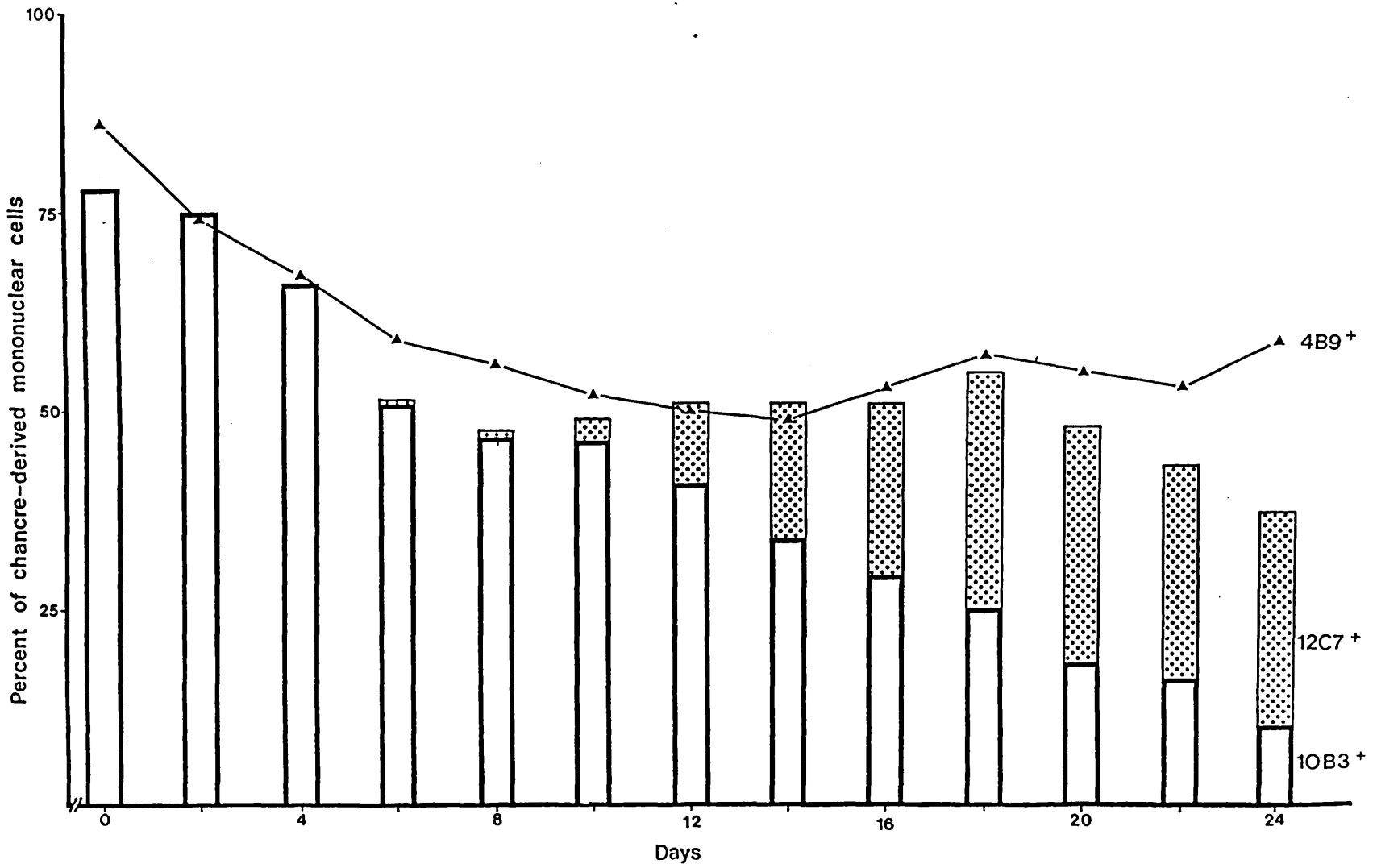

100
75
50
25
Percent of chancre-derived mononuclear cells
0
4
8
12
16
20
24
Days
4B9+
12C7+
10B3+

Figure 4.3

Data on the proportions of different cell types can be misleading and a measure of the absolute numbers of cells involved is of greater benefit (e.g. 96% of the lymphocytes in normal skin were shown to be T-cells, but the very small numbers of these cells may preclude their mounting a response as an effector cell type). The procedure by which cells were extracted from skin was rather inefficient, in that large numbers of mononuclear cells remained within the debris of the skin, and as such, absolute numbers of cells could not be counted. In order to provide such an analysis, the numbers of lymphocytes in the infiltrate were counted directly on stained sections of histological material prepared from local skin reactions removed on different days post-infection. The number of lymphocytes in the chancre is shown in figure 4.4. The results are in broad agreement with the analysis of Emery et al (1980a).

Using the values obtained in Figure 4.4 it was possible to estimate the numbers of cells of each different phenotype in the infiltrate at any time as shown in Figure 4.5.

This analysis clearly demonstrates that the first cells to infiltrate the injection site were $10B3^+$ T_a-cells although there were also some infiltrating cells belonging to the cell population(s) not recognised by the McAbs available. The $10B3^+$ T_a-cells precede an almost parallel rise in the numbers of sIg^+ cells in the infiltrate by about 1.5 days. After day 10 of the reaction the numbers of $10B3^+$ T_a- cells decreased almost as quickly as they initially infiltrated. The sIg^+ cells persisted in very high numbers until day 14 after which they also declined.

An unexpected finding of this study was the late infiltration into the lesion of large numbers of $12C7^+$ T_c/T_s-cells. These cells were not seen in normal skin but by day 24 of the local skin reaction

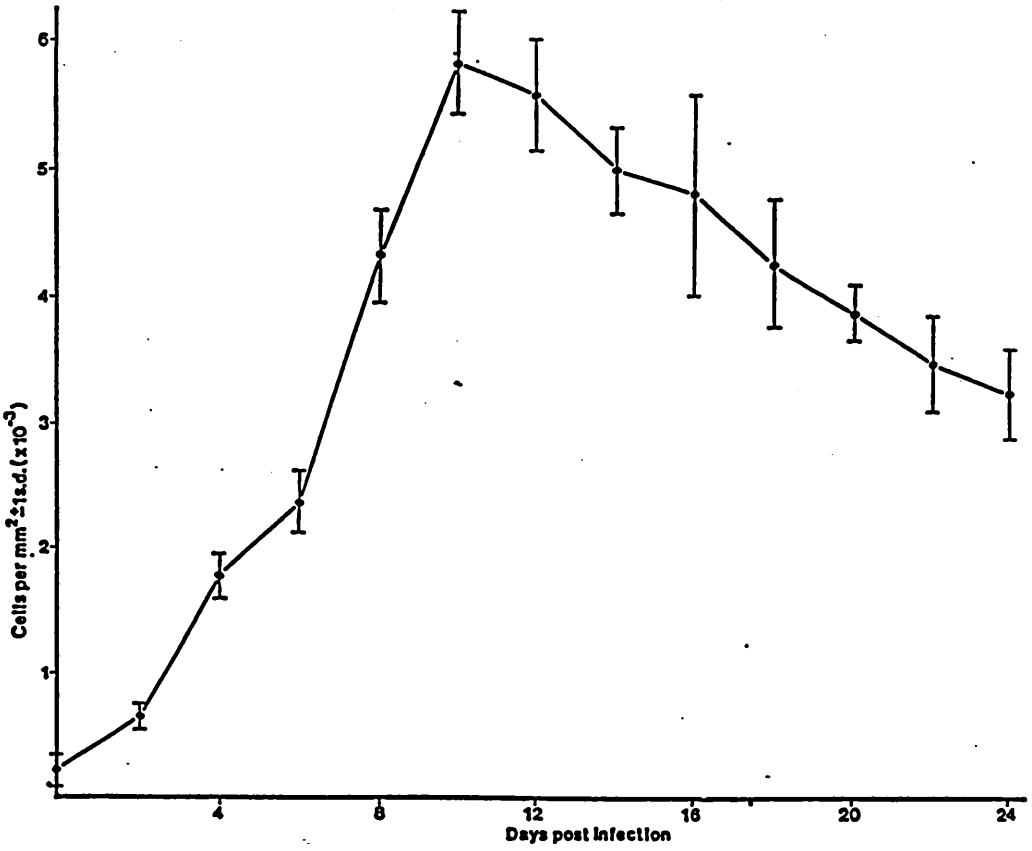


Figure 4.4

Figure 4.4 Graph of mononuclear cell numbers versus time after inoculation for chancres elicited by intradermal injection of 10^5 monomorphic trypanosomes, expressing GUTat 7.1, GUTat 7.2 or ILTat 1.3.

Each point represents the mean of three determinations (± 1sd), using data from lesions elicited by at least two VATs. In total, eight rabbits were used.

Figure 4.5 Graph of cell numbers versus time for subpopulations of lymphocytes isolated from chancres at various times after the intradermal inoculation with 10^5 monomorphic, monotypic bloodstream form trypanosomes.

Cell types shown are $4B9^+$ T-cells ■——■, $10B3^+$ T_a-cells ●——● , $12C7^+$ T_c/T_s-cells ▲——▲ and sIg^+ B-cells ◆——◆.

Chancres were elicited by trypanosomes expressing GUTat 7.1, GUTat 7.2, GUTat 7.13, ILTat 1.3 and ILTat 1.61. The results represent the pooled data from fifteen infected rabbits each of which received four inocula.

Each point represents the mean of three determinations, on lesions elicited by at least two different VATs.

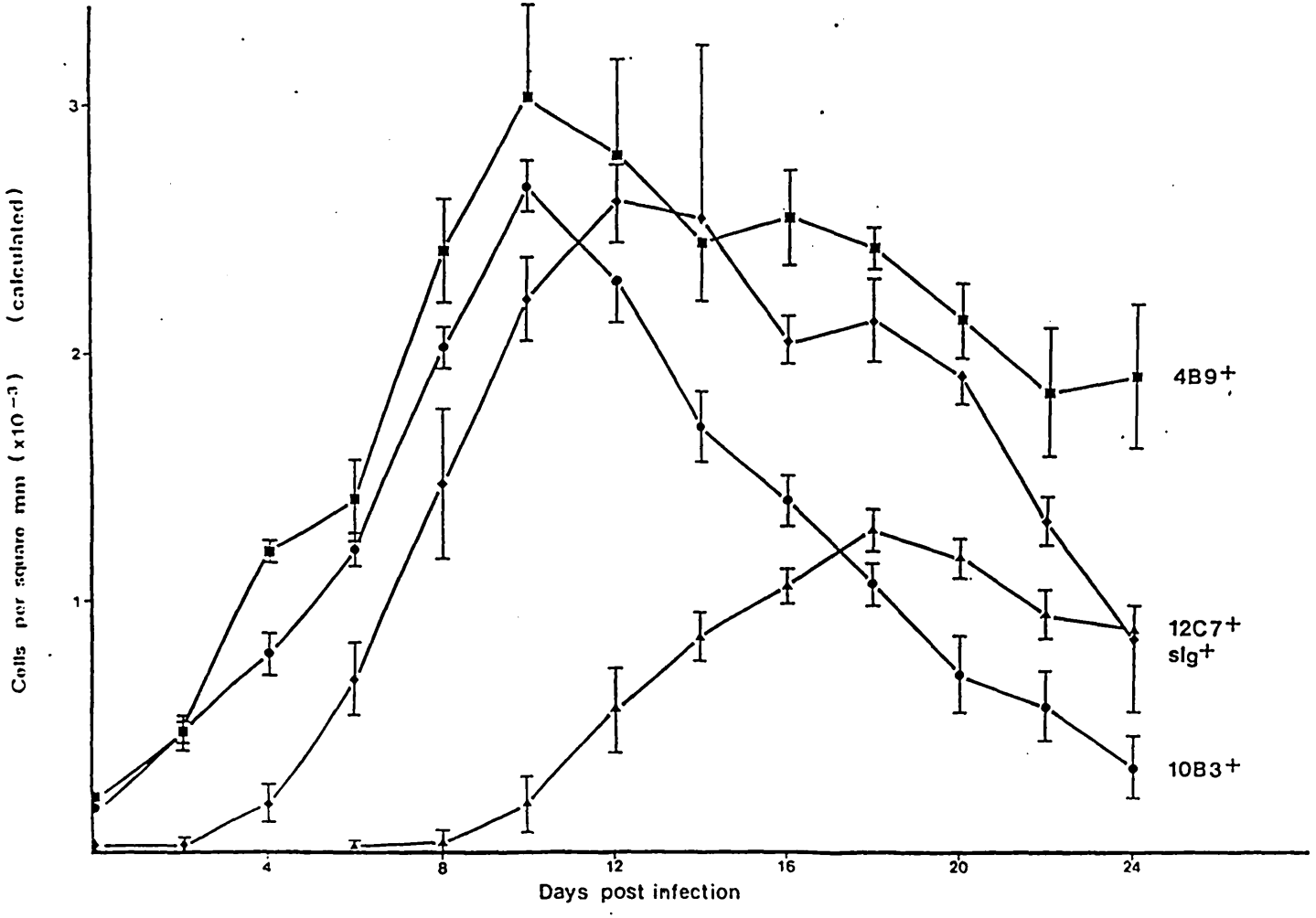

Cells per square mm (x10⁻³) (calculated)
3
2
1
4
8
12
16
20
24
Days post infection
4B9+
12C7+
sIg+
10B3+

Figure 4.5

they were the most prevalent lymphocyte subpopulation.

4.4 Discussion

Following the intradermal injection into rabbits of bloodstream forms of T. rhodesiense an inflammatory weal, the chancre, developed. The cells which infiltrate the chancre are predominantly lymphocytes (Emery et al 1980a). The results presented here have analysed this lymphocytic infiltrate in greater detail using a panel of McAbs directed against antigens on the surface of subsets of rabbit T-lymphocytes (de Smet et al 1983).

The initial finding of this work was that, of the few lymphocytes present in normal rabbit skin, the vast majority are $4B9^+$ T-lymphocytes. Of these T-lymphocytes, most were found to be $10B3^+$ T_a-cells and none were found to be $12C7^+$ T_c/T_s-cells. As McAb 10B3 consistently stained a lower percentage of the cells than McAb 4B9 it seems likely that another population of T-lymphocytes, to which no McAb was available, was present in normal skin. By analysing the composition of the infiltrate sequentially it has been possible to construct a picture of the changes in lymphocyte composition during the development of the chancre.

The initial rise in $10B3^+$ T_a-cells preceded a parallel infiltration of B-lymphocytes by about 1.5 days. The late and very marked rise in the numbers of $12C7^+$ T_c/T_s-cells coincided with the sharp decline in the numbers of $10B3^+$ T_a-cells although $12C7^+$ T_c/T_s-cells were seen before the peak of the reaction. These changes do not reflect the pattern seen in peripheral blood lymphocytes during the same period.

The chancres on which this analysis was performed were elicited by trypanosome populations expressing different single VATs. Monotypic trypanosomes expressing GUTat 7.1, GUTat 7.2, GUTat 7.13.

ILTat 1.3 and ILTat 1.61 were all used to elicit chancres for this analysis. No significant differences were noted either macroscopically or upon cell analysis between any of the VATs used. One possible drawback of the system used to isolate the cells from the chancre was that it selected for, or against, a particular class or subclass of cells. The isolation procedure was checked at a gross level by performing differential white cell counts on cells isolated from chancres and from stained sections of material from the same source. The differential white blood cell counts showed no significant difference between the two procedures (Table 4.1). In addition, the variability in the proportions of different subsets of T-lymphocytes isolated from reactions at different stages of development implies that no selective process was operating during the isolation procedure. All cell types tested have been isolated by this method.

The analysis presented here is limited by the fact that the absolute numbers of cells involved in the reaction at any given time were enumerated by calculation from non-specifically stained histological sections. This was necessary as none of the direct staining techniques of T-cells in sections, using the same McAbs as the present work, gave satisfactory results in on-section labelling. Despite these limitations, this technique provides a valuable additional perspective from which to view the progress of the reaction. For example, from the analysis of only the proportion data it was not obvious that T-lymphocytes were the first cell population to infiltrate the chancre.

Some interesting features emerge from the analysis of the lymphocyte composition of the chancre, although it is slightly complicated by the inability to define all the T-lymphocyte subpopulations.

The rapid and massive infiltration of $10B3^+$ T_a-lymphocytes appears to be the initial host reaction to intradermal challenge with trypanosomes. The parallel and delayed infiltration of B-cells suggests that these cells are being specifically recruited into the inflammatory site. Such a recruitment could be mediated by lymphokines secreted from the $10B3^+$ T_a-cells or other cell types (e.g. macrophages, Langerhans cells) in the skin. As only long slender trypanosomes were used it is possible that very little soluble VSG is present (Sendashonga and Black 1982). In this case macrophages may be of more importance as dendritic cells appear to play little part in the immune response to particulate antigens (Kapsenberg et al 1985). The decline in the numbers of $10B3^+$ T_a-cells is very rapid and the reason for this is unclear. One possibility is that no antigen remains at the site after the peak of the reaction. Possible support for this view comes from the observation that trypanosomes are rarely seen in the chancre after day 6-8. Any attempt at assigning a possible function to the $10B3^+$ T_a-cell population is, however, restricted by the lack of functional studies on cells bearing this phenotype (de Smet et al 1983).

Another striking feature of the chancre infiltrate was the late appearance of large numbers of $12C7^+$ T_c/T_s-cells in the reaction . Preliminary studies on the function of these cells in normal rabbits indicated that they may be analogous to the cytotoxic/suppressor subset of T-lymphocytes which has been defined in both human and murine systems (de Smet et al 1983). It seems unlikely that the $12C7^+$ cells in the chancre are T_c-cells for two reasons. Firstly, no cytotoxic action of such lymphocytes on trypanosomes has been reported or indeed would be expected as the

necessary host MHC antigens are not seen on trypanosomes. Secondly, a cytotoxic response against host cells which by either accident or design were displaying trypanosome antigens on their surface would have no effector capability against extra-cellular parasites.

If the $12C7^+$ $T_C/_S$-cells are suppressor cells it would be of interest to determine if they were antigen specific or non-specific. Antigen specific suppressor cells could be responsible for the decline in the chancre while the demonstration of a non-specific suppressor population would have implications for the more generalised immunosuppression found in trypanosome infections (Mansfield 1978, Hudson & Terry 1979). Eardley and Jayawardena (1977) suggested that such non-specific suppressor T-cells were active in T. brucei infections of mice, and that they mediated their effect through macrophages. Cells of this type, however, have been reported only from in vitro experiments in rodent systems and their relevance to human and/or cattle disease remains questionable.

Studies on inflammatory skin lesions in human systems have shown that most lymphocytic infiltrates have a helper/inducer (OKT4) rather than a suppressor/cytotoxic (OKT5/8) phenotype. Infiltrates with this characteristic have been found in contact dermatitis, granuloma annulare, DTH skin tests and atopic eczema (Haynes et al 1982, McMillan et al 1985, Zachary et al 1985). Unfortunately, all the above studies used a single biopsy technique (for ethical reasons) and small sample sizes prevented the extrapolation of data to portray the changing composition of such infiltrates.

The accumulation in a skin lesion of cells with the T_C/T_S phenotype has been demonstrated in the microscopic skin lesions of psoriatic patients (Hammar et al 1984) where they were more common in the epidermis than the dermis. As in the chancre, the ratio of T-cell subsets in peripheral blood was found to bear no relation to the

situation in the skin. The psoriatic lesions described by Hammar and colleagues contained very few B-cells which clearly differentiates the pathogenesis of this lesion from those seen in trypanosome induced lesions.

Further study of the changes in lymphocyte populations in the chancre would be profitable. Studies on the functional abilities of each of the subsets would seem to be particularly valuable. In addition, the approach used here of sequential biopsy would seem to be of value in the study of many other skin lesions and may yield information of a basic nature on the control of the inflammatory/immune processes in the skin.

5. THE PROLIFERATIVE RESPONSE TO DEFINED VARIANT ANTIGENS OF CELLS ISOLATED FROM THE CHANCRE

5.1 Introduction

Histological studies of inflammatory infiltrates are limited by the "photographic" nature of the information they provide; although they can define the components at a given moment in time, they are open to interpretation as to the likely "picture" at an earlier or later time. Even studies of sequential histology are limited by their lack of information about cell function. In the context of the chancre, histological studies, such as the one presented previously (Chapter 4), can yield little information as to the effector mechanisms involved, or to the stimuli which elicit chancre formation. Such information is only obtainable if the cells concerned can be isolated and manipulated *in vitro*. Even if the host cells involved can be placed in culture, studies of this kind in natural infections are complicated by the large number of antigens (VATs) present. Use of monotypic trypanosomes defines the antigenic stimulus and allows comparisons between different single antigens to be made; this is especially valuable if responses in primed or previously infected animals are to be investigated.

The definition of the cells infiltrating the chancre (in response to the trypanosome stimulus) is more difficult. The manufacture of monoclonal antibodies which recognise differentiation antigens on rabbit T-lymphocytes has aided such definition considerably (de Smet *et al* 1983). Using these antibodies it is possible to deplete mixed cell populations of one or more cell types in order to characterise a particular responding population. The positive selection of cells of a particular phenotype is also

feasible.

One problem of *in vitro* culture of cells from cutaneous lesions is the difficulty of removing cells from the collagenous matrix of the skin. A method is described here which allows mononuclear cells to be recovered, purified and cultured from local skin reactions. The VAT-specificity of such cell populations has been investigated by the addition of whole, fixed trypanosomes and purified VSG incorporated into liposomes, to *in vitro* cultures of cells isolated from local skin reactions.

5.2 Materials and Methods

5.2.1 Media and solutions

RPMI 1640 complete medium - This was made from the incomplete stock solution described in section 4.2.1. To 87 ml of this stock solution was added:

10 ml foetal calf serum (inactivated 56°C, 30 mins)

1 ml 7% (w/v) $NaHCO_3$

1 ml 200 mM L-glutamine

For cell isolation procedures the medium also contained 100U/ml penicillin (Glaxo, Greenford, England), 100 ug/ml streptomycin sulphate (Evans, Greenford, England) and 2mg/ml collagenase, type IV (Sigma, Poole).

After isolation, cells were cultured in the absence of collagenase, and without penicillin and streptomycin. The antibiotics used for culture were gentamycin sulphate, 200 ug/ml (Sigma, Poole) and fungizone, 2.5 ug/ml (Flow, Rickmansworth, England).

Balanced salts solution (BSS) - this solution contained:

NaCl	8.00 g/l
KCl	0.40 g/l
$MgSO_4.7H_2O$	0.20 g/l
$CaCl_2.2H_2O$	0.15 g/l
KH_2PO_4 (anhyd)	0.06 g/l
Na_2HPO_4 (anhyd)	0.20 g/l

The solution was filter sterilised by passing through 0.22 um pore size filter (Millipore, London).

Krebs Ringer phosphate (KRP) buffer - this solution contained:

NaCl	5.73 g/l
Na_2HPO_4 (anhyd)	3.56 g/l
NaH_2PO_4 (anhyd)	0.24 g/l
KCl	0.15 g/l
$MgSO_4$ (anhyd)	0.25 g/l
D-glucose	1.80 g/l

5.2.2 Cell isolation and culture - Cells were isolated from lesions by a modification of the procedure described in section 4.2.3. All reagents and instruments were sterilised before use and the medium used was RPMI 1640 complete medium as described above. Collagenase was added to this medium as described previously (section 4.2.2). The necessary severity of the isolation procedure resulted in a great deal of cell death (commonly 40-60%) and it was necessary to remove dead cells by a second round of hypotonic lysis with subsequent spinning through FCS (1000 x g for 1 min at room temperature) to remove cell ghosts. During isolation, large accretions of acellular debris were removed by filtration through nylon mesh (35um mesh size; Henry Simon, Stockport, England) which had been "welded" onto the cut

barrel of a 10ml syringe, by melting the cut end of the barrel in a bunsen flame and pressing the molten end on to a piece of mesh. The complete units were sterilised by microwave radiation (600 W for 10 mins).

Isolated cells were plated out (5 x 10^5 cells/well) in flat bottomed 96-well microtitre plates (Nunc, Denmark) and incubated at 37°C with 5% CO_2 in a humidified incubator.

5.2.3 Removal of adherent cells - Where stated, adherent cells were removed by passing the cell suspension over a Sephadex G-10 column. Column bodies were prepared in 10 ml syringe barrels plugged with absorbent cotton wool and sterilised by microwave radiation (600 W for 10 mins). Into such columns was poured 1.5ml pre-swollen Sephadex G-10 pre-equilibrated in balanced salt solution (BSS). The column was washed with at least 3 column volumes of pre-warmed (37°C) BSS before applying the cell suspension. Eluted cells were counted, spun down (550 x g for 10 mins) and resuspended in RPMI 1640 complete medium before being plated out as described above.

5.2.4 Cell depletion by antibody mediated lysis - Populations of cells expressing antigens recognised by McAbs 10B3 (T_a-cells) and 12C7 (T_c/T_s-cells), (section 4.2.2) were removed by lysis. Cell suspensions were incubated with the appropriate dilution of ascites fluid (10B3 - 1/50; 12C7 - 1/400) or donkey anti-rabbit whole immunoglobulin antiserum (1/50; SAPU, Law Scotland), in RPMI 1640 complete medium for 1 hour on ice. The cells were spun down (550 x g for 10 minutes at 4°C), and washed twice in RPMI 1640 complete medium (with spinning as above). Cells were resuspended in fresh guinea-pig serum (GPS) which had been absorbed with an equal volume of whole rabbit blood for 1 hr at room temperature, cells being removed by

spinning at 3000 x g for 2 minutes at room temperature. The antibody treated cells were incubated in GPS for 1 hr at 37°C. After washing the cells twice in RPMI 1640 complete medium the antibody and complement treatments were repeated.

The appropriate dilution of the McAbs was determined by titrating the antibodies on normal peripheral blood lymphocytes isolated as described in section 4.2.3. Lysis was assayed by immunofluorescent staining of cells (section 2.2.9) before and after lysis.

5.2.5 Preparation of antigens - Populations of trypanosomes expressing one of the VATs GUTat 7.1, ILTat 1.61 were purified from infected mouse blood as described in section 2.2.8. Populations were checked for antigenic homogeneity by immunofluorescence (section 2.2.9). Purified trypanosomes were spun down (550 x g for 10 mins) and resuspended in 1% formaldehyde in PBS for 10 minutes at room temperature. The fixed trypanosomes were washed in PBS (with 0.02% NaN_3) six times with spinning at 3000 x g for 1 minute at room temperature, before being resuspended in RPMI 1640 complete medium at a concentration of 10^8 trypanosomes/ml. Immunofluorescence was also performed on these fixed organisms to check that antigenic identity had been preserved. For stimulation assays of lymphocytes 20ul of the fixed trypanosome suspension (i.e. 2 x 10^6 fixed trypanosomes) was added to each well of the culture plate.

5.2.6 Purification of mfVSG - Membrane form variant surface glycoprotein (mfVSG) was purified from trypanosomes expressing GUTat 7.1 and GUTat 7.2 by the method of Jackson et al (1985). Briefly, trypanosomes were purified from the pooled blood of infected rats as described in section 2.2.2., except that large sintered glass columns were used. A sample of the trypanosomes eluted from the

column was smeared on microscope slides to check for antigenic homogeneity by immunofluorescence. The suspension of trypanosomes was spun down (550 x g for 10 minutes at 4°C) and resuspended in KRP buffer to a concentration of 2 x 10^9/ml on ice. The suspension was treated (on ice) with an equal volume of 10% (w/v) trichloroacetic acid and the precipitate centrifuged at 9000 x g for 10 seconds. The material in the pellet was resuspended in distilled, deionised water (4ml/10^{10} cells) using a Dounce homogeniser with a loosely fitting pestle. This suspension was extracted with 20 volumes of chloroform/methanol (2:1, v/v) with vigourous shaking for five minutes and stored overnight at 4°C. The extract was separated into two phases by the addition of 0.2 volumes of 0.9% NaCl solution and was centrifuged at 12,000 x g for 1 hour at 4°C. The upper aqueous phase was removed and dialysed against six changes of distilled, deionised water at 4°C over 72 hours. The retained material was freeze dried and the resulting mfVSG was stored desiccated at -40°C.

5.2.7 Western blotting - VSG preparations were analysed by western blotting. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% gels under reducing conditions as described by Laemmli (1970), electrophoresed for 3.5 hours (150V, 30 mA) using a ECPS 3000/150 power pack (Pharmacia, Uppsala, Sweden). The electrophoresed proteins were then electro-blotted (10 hours at 200 mA) on to a nitrocellulose membrane (Bio-Rad, Watford, England) in a Bio-Rad Transblot cell using a Bio-Rod 250/2.5 power pack. VSG was detected by the application of VAT-specific monoclonal antibodies to strips of nitrocellulose corresponding to tracks on the SDS-PAGE gel. After thorough washing in 1% sheep serum in PBS, horseradish peroxidase conjugated sheep anti-mouse immunoglobulin was applied to each strip (SAPU, Law, Scotland; used at 1/1000). After thorough

washing in PBS the VSG was visualised by immersing the strips in freshly made 0.01% (w/v) 3,3'-diamino benzidine solution containing 0.05 ml of 30% H_2O_2 per 100 ml. Strips were removed from this solution to distilled water as soon as bands had appeared. The strips were then dried and stored protected from light.

One strip of nitrocellulose, containing molecular weight markers, was stained with 0.5% (w/v) amido black in a solution of 5% (v/v) acetic acid, 50% (v/v) methanol for 15 minutes. Following destaining in 5% (v/v) acetic acid, 50% (v/v) methanol this strip was also dried and stored protected from light. The results of a typical blot of GUTat 7.1 mfVSG are shown on Plate 1.

5.2.8 Manufacture of liposomes - Liposomes with mfVSG incorporated into their lipid bilayer were made as follows. In 5 ml chloroform/methanol (Analar)(2:1, v/v) was dissolved 1 mg cholesterol (Merck, Germany), 10 mg egg lecithin (Lipid Products, Redhill, England) and 0.25 mg phosphatidic acid (Lipid Products). The solvent was then blown off under nitrogen to leave the other components coating the inside of the vessel. To the vessel was added 10 ml of 0.1M NaCl, 0.05M Hepes buffer pH 7.2, and upon gentle agitation liposomes formed spontaneously. To pre-formed liposomes was added 0.5 mg purified VSG in 0.5 ml NaCl/Hepes buffer. The liposomes were then washed five times in NaCl/Hepes buffer with spinning at 800 x g for 10 minutes at room temperature. Liposomes were manufactured using purified GUTat 7.1 and GUTat 7.2 mfVSG in addition to a control preparation which contained no protein.

Liposomes were tested for incorporation of VSG by agglutination using monospecific rat antisera against GUTat 7.1, GUTat 7.2 or ETat 1.8 diluted 1/50 in NaCl/Hepes buffer. After incubation for 1 hour at room temperature, large scale agglutination was only observed in

Plate 1 Photograph of western blot of GUTat 7.1 mfVSG.

Track A - Electrophoresed preparation of GUTat 7.1 mfVSG stained with McAb GUPM 23.2 (anti- ILTat 1.61) and visualised with HRP-conjugated anti-mouse IgG.

Track B - Electrophoresed preparation of GUTat 7.1 mfVSG stained with HRP-conjugated anti-mouse IgG.

Track C - Electrophoresed preparation of GUTat 7.1 mfVSG stained with McAb GUPM 27.1 (anti-GUTat 7.1) and visualised with HRP-conjugated anti-mouse IgG.

Track D - Electrophoresed preparation of GUTat 7.1 mfVSG stained with amido black.

Track E - Molecular weight markers stained with amido black (molecular weights in kD indicated by figures).

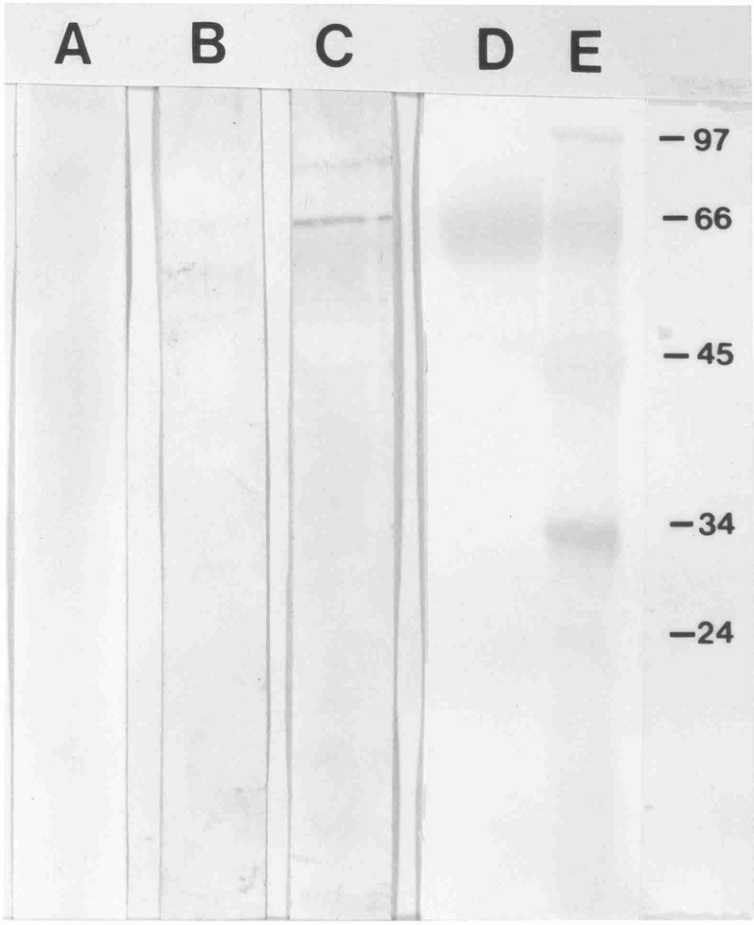


Plate 1

liposome preparations incubated with the homologous antiserum.

5.2.9 Cell proliferation assays - Cell proliferation in response to antigen was estimated by the incorporation of ^{14}C-Thymidine (Amersham International, Amersham , Bucks). Cells isolated from animals infected intradermally with GUTat 7.1 were cultured for 40 hours after the addition of 2 X 10^6 fixed trypanosomes expressing GUTat 7.1, ILTat 1.61, or an equal volume of RPMI 1640 complete medium as a control. In one experiment liposomes incorporating GUTat 7.1 or GUTat 7.2 mfVSG were added to cell cultures. Liposomes incorporating no protein were used as controls. Cells were also stimulated with 0.5ug/ml concanavalin-A (Sigma, Poole). After incubation, 50ul of RPMI 1640 complete medium containing ^{14}C-Thymidine (2uCi/ml) was added to each well of the culture plate. Four hours after the addition of radioactive thymidine the cells were harvested onto glass fibre filters using a semi-automatic cell harvester (Skattron, Stockholm, Sweden). After drying, the filters were placed in vials with 2ml "Ecoscint" scintillation fluid (National Diagnostic, Somerville, NJ, USA) and radioactivity counted in a liquid scintillation counter (LKB, Bromma, Sweden).

Cell proliferation was estimated by the calculation of a stimulation index (SI):

$$SI = \frac{\text{cpm experimental} - \text{cpm control}}{\text{cpm control}}$$

where cpm values were the mean cpm for quadruplicate cultures. Statistical testing of significance between stimulation indices was calculated using Student's t-test on the mean cpm values for each antigen.

5.3 Results

5.3.1 The proliferative response of mononuclear cells isolated from the chancre and the draining lymph node after four days of infection.

Rabbits were inoculated intradermally with 10^5 monomorphic trypanosomes expressing GUTat 7.1 at two sites on each flank. Four days later the local skin reactions and draining lymph nodes were excised and mononuclear cell cultures were prepared. Before being placed in culture, aliquots of the cells were subjected to cell depletion procedures, namely, passage over Sephadex G-10 to remove adherent cells or immune lysis of one subpopulation of cells using McAbs 10B3, 12C7 or anti-rabbit immunoglobulin antiserum. Unfractionated cells were also cultured in the presence of con-A, as were peripheral blood mononuclear cells from an uninfected rabbit. With the exception of the cultures which were treated with con-A, cultures were treated with fixed trypanosomes expressing GUTat 7.1 or ILTat 1.61. Control cultures of each cell preparation received only medium. The results are shown in Figure 5.1.

With the exception of the culture depleted of $10B3^+T_a$-cells, all chancre derived cell preparations showed greater proliferation in response to the homologous antigen (i.e. GUTat 7.1) than to heterologous antigen (ILTat 1.61), although significant proliferation to the latter antigen was observed. The cell population depleted of $10B3^+T_a$-cells did not proliferate in response to either antigen. The residual population was composed of approximately 30% sIg^+ cells and 70% null cells. Depletion of adherent cells from the population made little difference to the proliferative response; the responses to both GUTat 7.1 and ILTat 1.61 were increased slightly and this may reflect an enrichment of lymphocytes resulting from the depletion. The response of the cell population depleted of sIg^+ cells showed a

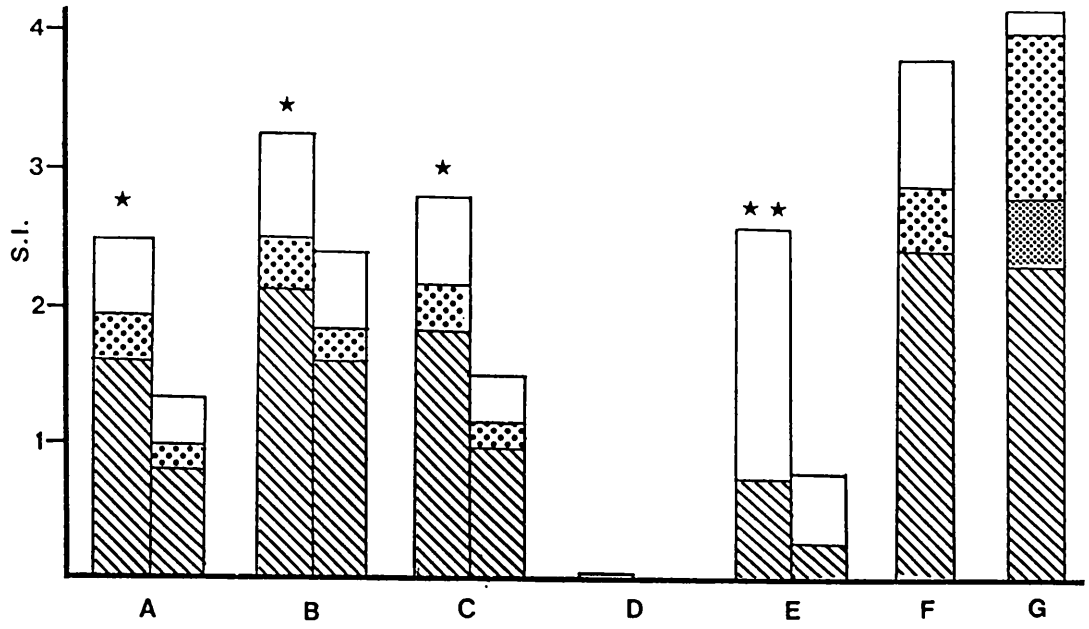
Figure 5.1 Plot of stimulation index (S.I.) of cells cultured from the chancre or draining lymph node four days after the intradermal inoculation of 10^5 GUTat 7.1. Cells were isolated and cultured from chancres (A-F) or the draining lymph node (a-f). Preparations G,g were of peripheral blood mononuclear cells. Cell preparations were depleted of specific cell types before being placed in culture.

Preparations A,a; F,f; and G,g were unfractionated; B,b - depleted of G-10 adherent cells; C,c - depleted of $12C7^+$ T_C/T_S-cells; D,d - depleted of $10B3^+$ T_a-cells; E,e - depleted of sIg^+ B-cells.

Preparations A,a - E,e were treated with fixed trypanosomes expressing GUTat 7.1 (left hand bar) or ILTat 1.61 (right hand bar). Preparations F,f and G,g were stimulated with con-A.

Significant differences in stimulation index between antigens are denoted by ★ ($p<0.05$) or ★★ ($p<0.01$).

An indication of the cell types present in each culture is given by the shading, where  = $10B3^+$ T_a-cells,  = $12C7^+$ T_C/T_S-cells,  =sIg^+ B-cells and  = unstained "null" cells.



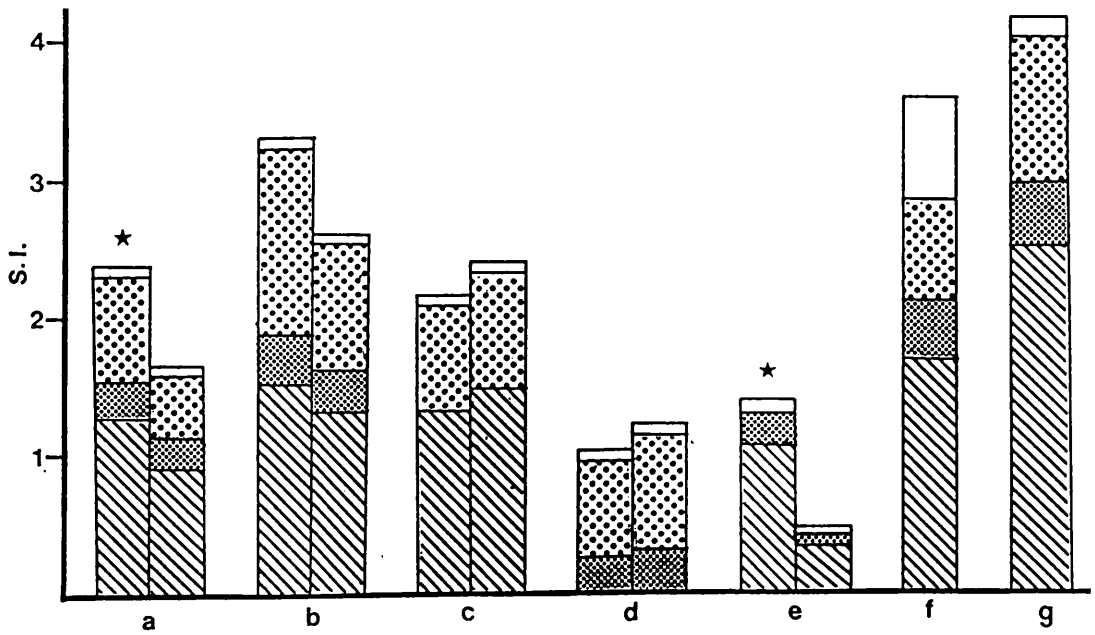


Figure 5.1

response to GUTat 7.1 equal to that of the unfractionated cell population, while the response to heterologous antigen was slightly diminished. The response of unfractionated cells to con-A was essentially the same as for normal peripheral blood lymphocytes.

Cells from the lymph node draining day 4 chancres differed from those in the lesion itself in both composition and response to antigen. Lymph node preparations contained many more B-cells (33% c.f. 10% in the chancre) and also comprised 10% $12C7^+$ T_C/T_S-cells, which were absent from the chancre. Unfractionated lymph node cells showed a less marked antigen specificity than their counterparts in the chancre and depletion of G-10 adherent cells, $12C7^+T_C/T_S$-cells and $10B3^+$Ta-cells removed any antigen specific response. Depletion of sIg^+ cells however restored a component of specificity for the priming antigen (GUTat 7.1). Cells isolated from the draining lymph node gave a proliferative response to con-A which was not significantly different from that of normal peripheral blood mononuclear cells.

5.3.2 The proliferative response of cells from the chancre and the draining lymph node eleven days after infection.

Rabbits were intradermally infected and, eleven days later, mononuclear cells were isolated from both the chancre and the draining lymph node and cultured as above. The cells were depleted by the same procedures as before (section 5.3.1) and stimulated with the same antigenic material. In addition, unfractionated cells from both sources were stimulated with liposomes incorporating GUTat 7.1 mfVSG, GUTat 7.2 mfVSG or free of added antigen. The results are shown in Figure 5.2.

Cells isolated from the chancre on day 11 displayed a broadly

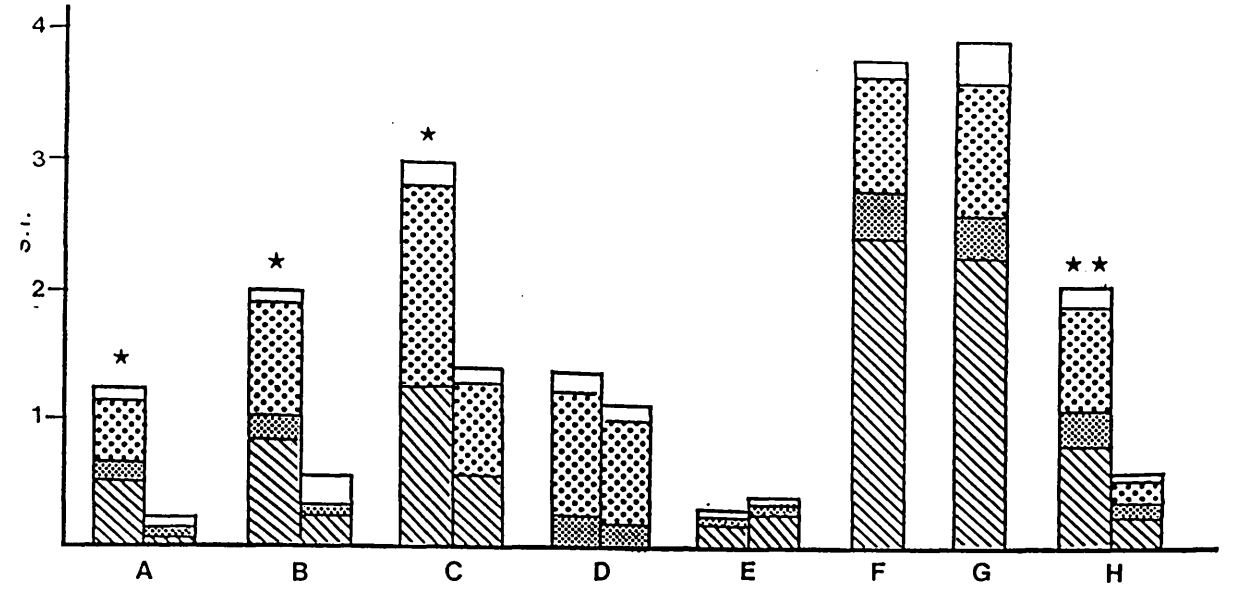
Figure 5.2 Plot of stimulation index (S.I.) of cells cultured from the chancre or draining lymph node eleven days after the intradermal inoculation of 10^5 GUTat 7.1. Cells were isolated and cultured from chancres (A-F,H) or the draining lymph node (a-f,h). Preparations G,g were of peripheral blood mononuclear cells. Cell preparations were depleted of specific cell types before being placed in culture.

Preparations A,a; F,f; G,g and H,h were unfractionated; B,b - depleted of G-10 adherent cells; C,c - depleted of 12C7$^+$ T_c/T_s-cells; D,d - depleted of 10B3$^+$ T_a-cells; E,e - depleted of sIg$^+$ B-cells.

Preparations A,a - E,e were treated with fixed trypanosomes expressing GUTat 7.1 (left hand bar) or ILTat 1.61 (right hand bar). Preparations F,f and G,g were stimulated with con-A. Preparations H,h were treated with liposomes incorporating GUTat 7.1 or GUTat 7.2 mfVSG.

Significant differences in stimulation index between antigens are denoted by ★ ($p<0.05$) or ★★ ($p<0.01$).

An indication of the cell types present in each culture is given by the shading, where  = 10B3$^+$ T_a-cells, = 12C7$^+$ T_c/T_s-cells,  =sIg$^+$ B-cells and  = unstained "null" cells.



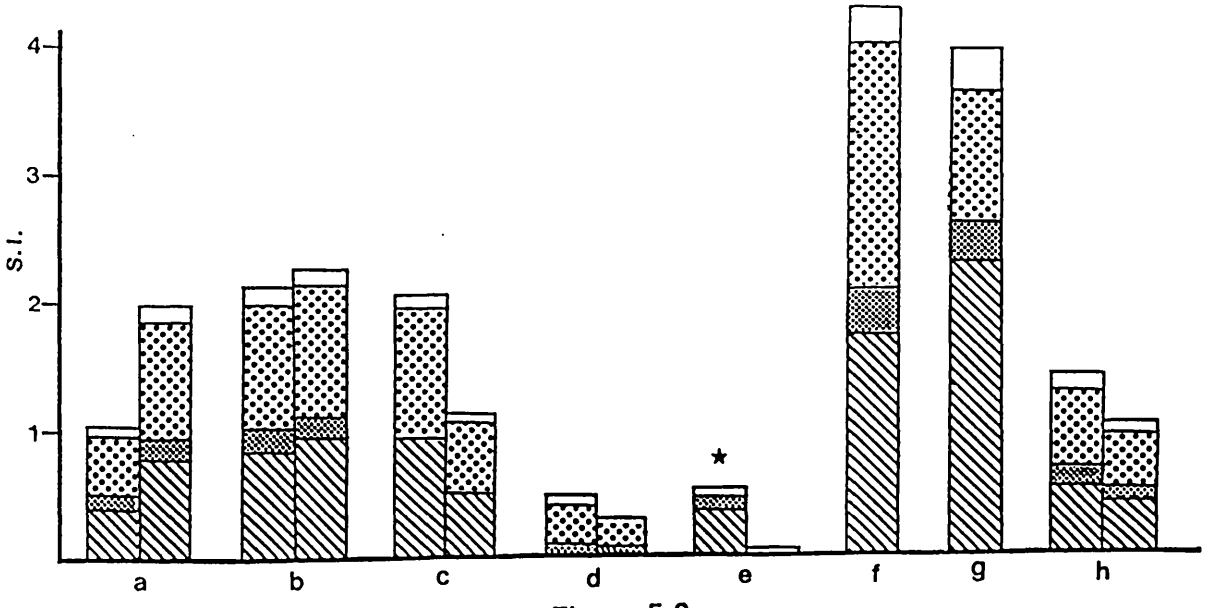


Figure 5.2

similar response pattern to those isolated on day 4, although the interpretation of the data was complicated by the appearance of $12C7^{+}T_{c}/T_{s}$-cells which were absent from the day 4 lesions. Unfractionated cells, G-10 adherent cell depleted populations and $12C7^{+}T_{c}/T_{s}$-cell depleted populations all showed a greater response to homologous antigen than to heterologous antigen. Cultures depleted of $10B3^{+}T_{a}$-cells or sIg^{+} cells proliferated equally well in response to either antigen. The antigen specific proliferative response of unfractionated cells was significantly increased ($0.01 < p < 0.05$) by depletion of G-10 adherent cells. Depletion of $12C7^{+}T_{c}/T_{s}$-cells significantly increased the response to both antigens. Unfractionated cells stimulated with VSG coated liposomes showed significantly greater proliferation to GUTat 7.1 than to GUTat 7.2 ($p < 0.01$). No difference was observed between con-A induced proliferation of cells isolated from the chancre and normal peripheral blood cells.

There was a marked difference in the pattern of responses of cells isolated from the draining lymph node. No significant difference in proliferative activity to homologous or heterologous antigen was observed for unfractionated cells, G-10 adherent cell depleted cells and $12C7^{+}/T_{c}/T_{s}$-cell depleted cultures. Cultures depleted of sIg^{+} cells did respond to GUTat 7.1 but failed to respond to ILTat 1.61. Unfractionated lymph node cells showed no significant difference in their response to liposomes incorporating GUTat 7.1 and GUTat 7.2 mfVSG. Lymph node cells and normal peripheral blood leukocytes showed no significant difference in their response to con-A.

5.3.3 The proliferative response of cells from the chancre and the draining lymph node twenty four days after infection.

Rabbits were intradermally infected and, twenty four days later, mononuclear cells were isolated from both the chancre and the draining lymph node and cultured as described above. The cells were depleted by the same procedures as before (section 5.3.1) and stimulated with the same antigenic material. The results of proliferation assays on these cell cultures are shown in Figure 5.3.

As in cultures of lymph node cells from day 11, significantly greater proliferation to homologous antigen than to heterologous antigen was observed only when the 24-day chancre cell cultures were depleted of sIg^+ cells. Although there was no significant difference in the proliferative response to the different antigens, the general level of response was significantly increased by depletion of G-10 adherent cells ($p < 0.05$) and by depletion of $12C7^+T_c/T_s$-cells. Depletion of $10B3^+T_a$-cells made no significant difference when compared to unfractionated cells. Interestingly, cells isolated from the chancre responded to con-A significantly less well than cells from normal peripheral blood.

Cells isolated from the draining lymph node on day 24 showed no significant difference in their response to homologous or heterologous antigen, irrespective of the cell populations present in the culture. Depletion of G-10 adherent cells or $12C7^+T_c/T_s$-cells did, however, raise the general level of response. The response of unfractionated lymph node cells to con-A was significantly lower ($p < 0.05$) than the response of normal peripheral blood leukocytes.

5.4 Discussion

The results presented here are of a preliminary nature and must be repeated and extended before any firm conclusions can be drawn. Nevertheless, the results do give some information on the sequence of immunological sensitisation following intradermal challenge with

Figure 5.3 Plot of stimulation index (S.I.) of cells cultured from the chancre or draining lymph node twenty-four days after the intradermal inoculation of 10^5 GUTat 7.1. Cells were isolated and cultured from chancres (A-F) or the draining lymph node (a-f). Preparations G,g were of peripheral blood mononuclear cells. Cell preparations were depleted of specific cell types before being placed in culture.

Preparations A,a; F,f; and G,g were unfractionated; B,b - depleted of G-10 adherent cells; C,c - depleted of $12C7^+$ T_C/T_S-cells; D,d - depleted of $10B3^+$ T_a-cells; E,e - depleted of sIg^+ B-cells.

Preparations A,a - E,e were treated with fixed trypanosomes expressing GUTat 7.1 (left hand bar) or ILTat 1.61 (right hand bar). Preparations F,f and G,g were stimulated with con-A.

Significant differences in stimulation index between antigens are denoted by ★ ($p<0.05$).

An indication of the cell types present in each culture is given by the shading, where  = $10B3^+$ T_a-cells,  = $12C7^+$ T_C/T_S-cells,  =sIg^+ B-cells and = unstained "null" cells.

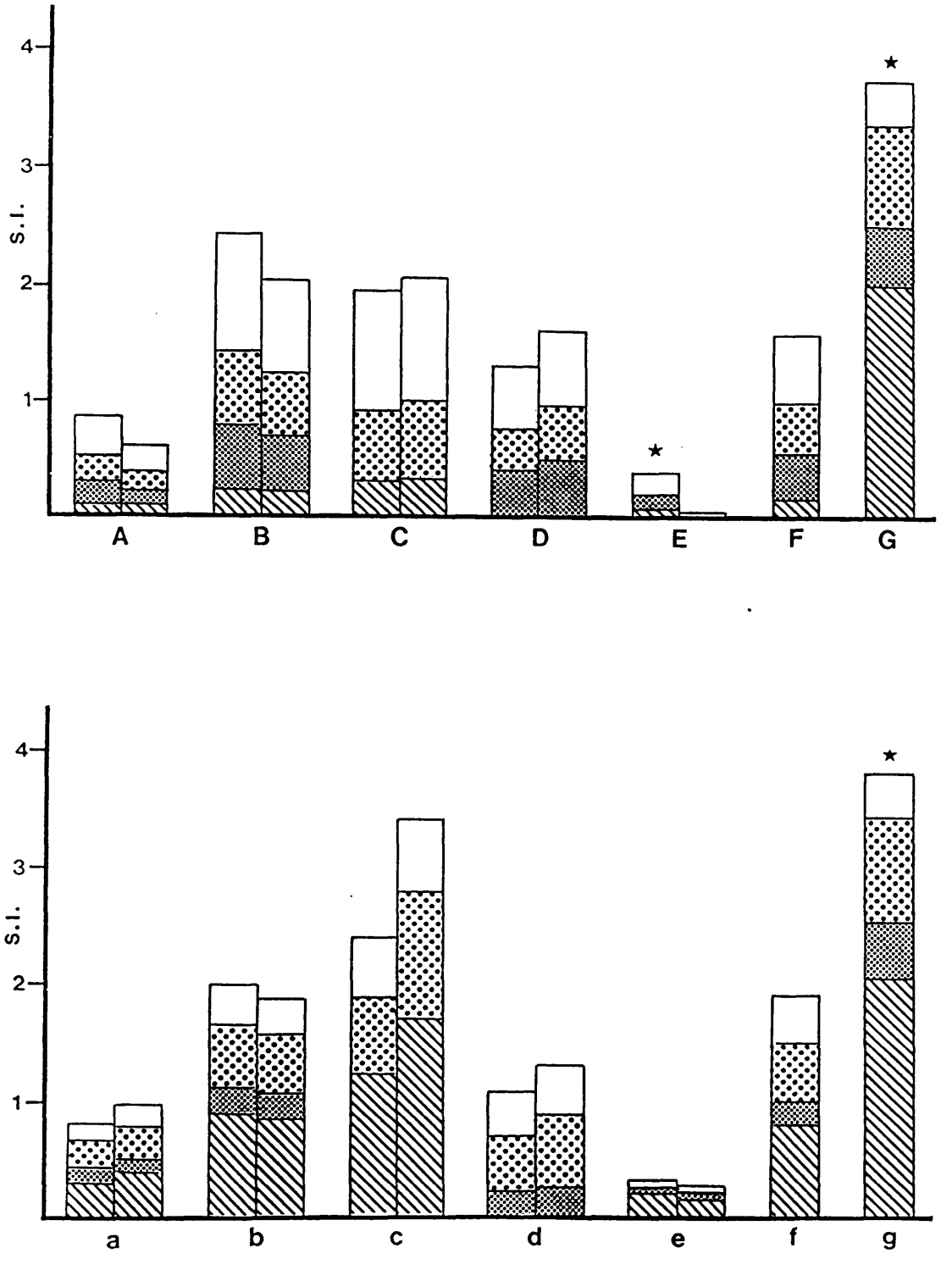


Figure 5.3

trypanosomes.

An important finding of these experiments is that viable leukocytes can be isolated from skin lesions in a condition which enables them to respond to antigen. This is important because the cell populations which infiltrate cuticular lesions are not generally reflected in either the draining lymph node or peripheral blood (Streilein 1983, this study section 4.3). Indeed, there may exist a distinct population of lymphoid cells which are specialised for effector functions within the skin (reviewed by Streilein 1983).

The results are rather difficult to interpret due to the different cell populations in each cell culture. In this respect the cells isolated from the chancre on day 4 present fewer difficulties as this population contained no $12C7^{+}T_{C}/T_{S}$-cells.

Cells isolated from day 4 chancres showed an absolute requirement for $10B3^{+}T_{a}$-cells in order to proliferate. These cells, however, may have acted in combination with null cells as the population depleted of sIg^{+} cells showed a response equivalent to that of unfractionated cells although as a result of depletion the latter population contained more than twice the number of $10B3^{+}T_{a}$-cells. It is interesting to note that sIg^{+} cells in the chancre at this time may be unable to proliferate in the absence of $10B3^{+}T_{a}$-cells. This could be an indication of a T-dependent antibody response to trypanosomes, as suggested by Dempsey and Mansfield (1983).

Cells in the draining lymph node on day 4 showed much less enhanced proliferation to the infecting VAT. Only unfractionated cells and sIg^{+}-depleted cells showed significantly greater proliferation to the homologous antigen than to an heterologous VAT. The difference in the response of the $10B3^{+}T_{a}$-cell depleted population between the chancre and the draining node is quite

striking. Lymph node cells did proliferate in the absence of $10B3^{+}T_a$-cells. This may reflect the participation of $12C7^{+}T_c/T_s$-cells in the proliferative response. An alternative possibility may be that B-cells in the draining lymph node are already undergoing a primary reaction, induced either in response to common antigens draining from the chancre or to a mitogenic stimulus. Such a response may explain why the response of sIg^{-} cells to the infecting VAT becomes apparent only when the sIg^{+} cells are removed. Evidence of B-cell proliferation in lymph draining from the chancre has been provided by Barry and Emery (1984).

Cells isolated from the chancre at the peak of the reaction on day 11 showed an enhanced proliferative response to the infecting VAT only when both sIg^{+} cells and $10B3^{+}T_a$-cells were present. Removal of either of these populations resulted in cells proliferating equally to both the infecting and heterologous VATs. This may be an indication of a co-operative response for the formation of VAT-specific antibody occurring within the chancre itself. Interestingly, this response was not abrogated by depletion of G-10 adherent cells. This could be an indication that a non-adherent accessory cell is present in the null cell population. An in situ antibody response in the chancre was suggested by Emery et al. (1980a); given the antigenic definition provided by the model system used in this study it should prove possible to confirm or deny this suggestion.

Removal of $12C7^{+}T_c/T_s$-cells or G-10 adherent cells increased the proliferation to both antigens by a greater amount than would be expected as a result of the cell concentration effect (i.e. increasing the relative proportions of the remaining cell types). This was perhaps an indication of a non-specific suppressor activity

in either or both of the G-10 adherent and $12C7^{+}T_{C}/T_{S}$ populations, although the normal proliferation of unfractionated cells to con-A does not support this view (see section 1.8).

Of the cells isolated from the draining lymph node on day 11 an enhanced proliferative response to the infecting VAT was observed only when sIg^{+} cells were removed. No other cell depletion treatment gave rise to an enhanced response to either of the VATs tested. The response of unfractionated cells to con-A was not significantly different from the response of normal peripheral blood leukocytes.

Cells from both the chancre and the draining lymph node on day 11 were also stimulated with liposomes incorporating GUTat 7.1 or GUTat 7.2 mfVSG. The responses of unfractionated cells from both sources showed the same pattern as for fixed trypanosomes expressing GUTat 7.1 and ILTat 1.61. This result demonstrates that the response patterns observed are not merely an artifact caused by the use of a restricted set of fixed trypanosome antigens.

Cells isolated from the chancre and draining lymph node on day 24 displayed broadly similar proliferative responses to both GUTat 7.1 and ILTat 1.61. The only notable difference between the two populations was that cells from the chancre which had been depleted of sIg^{+} cells proliferated significantly more to the infecting VAT than to the heterologous VAT; lymph node cells showed no such enhanced proliferation. The responses of both these populations was, however, small and no firm conclusion can be drawn from this difference.

Cells isolated on day 24 from both the lymph node and the chancre showed a significant drop in their ability to respond to con-A, when compared to normal peripheral blood leukocytes. This suggests that some mechanism of generalised immunosuppression was operating at this stage in the infection although responses to

parasite antigens appear to be present, but reduced. An alternative explanation may be that lymph node cells are responding to new antigenic variants arising *in situ* (Barry and Emery 1984), or derived from the systemic circulation (Ssenyonga and Adam 1975).

Overall, the results of cell cultures isolated from the chancre suggest an initial proliferation of VAT-specific T-cells (probably the $10B3^{+}T_{a}$-cell population or part of it), which may participate in the production of an antibody response. A lower level of proliferation seen in response to heterologous VATs may indicate a response to common antigens, mitogenic stimulation or heterologous VATs arising *de novo* in the chancre. The participation of common antigens in these responses could be explored by the *in vivo* use of VSG incorporating liposomes. Virtually all specificity for the infecting antigen was lost by day 24 which might suggest that a response was being mounted to VATs expressed during the systemic infection.

The pattern of responses displayed by cells isolated from the draining lymph node at different times after infection showed much less bias towards the infecting VAT. The overall pattern is consistent with a primary B-cell response to antigens other than the infecting VAT (common antigens, other VATs or a mitogen), which possibly masks an underlying T-cell response against the infecting VAT. This may be a reflection of a response against several systemic VATs, as suggested by Barry and Emery (1984). All specificity for the infecting VAT was lost by day 24 and at this time the response to con-A was depressed. The reason for this depression remains unknown.

The definition of both the antigenic composition of the infecting population and the phenotype of the cells which are responding to those antigens will enable many of the possibilities outlined above

to be tested. Some doubt is still present about the precise phenotype of the cell(s) responding to antigen in the chancre. The depletion of more than one population will help to resolve this. Further definition is possible by the positive selection of cells of a particular phonotype by flow cytometry. Initial experiments, using fixed cells, have been carried out using a FACS IV fluorescence activated cell sorter (Becton-Dickinson, Sunnyvale, CA., U.S.A). These experiments have demonstrated that positive selection of cells expressing a particular antigen is possible. Flow cytometry has not yet been used to select cells from the chancre for return to culture.

The availability of purified proteins (i.e. VSG) corresponding to the antigenic identity of whole trypanosomes also presents the opportunity to select cells which recognise those antigens. This can be done both positively or negatively, by "panning" for cells on plates coated with antigen (Wysocki and Sato 1978).

The use of an experimental system containing both defined antigens stimulating a response and defined cells responding to them is a largely unexplored area of parasitology. Using the system and techniques described here it should prove possible to define the sequence of immunological events which take place following intradermal infection with T. b. rhodesiense. The origins of the cellular defect(s) leading to immunosuppression may also be investigated more thoroughly, although the use of rabbits precludes cell transfer experiments as inbred strains of rabbits are not available.

6. Concluding Remarks

The work presented here has described a model system for the production in rabbits of local skin reactions against African trypanosomes. Using this model system it has been demonstrated that chancre formation was a VAT-specific phenomenon and that animals could therefore be immune to intradermal challenge with one VAT while responding, by the formation of a chancre, to similar challenge with a different VAT. Failure to produce a chancre when challenged intradermally correlated with a high titre VAT-specific humoral antibody response. In one case, a reduction in both the observed skin reaction, and in the serological response against the VAT used for challenge was observed. This reduction occurred only in late infection. The cellular infiltrate into local skin reactions was investigated using a panel of monoclonal antibodies which recognise determinants expressed on rabbit T-lymphocytes or subpopulations of these (de Smet et al., 1983). The initial infiltrate into the chancre was found to be predominantly comprised of T-lymphocytes which preceded a parallel rise in B-lymphocytes by 1.5 days. All of the T-lymphocytes isolated from normal skin were found to be $10B3^+$ T_a-cells and the T-cells isolated from chancres were virtually all of this type until day 6, when $12C7^+$ T_c/T_s-cells were first observed. This population steadily increased in proportion and by day 24, $12C7^+$ T_c/T_s-cells formed the largest cell population in the chancre infiltrate.

A method for the isolation and culture of cells from the chancre has also been described. This method involved collagenase digestion to release cells from skin and density gradient centrifugation to enrich the mononuclear cell fraction. Cells isolated by this procedure were cultured in the presence of antigens derived from the

infecting VAT or an heterologous VAT. Similar cell cultures were prepared using cells derived from the draining lymph node. Such cultures were assayed for proliferation which gave an index of the antigen specificity of the cultured cells. These studies demonstrated that the initial infiltrate into the chancre was a VAT-specific T-lymphocyte population and that the cells in the lymph node showed markedly less antigen specificity. At the peak of the reaction, these studies showed that VAT-specific cell proliferation was dependent on the presence of both the sIg^{+}B-cell and $10B3^{+}T_{a}$-cell populations, which is suggestive of an *in situ* T-cell dependent antibody response against the infecting VAT. Cells isolated from late reactions showed almost no specificity for the infecting VAT but proliferative responses including that to con-A, were depressed.

The model system described used intradermal inoculation of monomorphic bloodstream trypanosomes to elicit chancre formation. The use of such cloned lines, which stably express a single VAT, allowed the antigenic definition of the system. Such definition allowed subsequent work on serological and cell mediated responses to be more fully characterised. In the context of the immune response to trypanosomes, how valid is such a model?

As previously stated, the use of monomorphic bloodstream trypanosome lines allows antigenic definition of the immunological reaction. The intradermal inoculation of such trypanosomes also removes the variability and extra antigenic components inherent in cyclically transmitted infections. The drawback of this system, however, is the lack of short stumpy trypanosomes which are present in more natural pleomorphic infections. Stumpy form parasites have been reported to be more immunogenic than slender bloodstream forms although possibly only when they senesce (Black *et al.*, 1985). Slender form parasites were found to be unable to stimulate a

response against external epitopes on VSG as shown by their failure to generate VSG-specific antibody producing hybridomas (Sendashonga and Black 1982). These authors suggested that the lack of response against monomorphic trypanosomes was not due to an intrinsic lack of immunogenicity or to an immunodepressive effect. The work of Sendashonga and Black (1982) and Black et al., (1985) was performed in mice. Experiments in rats (Zahalsky and Weinberg 1976) and rabbits (this study, section 3.3) have, however, demonstrated that monomorphic trypanosomes do stimulate the formation of VAT-specific antibodies. It may be, therefore, that failure to demonstrate anti-VSG reponses in mice infected with monomorphic trypanosomes is a consequence of limitations on the absolute numbers of trypanosomes present which are imposed by the small blood volume of a mouse. Monomorphic trypanosomes are highly virulent, but this is offset to some extent by the size of the host (e.g. 2×10^9 parasites in a mouse of blood volume 2 ml represents a parasitaemia of 10^9/ml, but the same number of organisms in a rabbit of blood volume 200 ml represent a parasitaemia of 10^7/ml). The larger blood volume therefore gives the host extra time to make a response before fatal levels of parasitaemia are reached. Larger animals also have a greater antibody producing capability as a consequence of having a proportionately greater number of cells in their immune system. In a similar fashion, it has been demonstrated that the number of VATs expressed in any given host was roughly proportional to the size of the host (Barry 1986b). This was suggested as a reflection of an essentially constant rate of switching in populations which grew in absolute numbers in proportion to host size.

The lack of stumpy form trypanosomes in the populations used for chancre induction may be a realistic model early in the development

of the reaction. Both Willett and Gordon (1957) and Roberts et al., (1969) observed only long slender trypanosomes in chancres induced by T. rhodesiense and T. congolense respectively. The failure of the populations used in this study to generate stumpy form trypanosomes may however affect the pathogenesis of the disease in later infections and this must be taken into account when interpreting the results.

One further intriguing feature of the experiments on a model system was the complete cutaneous anergy shown by rodents in response to intradermal challenge with monomorphic trypanosomes. This non-responsiveness was clearly not due to suppressor cell generation or to the organisms leaving the skin too rapidly for a response to be made. These results serve, yet again, to highlight the dangers of extrapolating from rodent data to the situation prevaling in larger animals.

The serological findings of this study were very simple; a high titre lytic antibody response against the infecting VAT coincided with the peak, and subsequent decline of the chancre. The peak of reaction to one VAT coincided with the point in infection after which inoculation with the same VAT did not induce a chancre but resulted in the formation of an Arthus-type reaction. Inoculation at this time with an heterologous VAT elicited a reaction with the appearance of a primary chancre and induced a good serological response against the heterologous VAT. No interference with responses against challenge VATs was noted, with the exception of the response to GUTat 7.13 inoculation on day 16 in rabbits which already harboured infections with four other VATs. The responses to GUTat 7.13 (both skin thickness and lytic titre) were diminished when compared to controls.

If one extrapolates from the defined single-VAT situation

described here to the situation pertaining in the field it would seem reasonable to expect chancre formation to be a serodeme specific reaction. If this was the case then some form of serotyping could theoretically be performed using the local skin reaction as a test of identity or non-identity. Such tests are likely to be of limited value and in cases of related or overlapping serodemes such a system would always give equivocal results.

The sequential histological findings described here demonstrated that the initial infiltration into the chancre was composed almost exclusively of $10B3^{+}T_{a}$-cells. This infiltration was followed 1.5 days later by a parallel rise in the numbers of B-cells in the reaction. The $10B3^{+}$ cell population reached peak numbers at the macroscopically observed peak of the reaction and then declined sharply. Numbers of B-cells peaked two days later and thereafter declined more slowly. A most intriguing finding was the late infiltration (from day 6 onwards) of $12C7^{+}T_{c}/T_{s}$ cells. These cells rose steadily in numbers and by day 24 were the most prevalent cell type. If these cells are assumed to be equivalent to $OKT8^{+}$ cells in human systems such a profile is an unexpected finding. Cutaneous infiltrates of $OKT8^{+}$ cells are much less common than their $OKT4^{+}$ counterparts. Of the common infiltrative skin lesions, few have a majority of $OKT8^{+}$ cells, one exception to this being the microscopic lesions of psoriasis patients. (Hammar et al., 1984)

Preliminary functional studies on cells from local skin reactions were facilitated by the development of a technique to isolate mononuclear cells from skin, using collagenase digestion and density gradient centrifugation. Although this technique resulted in a high proportion of cell death, it has the advantage over procedures using trypsin, of leaving surface proteins, including receptors,

intact. Such cells are therefore available to be stimulated immediately after purification. This technique may be of value in other areas of dermatological research as it is a common finding that the cell proportions in infiltrative lesions bear little or no relation to the proportions found in blood or lymphoid tissues (Hammar et al., 1984, this study section 4).

Functional studies on cells isolated from the chancre demonstrated that early in the reaction, T-lymphocytes respond in a VAT-specific manner. The observed specificity for the infecting VAT was much more pronounced in the chancre than in the lymph node draining the lesion. Cell proliferation in cultures isolated from the chancre was abrogated by the depletion of $10B3^{+}T_{a}$-cells, which implicates these cells as either the responding cells or as inducer-type cells. The response of cells from the draining lymph node did not show such a dependence on $10B3^{+}T_{a}$-cells, which may be indicative of a primary T-cell independent antibody response in this site.

By day 11, cells from the chancre showed antigen specific proliferation only when both the $10B3^{+}T_{a}$-cells and $sIg^{+}B$-cells were present. This suggests that a VAT-specific T-cell dependent antibody response was being generated in situ in the chancre. No such responses have been reported for systemic immunity to trypanosomes. Indeed, the finding that athymic nu/nu mice control parasitaemia as well as their euthymic litter mates suggests that T-dependent responses are not essential to control systemic infections (Campbell et al., 1979, Clayton et al., 1979). These findings however do not preclude the formation of a T-dependent response in inflammatory infiltrates.

It is interesting to speculate on the role of the $10B3^{+}T_{a}$-cells in the early chancre infiltrate. Clearly, one possibility is that they participate in the formation of a co-operative antibody

response. If this was the case would the B-cell infiltration be distinct from the T-cell infiltration, or might they be 'recruited' by the latter population? If B-lymphocytes are recruited are they recruited in an antigen (VAT)-specific manner? Does such an antibody response require an antigen presenting cell?

The question of antigen presentation is a particularly intriguing one. As slender form trypanosomes do not release VSG (Black et al., 1982), it might be expected that no soluble VSG will be available to the immune system. In the absence of soluble antigen, Langerhans cells may not function as antigen presenting cells (Kapsenberg et al., 1984). Macrophages can present particulate antigen, but do not phagocytose trypanosomes in the absence of specific antibody. It is possible that the inoculated trypanosomes fix complement (Musoke and Barbet 1977) which, even in the absence of trypanosome-specific antibody may be responsible for the transient influx of neutrophils seen in the chancre (Akol and Murray 1982). It is interesting to note that the numbers of neutrophils observed are proportional to the number of trypanosomes inoculated, and this may be an index of the level of complement fixation. The presence of neutrophils in large numbers may cause enough trypanosome death (through the hostility of the environment which neutrophils can create) to subsequently induce the antibody-forming inflammatory cascade described.

The appearance of a large population of $12C7^{+}T_{C}/T_{S}$ cells in late reactions also poses certain questions. As mentioned previously (section 4) these cells are unlikely to be cytotoxic T-cells. If, however, they are suppressor T-cells, are they antigen specific or non-specific. The preliminary results presented here suggest that they do not proliferate in an antigen specific manner, although this

may be an effect of masking by other, larger populations. The response of cells from both skin lesions and draining lymph node to con-A was, however, depressed and this suggests that some form of suppressive mechanism was operating.

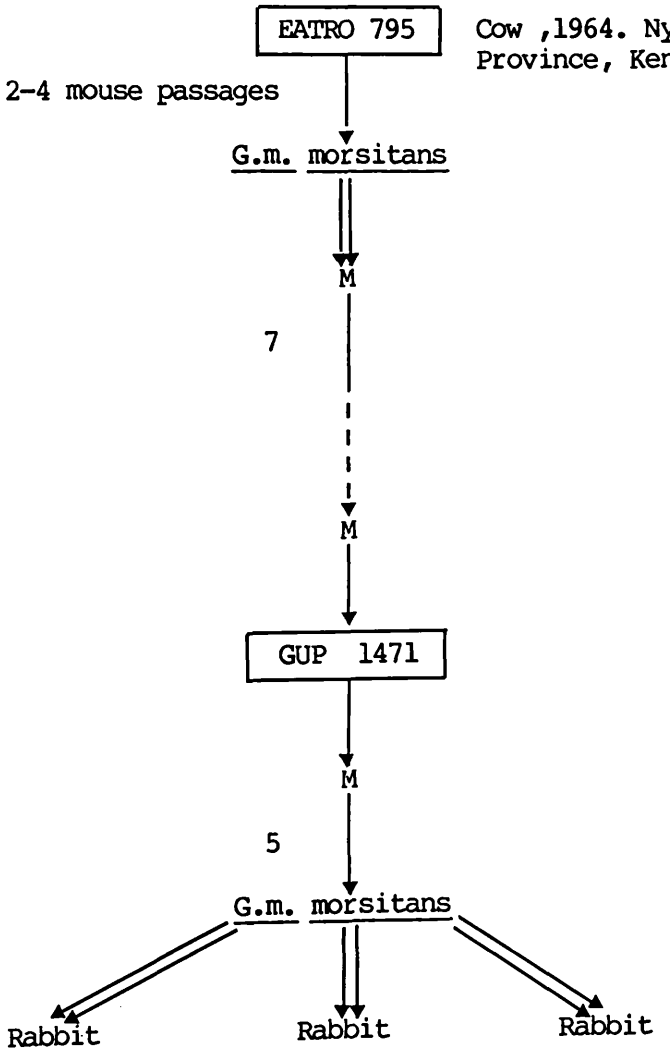
There are, therefore, many unanswered questions concerning the sequence of immunological events which take place in the chancre. It is in this situation that an antigenically defined system is expecially valuable as it allows most hypotheses to be rigorously tested. Only the continued use of such antigenically defined systems will facilitate the elucidation of the immune and inflammatory processes involved.

APPENDIX

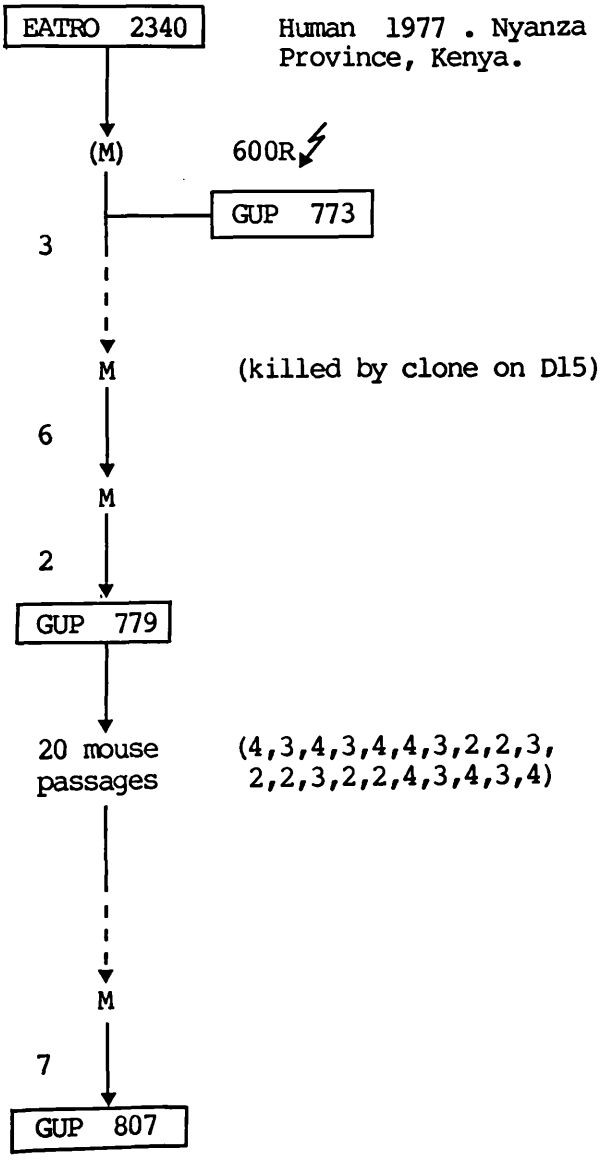
The histories of the trypanosomes used in this thesis

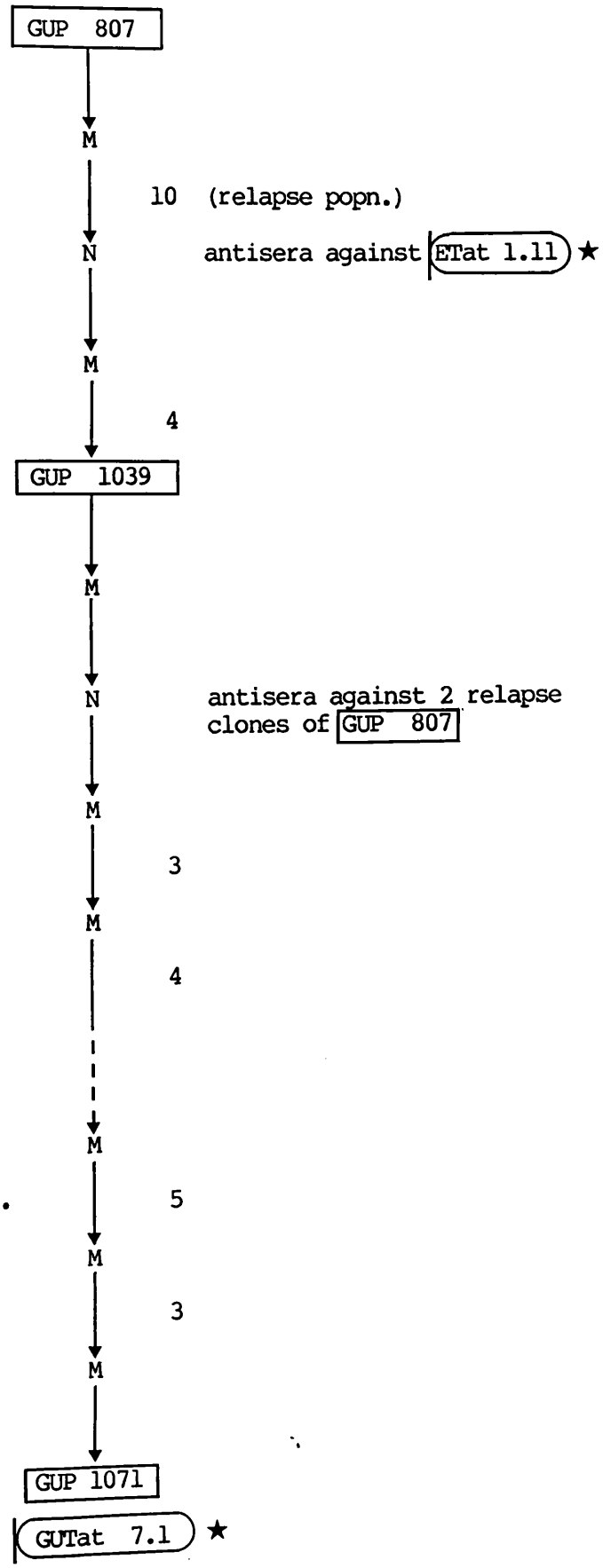
The pedigree digrams which appear on the following pages describe the life-histories of the trypanosome stocks used throughout this study. They are drawn according to the conventions of Lumsden *et al* (1973). Cryopreserved stabilate numbers appear in boxes, with primary isolates being designated by the source, date and place of isolation. Solid lines represent passage in animals, where (M) represents mice, and broken lines indicate cloning of trypanosomes. Double lined arrows represent cyclical transmission by tsetse flies. Stabilates containing VAT reference populations are designated by cartouches. Monomorphic bloodstream populations are indicated, (★). Neutralisation reactions *in vitro* are also shown (N) and the antiserum preparation used are stated.

History of cloned stock EATRO 795 used to infect rabbits

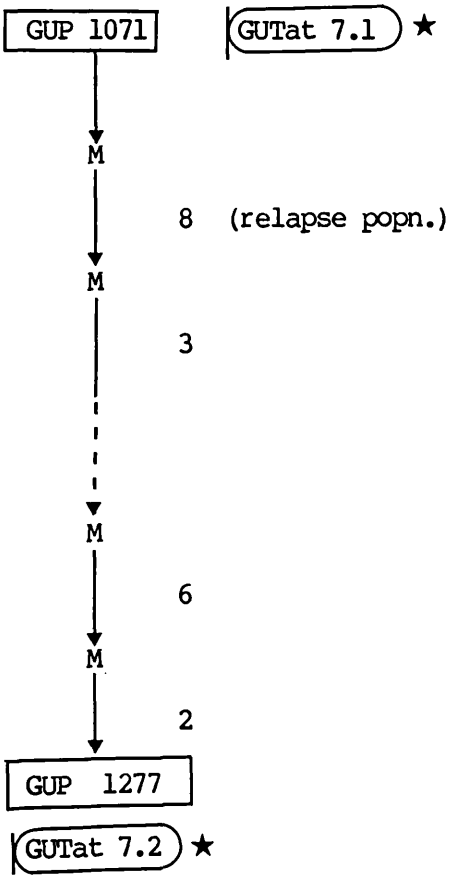


Derivation of cloned monomorphic line GUTat 7.1

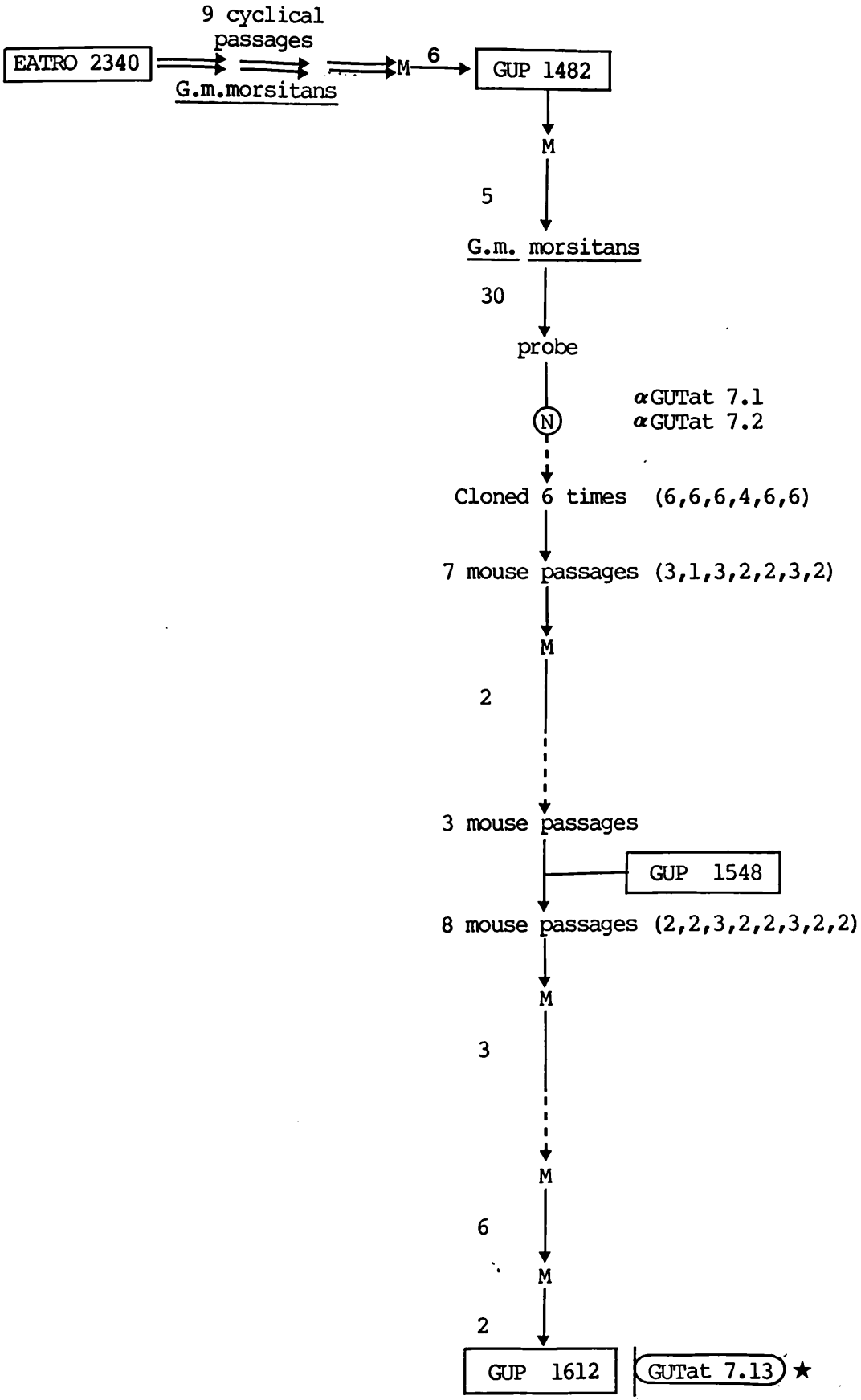



GUP 807
M
10 (relapse popn.)
N
antisera against ETat 1.11 ★
M
4
GUP 1039
M
N
antisera against 2 relapse clones of GUP 807
M
3
M
4
M
5
M
3
M
GUP 1071
GUTat 7.1 ★

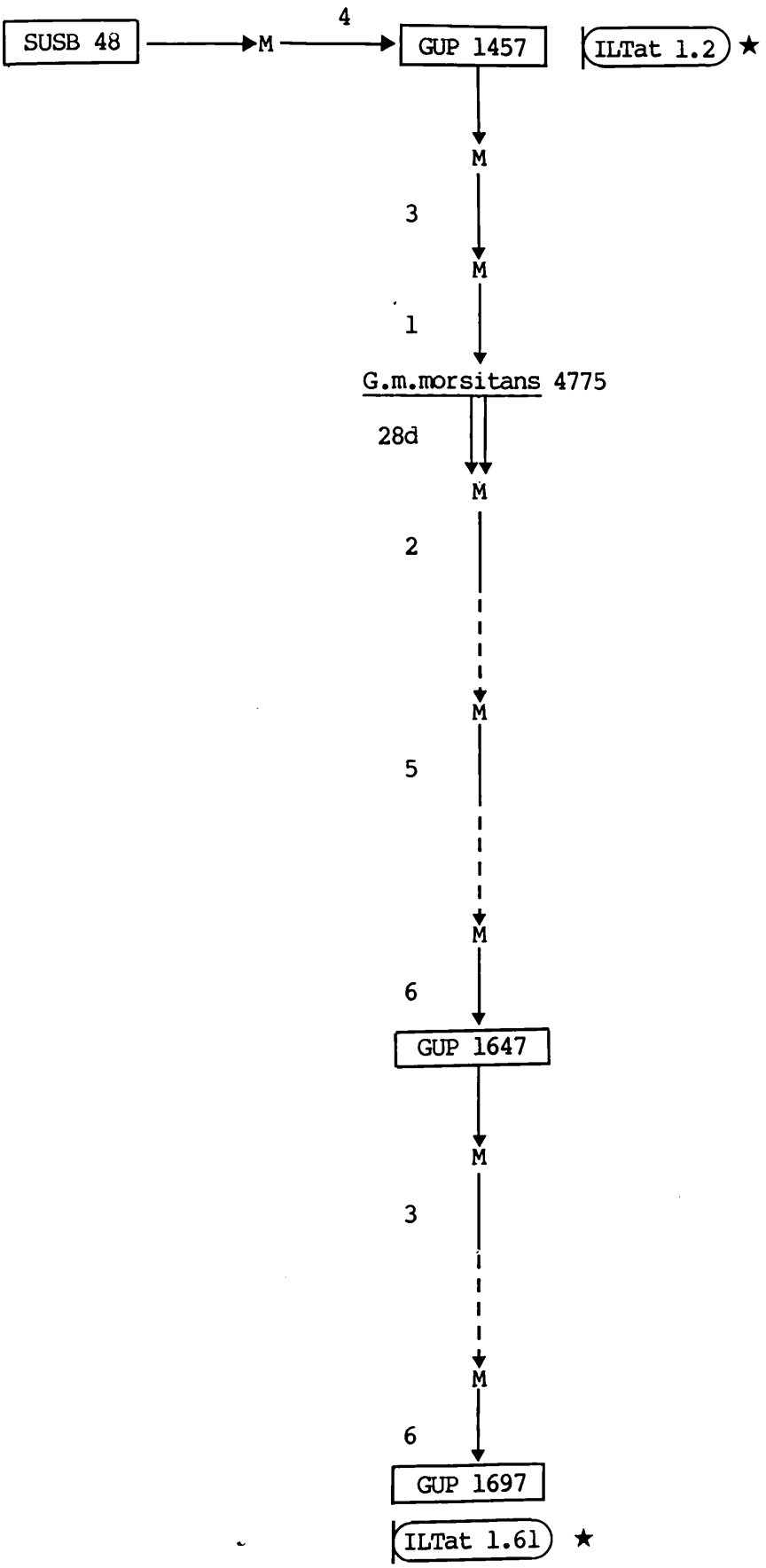
Derivation of cloned monomorphic line GUTat 7.2



Derivation of cloned monomorphic line GUTat 7.13



Derivation of cloned monomorphic line ILTat 1.61



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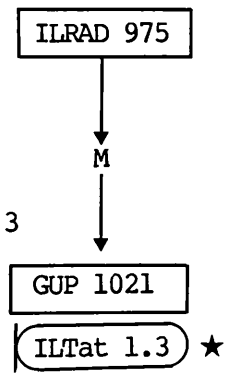

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