STUDIES OF PHAGOCYTIC ACTIVITY IN EXPERIMENTAL AFRICAN TRYPANOSOMIASIS

by

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CONTENTS

ABSTRACT

Various investigations were carried out on the importance of phagocytosis in African trypanosomiasis using laboratory rodents and rabbits.

The opsonic and macrophage cytophilic antibody activities of sera prepared from mice and rabbits infected with both chronic and acute strains of Trypanosoma brucei were studied using cultured peritoneal and alveolar macrophages. The attachment and phagocytosis of separated trypanosomes was quantified in the presence of immune and hyperimmune sera, and the effect of various chemicals and drugs was determined.

Opsonic and cytophilic antibody activity was demonstrated in the sera of infected mice and rabbits, the reaction being significantly strain-specific. The activity in acutely-infected mice increased from control levels during infection and could be correlated with parasitaemia, whereas in chronically-infected rabbits appreciably higher results were obtained, with the values rapidly rising and flattening out after the first two weeks of infection.

The establishment of these enhanced levels occurred during or after the rise in the titre of agglutinating antibody and appeared to be associated with the in vivo development of immune complexes.

Attachment of parasites to induced rabbit macrophages was not dependent upon variant specific antibodies, no correlation being recorded between adherence and the appearance of successive variant subpopulations during infection.

The effect of complement, serum immunoglobulin M and immunoglobulin G on trypanosome attachment was investigated, using selectively-depleted and separated immune and hyperimmune sera. This indicated the primary importance of immunoglobulin G in both opsonic and cytophilic antibody activity and the role of complement, possibly

mediated by both the classical and alternate pathways, on the specific attachment of trypanosomes to macrophage surface receptors.

Several pharmacologically active substances, most notably 5-hydroxytryptamine (serotonin) were used to enhance attachment, and the presence of immune complexes in the system, either free or passively sensitized to peritoneal macrophages, gave an increased response. The limiting factor in attachment appeared to be the number and availability of macrophage surface sites and their preferential susceptibility to specific chemicals and enzymes. The physical nature of the environment and the nature of macrophage-parasite contact were also of importance; high levels of attachment being recorded from tissue smears in the absence of immune sera.

An in vitro technique was used to monitor chemotaxis and a quantifiable estimation of cellular migration in the presence of cytotaxins and cytotaxigens was made. Trypanosome antigen-antibody complexes were shown to be similarly effective in generating specific cytotaxins from both normal and heat inactivated hyperimmune sera.

The serum levels of Cx-Reactive Protein in chronically infected rabbits were shown to rise and appeared to be a response to the pending inflammation. They reached a peak before external symptoms were evident and thereafter declined as tissue damage became more pronounced. Anti-inflammatory drug treatment during infection temporarily reduced the recorded levels of Cx-RP but did not affect the parasitaemia or the subsequent course of the disease.

Glossary of Abbreviations

AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BS	Barbital sodium buffer
BSS	Balanced Salt Solution
C'(1,2,3 etc.)	Serum complement (Components 1,2,3 etc.)
CF	Complement Fixation
CI	Chemotactic Index
CMI	Cell Mediated Immunity
CNS	Central Nervous System
Con A	Concanavalin A
CRP	C- Reactive Protein
Cx-RP	Cx- Reactive Protein
CVF	Cobra Venom Factor
EACA	Ɛ- aminocaproic acid
EDTA	Ethylene diaminetetraacetic acid dipotassium salt
ESR	Erythrocyte Sedimentation Rate
FCA	Freund's Complete Adjuvant
FDP's	Fibrin / Fibrinogen Degradation Products
FIT	Fluorescein Isothiocyanate
5-HT	5 - Hydroxytryptamine (serotonin)
IA	Immune Adherence
Ig (G,M,D,E,A)	Immunoglobulin (G,M,D,E,A,)
i/m	Intramuscular
i/p	Intraperitoneal
IU	International Unit
i/v	Intravenous
K	Phagocytic Index
KAF	C3b Inactivater
2-ME	2 - Mercaptoethanol

MIF	Migration Inhibition Factor
MPS	Mononuclear Phagocyte System
MRBC	Mouse Red Blood Cells
MW	Molecular Weight
NCS	Normal Calf Serum
NGPS	Normal Guinea Pig Serum
NMP	Normal Mouse Plasma
NMS	Normal Mouse Serum
NRS	Normal Rabbit Serum
P	Properdin
PBS	Phosphate Buffered Saline
PCMB	p -chloromercurybenzoate
PMNG	Polymorphonuclear Granulocytes
PSG	Phosphate Saline Glucose
PZ	Properdin - zymosan complex
R (1,2,3 etc.)	Serum deficient in Complement Components (1,2,3 etc.)
RES	Reticuloendothelial System
RF	Rheumatoid Factor
RHS	Reticulohistiocyte system
RP	Properdin deficient serum
RT	Room Temperature
S	Svedberg Unit
s/c	Subcutaneous
SRBC	Sheep Red Blood Cells
TCM	Tissue Culture Medium (Medium 199)
u.v.	Ultra violet

PART I. INTRODUCTION

1. 1. Object of Study

The purpose of the study was to investigate the *in vitro* efficacy of the cellular defence system of animals infected with experimental African trypanosomiasis and to assess its importance in the immunopathology of the disease process.

1. 2. Trypanosomes and Trypanosomiasis

(1) Taxonomy

The general classification of trypanosomes as given by Hoare (1966, 1972) will be followed. Classically, the *gambiense* and *rhodesiense* trypanosomes were regarded as distinct species (reviewed by Baker 1974), and later suggestions, recently revived by Ormerod (1967), have been made whereby the *brucei*, - *gambiense*, - *rhodesiense* complex should be considered as one species. It is intended to follow the compromise of Hoare (1970), Lumsden (1970) and Baker (1974), where the *brucei*, *gambiense* and *rhodesiense* trypanosomes are regarded as subspecies of *Trypanosoma brucei*. *Trypanosoma rhodesiense* may also be considered as a virulent nosodeme (clinical race) of *T. gambiense* (Hoare 1970, Goodwin 1970).

Following the present convention of WHO, the subgeneric term - *Trypanozoon*, encompassing the former *brucei* and *evansi* sub groups (Hoare 1972) - will not be used.

The major species to be considered, *Trypanosoma* (*Trypanozoon*) *brucei brucei*, Plimmer and Bradford 1899 (Hoare 1966, 1972) will be referred to as *Trypanosoma brucei*.

(11) The Importance of Trypanosomiasis

"Trypanosomiasis, with its toll of human and domestic animal life, was (and still is) of particular importance in impeding the progress of African development" (Desowitz 1970).

African trypanosomiasis is a generalised debilitating disease of both man and cattle. Continuous attempts have been made during this century to control the spread, and minimise the social and economic consequences, of this disease.

WHO, in considering the major health problems facing man and his domestic animals, believed that trypanosomiasis was of fundamental importance, capable of being ranked alongside malaria, cancer and heart disease (Kershaw 1970).

Since the early observations of Bruce and Livingstone (1861), much information has been detailed concerning the epidemiology of tsetse-transmitted trypanosomiasis; yet present-day knowledge of human and animal pathology and of the various pathological processes themselves, remains surprisingly sparse (Losos & Ikede 1972, Goodwin 1974). It has become increasingly evident that the nature of the disease produced in different hosts by different causative organisms can vary considerably (Goodwin 1970). This, partially due to variations in the ecological requirements of the infective parasites, and alterations to the general immune response of the host (Gray 1976), indicate the variation and indeed, the complexity which face present investigation. Up to 4 million square miles of Africa south of the Sahara, which support several Glossina species, are effectively devoid of any form of viable animal husbandry (Goodwin 1974). Nagana, the classical wasting disease of cattle, caused by Trypanosoma congolense or Trypanosoma vivax is regarded as the most serious problem. T. brucei, for long considered to be responsible, appears not to cause appreciable disease symptoms (Goodwin 1970, Killick-Kendrick 1971), despite the possibility of high natural infections being present. T. brucei infections are, however, often found mixed with the more severe T. congolense and T. vivax (Losos & Ikede 1972).

A large number of significant contributions have been made during the present century towards an understanding of African trypanosomiasis. The hoped-for development of an effective vaccine has not, however, materialized, due to the presence of antigenic-dissimilarity between many of the parasites and the problems of existing resevoir hosts. As yet, the political and economic implications of mass-vaccination of both cattle and man have not been realistically considered and until then, trypanosomiasis will remain a serious menace to man, domestic animals and the wild game of Africa (Goodwin 1970).

Improved, ecologically-balanced, vector control is occurring. Some 5 or 6 species of Glossina can be considered of major importance, occupying various and varying ecological niches (Jordan 1976). Effective control measures need, therefore, to be effective over the whole range of present species' distribution. As Jordan (loc cit) has indicated, tsetse flies are, in many instances, acting as conservation agents, in monitoring vast tracts of land which could eventually be more profitably utilized by man. There exists a need for a rational integration between control and development. Just as the tsetse fly is no respector of artificial boundaries, so effective and realistic control cannot be effected without regard to overriding political and economic considerations.

The causative organisms of African human trypanosomiasis (sleeping sickness) are T. gambiense and T. rhodesiense (which are morphologically indestinguishable from T. brucei). The former tends to cause a chronic disease (Gambian Sleeping Sickness) and predominates in the western half of tropical Africa; the latter produces a disease which runs an acute course (Rhodesian Sleeping Sickness) and is restricted to the eastern and central areas of tropical Africa (Baker 1974).

de Raadt (1976), has indicated how the especial problem of human trypanosomiasis has altered over the last century. Early reports, despite little epidemiological information, suggested small, comparatively isolated village communities where separate disease isolates could run independent courses. With the development of increased and improved communication, a corresponding alteration in disease transmission occurred, with characteristic epidemic outbreaks; e.g. those occurring along the Congo river and north of Lake Victoria at the turn of the century. With increased knowledge and application, epidemic control was initiated and a programme of eradication, later modified to control, was started. This progress is presently continuing, but the problem of local endemicity is still present and re-surgence to epidemic proportions can occur (Baker 1974, Bray 1974). The reasons for epidemic re-activation are various (de Raadt 1976); but with the increased sophistication of emerging Africa, developing and maintaining improved forms of social service, the potential spread of human, and indeed cattle, trypanosomiasis, is perhaps more important than ever. Thus Lumsden (1970), noted that "the impact of trypanosomiasis retarding the development of Africa increases rather than diminishes".

Endemic foci remain and epidemics flare up. Social, economic and political instability coupled with improved communication and the desire to utilize present resources can all be effective in the spread of the disease. As Goodwin (1974), has indicated, "Trypanosomiasis has much the same hold on the African continent today as it had at the beginning of the century".

1. 3. The Mononuclear Phagocyte System

1.3.1. History and Nomenclature:-

It was over 100 years ago when it was first realised that metazoan organisms possess large numbers of amoeboid-like cells in their blood and tissues, which are capable of actively migrating and avidly engulfing foreign matter in their environment. In 1863, von Recklinghausen described mononuclear cells derived from fixed connective tissue in an inflamed cornea; and three years later von Kupffer described the phagocytes of the liver. In 1892, Metchnikoff proposed a classification - The Macrophage System - whereby the actively phagocytic cells or macrophages (lit "big-eaters"), could be distinguished from the less active cells capable of ingesting only small particles - the microphages or polymorphonuclear leucocytes (polymorphs). He was the first to recognise the interrelationships between the phagocytic cells found in various organs and the connective tissues, and by demonstrating their defensive role, especially in inflammatory reactions, established the cellular theory of body defence (Vernon-Roberts 1972).

Aschoff (1924), conceived the term 'Reticulo-Endothelial System' (RES), whereby several types of cell sharing a common morphology, origin or function, could be grouped to form a distinct system (Rowley 1962, van Furth 1970); these cells could be ranked in an order of phagocytic activity. Aschoff's somewhat arbitrary classification has been widely criticised (van Furth et al 1975). Thomas (1949), indicated that many cells such as muscle, bone, Schwann and epithelial cells could acquire the "histiocytic state" under certain conditions. Once this histiocytic state was established, it became stable and further differentiation could then occur. He proposed the term 'Reticulo-Histiocyte System' (RHS). It has recently been suggested (Langevoort et al 1970, van Furth et al 1972, van Furth et al 1975), that it would be more advantageous to regard all highly phagocytic

cells and their precursors as being in one system viz: 'The Mononuclear-Phagocyte System' (MPS). This collective term may be used to embrace the cells generally referred to as macrophages or their progenitors (Nelson 1972; see Wilkinson 1974b).

Macrophages have been referred to by a variety of synonyms (Taliaferro & Mulligan 1937), such as 'clasmatocytes', 'adventitial cells' and 'histiocytes' (Kiyona 1914), although the latter term is more properly reserved for connective tissue macrophages only. Macrophages are found in two main forms - free and fixed. The former can move throughout the tissues, the latter tend to be more permanently positioned; often lying along the walls of blood and lymph channels in direct contact with circulating blood or lymph.

The free macrophages include those of the connective tissue, those found in serous cavities and in the synovial cavities of joints (Carr 1973), ones found in inflammatory exudates and the pulmonary alveolar macrophages. They are also located within the spleen, bone marrow and lymph nodes where they lie in the interstitial spaces in close association with the reticular cells to which the fixed macrophages are attached. The reticular cells themselves and the follicular dendritic cells of the spleen and lymph nodes are not usually considered as macrophages (Steinman & Cohn 1973).

The fixed macrophages are found in the liver (Kupffer cells), lining the hepatic sinusoids, the bone marrow where they may again line the sinusoids but may also be scattered; the spleen, in the red pulp and within the Malpighian follicles of the white pulp, the lymph nodes particularly in the circular and medullary sinuses, the placenta and the CNS (Oehmichen 1975). Osteoclasts in bone tissue may be formed by the coalescence of mononuclear phagocytes (van Furth 1970). Macrophages also form part of the lining of the blood sinusoids in the adrenal cortex and are scattered in the blood spaces of the medulla. They are

also distributed along the blood and lymph channels and in the connective tissues of the pituitary, ovary and testis, and small numbers are found in the cortex and medulla of kidneys.

1.3.2. Derivation and Transformation:

Allogeneic implantation studies and kinetic investigations have indicated that mononuclear phagocytes are derived from immature precursor cells situated in the bone marrow, are transported via the blood (as monocytes) and may eventually become tissue macrophages (Balner 1963, Goodman 1964, Vernon-Roberts 1972, Adams 1974). Volkman & Gowans (1965a), employing the 'skin window' technique of Rebuck & Crowley (1935), demonstrated that inflammatory-macrophage precursors emigrate from the bone marrow, and to a lesser extent the spleen, and can congregate and further proliferate at sites of non-bacterial inflammation (Volkman & Gowans 1965b, van Furth 1975). Under pathological conditions, the majority of macrophages are derived from mildly phagocytic blood monocytes and from extensive local multiplication of previously-formed macrophages (Ebert & Florey 1939, Nelson 1972). Macrophage-proliferation appears to be stimulated by the presence of blast cells and inhibited by mature differentiated macrophages (Gottlieb & Waldman 1972).

Studies by van Furth et al (1970), have shown that in the mouse, the promonocytes of bone marrow are proliferating cells, whilst under normal conditions, the monocytes of both bone marrow and blood are non-dividing (van Furth & Cohn 1968). The multiplicative promonocytes probably divide only once, each individual giving rise to two monocytes (van Furth et al 1973). The promonocytes themselves are formed from monoblasts which in turn are derived from committed haemopoietic stem cells in the bone marrow (Cudkowicz et al 1964, Goud et al 1975, van Furth et al 1975). The 'thymic-derived macrophages' of Miller & Mitchell (1968), may represent 'reconditioned' bone marrow macrophages

which have migrated to secondary lymphoid tissue (Vernon-Roberts 1972, Warr & Sljivic 1974).

At the International Conference on Mononuclear Phagocytes (Leiden 1970), it was proposed that a macrophage should be defined as "a mononucleated tissue cell, derived from a circulating monocyte that originated in the bone marrow, which adheres to glass or plastic surfaces, is characteristically highly phagocytic or pinocytic and possesses an undulating membrane " (Fishman 1972).

Monocytes and macrophages may transform to epithelioid cells and giant cells in vitro (Lewis 1925, Vernon-Roberts 1972). In vivo transformation is, however, more equivocable. Giant cells which have been observed in the spleen following intravenous injection of carbon (Potek & Bernick 1960), may well arise by macrophage fusion (Gillman & Wright 1966), although positive evidence for the transformation of monocytes to cells other than macrophages is lacking (Vernon-Roberts 1972, Leibovich & Ross 1975).

Monocytes are the immediate precursors of inflammatory macrophages. In the absence of inflammation there appears to be a limited monocytic recirculation with a proportion migrating into the tissues and transforming to mature macrophages (Vernon-Roberts 1972). Similar transformation has been noted in vitro (Sutton & Weiss 1966). Blood monocytes are often considered as immature macrophages rather than precursor cells as cell division seldom occurs, except, perhaps in inflammatory tissues (Spector 1969).

Bloom (1927), suggested that lymphocyte-macrophage transformation could occur in vitro and Rebuck et al (1958; 1960; 1964), believed that small lymphocytes could transform to macrophages within 21 hours of reaching inflammatory sites. They implied the necessity for an applied antigenic stimulus. Vernon-Roberts (1969), also suggested

that in the presence of viable polymorphs, peritoneal lymphocytes could transform to macrophages, and it has been suggested that under conditions of direct MPS stimulation, circulating lymphocytes may give rise to typical macrophages (Howard et al 1969, Benested et al 1971, Gottlieb & Waldman 1972). Medawar (1940), indicated, however, that transformation was unlikely and this has been subsequently confirmed by ultrastructural investigations (Carr 1973), and by the work of van Furth & Cohn (1968). If any transformation does occur in normal physiological conditions it probably makes only a minor contribution to the total supply of macrophages (Nelson 1972).

1.3.3. Structure:

Macrophages are morphologically heterogenous cells. They vary considerably in shape, size (20 - 50 μm) (Cohn 1968), and in cytoplasmic and nuclear appearance, according to location, degree of activity and whether they are fixed or free (Taliaferro & Mulligan 1937). A lot of the early morphological information was determined by the use of vital dyes and metallic impregnation techniques (Marshall 1956). Most macrophages possess rounded or indented nuclei with either condensed or vesicular chromatin material. The vacuolar cytoplasm, often containing ingested material, may be basophilic or eosinophilic (Vernon-Roberts 1972). Macrophage monolayers (Jacoby 1965), are often motile with cells exhibiting a marked activity of the peripheral cytoplasm which tends to be devoid of organelles, and large, fan-shaped cytoplasmic projections, or hyaloplasmic veils, may extend from the edges. Ridges and flanges cover the whole surface of peritoneal macrophages (Carr et al 1969), which become more prominent following lipid stimulation, suggesting a relationship between their appearance and phagocytic activity. The amorphous, acid mucopolysaccharide-containing cell surface may be responsible for adhesion. ATPase

activity can occur here (North 1966), and may be inhibited by the use of sulphydryl poisons (which also prevent adherence to glass) and trypsin. The prominent centrosome and Golgi apparatus are often situated in the cytoplasm in the nuclear indentation (hoff). A varying number of primary and secondary lysosomes with associated electron-dense granules are invariably present.

Mononuclear phagocytes, unlike polymorphonuclear leucocytes, are not fully mature upon leaving the marrow, but continue to differentiate outside it (Fedorko & Hirsch 1970). The small immature monocytes are less heterogenous than the mature macrophages (Cohn 1968, Adams 1974). They possess smaller nuclei, have less cytoplasm and smaller cell processes, mitochondria and fewer cytoplasmic dense bodies. During in vivo and in vitro development both morphological and physiological alterations occur with substantial cellular enlargement and increases in mitochondria, golgi profiles, endoplasmic reticulum and lysosomes (Cohn 1968). This is associated with alterations in respiratory rate, protein synthesis, glucose utilization and changes in phagocytic and microbicidal capacities (Dannenberg 1968, Adams 1974).

1.3.4. Function:

Perhaps the major property of mature macrophages is their extensive capacity for endocytosis, usually visualized as phagocytosis of particulate matter (Vernon-Roberts 1972, Nelson 1972). Other cells such as polymorphs, tumour cells, Schwann, smooth muscle and some epithelial cells can, under appropriate conditions, be induced to endocytose particles, yet they lack both the capacity and, more importantly, the highly-developed discriminative ability possessed by macrophages (Vernon-Roberts 1972). They are frequently motile and are invariably equiped with a highly active cell membrane, seen as cytoplasmic extensions, ridges and microvilli. They significantly

contain large numbers of lysosomes and high levels of acid phosphatase (Turk et al 1966), and readily degrade ingested material (Klebanoff & Hamon 1975); they may store foreign matter from colloidal solutions (Taliaferro & Mulligan 1937, van Furth et al 1975) and can harbour and disseminate certain microorganisms, resistant to the normal digestive processes (Jones 1974, Draper & D'Arcy Hart 1975, Jones 1975). They do not appear to synthesize antibody (Leake & Myrvik 1968, Nelson 1972) (cf: Osawa et al 1971 quoted in Solotorovsky & Suderberg 1972), although they may retain and subsequently release previously formed serum antibody (Hunt & Myrvik 1964). They may play an important role in immunogenic induction either by antigen processing, perhaps involving RNA, or by retaining antigen fragments on their surface (Mitchison 1969, Unanue et al 1969, Askonas & Jaroskova 1970). Immunogenicity, however, cannot be simply equated with macrophage activity (Byrt & Ada 1969, Gottlieb & Waldman 1972). Antigen-retention by macrophages may serve in the generation of memory cells (Blanden 1968, Humphrey 1969). Macrophages may also be peripoletically and emperipoletically involved in T and B lymphocyte co-operation, and may modulate the immune response, suppressing or regulating the humoral antibody response and ensuring the induction of delayed hypersensitivity (Gottlieb & Waldman 1972). Degredation of antigen may be important in preventing immunological paralysis. Macrophages alone, or in association with lymphocytes, have been implicated in cell-mediated cytotoxicity (Evans & Alexander 1970, Leibovich & Ross 1975); this may in some circumstances be independent of phagocytosis (Temple et al 1973).

Macrophages characteristically possess specific receptor sites for immunoglobulins, and other serum factors (Nelson 1972), and can synthesize certain complement components (Phillips & Thorbecke 1966) and interferon (Glasgow 1966, Wagner & Smith 1968). Macrophages are also involved in the inactivation of bacterial endotoxins, resistance

to shock, prevention of irradiation sickness (Perkins et al 1966), bile-pigment formation and the metabolism of lipids (Day 1964, steroids (Berliner et al 1964), cholesterol, iron and proteins (Cohn 1968). They have been suggested as being involved in the aetiology of artherosclerosis (Day 1964), hypercholesterolaemia, lipoidoses, haemolytic anaemias (Vernon-Roberts 1972), amyloidosis (Shirahama et al 1971) and neoplastic diseases such as monocytic leukaemia (Bainton 1975) and malignant histiocytosis (van Furth et al 1975), and they can act as direct effectors of damage in neurological auto-immune diseases (Nelson 1969, 1972).

1.4. Phagocytosis and the Role of Opsonins

(a) Preliminary

The process of phagocytosis *per se* has existed from the first unicellular animal, and has been retained to a certain extent by all metazoans (Prowse & Tait 1969, Scott 1971). Indeed, during the embryonic development of tripoblastic animals, all three germ layers are at some stage capable of phagocytosis. In man and other vertebrates, the successful resistance against bacterial infection depends largely upon the efficiency and discriminatory ability of phagocytic cells (White 1968).

Phagocytosis may be divided into various phases (Rabinovitch 1967, Jones & Hirsch 1971, Jones *et al* 1972, Griffin & Silverstein 1975). Boyden (1962b), recognized three responses of a phagocytic cell towards a foreign particle - viz: chemotaxis, phagocytosis and digestion. He believed that the selectivity of the general phagocytic response applied not only to particle uptake, but also to migration of the phagocyte. He considered it improbable that the phagocyte relied on two different mechanisms for recognition; regarding both chemotaxis and actual uptake as being the consequence of similar stimuli, such that in many instances, phagocytosis could be regarded as a continuation of chemotaxis, even after surface contact had been made (see Section 1.6.). He did not, therefore, distinguish between attachment and engulfment. Carr (1973), also divided phagocytosis into three phases, but, like Vernon-Roberts (1972), distinguished between attachment and ingestion which he considered to have different controlling factors. He regarded chemotaxis not as an integral part of the phagocytic response, but merely as a precursor. Hirsch (1965), suggested that the initial requirement for phagocytosis was a means

of contact between the phagocytic cell and the foreign material. He recognized two mechanisms whereby this could be effected; firstly the transfer of the particle to fixed phagocytes and secondly, the movement of free phagocytes to the particle. Contact may be a result of random hits, both specific and non-specific chemotaxis (Florey 1962), or physical trapping (Wood 1960); its efficiency may be increased by the filtration systems present in certain reticulo-endothelial organs.

After initial contact, a host of factors become operative. Firstly, phagocytosis being an energy-requiring process (Baldridge & Gerard 1933), a source of suitable carbohydrate and a functioning glycolytic pathway are needed. Physico-chemical features of the particulate surface, such as bacterial encapsulation and the resultant nature of surface forces (Fenn 1922), leading to attraction, indifference or repulsion, all play a part. Various physical and chemical environmental factors are important, especially the presence or absence of serum antibodies or complement (opsonins), and surface phagocytosis (Wood et al 1946). Phagocytic rates may be influenced by salt concentration, pH, divalent cations and temperature (Hirsch 1965).

The separation of attachment and engulfment has been clearly demonstrated (Rabinovitch 1967, Rabinovitch 1970, Vernon-Roberts 1972). Gluteraldehyde-treated red cells can specifically attach to macrophages and this does not require serum factors or divalent cations. Ingestion, however, is initiated by serum or specific antibodies directed against red cell antigens or protein antigens bound to the red cells and requires divalent cations (Rabinovitch 1967). The attachment of serum-opsonized red cells also requires cations (Cohn 1968), but stored red cells are capable of adhering to macrophages in the

absence of both serum and cations (Vaughan & Boyden 1964). The ingestion phase, but not attachment, can be inhibited by cooling or by the judicious use of metabolic inhibitors such as iodoacetate, suggesting that the latter process does not require energy (Vernon-Roberts 1972).

(b) Recognition

Particle-recognition is an essential requirement for phagocytosis and varies according to the nature of the particle, but is, in many instances, thought to involve antibody and/or complement factors. Wright & Douglas (1903), introduced the term 'opsonin' as it applied to heat-labile substances of normal mammalian serum, which could render certain bacteria capable of being phagocytosed by leucocytes. They were considered to be non-specific, since normal serum, from non-immune animals, was often found effective. It is considered that the heat lability of certain opsonins is due to the promoting effects of complement, and that the opsonic nature of non-immune sera is caused by naturally-occurring antibodies. In many cases, opsonins appear to be integral factors essential for effective phagocytosis. The recognition and subsequent phagocytosis of inert particles such as carbon, however, may require substances other than specific serum or cell-coating antibody (Hirsch & Strauss 1964, Brogan 1966).

Boyden (1962b), investigating the specificity of the phagocytic response, suggested that the fine discrimination between foreign and indigenous material was not due to differences in electrostatic charge, as the normal minimal variations would be too small. He further considered that if chemotaxis was indeed regarded as an integral part of phagocytosis, then the distances involved would be too great to implicate electrostatic forces. He suggested that phagocytic discrimination relied to a large extent upon structural differences

between unfamiliar environmental macromolecules (Boyden 1962b, 1966). It is known that the immune system is also able to respond positively, either by antibody production or by a hypersensitive reaction to abnormal macromolecular configurations (Vernon-Roberts 1972). The ability of particles to be phagocytosed by macrophages may be determined by the relative hydrophobicities of particles and the ingesting cell (Wilkinson 1974a). The actual adhesion may be mediated by immunological factors in the form of specific receptor sites, or non-immunological profiles, or may be due to purely physico-chemical forces, with the various adhesive and repulsive forces being dependent upon a variety of factors including differences in surface charge. The large number of microvilli present on the macrophage surface could facilitate cationic bridging. Indeed it has been suggested that free Ca^{++} ions are an important adjunct for some forms of attachment (Carr 1973).

(c) Structure and Function of Macrophage Surface

Macrophages themselves are capable of adhering to a variety of surfaces; this may well be due to the presence of an acid mucopolysaccharide surface layer. North (1966), has indicated that the surface layers of guinea pig macrophages are involved in active processes, and that ATPase activity, utilizing both Ca^{++} and Mg^{++} ions, can be inhibited by treatment with trypsin or sulphydryl compounds. He suggests that both surface attachment and particle-ingestion are part of an overall mechanism of energy-dependent membrane movement, employing phosphate-bound energy. The macrophage membrane itself is composed of a matrix possessing lipid and protein organized into specific functional areas, e.g. antigen receptors, areas rich or poor in endocytic activities, and various surface antigens (Aoki et al 1969, Unanue 1972). Both Kupffer cells and free macrophages in general have a thick filamentous bilayered coat, the outer layer of which can be easily removed (Carr 1973).

It may be this mucopolysaccharide/acidic glycoprotein layer (Diluzio & Flemming 1971), which is functionally important (Emeis & Wisse 1975). The cytoplasmic zone below this membrane coat tends to be free of organelles, with only a few fibrils present (Carr 1973). Both interiorization and surface membrane release is common (Nachman et al 1971, Gallily & Schroit 1975), and new membranes may be synthesized from amino-acids, sugars, lipid precursors and plasma cholesterol (Werb 1975).

Curtis (1969), indicated that when cells collide in culture, they tend to separate unless an adhesive force is present to prevent aggregate break-up. The various forces which may contribute to repulsive/adhesive activity between cells and particles include electrostatic (Curtis 1967), hydrophobic/philic (van Oss & Gillman 1972), zeta potential (Bangham 1964), van der Waals' (Curtis 1969) and covalent bonding (Rabinovitch 1970). Thrasher et al 1973), have indicated that phagocytosis is enhanced when the phagocyte surface becomes more hydrophilic, i.e. the surface tension is lowered, or conversely when the substance or organism to be ingested is made more hydrophobic (van Oss & Gillman 1972). Indeed, the capsular coat of certain streptococci render them more hydropilic than unencapsulated organisms (van Oss & Gillman 1972). Raffel (1961), suggested that phagocytosis may be due to a decrease in phagocyte-surface energy brought about by substances diffusing from the microbe. Thus detergents in vivo have the same effect. Metal cations, tannic acid, protein and polypeptide sensitization, and receptor-molecule interaction all increase attachment (Rabinovitch 1967, 1970; Berken & Benacerraf 1966, Lo Buglio & Rinehart 1970).

(d) Role of Antibodies and Complement (C')

Phagocytosis may result as a consequence of cytophlic antibodies (see Section 1.5.). These may be detected in the sera of both immunized and non-immunized animals (Nelson 1969), and are often in equilibrium with globulins in the surrounding fluid (Carr 1973). In some instances, however they can be found attached to certain cells

but are not present in sera (Vernon-Roberts 1972). Most cytophilic antibodies have been identified as immunoglobulin G (IgG), although some immunoglobulin M (IgM) fractions have been found. Cytophilic antibody receptors on the macrophage surface contain sulphydryl (-SH) groups and phospholipid (Howard & Benacerraf 1966, Davey & Asherson 1967), and proteolytic enzyme treatment of the macrophage can often increase their adherence (Davey & Asherson 1967). Like direct opsonins, cytophilic antibodies play a prime role in the recognition of foreign material and thus affect the discriminative capacities of phagocytic cells.

Attachment involving opsonins can occur in a variety of ways; antigen-antibody complexes may be formed which attach to specific cell receptors; cytophilic antibodies can adhere to cell receptors facilitating subsequent particle-antibody attachment; non-cytophilic antibodies may bind to specific sites following configurational changes due to antigen contact, and normal complementary profile-binding may occur without the intervention of serum or immune factors, and various combinations of all the above mechanisms, with or without the added presence of complement, may be operative (Carr 1973).

The term 'opsonin' has been used by different authors with different meanings, some applying it only to non-specific heat-labile components of normal serum (Maaløe 1946), others to immune antibody (Boyden 1963), and others to both antibody and complement (Boyden et al 1965). It is now generally taken as being any substance or complex of substances in serum which reacts with foreign particles to render them susceptible to phagocytosis. A great deal of confusion has arisen as to the relative roles of the various substances, due to the multitude of techniques and test materials, not always complementary, which have been employed. Four general statements, however, can be made: firstly; most biological particles are not phagocytosed

in vitro to any great extent unless they are pretreated with serum (Mudd et al 1934, Hirsch & Strauss 1964). Secondly; the opsonic activity of immunised animals is often higher than that of normal animals (Boyden et al 1965). Thirdly; in some cases, the opsonic power of both immune and normal sera is little affected by heat inactivation -: 56°C. for 30 min. (Hirsch & Strauss 1964), and fourthly; sometimes it is either completely eliminated (Hirsch & Strauss 1964), or partially impaired by heat treatment (Howard & Wardlaw 1958, Rowley & Jenkins 1962). Boyden et al (1965), considered it unlikely that the phagocytes themselves could provide the necessary heat-labile components of complement to effect opsonization. It is possible that heat treatment of serum may cause the production of, or unmask, phagocytic inhibitory substances. Inactivation at 56°C. can certainly change bactericidal antibodies (Muschel & Treffers 1956), and may well destroy certain natural and immune antibodies as well as complement (Cohn & Pappenheimer 1949). It also needs considering that the mechanism of opsonization obtained with one type of phagocytic cell need not necessarily apply to other phagocytes nor even to similar cell-types (Vaughan & Boyden 1964).

Patterson & Suszko (1966) and R.A. Nelson (1953), suggested a relationship between immune adherence (IA) and phagocytosis. In primates, IA to erythrocytes (or to platelets in non-primates), may aid the phagocytosis of microrganisms. Nelson (loc cit) considered that the attachment to polymorphs of encapsulated bacteria sensitized with both antibody and complement was analogous to IA, although he did recognise that several different methods of opsonization may exist. Unlike Boyden (1962b), he regarded the quantities of complement components produced and liberated from macrophages, as sufficient to induce some form of attachment of antibody-sensitized organisms, and it is now realised that macrophages are capable of synthesizing at least one complement component - (C3) (Vernon-Roberts 1972). Interestingly,

it has been suggested that the aggregation of primate platelets may also enhance phagocytosis (Mustard & Packham 1970).

The role of complement in phagocytosis, however, still remains controversial - with it in many cases acting as an accessory factor to the normal or immune opsonins present (Bonnin & Schwartz 1954), while some reports indicate it has a negligable or even a depressing effect (Boyden et al 1965). In 1904, Neufeld & Rimpau described sera substances from animals immunized with bacteria which were capable of promoting phagocytosis, and were heat stable at 56°C. They termed these, 'bacteriotropins'. They later indicated that opsonic activity was due to a specific heat-stable component (antibody) and a heat labile, non specific component (like complement). It has been suggested that 'bacteriotropin' may well be antibody (Ward & Enders 1933). The possible role of properdin, or properdin-like substances cannot, however, be entirely ignored as their effects have been implicated in other opsonic systems (Biozzi & Bier 1960, Rowley 1962). Heat-labile components in human sera are necessary for efficient phagocytosis in osteomyelitis infections; in some instances weak heat stable opsonins may be present which require heat-labile serum complement for full effectiveness (Williams et al 1969).

A major part of the host defence against infection is concerned with altering particle surfaces to form adhesive properties. Immune IgG or complement, the latter activated by either the classical or alternate pathways, can increase adhesiveness (Hirsch & Strauss 1964, Huber et al 1968, Mantovani et al 1972). The classical complement pathway may in some instances function to activate the alternate pathway, which in turn may be responsible for opsonization (Diamond et al 1974). IgG may initiate ingestion, whereas complement is largely responsible for adherence (Mantovani 1975), although the complement receptors of activated macrophages can mediate both attachment and ingestion (Bianco et al 1975).

(e) Attachment and Ingestion

Ingestion may be brought about either by certain molecules acting as triggers or by a bonding interaction between particle and phagocyte which enables ingestion to occur (Jones 1975a). A trigger mechanism could involve IgG-antigen complexes and require ATP. Conversely, the other method would involve a passive in or e-vagination of the plasma membrane. Thus IgG molecules which were attached to surface antigen would form a molecular bridge attracting adjacent IgG molecules, causing surface membrane to spread around the antigen. It is probable, however, that a signal which initiates ingestion via one type of receptor is not necessarily transmitted to other mediating receptors (Griffin et al 1975). This suggests the discrete nature and relative specificity of receptor sites (Loor & Roelants 1974, Davies et al 1975).

Whichever method predominates, the formation of a phagocytic vacude requires complete membrane fusion, which presumably requires specific stimuli (Jones 1975a); the ingestion phase itself cannot be affected by glycolytic inhibitors such as iodoacetate or fluoride (Cohn 1970, Karnovsky et al 1970). During adhesion the Fc portion of the IgG molecule attaches to a trypsin-resistant recepter on the macrophage surface (Huber et al 1968), and the ingestion phase is initiated (Berken & Benacerraf 1966, Jones et al 1972). In mycoplasma, bacterial and certain other parasitic infections, the $F(ab^1)_2$ portion of the IgG molecule appears ineffective, although $F(ab^1)_2$ fragments of rabbit anti-mouse IgG can attach to red blood cells but do not promote ingestion (Griffin & Silverstein (1975); and Steinman & Cohn (1972), have indicated that although immune aggregates containing $F(ab^1)_2$ fragments can attach to mouse peritoneal macrophages at $4^{\circ}C.$, this may be non-specific as the receptor site responsible may well be the same as for Fc fragment attachment. The attachment and ingestion of particles via these specific immunoglobulin receptors has been termed

'immunologic phagocytosis' (Rabinovitch 1970). Peptide $F(ab^1)_2$ fragments can also fix on the surface of B lymphocytes (Johnson et al 1975, Kulberg et al 1976).

Lay & Nussenzweig (1969), showed a Ca^{++} - dependent opsonizing effect of IgM on sheep erythrocytes, and IgM may be of importance either with or without complement (Lay & Nussenzweig 1968, Allen & Cook 1970). Obviously, trypsin alteration of the complement receptors may block attachment as binding may be mediated via the C3 receptors (Lay & Nussenzweig 1968, Huber et al 1968). In many instances it appears that IgG alone is relatively ineffectual in promoting attachment, but may be capable of initiating ingestion, although this may not hold for all systems (Rabinovitch 1967). Most particles which attach 'non-immunologically' are ingested, unless phagocytosis is positively inhibited. The ingestion process itself is distinct from that seen in 'immunologic phagocytosis', although occurring in induced phagocytes, it tends not to be blocked by antimacrophage serum (Holland et al 1972).

Attachment without ingestion may occur (Allen et al 1971). Certain particles possess anti-ingestion substances, the removal or alteration of which by e.g. trypsin or chymotrypsin, leads to phagocytosis (Rabinovitch 1970, Jones 1975a). Facultative phagocytosis can occur, whereby cells not normally phagocytic, are able to ingest large particles - again this is typically 'non-immunologic', these cells are often very poor in lysosomes (Ginsburg 1975). This type of phagocytic inducement can be seen in Toxoplasma gondii infections, where a surface environment may be present which can induce spreading attachment and membrane fusion.

Griffin & Silverstein (1975), suggest that there may be special membrane receptors other than those for Fc and C3, which can specifically recognise certain bacterial surface components; thus neutrophils and macrophages, although both possessing these receptors, may respond

differently to the same particle or micro-organism. Under suitable conditions, antibody-binding can initiate various functions including phagocytosis and contact-mediated lysis of antibody-coated target cells (Huber & Holm 1975). Both human monocytes and tissue macrophages possess receptor sites for IgG (Huber & Fudenberg 1968, Huber et al 1969). Experiments employing human red blood cells coated with IgG subclasses have indicated the specificity of IgG1 and IgG3 (Jones et al 1965, Roitt 1972), the activating determinants being localized within the CH_3 domain of the IgG heavy chain (Hay et al 1973). In addition, binding sites for the activated third component of complement, C3, have been detected (Huber et al 1968, Lay & Nussenzweig 1968). Macrophage complement receptors are capable of recognising particle-bound C3b (Griffin et al 1975), and in some instances the C3b-inactivator-cleaved third component - C3d (Wellek et al 1975, Stossel et al 1975). Reynolds et al (1975), however, determined that although alveolar macrophages do possess C3d receptor sites, these do not appear available to macrophages when whole serum is used as the complement source. (See Fig. (33), Section 3.9.1.)

Antigen-IgG complexes can both attach to and be engulfed by macrophages. Van Oss et al (1974), measured the interfacial free energy of the Fc part of IgG and concluded that alone, its surface area has too high a kinetic energy to be phagocytosed. Antigen-antibody complexes, however, with three or more IgG molecules have a lowered kinetic energy. If complement is present only two IgG or perhaps one IgM molecule may be required. Complexing, therefore, probably increases the energy of IgG binding to receptors (Philips-Quagliata et al 1971). Cytophilic IgG antibodies may adhere singly to macrophages as this type of attachment appears to be of a higher specificity and has a higher binding energy (van Oss et al 1974).

Arend & Mannik (1975), showed that macrophages from several species possess receptors for IgG but not for other Ig's, although Lay & Nussenzweig (1969), indicated that mouse macrophages may interact with

mouse 19S IgM-coated red blood cells in the presence of Ca^{++} and the absence of complement (Hoy & Nelson 1969, Lay & Nussenzweig (1969). Interestingly, Rhodes (1973), suggests that guinea pig splenic cells possess a receptor for 7S IgM but not for 19S IgM. Several separate and discretely spaced receptors have been described from macrophage surface membranes; complement, IgG and IgM have been individually identified and there may be separate ones for inert and denatured particles (Lo Buglio et al 1967, Lay & Nussenzweig 1965, 1969). Specific rosette-forming reactions can occur between alveolar macrophages and red cells which appear to require neither antibody nor complement, although a membrane-associated 'receptor-type' mechanism is apparently used (Lalezari et al 1974). In mice, there may be macrophage receptor sites for an a, globulin which is similar to cytophilic antibody (Nelson 1970). Jenkin & Rowley (1961), claimed that the clearance of both bacteria and inert particles depended largely upon serum opsonins and Boyden (1962b), considered that inorganic particles can adsorb opsonins and their uptake is dependent upon coating with specific serum components. Biozzi et al (196)), however, were unable to demonstrate opsonins for carbon, and later studies concerned with the clearance of Salmonella, red cells and carbon particles, indicated that serum opsonins were important in the first two cases only (Lirenman et al 1967, Vernon-Roberts 1972). Attempts to show specific recognition for the phagocytosis of protein molecules have proved inconclusive, and the lack of heat-stable opsonins for colloidal gold has also been recorded (Filkins et al 1966), but non specific factors including natural antibodies may be operative. Differences in modes of uptake may be significant, but has as yet, been little considered (Longstaffe 1975). Different macrophage populations may exhibit selective specificity. Walker (1974), separated oil-induced rabbit peritoneal exudate cells into five density subclasses using Ficoll discontinuous gradients, these subclasses, composed largely of macrophages, differed in their antigen-binding properties and in their ability to form immunogenic RNA species.

Howard & Wardlaw (1958), and Wardlaw & Howard (1959), have shown that the bacteria Staphylococcus aureus, Corynebacterium murium and Streptococcus pyogenes are more successfully phagocytosed in the absence of serum whereas Escherichia coli and Proteus mirabilis are phagocytosed in the presence, but not the absence of sera, and Streptococcus pneumoniae is not phagocytosed in either the presence or absence of sera. The opsonic factor for E. coli in human serum could be partially removed by heat inactivation, absorption with bacteria or with antigen-antibody complexes, suggesting the importance of both naturally occurring antibody and complement. Normal serum factors may also be involved in the phagocytic uptake of Staphylococcus albus in mice and E. coli in rabbits (Benacerraf et al 1959, Cohn 1962). Jenkin & Rowley (1963), found that avirulent strains of Salmonella typhimurium were cleared at a faster rate by mouse phagocytes, than virulent strains. The opsonins involved include both natural and immune antibodies, but phagocytosis itself can not be related to the serum complement titre (Stiffel et al 1964). Complement is important, though not essential for E. coli uptake in mice, indeed immune antibody alone may be just sufficient (Spiegelberg et al 1963).

Ingestion may occur in a variety of ways. If the particle to be phagocytosed is small, then it may sink into the membrane, enclosure being effected by invagination. Carbon particles are often swept by a ruffling movement into small vacuoles which then pass into the cell (Karrer 1961). If the particle is larger, then enclosure is effected by pseudopodal activity surrounding the particle (Vernon-Roberts 1972). If the mass to be phagocytosed is larger still, then a close application between particle and cell occurs, and other cells are often intimately involved (Carr 1973). Small interlocking processes may be extruded or exterior lysozyme secretion may take place (Curran & Clark 1964). It has been observed that when polymorphonuclear granulocytes (PMNG) are engaged in phagocytosis, they tend to clump and both intertwine and adhere to each other (Lockwood & Allison 1966). With particles between 2 - 10μm, normal phagocytosis and process intrusion may occur

(Shirahama et al 1971), and macrophages can protrude processes into damaged hepatic epithelial cells. Particles may also enter macrophages lysosomotropically (Sbarra et al 1962).

Stossel & Hartwig (1975), indicated that the migration of surface membrane around a particle is accomplished by the extension of hyaline pseudopodia, which appear as a local transformation of the ectoplasm lying beneath the plasma membrane. This may be initiated by the presence of divalent cations (Stossel 1973). Particle encirclement causes membrane invagination, which after pseudopodal fusion, forms an inverted vesicle, the phagocytic vacuole or phagosome (Hirsch 1965). During the formation of the vesicle, the digestive phase proper is initiated (Vernon-Roberts 1972), and a variety of cytoplasmic particles fuse with the vacuolar membrane and degranulation occurs (Robineaux & Frederic 1955, Hirsch & Cohn 1960). When antibody, antigen and complement interact, a substance, lysolecithin, may be produced which can be released inside the vacuole and may act on the adjacent membrane and lysosomes, causing consequent enzyme release. Not only are serum opsonins important in promoting ingestion, but they may, perhaps play a role in determining the fate of intracellular bacteria (Rowley 1958, 1962, Mackaness 1960, Jenkin 1963, Jenkin & Rowley 1963, Blanden et al 1966). Macrophages are considered to contain and/or produce several complement components, and it is possible that for complete effectiveness, from membrane-bound C3 to vacuolar lysis, the components from C5 to C9 are required, and that 'lysolecithin' is merely a reflection of full complement activation (Di Luzio & Flemming 1971).

(f) Macrophage Activation

The phagosomes themselves are devoid of hydrolytic enzymes and fuse with small membrane-bound bodies with electron dense cores (Leake & Myrvik 1968, 1970) - the pre-existing primary lysosomes, present in the macrophage cytoplasm. The resulting secondary lysosome or phagolysosome

contains both ingested material and hydrolytic enzymes (North 1966). Further fusion with other very small secondary lysosomes may also occur (Carr 1973). Macrophages display changes in metabolism during ingestion. Dannenberg et al (1963), recognised an initial 'protoplasmic excitation' phase caused directly by particle attachment and ingestion, resulting in macrophage 'activation'. Activated macrophages are capable of ready ingestion, increased mobility and pseudopod formation; they can attach more readily to glass and their lysosomal enzymes are increased in effectiveness. They can also kill ingested micro-organisms at an increased rate,with their kinetics and energy requirements being altered. Phagosome formation itself, is known to be dependent upon ATP production (Oren et al 1963). A second 'protoplasmic adaptation' phase follows, resulting in an enzyme increase (Carr 1973). Hirsch (1965), reported increases in the rates of glucose, lactate and oxygen uptake and in glycogenesis, although this could be largely reflected in the activation of cytoplasmic oxidases, rather than respiration. There is an increased turnover rate for neutral lipids and phospholipids suggesting changes in membrane synthesis (Karnovsky et al 1966), and indeed both oxygen consumption and carbon dioxide production vary, as does the energy needed for the re-synthesis of new cell membrane (Oren et al 1963). It is known that alveolar and peritoneal macrophages differ in their respiratory requirements, deriving energy aerobically and anaerobically respectively (Karnovsky 1962), although functional activity depends upon the integrity of anaerobic respiration (Cohn 1970, Oren et al 1963). After in vitro exposure to bacteria both oxygen uptake and glucose oxidation increase via the hexose monophosphate pathway, due to a particulate NADPH oxidase which is more efficent in activated macrophages (Rossi et al 1975). When cell surface agents such as concanavalin A (Con A) or phospholipase are added, similar effects are noted, although Con A treatment of mouse peritoneal macrophages

inhibits the absorption and ingestion of latex particles (Friend et al 1975). There is an enhancement of hydrogen peroxide production which may, via peroxidative systems, be effective in killing interiorised bacteria (Klebanoff & Haman 1975). Both phagocytosis and macropinocytosis require metabolic energy and are inhibited by cytochalasin B whereas the pinocytotic ingestion of very small particles may require thermal energy only (Allison & Davies 1975). Glycolytic inhibitors capable of blocking bacterial ingestion do not block degredation (Cohn 1963, Carr 1973). Brogan (1966), investigating human polymorphs, distinguished between serum dependent phagocytosis which does not rely on cell glycolysis and serum-independent phagocytosis which does rely on glycolytic energy.

(g) Intracellular Events

After ingestion, micro-organisms may be enzymatically digested, or may multiply within the cell cytoplasm, or antigenic-contact may initiate the production of specific antibodies by other, immunologically-competent cells (Vernon-Roberts 1972). Both antibacterial products and hydrolytic enzymes are carried within lysosomes (Hirsch 1965), bacterial inactivation itself may be largely mediated by acidic conditions within the phagolysosomes (Auzins & Rowley 1962), although this may not be significant (Looke & Rowley 1962). In addition to lysozyme, a low molecular weight (MW) protein effective in hydrolysing acetyl-aminopolysaccharides, lysosomes contain acid phosphatase, lipase, cathepsin, acid ribonudeose, β-glucuronidase, aminopeptidase, succinic dehydrogenase, neuranimidase, hyaluronidase, aryl sulphatase and various esterases and proteases (Vernon-Roberts 1972, Cohn & Wiener 1963). Several specific antibacterial agents are known with concentrations and combinations varying between and within different populations (Cohn 1964). The 'availability' of lysosomal enzymes may be important (Leake & Myrvik 1906, Fauve & Delauney 1966), with the average intracellular survival

rate for most bacteria being about 10 - 15 minutes, although some atrains can more easily resist bactericidal action (Hirsch 1965). Neutrophils possess lysozyme and phagocytin, alveolar macrophages lack phagocytin and it is possible that peritoneal macrophages and eosinophils may lack lysozyme (Cohn & Hirsch 1965), although McClelland et al (1975), consider both macrophages and monocytes (but not lymphocytes), capable of synthesizing lysozyme. It is possible that mononuclear phagocytes may be less efficient at killing than polymorphs (Mackaness 1960), and this may be related to the more numerous examples of intracellular survival in mononuclear cells than in polymorphs, although this need not always hold true (Holmes et al 1966). Macrophage populations may also show a heterogeneity with respect to bactericidal capacity (Mackeness 1960, Rowley 1966, Pavillard & Rowley 1962). In carrier states and chronic infections, micro-organisms may remain viable, localized intracellularly in less active phagocytes.

(h) Response to Infection

The phagocytic response of the host against infection depends on a multitude of factors, such as anatomical situation, opsonins, enzyme efficiency, as well as the type of bacteria - whether or not they can resist digestion and thus be disseminated (Boyden 1962b), and general deficiencies in macrophage structure and metabolism. The effects of host hormones (White & Marshal 1951), and blockade are also important; as is whether or not the phagocytes have been activated; indeed macrophages develop differently in vivo according to the nature of the inducing agent - thus cells can alter in size, motility and in enzyme specificity (Cohn & Benson 1965, Biozzi et al 1955). Chemical depletion (Rich & Mckee 1939) and splenectomy can also cause a lowering of resistance. The inoculative route will affect susceptibility (Dutton 1955). Interference with phagocytosis itself may be purely mechanical, or may be due to adsorption of opsonins (Coombs & Smith 1968),

or certain toxins or leucocidins may have direct toxic effects on the phagocytes (Keppie et al 1963, Morse 1965). The efficiency of the RES can be enhanced or depressed by the administration of non-specific stimuli, thus bacterial endotoxin can induce metaplasia and increase both the ingestive and bactericidal properties of phagocytes (Rowley 1962, 1966), and cortisone can depress activity (Hirsch & Church 1961).

Phagocytosis and pinocytosis are generally considered to be identical processes, differing only in the size of the phagocytic entity and the quantity of ingested liquid; pinocytosis may refer to the uptake of dissolved substances by means other than diffusion or related permeability effects (Lewis 1931, Vernon-Roberts 1972). The generalized term, endocytosis is often used to encompass both terms. Pinocytotic vesicles may migrate within the macrophage cytoplasm, where they are progressively reduced and absorbed (Robineaux & Pinet 1960), or pinocytosed protein may be segregated within lysosomes and digested normally. Allison & Davies (1975) and Jones (1975a), consider phagocytosis to be the attachment and ultimate ingestion of particles larger than 0.1 μm diameter. Smaller particles such as protein and viruses are considered to be ingested pinocytotically. If the vacuole diameters lie between 0.3 - 2 μm, it is termed macropinocytosis, and if between 70 - 100 nm, micropinocytosis (rhopeocytosis)(Allison & Davies 1975). Macropinocytosis may be important in the formation of macrophage lysosomes (Cohn 1968), and may be temperature and energy dependent (Cohn 1966), requiring ATP (Cohn & Parks 1967). Both macro- and micro-pinocytosis may be important in the uptake of antigen (Robineaux & Pinet 1960) , and of antigen-antibody complexes (Sorkin & Boyden 1959).

Parasites can enter host cells by endocytosis, microbe-membrane fusion or by direct penetration of the plasma membrane (Norrby 1971, Jones 1974). The latter mechanism, although uncommon, may be responsible for microsporidial entry, and there have been reports of viral fusion

(Jones 1974). It was at one time considered that Toxoplasma gondii could also traverse the cell membrane (Norrby et al 1968) may be by using secreted enzymes (Norrby 1971), however, it is now considered that entry is effected phagocytically (Pulvertaft et al 1954, Hirsch et al 1974). Indeed toxoplasmas are capable of inducing a phagocytic response in fibroblasts and other normally non-phagocytic cells. Multiplication of the parasites within macrophages can be suppressed in immunized animals. This locally acquired cellular immunity may be triggered immunologically, involving interactions between specific antigen and factors produced from sensitized lymphoid cells (T cells?) (North 1973). The macrophages show enhanced microbicidal activity (Nelson 1972) - become activated - the effects, however, are translated non-specifically (Mackaness 1962). Indeed, it is believed that the phagocytosis of one type of particle may trigger generalised cellular events resulting in indiscrimate phagocytic uptake of other membrane-associated particles (Griffin & Silverstein 1975). Rabinovitch & Gary (1968), found that macrophages which ingested staphylococci could also engulf gluteraldehyde-treated red cells at faster than control rates. It is possible, however, that the stimulus which initiates ingestion is confined to the segment of plasma membrane immediately adjacent to the particle being ingested, thus allowing the retention of a certain degree of local specificity.

Macrophage activation may also, along with humoral factors, be responsible for the control of Leishmania donovani and Leishmania enrietti infections, in mice (Mauel et al 1974). Leishmanoids cannot actively penetrate cells, they are engulfed (Alexander 1976), but they may, like toxoplasmas, be able to induce phagocytosis. Leishmania mexicana does not inhibit secondary lysosome fusion to the parasitophorous vacuole as does T. gondii (Hirsch et al 1974), nor can it traverse the vacuolar membrane like Trypanosoma cruzi. It does, however, appear to inhibit lysosomal hydrolases and its entry into host cells

may be partially prevented by cytophilic antibodies (Alexander 1976).

Non-pathogenic viruses are largely cleared by liver macrophages (Mims 1964), with again serum opsonins perhaps being important. Macrophages are capable of synthesizing interferons - cell proteins which can prevent viral replication, by indirectly acting on protein synthesis (Vernon-Roberts 1972). Interferon may be produced in response to infection, and spontaneously, by cultured macrophages or in response to bacterial endotoxin (Wagner & Smith 1968).

Inorganic particles can be sequestered within macrophages, certain remain enclosed within phagolysosomes and appear to have little effect on phagocyte activity. Some particles such as silica, asbestos, perhaps coal dust, fibre glass etc. can be toxic (Allison et al 1966), perhaps due to lysosomal enzyme release and macrophage rupture. Antigen-antibody responses may also be important and hypersensitivity reactions may occur. Carbon clearance can be affected by variations in binding onto a plasma component (Gabrieli et al 1967), and partially due to the exhaustion of plasma opsonin (Jeunet et al 1969); it is also affected by the rate of leakage between endothelial cells and out of the circulation (Carr 1968). Carbon particles may be trapped by platelet aggregates (Singer et al 1969). Interestingly, vital staining - the concentration of generally non-toxic dyes in macrophage cytoplasm, may differ from simple endocytsis (Tanaka 1961), and involve direct penetration of the cell or vacuolar membranes and subsequent localization within the cytoplasm and mitochondria. Repeated doses of the vital dyes, trypan blue or Evans' blue can cause neoplastic changes in macrophages and lympho-reticular tissues (Vernon-Roberts 1972).

Certain organisms which are capable of evading the normal killing and digestive mechanisms of phagocytic cells (Hirsch 1972), can do so as a result of possessing surface characteristics which can inhibit attachment; viz: the polysaccharide capsules of pneumococci and the

hyaluronic acid and M protein of streptococci (Cohn & Hirsch 1965). Indeed the pathogenicity of pneumococci is directly dependent upon its possession of a type-specific capsule (Enders et al 1936). The opsonic effect of antibody and complement modify its effectiveness. Mycoplasma pulmonis can attach to the macrophage surface, but due to the presence of a cell-wall protein, ingestion can be avoided (Jones et al 1972). This antiphagocytic surface covering can be removed by proteolysis (Jones et al 1972). It does not attach in suspension in vitro, but only in spread, stationary cultures. Surface contact via protein receptors (Huber et al 1968), sialic acid receptors (Sobeslavsky et al 1968), or by divalent cationic bridging (Lay & Nussenzweig 1969), have been recorded. Mycoplasma pneumoniae is not phagocytosed without opsonization, requiring either complement (via either the classical or alternate pathways), or, less effectively, antibody (Bredt 1975, Powell & Clyde 1975). These, and related observations, promulgated the idea that successful phagocytosis almost invariably depended upon the presence of type-specific opsonins (Zinsser et al 1939). The recognition, however, of both non-specific natural antibodies and complement components present in the circulation, together with observed differences between in vivo and in vitro experiments, have indicated the complexities of effective opsonization (Vernon-Roberts 1972).

If Friedländer's bacteria are injected into the lung of a non-immune rat, phagocytosis is demonstrable in the infected alveoli (Sale & Wood 1947). It may not, however, be possible to detect specific antibodies in either the tissues or circulation, although antibody production may begin almost immediately after immunization (Wood 1960). It was thus postulated that some factor is missing in vitro, which permits phagocytosis to occur in the tissues of the host. The non-specific mechanism which allows in vivo phagocytosis in the absence of antibody has been termed 'surface phagocytosis' (Wood et al 1946,

Wood 1960). Surface phagocytic mechanisms have been demonstrated for both monocytes and granulocytes and with group A haemolytic streptococci infections a significant correlation exists between virulence and susceptibility to surface phagocytosis both *in vitro* and *in vivo* (Wood 1960).

If phagocyte-bacteria mixtures are allowed to react on the surface of freshly excised tissues or on various inert surfaces, phagocytosis occurs. The behaviour of the cells can be visualised and the phagocytes can be seen engulfing the encapsulated bacteria by trapping them against the tissue surfaces. It is also probable, that if a dense exudate of phagocytic cells is present, they can use each others' surfaces for bacterial trapping (Lockwood & Allison 1966). Phagocytosis accomplished by this means has been referred to as 'intercellular surface phagocytosis' (Wood 1960). If organisms are caught in the interstices of a fibrin clot, they can be similarly phagocytosed in the absence of antibody (Smith & Wood 1949). This latter method could be of significance in the elimination of organisms and substances which are known to affect the clotting mechanism of the host.

Certain facultative and obligate parasites can remain within endocytic vacuoles during their intracellular existence. They further appear capable of deriving metabolites from host cells. *Mycobacterium tuberculosis* and *T. gondii* can inhibit the process of lysosomal fusion with the vacuole (Armstrong & D'arcy Hart 1971, Hart *et al* 1972, Jones & Hirsch 1972). *Mycobacterium lepraemurium* allows lysosomal fusion, but, perhaps due to the peptidoglycolipid capsular coat (Draper & D'Arcy Hart 1975), destruction is directly resisted (Draper & Rees 1970, Hart *et al* 1972). Some stercorarian trypanosomes can multiply within macrophages, but it is not clear whether they induce vacuolar-membrane lysis or inhibit lysosomal fusion. Certain eimerian stages

may be transported within macrophages (P. Long, pers. comm.). In many cases, parasites may be altered by humoral factors leading to enhanced lysosomal fusion, microbicidal killing and subsequent digestion.

Opsonization and phagocytosis play an important part in protection against malaria (Chow & Krier 1972, Zuckerman et al 1973), and macrophage hyperactivity (activation) occurs (Brown 1971, Brown & Hills 1971), whereby some protection may be afforded against other strains or species of Plasmodium. Opsonic activity increases as the level of parasitaemia decreases and appears to be variant specific in Plasmodium knowlesi infections (Brown et al 1970 a & b), although Cantrell et al (1970a) indicated that recovery of rats from P. berghei-infections was primarily due to acquired immunity effective without phagocytic hyperactivity.

(i) Digestion

Degradation products such as bacterial macromolecules may either be excreted into the medium or utilized by the cell for its own synthetic purposes (Hirsch 1965). Although, Cohn (1963), studying the fate of the bacteria Bacillus subtilis and E. coli in phagocytic cells found no significant incorporation of low MW products from bacteria into the phagocyte structure. The exocytosed end-product of digestion is a large, irregular, electron dense residual body with myelin whorls, phospholipid and maybe ferritin (Carr 1973). Various other indegestible materials and lysosomal enzymes are also expelled (Cohn & Wiener 1963). Macrophages can continuously secrete lysozyme and acid hydrolases (Allison & Davies 1975), and stimulated macrophages also secrete a fibrolysin capable of converting serum plasminogen to plasmin (Unkeless et al 1974). Macrophages from BCG-immune guinea pigs can release a listericidal factor against Listeria monocytogenes (Bast et al 1974). Human leucocytes when digesting staphylococci discharge extracellular histamine and the lysosomal enzymes, muramidase and

cathepsin. They may, occasionally, egest bacteria and discharge the enzymic contents of lysosomes (Crowder et al 1969). Secretion from phagocytic cells may be important in chronic inflammation (Gordon 1975).

1.5.1. Cytophilic Antibody

Cytophilic antibody is the term applied to globulin components of antiserum which become attached to certain cells in such a way that the cells are subsequently capable of specifically adsorbing antigen (Boyden 1964, Nelson & Boyden 1967). In 1960, Boyden & Sorkin described a class of rabbit antibodies which were able to passively sensitize rabbit spleen cells, thus rendering them capable of adsorbing radio-isotope-labelled antigen. The responsible serum antibody appeared to have a strong affinity for both antigen and splenic cells. Macrophage-specific cytophilic antibody activity was subsequently demonstrated in guinea pigs. Treatment of polymorphs or lymphocytes did not confer on them this capacity to adsorb particulate antigen (Boyden 1964, Jones et al 1965) (cf: Fitzpatrick et al 1967). Polymorph sensitization may, however, be effected by levels of cytophilic antibody, perhaps in conjunction with complement or other serum components, which are too low to be detected, but are nevertheless sufficient to initiate phagocytosis (Howard & Benacerraf 1966, Boyden et al 1965). Cytophilic antibodies can attach to the pulmonary and peritoneal macrophages of mice and rabbits and, in addition, to the blood monocytes of guinea pigs (Howard & Benacerraf 1966). No attachment has, as yet, been evidenced using eosinophils, neutrophils, ileal mucosal cells or peritoneal serosal cells (Boyden 1964, Berken & Benacerraf 1966). Aggregated IgG can attach to B-cells (Basten et al, Dickler & Kunkel 1972) and immune complexes may bind to mononuclear cells, perhaps mediating antigen-specific cytotoxicity (Greenberg et al 1973).

Evidence indicates that mouse and guinea-pig macrophage cytophilic antibodies are contained in the slow 7S γ_2 globulin (IgG2) fraction of sera. Cytophilic activity may also reside in the 19S globulin fraction (Nelson & Boyden 1967, Guercio et al 1969), being susceptible to 2-mercaptoethanol treatment (Berken & Benacerraf 1966). Human β_2

micro-globulin may also bind cytophilically to mouse lymphocytes and polymorphs (Anderson et al 1975) and 7S IgM may well be cytophilic for specific macrophages, perhaps implicated in T-B cell collaboration (Feldmann 1972, Feldman et al 1973). Most guinea pig cytophilic antibody is found in association with complement-fixing antibodies (Bloch et al 1963), although there appears to be no relation between these two activities (Nelson & Mildenhall 1968); indeed Boyden & Sorkin (1960), indicated that cytophilic activity was not destroyed by heat-inactivation of C', and Gowland (1968), suggested that when serum with high titres of cytophilic antibody was injected into normal guinea pigs, its activity rapidly declined, whereas that for C' fixation (CF) was maintained for much longer. Berken & Benacerraf (1966), however, indicated that the pepsin-sensitive macrophage attachment site for IgG_2 molecules is the same as that for CF. Nelson & Boyden (1967), suggested that cytophilic activity and CF were not mutually exclusive, but that individual IgG_2 molecules could possess sites for either one or the other function, but not necessarily both. Separate and distinct membrane-receptor sites may be present on guinea pig peritoneal macrophages, capable of recognising specific IgM-C' components (Huber et al 1968, Wellek et al 1975).

The Ig class specificity for attachment remains uncertain. It is not clear whether IgG_2 is functionally homogenous (Berken & Benacerraf 1966, Leslie & Cohen 1974b), or whether there is a cytophilic sub-population (Gowland 1968). IgG_2 and IgM may bind to common or separate receptors, more likely the latter (Lay & Nussenzweig 1969). Attachment of cytophilic antibody to the macrophage surface is via the H-chains of the Fc fragment of the IgG_2 molecule (Berken & Benacerraf 1966, Liew 1971, Askanase & Hayden 1974, Thrasher et al 1975). The site itself, however, is imperfectly understood. Trypsinization does not affect the attachment site of guinea pig macrophage cytophilic antibodies,

whereas it abolishes the activity of spleen cell cytophilic antibody (Sorkin 1964). In mice, the early cytophilic antibodies produced in response to sheep erythrocyte stimulation fail to attach to trypsin-treated macrophages, those however, which develop after repeated antigenic challenge (hyperimmune antibodies), attach equally well to trypsin-treated as to untreated mouse macrophages. The early, and natural antibodies present can be further removed from macrophage surface receptor sites following trypsinization, whereas hyperimmune cytophilic antibodies appear relatively resistant. It has been proposed that there may be two distinct macrophage receptors and two distinct classes of mouse-cytophilic antibody (Nelson & Boyden 1967), with perhaps different functions (Levenson et al 1970).

Differences between guinea pig alveolar macrophage receptor sites for cytophilic anti-sheep red cell antibodies and aged erythrocytes in the absence of antibody have also been noted (Howard & Benacerraf 1966, Vaughan 1965b). The former were resistant to the action of proteolytic enzymes, whereas the latter were susceptible - further suggesting that there are separate and discrete receptors for the binding of antibody and effete red cells (Lalezari et al 1974).

Macrophage receptors for cytophilic antibody are destroyed by agents which specifically react with free-SH groups (iodoacetamide), by oxidising agents and by compounds reacting with free-NH_2 and - SH groups (formalin). They are not generally affected by high concentrations of mercaptoethanol (Howard & Benacerraf 1966, Berken & Benacerraf 1966, Davey & Asherson 1967).

Berken & Benacerraf (1966), have suggested that the macrophage-antibody bond is relatively weak (Leslie & Cohen 1974a), as they required large amounts of IgG_2 to approach site-saturation and they found the rate of cytophilic-antibody loss from the surface receptors into the medium or into the macrophage itself, to be high at 37° C.

Surface IgG-capping on guinea-pig alveolar macrophages has been noted at this temperature (Thrasher et al 1975), indicating that binding of cytophilic antibodies occurs at well-defined sites. When cytophilic antibody has reacted with antigen, its binding strength is considerably increased, perhaps due to allosteric changes in the antibody molecule or stabilizing interactions between adjacent antibodies (Berken & Benacerraf 1966, Thrasher et al 1975).

The role of cytophilic antibodies in delayed-type hypersensitivity reactions appears equivocable (Coombs & Smith 1968, Nelson 1969). Macrophages form a large proportion of the cells found in delayed skin reactions (Nelson & Boyden 1967); in vitro migration can be antigenically inhibited (David et al 1964, David 1970), and the specific in vivo 'macrophage disappearance reaction' appears typical of delayed hypersensitivity states (Nelson & Boyden 1963). Associated cell-bound antibody has not, however, been detected (Boyden 1964). There has also been a general lack of success in the proposed transfer of the hypersensitive state to normal animals by means of cytophilic antibody (Asherson & Zembala 1970). This could, perhaps, be due to competing classical antibodies present in the whole serum of delayed-type hypersensitive animals (Boyden 1957, Nelson & Boyden 1967). Macrophage cytophilic antibodies may, however, be involved in contact sensitivity (Askanase & Hayden 1974) and have been detected following skin grafting (Nelson 1969). Anaphylactic reactions involving macrophage cytophilic antibody appear unlikely (Boyden 1964, Nelson & Boyden 1967, Coombs & Smith 1968).

The precise function, therefore, of cytophilic antibodies remains unclear. Although they may be involved in delayed-type hypersensitivity, their main function appears to be with specifically enhancing the contact and engulfment phase of phagocytosis by macrophages, perhaps most importantly in situations where free serum opsonins are not

readily available, such as tissue spaces and on serous and mucous surfaces (Coombs & Smith 1968). Berken & Benacerraf (1966), indicated that the selective absorption of cytophilic antibodies could enhance the normal phagocytic potential of the macrophage. Indeed Rowley et al (1964), consider that the cellular immunity expressed against Salmonella typhimurium in mice can be solely explained by envisaging a 19S cytophilic antibody passively allergizing cells.

1.5.2. Passive Sensitization

There exist several recognized mechanisms whereby lymphoid cells can acquire antibody on their surfaces (Uhr 1965). Mäkela & Nossal (1961) showed that some lymphoid cells from rats immunized with Salmonella flagella could show specific adherence of Salmonella to their surfaces. They suggested the adherence represented an antigen-antibody reaction between the bacterial surface antigen and cell-bound antibody formed by the plasma cell itself. Macrophages, lymphocytes and certain other plasma cells can acquire 'cytophilic' antibodies on their surface (Boyden & Sorkin 1960, Boyden 1964). This activity has been demonstrated in the serum of guinea pigs which had been previously injected with sheep red cells mixed with Freund's Complete Adjuvant. Sheep erythrocytes could adhere, in the absence of serum, to normal guinea pig macrophages which had been treated in vitro with such serum (Boyden 1964). Reagin and reagin-like antibodies can also passively allergise or sensitise certain cells - most notably basophils and mast cells - although others may be involved. Indeed Fitzpatrick et al (1967), consider that reaginic antibody can act cytophilically for not only blood basophils but also for polymorphonuclear leucocytes. Uhr (1965) described a further method of sensitization, whereby antigen-antibody complexes were able to passively sensitize lymphocytes, plasma cells and macrophages, such that these cells could bind specific antigen to their surface.

Macrophages, lymphocytes and a variety of other guinea pig cells were passively sensitized with antigen-antibody complexes made up of Salmonella paratyphi B flagella and specific antisera. Adherence-positivity was detected by the addition of motile S. paratyphi B or S. adelaide. This form of passive sensitization, by antigen-antibody complexes, was considered to be dissimilar to surface sensitization of plasma cells following immunization, and to cytophilic antibody sensitization (Uhr 1965).

1.6. Chemotaxis

Chemotaxis is the determined directional response of a cell or organism towards chemical substances in the environment. It is said to be positive if the cell moves towards higher concentrations of such substances; negative, if it moves away (Dixon & McCutcheon 1936, McCutcheon 1946). Chemotaxis is manifested not by velocity of movement, but by its direction (Ramsey 1972). It is, therefore, possible for a cell to move as rapidly in a field lacking any known chemotactic substances as when it is responding to a chemostactic stimulus. Chemotactic agents are themselves capable of enhancing random migration (Keller & Sorkin 1966, 1968), with stimulation effecting both increased locomotion and changes in direction. Cells may well respond to repeated stimuli and consequently the migration direction can be reversed (Cornely 1966, Ramsey 1972). Thus cell re-orientation may be possible (Keller & Sorkin 1968).

Clark et al (1936), observed that macrophages could migrate towards areas of injury and Jacoby (1937 - quoted in Wilkinson 1974b), noticed macrophage-congregation around damaged cells. The migration of phagocytes into sites of infection is an essential feature of the tissue response in various disease and damaged states (Harris 1960, Symon et al 1972), and, although early work indicated the importance of this feature, it was not until fairly recently that any knowledge of the actual factors which elicit such attraction was determined. The various effector systems present within an acute inflammatory response have been shown to be inter-dependent. Particle-phagocytosis has been indicated as causing a decrease in both random and directional cellular migration (Keller et al 1975). Similarly, impaired chemotaxis may be considered as a possible cause of increased susceptibility to infectious diseases (Mowat & Baum 1971, Wilkinson 1974b, Keller et al 1974).

The techniques of Harris (1953), and Boyden (1962a), permitted in vitro identification of chemotactic stimuli and responses (Jungi 1975, Keller et al 1975). Monocytes and granulocytes can react to a number of similar stimuli (Harris 1960); these chemotactic substances: antigen-antibody complexes, bacteria, endotoxins etc. may be effective due to the formation of mediators (Keller & Sorkin 1967). Those which exert a direct stimulating effect on cells have been termed cytotaxins and those which induce the formation of cytotaxins, cytotaxigens. Certain cytotaxins have been identified which are specific for one cell-type only (Keller & Sorkin 1967, Wilkinson et al 1969). Hence it has been proposed that those substances such as casein, capable of attracting both polymorphs and macrophages may possess two or more specific cytotaxins (Sorkin et al 1972).

Of the cytotaxins, there exist endogenous substances such as may be present in normal and immune sera, the split products of C3 and C5 (C3a and C5a), $C\overline{567}$ (Ward 1967), and those in exudate fluids and post-granular supernatants from neutrophils. The exogenous components include bacterial culture filtrates, peptone, casein and certain lipids (Turner et al 1975). The cytotaxigens, which may either stimulate the production of, or unmask, heat stable (56°C.) cytotaxins (Boyden 1962a), include macrophage and neutrophil granules, plasmin, plasminogen and human kallikrein (Gallin & Caplan 1974), trypsin, C3 convertase, and various exogenous substances such as antigen-antibody complexes (Boyden 1962a), endotoxin, aggregated γ globulin (Keller & Sorkin 1965b), tuberculin and zymosan (Cornely 1966). These cytotaxigens often require prior incubation in normal sera, plasma or with heat-labile C' components, before chemotactic mediators may be generated (Keller & Sorkin 1965a). A comprehensive catalogue of chemotactic factors, both cytotaxins and

cytotaxigens, specific for neutrophils, eosinophils, macrophages, basophils and lymphocytes, has been compiled by Wilkinson (1974b).

The hypothesis has been presented, that cell-specific cytotaxins can cause, within certain limits, the accumulation of varying proportions of different cell types in different types of lesions and at different stages within a given lesion (Keller & Sorkin 1968). Directional attraction coupled with leucocyte-trapping, resulting from negative gradients, cytotaxin-induced immobilization or inhibition, can provide a fine control on the number and type of cells accumulating at any specific site (Keller & Sorkin 1966, Becker & Ward 1967, Keller et al 1974). Phagocytosis itself can promote the generation and release of cytotaxins and cytotaxigens as well as specific inhibitors (Borel 1970, Goetzal & Austen 1972). An intimate, functional relationship between chemotaxis and phagocytosis has been recognized for some time (Boyden 1962b, Wilkinson 1974b, Keller et al 1975).

Cytotaxin macromolecules themselves (Wilkinson 1974a), are believed to effect chemotactic recognition by leucocytes, by penetrating the hydrophobic portion of the membrane bilayer, perhaps mediated via reversible conformational changes in the protein molecules (Wilkinson 1973). Directional migration may be a result of leucocyte cation alterations leading to activation of cellular cytoplasmic contractile systems (Wilkinson 1975, Senda et al 1975).

Several cytotaxins have been found in serum (Keller & Sorkin 1967, Taylor & Ward 1967), with perhaps C' components playing the major functional role (Ward et al 1965, Ward 1967, Wagner et al 1974). Antibodies which are deficient at fixing C', such as guinea pig IgG_1, are often less efficient at inducing chemotactic activity than are C'-fixing antibodies (guinea pig IgG_2) (Keller & Sorkin 1968). In addition, alternate pathways may exist for the formation of effective chemotactic substances (Gallin et al 1975) and the kallikrein and

plasmin systems have also been implicated in cytotaxin generation (Taylor & Ward 1967, Kaplan et al 1972, Keller et al 1975). Chemotactic activity has been correlated with the observed interrelationships between blood coagulation, fibrinolysis, the kinin sequence and both C' pathway systems (Keller et al 1975).

Many tissues or tissue products are chemotactic (McCutcheon 1946, Jacoby 1965, Ryan & Hurley 1966), and cytotaxins and/or cytotaxigens may be released from damaged or dying cells (Keller & Sorkin 1968, Keller et al 1975).

In vitro chemotaxis is displayed by granulocytes, monocytes and macrophages. The latter, although exhibiting a more directional response, tend to respond at a slower rate to chemotactic stimuli, under most conditions (Harris 1953). Lymphocytes appear not to readily respond (McCutcheon 1955, Wilkinson 1974b), although this may be a reflection of test procedures (Harris 1953, Keller & Sorkin 1968). A cytotaxin may be released from primed lymphocytes upon contact with antigen (Ward et al 1969, 1970, 1971). The random migration of normal macrophages in vitro may be inhibited by a soluble factor (MIF), produced by antigen-primed lymphocytes from animals exhibiting delayed-type hypersensitivity (David et al 1964). Polymorphs may under certain conditions be capable of releasing in the absence of serum factors, a substance able to stimulate locomotion and exert a chemotactic effect on other polymorphs (Zigmond & Hirsch 1973).

1.7. C-Reactive Protein

C-reactive protein (CRP) is a protein which tends to appear in the blood fluid of man early on in the course of many infections, following inflammation or where tissue damage or necrosis has occurred (Raffel 1961). It was first described by Tillet & Francis (1930), who found that the somatic C-polysaccharide of pneumococcus reacted, in the presence of Ca^{++}, to form a precipitate when added to acute phase sera of individuals suffering from pneumococcal pneumonia. No reaction, however, was elicited with sera obtained from the same patients during convalescence. CRP was also found to fix complement during its reaction with the polysaccharide. Thereafter it was shown that this reaction was not specific but could be noted in the sera of patients with acute rheumatic fever, staphylococcal osteomyelitis and sub-acute bacterial endocarditis (Anderson & McCarty 1950). CRP also appears in Rhesus monkeys infected with pneumococci, but not in rabbits or mice.

Lofstrom (1943), described a substance present in acute phase sera which caused the non-specific swelling of the capsules of certain pneumococcal strains. He considered this substance to be CRP.

Abernethy & Avery (1941), demonstrated that this reactive substance could be inactivated when sera was heated above 65°C. and that it was precipitated from serum by salts, along with the albumin fraction. They suggested it was probably proteinaceous. It was later shown to be a 7.5 S protein with the electrophoretic mobility of a β-globulin. It is not an antibody but a normal plasma protein, usually at concentrations too low to be generally detected (viz: > 0.5mg./100ml.). It has a MW of 200,000, composed of identical subunits each of 21,500 MW.

Hedlund (1947), reported the appearance of CRP in the sera of patients with appendicitis, diseases of the biliary ducts, acute hepatitis, disorders of the urinary tract, myocardial infarction, pulmonary embolism and neoplasms. Although the origin and function of this protein is still

obscure, its appearance does denote the presence of an inflammatory reaction. It forms one of the 'acute-phase proteins' and its estimation is thus useful in determining measurements of 'illness' (Coombs & Gell 1967); and it is now regarded as being a sensitive index of rheumatic fever (Anderson & McCarty 1950). CRP appears very rapidly in the sera of infected animals and develops with the inflammatory reaction, whether pneumococcal or other, persisting only during the activity of that lesion. As a non-specific indicator of inflammation, CRP can be compared with ESR or pyrexia. CRP usually rises, however, before ESR or temperature changes can be detected, and subsides whilst ESR levels remain raised (Kindmark 1972).

In 1969, Ganrot & Kindmark investigated the phagocytosis of the bacteria Gaffkya tetragenaa by isolated human neutrophils in vitro. They found that the addition of pure human CRP considerably enhanced the phagocytic activity of the cells, and further suggested that CRP might be of importance in vivo in the cellular defence against bacteria.

In the blood of rabbits, during acute infection, the acute-phase protein produced in known as Cx-reactive protein (Cx-RP) - (reacting with a pneumococcal carbohydrate (Cx), related to that in man). By immunofluorescent staining, CxRP has been demonstrated in damaged rabbit myocardial tissue. The protein may be a result of local tissue breakdown, or is, more likely, representative of a local accumulation from the bloodstream. Studies based on specific radioactive labelling of amino acids and their incorporation into various proteins indicate that CxRP is made exclusively in the liver. Although Kushner & Kaplan (1961), located the origin of CxRP at the site of inflammatory lesions, where it appeared after a latent period of 8 - 10 hours, and in tissue elements undergoing necrotic change. Blockade of the RES by the intravenous injection of colloidal thorium dioxide (thorotrast), prevents CxRP from appearing - which normally occurs following the injection of pyrogens.

CRP is antigenically complex, possessing several properties. Its capacity to precipitate with polysaccharide can be absorbed out of sera containing it, but still leaving material that precipitates with a CRP antiserum prepared in the rabbit (Hedlund 1959); and its capacity to agglutinate sheep red blood cells coated with Cx-polysaccharide, may be due to another substance present (Fishel et al 1960).

Thomasson et al (1973), examined the levels of CxRP in experimental African trypanosomiasis in rabbits infected with T. congolense. CxRP was detected by testing the rabbit sera with goat anti-human CRP serum. It is known that antihuman CRP cross-reacts strongly with CxRP and does not cause precipitation of rabbit serum components other than CxRP. The results indicated that the CxRP levels rose during infection, becoming detectable early on in the course of the disease, reaching a maximum and then slowly declining as tissue necrosis became more severe.

CRP is therefore an antibody-like substance which initially appeared to be specifically induced by antigens, but whose production was subsequently shown to be dependent upon the species of animal and by a number of non-specific stimuli. Wood (1953), immunized rabbits with human serum proteins and demonstrated the early appearance of CxRP and an association between its titre and that of the specific antibodies subsequently produced. This correlation, however, appears fortuitous, since suppression of the antibody response by steroids does not affect the induction of CRP (Hokama et al 1960).

1.8. Immunity to Trypanosomes

Immune reactions are a sensitive and often ready means for the study of both the behavioural and physiological requirements of parasites in an infected host. The problem of African trypanosomiasis has seen a concentration on immunological investigations, most notably the humoral response of the host to salivarian brucei-subgroup trypanosomes; though recent investigations on the immunity to the Nagana-species, vivax and congolense have proved useful (Vickerman 1974). The antigenic structure of infective species and their inter and intra-specific variability has received a lot of attention, and indeed the idea of immunizing against pathogenic trypanosomes has been prominent throughout the present century (Gray 1976). The problems of resevoir infections, natural immunity in wild stock and the general inability of man and cattle to mount an effective immune response are all problems besetting immunoprophylactic development. The means whereby trypanosomes can evade both the hosts' natural and acquired immunity, either by structural modifications or by direct or indirect damage to the hosts' immune system, and the inter-relationships between these various methods, are paramount in an understanding of trypanosome immunopathology.

(i) Antigenic Structure of Trypanosomes and the Host-Humoral Response

The characteristic feature of salivarian trypanosome infections is the periodic waves of parasitaemia which occur in the host (Franke 1905); each wave succeeding its predecessor at an interval of a few days (Gray 1970, Lumsden 1972, Wilson & Cunningham 1972). These waves are believed to represent the multiplication of populations of different antigenic types, followed by their destruction by host-antibody response. The persistence of the infection is a consequence, therefore, of the ability of the trypanosome population to avoid the immune response, by repeatedly changing its antigenic structure (Lumsden 1972). The problem

of antigenicity is of great importance as the present inability to produce an effective vaccine has been largely attributed to this antigenic variation of the trypanosome (Desowitz 1970, Gray 1975). The literature concerned with both immunity and antigenic variation in African trypanosomiasis has recently been reviewed by de Raadt (1974) and Terry (1976).

Trypanosomes are composed of a large number of complex antigenic components; the challenge to the host is both quantitatively and qualitatively variable with constant alterations in antigen concentration and type, occurring throughout infection. Common or "stable" antigens are situated within the trypanosome, and consist of largely cytoplasmic and nuclear components. They tend to remain common to the same trypanosome species throughout its development (Lumsden 1972) and remain unchanged (de Raadt 1974). They may also be common over a broader spectrum (Schoenaers et al 1953, Gray 1960, Weitz 1970). Common antigens can elicit the formation of antibodies detectable by complement fixation, indirect agglutination, the fluorescent-antibody technique and precipitation. In T. brucei infections several common antigens have been identified, either as nucleoproteins or as having a cytoplasmic origin (Brown 1963, Brown & Williamson 1964). They are released at the crisis of each parasitaemic wave, thus being repeatedly brought into the circulation every few days (de Raadt 1974): they are thence eliminated from the bloodstream either by antibody action or by catabolism - hence belying their term - "stable".

Variant antigens are population specific. The phenomenon of antigenic variation can be seen in T. brucei infections, where different strains can produce different series of variants in infected hosts (Gray 1976). It is also present in T. vivax and T. congolense infections in cattle (Wilson & Cunningham 1972, Jones & Clarkson 1974), although there are some differences between these and the variation produced by

brucei subgroup infections (Vickerman 1974). Variant antigens can elicit protecting, agglutinating, lysing and precipitating antibodies. They are considered to be located on the surface coat of the trypanosome (Vickerman & Luckins 1969, Lumsden 1972, Cross 1973, Brown et al 1973, Vickerman 1974, Njogu et al 1974, Barry & Vickerman 1975); and are believed to be low molecular weight glycoproteins. The carbohydrate part acts as the antigenic determinant while the protein confers chemical heterogeneity (Njogu & Humphryes 1972, Allsopp & Njogu 1974, De Souza 1975). Earlier reports by Williamson & Desowitz (1961) and Desowitz (1970) indicated, however, that trypanosomes are virtually devoid of any polysaccharide material which could show antigenicity and that most antigens are proteinaceous. It is presently believed that the trypanosome surface coat is a secretion product of the cell (de Raadt 1974) and that the surface variant antigens are located in this layer (Vickerman & Luckins 1969). The surface coat of T. brucei can be released from the plasma membrane following cell rupture and appears to be effective in antigenic variation (Cross 1973). Furthermore, trypanosomes can retain their agglutinability with variant specific antisera after they have lost their surface coat - perhaps due to the continued cellular secretion of non-organised antigen. Coat transformation would also expose "common antigens", normally masked by the variant antigens (Barry & Vickerman 1975). There exist several theories which attempt to explain the mechanisms controlling antigenic variation (Watkins 1964, Gray 1965, Desowitz 1970, de Raadt 1974). Due, however, to the regular and predictable appearance of antigen serotypes (Gray 1965, Desowitz 1970), the tendency - in brucei subgroup infections at least - to revert to a basic antigenic type, and the observed large numbers of amino acid substitutions between sequential variant antigens - a theory involving the sequential expression of one of a whole series of antigen determining loci present in each trypanosome genome is currently favoured (Vickerman 1969, Wright & Hales 1970, Cross 1973).

In 1963, Weitz suggested the presence of soluble trypanosomal-antigen products which could be detected in the circulating plasma of infected animals. This 'exoantigen', was found to be precipitating, agglutinating (Desowitz 1970), and protective (de Raadt 1974). Allsopp et al (1971) were able to indicate the general similarity of the dectable 'exoantigen' with the 4S antigens of Williamson & Brown (1964), Njogu & Humphryes 1972). Allsopp et al (loc cit), considered that 'exoantigens' were not actively secreted by the trypanosome, and were composed largely of surface coat material and agglutinogens. Indeed they may be surface coat material released from plasmanemes (Wright et al 1970), which in turn are released from trypanosomes (Vickerman 1974). They could also partially represent free metabolites and common antigens released upon lysis of the trypanosome itself.

A striking feature of the humoral response in African trypanosomiasis is the increase in γ globulin levels and the decrease in serum albumin (Trinaco et al 1953, Mattern 1964). IgG (7S γ globulin) concentrations may rise slowly during infection and fluctuate; indeed Houba et al (1969) were able to find only very slight increases in IgG in T. brucei infections of rhesus monkeys, and actual reports on the amount of γ globulin - especially IgG - present within infections, vary considerably (Desowitz 1970). IgM (19S macroglobulin) levels, however, increase dramatically (Goodwin 1970, Clarkson 1975). The ratio of IgG to IgM has typically been found as 1:3 (McKelvey & Fahey 1965). A proportion of the IgM molecules appear directed against the surface antigens, although a great deal of both IgM and IgG is non-specific (Freeman et al 1970). The IgM increase is a typical early feature of brucei sub-group infections, and it may rise to four times the normal level (Mattern 1964), though it can revert following chemotherapy. The increase has been partially explained by the regular production of variant populations into the circulation, each one of which causes the stimulation and increased production of antibody (de Raadt 1974). As

well as anti-trypanosomal antibodies, the IgM fraction also contains heterophile, anti-immune complex antibodies (Houba & Allinson 1966, Goodwin et al 1972, de Raadt 1974), and anti-tissue autoantibodies (Mackenzie & Boreham 1974a); it may also play a part in B lymphocyte reception and as an effector mechanism in cellulor co-operation (Allinson 1974). Mattern et al (1967), indicated that a proportion of IgM produced during late infection, especially in human trypanosomiasis, was 7S IgM - approximately 5 - 10% (Houba et al 1969) - compared with 19S IgM (Frommel et al 1970). IgM is produced locally within the CNS, with perhaps the morular cells of Mott being responsible for abnormal synthesis (Greenwood & Whittle 1973). IgM responses may also involve the complement system through either the classical or alternate pathways (Vickerman 1974). Nagle et al (1974), have implicated both pathways and have detected IgM, properdin and C3 in T. rhodesiense-infected monkeys with proliferative glomerulonephritis. Preliminary work indicates that the general serum complement levels in human trypanosomiasis appear depressed during the early part of the infection, but are normal later on (Greenwood 1974).

(ii) Cell-Mediated Immunity and Immunosuppression

Lumsden (1970), considered that there was no evidence for cell mediated immunity (CMI) in salivarian trypanosome infections. Nevertheless, Tizard & Soltys (1971a), were able to elicit both Arthus reactions (an humoral response) and delayed-type skin reactions in infected rabbits by the administration of trypanosome antigen. They were further able to demonstrate CMI in normal rabbits which had received splenic cells from infected animals. de Raadt et al (1967), however, were unable to demonstrate delayed-type skin reactions in man; and Mansfield & Kreier (1972), were similarly unable to detect delayed hypersensitivity reactions in rabbits which were skin-tested with sonicated antigen, although they did detect Arthus reactions.

de Raadt (1974), considered that the chancre response to T. rhodesiense was an Arthus phenomenon, rather than CMI. He notes that histopathologically it is often difficult to satisfactorally differentiate between CMI and the Arthus reaction. Aiyedun & Amodu (1973), using T. gambiense infections in rabbits were, like Tizard & Soltys (1971a), able to transfer delayed hypersensitivity from infected to clean animals. Splenic transfer, however, only elicited a response when it was introduced intravenously, not intraperitoneally. Mansfield & Wallace (1974), undertook various investigations - skin tests, lymphocyte stimulation, lymphokine assays etc. - for CMI to mycobacterial protein in T. congolense-infected rabbits, and they concluded that T-cell functions were probably being depressed during trypanosomiasis. Viens et al (1974), working on T. musculi infections in CBA mice considered that a thymus-dependent response could inhibit parasite reproduction, and that thymus-independent antibody could remove newly formed parasites. However, antibody appeared to have little effect on the elimination of parasites from the blood at the end of infection (Targett & Viens 1975). Bungener (1975), indicated, however, that parasitaemia could be terminated by antiserum, but the mouse found difficulty in actually producing and maintaining sufficient antisera in the circulation. Viens et al (1975), suggest that the initial dividing parasites stimulate thymus-dependent ablastin formation and thymus independent (B cells alone) cells which produce trypanocidal antibody and/or activate macrophages. After the first crisis and the typical stable plateau phase has been reached, T cells appear to be sensitized by the adult trypanosomes, which are removed by activated macrophages.

Greenwood et al (1973), investigating immunosuppression in Gambian trypanosomiasis, found that both the induction and expression of cellular immunity were impaired, whereas the humoral responses appeared less affected. Goodwin et al (1972), found that the immune

response of T. brucei-infected mice and rabbits was diminished when challenged with sheep erythrocytes. Similar findings were made by Freeman et al (1974). Urquhart et al (1973), infected rats with T. brucei followed by Nippostrongylus brasiliensis. They found that the normal immune response to the nematode seemed impaired, and that there was a delay in expulsion. They also found that doubly infected rats failed to produce reaginic antibody and there was no increase in the number of intestinal mast cells. There was little change in the CMI response to oxazolone. Murray et al (1974a; b), investigated the role of macrophages and T and B lymphocytes on the nature of immunosuppression in T. brucei infections of mice. They noted the general expansion of the MPS and an increase in activity. The immunogenic potential of macrophages appeared unaltered. They concluded that the only evidence relating the MPS to immunosuppression was an increased hepatic uptake of particulate antigen and a possible selective failure of splenic uptake. This might have an effect on spleen cell production of antibody. The lymphocyte-response was, however, decreased; with immunosuppression being closely associated with the presence of living trypanosomes (Jennings et al 1974), possibly mediated through a B-cell defect. Various theories have been proposed to explain immunosuppression (Goodwin 1970, Urquart et al 1973, Terry et al 1973), not all of which have been purely immunological (de Raadt 1974). Murray et al (1974b), found a gross alteration in B-lymphocyte histology and suggested a defect in the B-cell population; a change in T-cell function was not precluded but an early CMI response to oxazolone could be initiated in infected mice (Urquart et al 1973, Longstaffe 1974). T-cell failure might ultimately occur in the terminal stages of the disease. They proposed that trypanosomiasis causes the elaboration of a substance which aspecifically stimulates B-lymphocyte multiplication, preventing their participation in immune responses. Terry et al (1973), suggested that

the heightened humoral response - notably the increase in IgM - coupled with immunosuppressive activity, could be due to defective T-cells not co-operating in thymus-dependent processes and also failing to regulate B-cell immunotransformation. Antigen-sensitive B cells could be committed to antibody production, but clonal development and the genetration of memory cells could be inhibited (Hudson et al 1975). Vitetta & Uhr (1975), have indicated the presence of IgD - as a major class of cell surface immunoglobulin on the peripheral lymphocytes of adult mice. Both IgM and IgD are often bound together and if, as seems possible, receptor IgD is concerned with determining pathway differentiation (Uhr 1975), immunosuppression may be a reflection of immunocompetent cells failing to possess one or other of these receptors. Thus small lymphocytes with both IgM and IgD may be required through T-cell dependent processes for the generation of IgM plasma cells and IgM memory cells.

Due to the increasing importance of immunosuppression as a contributory factor to the pathogenesis of the disease, this topic is currently receiving more attention (Murray et al 1974c). The presence of 7S IgM during trypanosome infections and its known affinity for B lymphocytes in leukaemic infections (Natvig et al 1975), as well as the inter-relationships between surface bound IgM, IgD and IgG indicate the importance of further investigations concerned with the production and effect of immunoglobulins (Marchalonis et al 1975, Uhr 1975).

Immune complexes have been implicated in trypanosome infections and both complement and immunoglobulin receptor sites have been detected on T and B lymphocytes (Sobel & Bokisch 1975). Membrane-bound C3 and C4 can cause ultrastructural changes in the surface layers of cultured T. cruzi (Anziano et al 1972). Investigations, therefore, on the detection of immune complexes in blood and tissue, and on the identification of their components (de Raadt 1974), related to a consideration of their activity within the context of the immune system as a whole, could prove useful (Gottlieb & Waldman 1972).

Macrophages could be of importance in not only cytophilic and opsonophagocytosis, but also in specific and non-specific activation (Keller 1975), either through antigen-sensitized lymphocytes or directly via trypanosome antigens. Their possible role in contact cytotoxicity and in antigen-handling could also be affected during trypanosome infections.

1.9. Pathology of Trypanosome Infections

Several substantial reviews have been made concerning the pathology of African trypanosomiasis (Ormerod 1970, Losos & Ikede 1972, Goodwin 1974); the following account deals with the general pathology of rabbits infected with T. brucei, with special emphasis on cellular involvement.

The blood parasitaemia of infected rabbits remains low with the course of infection usually running from between 4 and 8 weeks, occasionally longer, with infrequent and late asymptomatic cases occurring (Losos & Ikede 1972).

The first signs of infection, usually apparent between the second and fourth week, are oedematous and erythematous involvement of the ears, eyelids, external nares and scrotum. This is followed by exudative dermatitis and skin lesions accompanied by mucopurulent rhinitis and conjunctivitis; with recurrent keratitis and ophthalmitis being common. This progressive necrosis usually occurs after about 4 weeks. The rabbit shows muscular wastage, emaciation and finally dies (Goodwin & Hook 1968). There is apparently little evidence of CNS involvement.

Boreham (1968a), describing the presence of kinins in T. brucei-infected rabbits, suggested they were released as a result of immune complex formation and could be responsible for alterations in vascular permeability leading to the typical oedematous symptoms. Goodwin (1971), Goodwin & Hook (1968) and Goodwin et al (1973), have demonstrated vascular lesions which develop during trypanosomiasis, with the damage first appearing shortly after the allergic release of kinins and other pharmacological substances. The main arteries in the ears of experimentally-infected rabbits become irregularly constricted through autonomic nervous mediation, resulting in anoxia and tissue damage. Congestion of venous drainage from muscles and the viscera may also occur.

Mononuclear cells can adhere to the vascular endothelium, often as a result of trypanosome damage (Goodwin 1971, Losos & Ikede 1972) and the resulting obstructions may seriously modify blood flow, blocking the vessel, causing tissue anoxia (see Section 1.10).

Both autoagglutination and anaemia, associated with increases in plasma volume (Boreham 1968b), are common in infections (Zuckerman 1964). Boycott & Price-Jones (1912), observed a reduction in red and white blood cells (with a relative decrease in granulocytes and an increase in mononuclear cells (Ormerod 1970)), and of haemoglobin. They considered that red cell destruction occurred in the lymph nodes and to some extent in the bone marrow. Reticulocytosis, as well as polychromasia and normoblast development are typical responses to infection (Boreham 1968b, Losos & Ikede 1972).

Jenkins et al (1974), consider that the observed anaemic partially involves a microangiopathic haemolytic mechanism. Plasma plasminogen levels decrease and fibrinogen degradation products, which can effect haemostatic processes, increase (Boreham & Facer 1974), causing micro-circulatory insufficiency. Increased phagocytosis of normal erythrocytes occurs, especially in the spleen. Erythrophagocytosis may be an immunological phenomenum, with the red cells being coated with complement components (Woodruff et al 1973), immune complexes or antigen (Herbert & Inglis 1973). Autoimmune processes may also be involved (Mackenzie 1973, Facer 1974).

Serological alterations during infection involve marked increases in Ig, serum lipid and cholesterol and a fall in albumin (Goodwin & Guy 1973, Facer 1974, Diehl & Risby 1974). As the infection continues then blood and tissue-fluid pyruvate levels increase (Goodwin & Guy 1973). Fibroblasts become filled with lipid droplets and cease to manufacture collagen. If, as is suggested, the lipid accumulation is due to the high pyruvate concentrations, then degenerative connective tissue changes

could be directly mediated by parasite metabolism. Renal insufficiency also occurs and urea and creatinine levels are raised (Goodwin et al 1973); death in rabbits may be partially due to renal failure mediated by immune complexes (Goodwin & Guy 1973, Itazi & Enyaru 1973, Nagle et al 1974).

Early investigations indicated that brucei sub group trypanosomes localized outside blood vessels, in tissues and associated inflammatory reactions, as well as in the plasma (Goodwin 1974). Generally, trypanosome distribution depends upon the duration of disease (Losos & Ikede 1972), such that in severely acute infections, as seen in laboratory rats or mice, little tissue invasion occurs, massive numbers of trypanosomes being found in the blood (Losos & Ikede 1972). In chronic forms of the disease, solid tissue invasion occurs and the trypanosomes present in the blood may have very little to do with the disease process itself (Durham 1908, Goodwin 1971).

The spleen and lymph nodes of infected rabbits become enlarged (Hu 1931, Ormerod 1970), although this is not as pronounced as in mice, rats and guinea pigs. The observed splenomegaly is probably largely due to an increase in active phagocytic activity, Ig-synthesis and extra-medullary erythropoiesis (Facer 1974). There is an associated increase in the numbers of plasma cells, macrophages and large lymphoblasts and a corresponding reduction in small lymphocytes (Hu 1931). Macrophages found in cutaneous lesions are often derived from splenic tissue (Goodwin 1970).

Intercellular invasion and multiplication by trypanosomes is often accompanied by endothelial hypertrophy and hyperplasia. Necrosis of the parenchymous tissues of the myocardial and skeletal muscle fibres takes place, perhaps affected by pyruvate accumulation (Goodwin 1970) and is accompanied by the infiltration of fluid exudates, monocytes and polymorphs (Losos & Ikede 1970, Ormerod 1970).

1.10. Phagocytosis and Trypanosomes

The phagocytic activity expressed in non-pathogenic infections of rodents and in African trypanosome infections possess certain similarities. In both instances, phagocytic cells appear to play an active role in regulating and partially controlling the pathological process and may initially contribute towards the effectiveness of host humoral antibody response. The role of macrophages, in particular, differs from that seen in obligate intracellular parasitism as exemplified by Leishmania spp. and T. cruzi (see Section 1.4.). It is intended, therefore, to consider the literature concerned with the phagocytic activity in T. lewisi infections and to compare and contrast this with the largely neglected area of phagocytosis in African trypanosomiasis.

There have been a number of reports concerning the interaction between trypanosomes and the phagocytic cells of rabbits and rodents. Much of the early information is mainly observational, most of the experimental work having, until recently, been performed on T. lewisi infections in rats. There has been little or no direct observations on natural infections of African trypanosomes (see Losos & Ikede 1972), and little attempt to actively investigate the processes involved.

Early observations indicated that the phagocytosis of trypanosomes could occur in circulating leucocytes - predominately the large mononuclear cells (monocytes), rather than the polymorphonuclear leucocytes - and in fixed-tissue macrophages; and this, it was considered, could be an important factor in the resistance of the host (Taliaferro 1930). Laveran & Mesnil (1901) observed the phagocytosis of T. lewisi by the peritoneal cells of rats which had been both passively and actively immunized; and Levaditi & Sevin (1905), noted the process in animals naturally immune to T. paddae (T. brucei).

Mesnil & Brimont (1909), recorded the rapid phagocytosis of T. brucei injected into the peritoneum of mice and suggested that immune sera might

be of importance. Early *in vitro* work by Levaditi & Mutermilch (1910) similarly confirmed the effect of immune serum. These workers were further able to divide the phagocytic process into two distinct phases - firstly attachment, which they regarded as a simple physico-chemical phenomenon, occuring between serum-sensitized trypanosomes and randomly-encountered leucocytes; and secondly, incorporation, which, unlike attachment appeared to be a vital phenomenon, affected by damage to the phagocyte (Raffel 1961, Wilkinson 1974a). The delineation of these two processes - attachment and ingestion - has recently been re-considered by Jones *et al* (1972) in studies concerned with the *in vitro* factors capable of influencing the attachment and ingestion of *Mycoplasma pulmonis* by mouse macrophages.

Mesnil & Brimont (1909), also observed that *T. brucei* were often attached to mouse macrophages by their posterior, non-flagellated end. Similar findings were reported by Kloetzal & Deane (1970) on *T. lewisi* in rats. Takayanagi *et al* (1974a), working with *T. gambiense* and rat macrophages, however, indicated that attachment tended to be via the anterior, flagellated part of the parasite. They moreover suggested that the free flagellum contained certain agglutinins they considered responsible for phagocytosis (Takayanagi *et al* 1973a).

Mesnil & Brimont (1909), upon injecting *T. brucei* and immune sera into the peritoneal cavities of mice, noted that substantial phagocytic activity occurred between seven and ten minutes after inoculation, and that maximum activity was recorded between ten and thirty minutes. By this time (30 min.), approximately 50% of the phagocytic cells contained endocytosed parasites. Levaditi & Mutermilch (1910), suggested that the whole process of *in vitro* phagocytosis of *T. brucei* by guinea pig exudate cells took approximately five minutes. Takayanagi *et al* (1974b), again using an *in vitro* system found that cultured rat macrophages could firmly attach motile *T. gambiense* after about five minutes, but that

ingestion took between thirty and sixty minutes. Obvious differences can exist between *in vivo* and *in vitro* situations (Whitby & Rowley 1959) and it is necessary to distinguish carefully between attachement and ingestion as these are, in many instances, mediated by different serum components (Mantovani 1975).

Laveran & Mesnil (1901), Roudsky (1911) and Delanoë (1912), all considered phagocytosis to be an essential mechanism in ridding the host of *T. lewisi* infection. Delanoë (*loc cit*), using a susceptible strain of mice which could develop an acquired immunity, emphasized that phagocytic activity in infected animals, occurred throughout the body, and was not merely localized in the peritoneal cavity (Mesnil & Brimont 1909). Taliaferro (1930) maintained that the lytic antibodies produced in *T. lewisi* infections effected the major trypanocidal action and that both lysis and phagocytosis should be considered as expressions of the same mechanism. Regendanz (1932) believed that the reproduction-inhibiting factor- ablastin (Taliaferro 1932) - which is produced in *T. lewisi* infections, was the only acquired humoral antibody (see Coventry 1930), and that after its production, a generalized non-specific phagocytosis could account for the eventual removal of all parasites.

Sauerbeck (1905), when studying *T. brucei* infections- with its markedly different immunological and pathological picture - in rats, guinea pigs, rabbits and dogs, maintained that the chief pathological picture of the disease was the stimulation of the macrophage system and that phagocytes could be seen containing trypanosomes in various stages of digestion.

Thereafter little confirmatory work was done on the phagocytic activity in pathogenic infections, although further investigations continued, following Laveran & Mesnil's (1901) initial and Taliaferro's (1924, 1932) and Coventry's (1930) subsequent observations on the

immunopathology of T. lewisi infections. The problem of whether phagocytosis assumed an active role in T. lewisi infections (Laveran & Mesnil loc cit), or whether it merely played a part in the control of the infection, by being operative in clearing lysed particles from the blood stream following the second, infection-terminating crisis (Taliaferro 1924) was not, and indeed is still not, fully resolved.

Lange & Lysenko (1960) indicated that the phagocytosis of T. lewisi by normal rat peritoneal exudative cells could occur in vitro; and they suggested the presence of a heat-labile opsonin in immune serum which could have an enhanced phagocytic effect over that produced by normal rat serum. This "phagocytic enhancing antibody" could be absorbed out of immune serum by living trypanosomes. This observation, therefore, produced an added complication. Taliaferro (1924) had been able to show both an ablastic (reproduction-inhibiting) and a lytic activity; while Laveran & Mesnil (1901) in their early observations of phagocytosis had demonstrated an agglutinating activity; now Lange & Lysenko (1960) - appearing to amplify Laveran & Mesnils' conclusions concerning the importance of phagocytosis - indicated the presence of opsonic activity - the stimulation of phagocytic activity in the presence of antisera.

Patton (1972) confirmed the findings of Lange & Lysenko (1960) in that immune serum enhanced the phagocytosis of T. lewisi by rat peritoneal macrophages. He suggested, through experimental use of dexamethasone-treated animals, that phagocytic cells could both reduce the number of trypanosomes which initiate parasitaemias and also augment the protection given by the antibodies which arise during infection. Passive transfer of hyperimmune serum could elicit protection in normal but not in dexamthasone-treated rats, unless the latter were also supplied with peritoneal exudate cells. The subsequent intraperitoneal inoculation of trypanosomes in such animals resulted in extensive agglutination, phagocytosis and lysis in the peritoneal cavity.

Kloetzal & Deane (1970) were also able to demonstrate opsonic activity in the sera of T. lewisi-infected rats which accorded with the results of Patton (1972). Adherence was partially inhibited by 0.1M EDTA (which can effect the conversion of antibody-complexed C3b to C3d (Bianco et al 1975, Griffin et al 1975)) and it was suggested that the IgM component of serum was of importance in phagocytic activity.

Both Kloetzal & Deane (1970) and Lange & Lysenko (1960), showed that the serum-mediated adherence and phagocytosis of T. lewisi was only effective when immune sera taken at least eight days post infection was used. This accorded with the findings of Meyers & Lysenko (1953), who reported no appearance of serum-agglutinin in T. lewisi infection until about the eighth day - the time of the first crisis (Coventry 1930, D'Alesandro 1970).

Both in vivo and in vitro work on T. lewisi infections indicate, therefore, that several trypanocidal mechanisms can exist - agglutination, lysis and phagocytosis. Taliaferro (1932) suggested that the mode of action may depend upon the trypanocidal-antibody titre - if high, then lysis predominates, if low, then phagocytosis is more important. However, immune sera is needed for effective opsonophagocytosis (Lange & Lysenko 1960, Patton 1972). Although ablastin and the trypanocidal antibodies appear to be of major importance, the effect of phagocytosis has still not been properly assessed. It may play a secondary, subsidiary role, effecting a generalized mopping-up activity, or it may be involved in initial destruction and primary removal of parasites. Taliaferro's (1938) early work on splenectomised and blockaded rats which had been passively immunized and infected gave similar results to that found in the dexamethasone-treated animals of Patton (1972). In both instances, the importance of trypanosome pre-opsonization was emphasized. It could be suggested that severe depression of the "reticulo-endothelial" system may interfere with

in vivo interactions between antigen and antibody, or could affect plasma opsonic activity (Pisano et al 1968).

Several recent reports have been made by Takayanagi and his co-workers on the in vitro phagocytosis of T. gambiense by rat macrophages (Takayanagi et al 1974a, Takayanagi 1974b, Takayanagi & Nakatake 1975). They emphasized the importance of homologous antisera in effecting attachment, and suggested that the agglutinating antibodies produced during infection, were responsible for phagocytosis. By the passive transfer of specific immunoglobulin from infected mice and rats to non-immune recipients, short-term protection could be elicited upon challenge with T. gambiense (Takayanagi et al 1973 a,b, Takayanagi & Enriquez 1973). Accordingly, they suggest that the immunoglobulins facilitated in vivo phagocytosis (Laveran & Mesnil 1901, Lange & Lysenko 1960). By various methods of separation, the antigenic components responsible for agglutination when used for immunization gave antibody capable of enhancing in vitro phagocytosis. They indicated that antibody-agglutinating activity paralleled phagocytic activity and thus, they suggested, the antigenic components and the antibodies involved may well be the same for both activities.

They were later able to block the attachment of T. gambiense to macrophages by the addition of a soluble fraction of homogenised parasites or an insoluble fraction of homogenised macrophages (Takayanagi et al 1975). They suggested that antigen-antibody complexes were developed which preferentially bound to the macrophage surface, thus preventing parasite-attachment. They equated this activity with the presence of agglutinating antigens. This is at variance with the suggested role of Salmonella antigen-antibody complexes in passively sensitizing macrophage surfaces and thus enhancing phagocytosis (Uhr 1965).

Neither the attachment nor ingestion of T. gambiense by rat macrophages was decreased by heat-inactivation of immune sera, nor were

they enhanced by the addition of complement (fresh guinea pig serum) (Takayanagi et al 1974b). The authors further suggest that trypanosome attachment to the macrophage surface differs from the mechanism of immune adherence (Zuckerman 1964). Lange & Lysenko (1960), found a significant decrease in the phagocytic uptake of T. lewisi using heat inactivated immune serum (despite heating to only 46°C. for 30 mins!) Similar, although less clear-cut effects were seen by Kloetzal & Deane (1970).

Most of the in vitro work connected with attachment and phagocytosis of trypanosomes by macrophages has been concerned with the opsonization of trypanosomes by antisera, rather than sensitization of the macrophage surface. Bloodstream forms of T. brucei are rarely phagocytosed in the presence of normal mouse serum, but are actively and rapidly engulfed in the presence of homologous antiserum (Lumsden & Herbert 1967); this may be primarily due to surface opsonization. Interestingly, culture forms of trypanosomes which lack the surface coat appear readily taken up by macrophages even in the absence of immune sera (Vickerman 1974).

The possible effects of macrophage cytophilic antibody activity (Boyden & Sorkin 1960), in T. brucei infections was first considered by Tizard & Soltys (1971b) in rabbits. They detected cytophilic antibodies in the sera of rabbits infected with a mouse-adapted strain of T. brucei (Shinyanga III), which reached a maximum titre one week past infection, and thereafter steadily declined. The presence of cytophilic activity was associated with high titres of agglutinating antibody. The antibody concerned, however, did not appear to be IgM, nor was complement required for its activity; it seemed specific for macrophages. Vasquez et al (1975), have detected cytophilic antibodies in T. brucei-infected mice. They appeared very early on in the course of infection and were directed against 'stable' trypanosome antigens. (They were also directed against the surface antigens of culture-form trypanosomes.)

A number of recent *in vivo* observations on the activity of phagocytic cells in pathological processes have been made (Goodwin & Hook 1968, Goodwin 1971). In a study of the effect of *T. brucei* on blood vessels in rabbits, using the regenerative ear-chamber technique, Goodwin (1971), demonstrated the presence of mononuclear phagocytes on the endothelium of damaged venous channels which, in many instances, caused obstruction and blockage. He noted the general concentration of trypanosomes in the tissue spaces and further observed the trapping and digestion of some trypanosomes by phagocytic cells. The large mononuclear cells appeared to play an important part in the pathology of the infection, both by immobilising and removing parasites from the tissue spaces and also by adhering to the walls of small blood vessels, thereby contributing to occlusion, stasis and thrombosis. In a later study of the effects of *T. brucei* infections on connective tissue growth in rabbits, Goodwin *et al* (1973), showed that tissue, which formed around implanted plastic tissue cages, contained areas rich in both parasites and mononuclear cells.

The number of leucocytes present in infected animals may show a cyclical rise and fall, similar to the periodic nature of parasitaemia and associated with the different relapse populations produced during infection. During parasitaemic remission the phagocytic cells appear vacuolated, significantly larger and full of débris (Goodwin 1970). This débris probably consists of both partially-digested trypanosomes or complexes and damaged autochthonous tissue, including erythrocytes, which have been rendered immunologically recognizable.

Goodwin (1970), indicated that the initial changes in tissue physiology and vasculitis in rabbits infected with chronic *T. brucei*, first became obvious around the 14th day of infection. By this time the extravascular tissue was heavily infiltrated with mononuclear cells and endothelium-lining phagocytes had collected. Increased phagocytic

activity was associated with the production of each successive antigenic variant, and this in turn with the production of quantities of largely non-specific immunoglobulin.

In the spleen, bone marrow and lymph nodes of rabbits, extravascular leishmanoid-like forms have been observed both phagocytosed and agglutinated between cells. Inflammatory changes in the CNS have been associated with the perivascular accumulation of mononuclear cells, trypanosomes and occassionally cells of Mott. Endothelial hypertrophy and hyperplasia was commonly accompanied by mononuclear infiltration and necrotized myocardial and skeletal muscle fibres were associated with the presence of inflammatory exudate fluid and occassional polymorphs. The mononuclear cell response and the typical tissue changes led Losos & Ikede (1972), to suggest the possible involvement of a cell mediated immunologic mechanism.

Murray et al (1974d), investigating the pathology of rats chronically infected with T. brucei noted a similar picture. There was an overall increase in the MPS of the liver, spleen and lymph nodes, and sinuses tended to be packed with migrating macrophages. The spleen itself contained large numbers of both red cells and what appeared to be ingested trypanosomes. Typically, the lymphoid system was grossly depleted, a feature common in trypanosome infections. A large number of activated macrophages, showing increased non specific and erythrophagocytic activities, characteristically accumulated at inflammatory and infective sites. The microcirculation was impaired, small channels being blocked, affecting thrombus formation and leading to an increased vascular permeability.

Fiennes (1970), examined chronic trypanosomiasis in cattle infected with T. congolense, and similarly considered that the most characteristic pathological change was the increased involvement of the 'lymphoid-macrophage' system. The endothelium of blood vessels was hypertrophied

and denuded due to the intense mobilization of macrophages and thromboses were common. Fiennes (loc cit), also indicated that although polymorphs and monocytes were active in attacking both intact and disintegrated trypanosomes, they required the presence of humoral opsonins.

The fixed macrophage has been implicated in various "antigen-handling" properties in trypanosome infections (Longstaffe 1975), and the mononuclear phagocyte system has been investigated with regard to its possible significance in immunosuppression in T. brucei infections in mice (Murray et al 1974, a,b,c). There have, however, been few reports on the phagocytosis of either brucei sub group trypanosomes or of the other pathologically-important trypanosomes, T. vivax and T. congolense (Fiennes 1946); a similar paucity of information exists concerning the other species of Trypanozoon, T. evansi and T. equiperdum (Kuhn 1938). Some indications have been made concerning the antigenically variable surface coat in T. brucei, and its role in mediating antibody attachment, (Takayanagi et al 1974a, Vickerman 1974). Similarities could exist in T. vivax and T. congolense infections, both of which show a continuous and ordered antigenic variation (Wilson & Cunningham 1972, Jones & Clarkson 1974). The possibility of antiphagocytic surface activity - akin to the situation in encapsulated pneumococcal infections in mice (White 1968), hyaluronic acid encapsulation in Group A streptococci (Hirsch & Strauss 1963, Baughn & Bonventre 1975), or as found in cell-wall defective bacteria (Koller et al 1975), cannot be overlooked.

Little delineation has been made between opsonic and macrophage cytophilic activity in either T. lewisi or T. brucei-sub group infections. In both cases, however, serum antibodies appear important in effecting cytophilic or opsonophagocytosis, but little clear indication as to specificity or immunoglobulin class - whether IgG or 7S or 19S IgM

(Frommel et al 1970), or of the role of complement components in immune-adherence reactions (Nelson 1953), has been made. Opsonins may also be of importance in initiating not only phagocytosis but also subsequent intracellular killing (Rowley 1962, Sethi & Pelster 1973).

PART 2. MATERIALS AND METHODS

2.1. Animals

Male, outbred T.O. mice weighing 18 - 25g. were obtained from Animal Suppliers Ltd., Hertfordshire, and male CD-1 strain mice from Charles River U.K. Ltd., Kent.

Inbred, CBA mice of both sexes were originally obtained from Animal Suppliers Ltd., Hertfordshire, and subsequently bred in the laboratory.

Male, outbred albino CD rats weighing 200 - 220g. were obtained from Charles River U.K. Ltd., Kent.

Male, New Zealand White rabbits weighing 2.5 - 3.0kg. were obtained from 'Morton Commercial Rabbits', Parsonnage Farm, Essex.

All animals were fed on a standard laboratory diet with water freely available. The rabbits were kept for at least seven days before use.

2.2. Trypanosomes

(a) Trypanosoma brucei 427

Strain 427 was isolated from a sheep in South East Uganda in 1960. It was initially passaged in mice and has since been frozen and maintained in both mice and rats.

(b) Trypanosoma brucei S42

Strain S42 was isolated from a female warthog in Tanzania in 1966. It has been frozen and syringe-passaged in mice, rats and chimpanzees; and cyclically transmitted through mice and Glossina morsitans.

Both laboratory strains were originally obtained from the Lister Institute of Preventive Medicine, London and stabilates cryopreserved in liquid nitrogen at Silwood Park, Ascot (Lumsden & Herbert 1973). Maintainance was by the syringe-passage of infected blood through albino T.O. mice every two or three days.

(c) Trypanosoma brucei Etat 4 (cloned strain)

Obtained by the isolation of a single organism from a relapse population of an infection initiated by T. brucei TREU 164. (Allsopp et al 1971, Lumsden & Herbert 1975). Since passaged in mice.

Stabilate infection was obtained courtesy of Prof. W.H.R. Lumsden, London School of Hygene and Tropical Medicine. Preserved in liquid nitrogen at Silwood Park.

2.3. Infections

(a) Mice and Rats

Mice and rats were infected by intraperitoneal inoculation of trypanosomes in whole blood, or from diluted whole stabilate. T. brucei 427 and T. brucei Etat 4 produced acute infections, death resulting within a few days. T. brucei S42 ran a more chronic course, capable of lasting several weeks.

(b) Rabbits

Rabbits were infected with T. brucei 427 by intravenous, intramuscular or subcutaneous injections of between 1.0×10^7 - 5.0×10^8 organisms. Infected, whole blood from highly parasitaemic mice or rats, or, more usually, trypanosomes which had been previously separated from blood components on a cellulose column and washed in buffer, were used (see Section 2.5.).

One rabbit was subcutaneously inoculated with 8.3×10^7 trypanosomes, T. brucei Etat 4, in whole mouse blood.

Both strains produced chronic infections, usually killing between five and eight weeks post infection. Occassional animals, infected with T. brucei 427, became asymptomatic and aparasitaemic after four or five weeks.

2.4. Preparation and Collection of Sera and Plasma

2.4.1. Preparation

(a) Infected Sera

Whole blood obtained from passage mice, infected with T. brucei 427 or S42, was diluted in lithium heparinised (150 IU/ml) tubes, with 0.85% saline, to give the appropriate concentration of trypanosomes. Each of twelve mice was inoculated with 100 trypanosomes of T. brucei 427 and each of thirty six mice with 400 trypanosomes of T. brucei S42. Sera were collected from day 0 and thereafter every 24 or 48 hours, until all the mice had been killed or had died.

Normal, non-infected mouse serum was similarly collected.

Sera from rabbits infected with T. brucei 427 or Etat 4 were collected at weekly or biweekly intervals until the death of the rabbit or terminal parasitaemia had been reached.

Control rabbits were given a sham, subcutaneous inoculation of 1.0ml of 0.85% saline.

(b) Hyperimmune Sera

Hyperimmune sera were prepared by immunizing mice with an intraperitoneal injection of 0.1ml of an equal parts emulsion of separated, dead trypanosomes - T. brucei S42 or 427 (2.0×10^8/ml, which had been previously disrupted by repeated freezing and thawing), and Freund's Complete Adjuvant (F.C.A.) (Difco). The injection was repeated seven days later and after a further seven days, the sera were collected.

Mice were similarly treated with F.C.A. alone.

Hyperimmune rabbit sera were prepared from rabbits intramuscularly inoculated with 2.0ml of an equal volume suspension of dead, disrupted trypanosomes - T. brucei 427, at a concentration of between 2.0×10^8 and 6.0×10^8/ml, and F.C.A. (i) Inoculations made on days 1, 5, 8 and 20. Sera collected 23 days after initial inoculation; (ii) rabbits immunized on successive weeks for five weeks and sera collected on week six.

2.4.2. Collection

(a) Sera

Mice were deeply anaethetised with chloroform and bled by cardiac puncture. Samples of rabbit blood were obtained from the marginal ear vein at intervals throughout infection (Herbert 1973). Blood was allowed to clot at 37°C. for 4 h. Serum was removed by centrifugation at 1500 x g for 10 min. and 0.3ml aliquots were stored at -20°C. until required. All sera were used within six weeks of collection.

All hyperimmune sera were tested for agglutinating antibodies (Cunningham & Vickerman 1962), within seven days of preparation.

Infected and non-infected control rabbit sera were similarly tested. The antigenic type of trypanosome populations used was also determined by this method, employing specific test antisera.

(b) Plasma

Blood samples were taken from CD-1 mice by cardiac puncture. The anticoagulant used was EDTA at a concentration of 2mg/ml of blood. Centrifugation was at 1500 x g for 10 min. and individual 0.5ml aliquots were prepared. Before use, the plasma samples were re-centrifuged at 1500 x g for 30 min. to remove any residual blood platelets.

2.5. Separation of Trypanosomes

Heavily infected mice or rats were deeply anaethetized and bled from the heart three days after infection. Trypanosomes were separated from host blood-cell components by the method of Lanham (1968), on a Whatman's DEAE-cellulose column. Buffered phosphate saline glucose (PSG), pH 8.0, was used as the eluant and separations were carried out in a Buchner Funnel with a No.I sintered-glass support. The lightly centrifuged blood supernatant containing the trypanosomes was layered on the surface of the column with a Pasteur pipette and the eluate collected in a flask. The motile trypanosomes, free from blood cells, were washed three times in PSG and re-suspended to the required concentration.

2.6. Estimation of Parasitaemia

(a) Mice and Rats

Parasitaemia was adjudged subjectively by a wet film examination of whole blood, on a 1+ to 4+ scale.

1+ < 10 trypanosomes per field (x480 magnification)

2+ $\leq$ 10 - 50 " " " "

3+ $\leq$ 50 - 100 " " " "

4+ > 100 " " " "

(b) Rabbits

Parasitaemia was assessed by microscopical examination of a wet film preparation of whole blood. The number of trypanosomes observed in 30 fields at a magnification of x480 was determined.

(c) Trypanosome Counts

Separated, washed trypanosome suspensions were diluted with PSG and counted on an Improved Neubauer haemocytometer, counting as for erythrocytes.

2.7. Tissue Culture of Cells

2.7.1. Extraction and Culture of Peritoneal Cells

(a) Mice

Mouse peritoneal exudate cells were obtained from uninfected CD-1, CBA and infected and hyperimmune CD-1 animals, without prior inducement (Stuart et al 1973).

Mice of a standard weight and age (between 35 - 40 days) were used throughout. Older animals tended to possess a variable macrophage population and a high proportion of mast cells (Stuart et al 1973), whilst younger individuals tended to haemorrhage internally when killed, thus contaminating the cellular peritoneal fluid.

The mice were killed by rapid dislocation of the neck and the fur dampened with alcohol. The ventral skin was pulled back over the abdominal wall. 1.5ml of tissue culture medium, single strength (TCM) (Wellcome Reagents Ltd.), containing penicillin (200 units/ml) and streptomycin (100 µg/ml) was administered intraperitoneally along the mid-ventral line and circulated gently within the peritoneal cavity. Heparin was omitted as earlier results indicated that macrophage vacuolation could occur. High doses of heparin are also known to depress complement activity (Brogan 1966). Both the single strength medium and the concentrate were used. The latter was diluted aseptically with deionised water and the recommended amounts of sterile sodium bicarbonate (Wellcome Reagents Ltd.) added. No antibiotics were used. No differences were observed between the two media, so the ordinary, single-strength medium was usually preferred.

Within 2 min. of injection the clear, straw-coloured peritoneal exudate (approximately 1.0ml) was aspirated and dispersed into a sterile, siliconized graduated cylinder kept on ice. The cells were then diluted with TCM, containing 10% foetal or inactivated calf serum (Wellcome Reagents Ltd.) as a supplement, such that the final concentration of

Plate A.

Plate B.

Plate C.

Plate A. *In vitro* culture of mouse peritoneal macrophages. Materials required.

Plate B. *In vitro* culture of mouse peritoneal macrophages. Injection of TCM along the mid-ventral line.

Plate C. *In vitro* culture of mouse peritoneal macrophages. Extraction of peritoneal exudate fluid.

cells approximated 1.0 x 10^6 / ml (counting on a haemocytometer). Macrophages tended to make up between 40 and 60% of the cell population, the rest being lymphocytes, polymorphs and mast cells (Jaroskova 1974).

One ml of the final solution was added to individual sterile Leighton tubes containing a 9 x 35mm flying glass coverslip. The tubes were stoppered and incubated in air at 37°C. for 24 hours, during which time the macrophages present had settled and attached to the surface of the coverglass. In certain instances, each flying coverslip was divided into two before seeding with the peritoneal suspension, thus enabling direct comparisons to be made between treatments.

In one experiment (see Section 3.1.6), the washed exudate cells from the peritoneal cavity of hyperimmune mice were re-suspended in 5.0ml of cold TCM and gently centrifuged at 500 x g for 4 min at 4°C. The supernatant was discarded and the washing procedure repeated five times *in toto*. After the final wash, the cells were re-suspended to their optimum concentration and cultured normally.

New glassware was washed in commercial detergent (Lab-Brite BHC) before use, rinsed in tap water and then re-washed in running tap water for 24 hours. It was then rinsed in distilled water and dried in a hot-air oven (Whitby & Rowley 1959). Thereafter, glassware was cleaned by prolonged washing in distilled water and oven-dried. Glassware and instruments were foil wrapped or placed in cannisters, and autoclaved at 15lbs / square inch for 20 min., and then subjected to ultra violet (U.V.) light for several minutes. All work was carried out in a sterile cabinet and normal, sterile precautions were taken throughout.

(b) Rabbits

The number of peritoneal cells in healthy rabbits tends to be too low to make the use of untreated animals economical (Stuart *et al* 1973). The macrophage population was, therefore, enhanced by the use of an intraperitoneal inducing agent (Solortorovsky & Sodeberg 1972).

Rabbits were given an intraperitoneal injection of a mixture of 1.0ml F.C.A. and 50.0ml sterile liquid paraffin (B.P.) (B.D.H. Chemicals Ltd.). Very large increases in the number of macrophages were obtained, animals being left for five days before harvesting.

After the required time, the rabbits were heavily anaethetised with an intravenous injection of 2.5ml of veterinary nembutal (Abbott) or sagatal (Veterinary Drug Co. Ltd.). The fur over the abdominal wall had been previously shaved to permit the inducing injection and the exposed skin and the rest of the undersurface was dampened with alcohol. Two hundred ml of sterile 0.85% saline were injected into the peritoneal cavity after the abdominal skin had been bluntly dissected and reflected. Neither heparin nor antibiotics were administered.

The fluid was circulated throughout the peritoneal cavity by gentle massage of the body wall and then either aspirated using a 50ml syringe with a 20 x 1" needle connected to a cannula, or a small incision was made in the anterior abdominal wall and the fluid drained into a sterile, graduated cylinder kept on ice. The peritoneal fluid was removed within four min. of its introduction.

The cell suspension was transferred to siliconized centrifuge tubes and centrifuged at 300 x g for 5 min. The layers of paraffin oil and saline were removed and the cells re-suspended in 2.5 - 3.0ml of cold TCM. They were gently agitated and re-centrifuged at 200 x g for 2 min. The cells were re-suspended in TCM such that their concentration approximated 1.0×10^6/ml. Inactivated calf serum was added to a 10% concentration as a serum-supplement. The yield of cells obtained from induced animals was , counting on a haemocytometer, approximately 1.5×10^8 total. This compared favourably with the recoveries of 200×10^6, obtained by Stuart et al (1973).

One ml of the diluted cell suspension was used to seed individual flying coverslips in Leighton tubes. These were stoppered and incubated

in air at 37°C. for 5 hours, by which time the macrophages had settled and adhered, and could be used.

2.7.2. Extraction and Culture of Alveolar Macrophages

A modified form of the technique of Stuart et al (1973) was adopted.

Albino CD-1 mice were killed by rapid dislocation of the neck and the fur over the pulmonary cavity was swabbed with alcohol and the cavity dissected open. The animal was totally exsanguinated by cardiac puncture. The two lobar lungs were then carefully removed and the main bronchii and blood vessels discarded.

The lung tissue was transferred to a sterile petri-dish containing 5.0ml of cold TCM without heparin, but with penicillin and streptomycin. It was then chopped into small fragments (2mm cubes or less) and, using a sterile Pasteur pipette, the pulmonary cells were washed free. This was continued for about 10 min. The resultant mixture was then filtered through a single layer of sterile, surgical gauze into a siliconized centrifuge tube, and the cells were sedimented by gentle centrifugation at 300 x g for 5 min. and resuspnded in fresh, cold TCM at a concentration of 1.5×10^6/ml. Ten per cent inactivated calf serum was added as a supplement. Slight contamination with red cells was inevitable and accepted. Grossly contaminated samples, however, were discarded as erythrophagocytosis may have interfered with further particle ingestion (Stankova et al 1975). Lower yields from individual mice were obtained for pulmonary exudate cells than for peritoneal cells, thus combined lavages from at least three individuals were used.

Individual Leighton tubes containing a 9 x 35 mm flying glass coverslip were seeded with 1.0ml of the cell suspension and incubated in air at 37°C. for 24 hours.

2.7.3. Measurement of Macrophage Viability

Macrophages were assessed morphologically and for their ability to adhere to and spread out on glass surfaces (Jacoby 1965). Cell viability was determined at the beginning of experiments by the trypan blue dye exclusion test (Bast et al 1974). 0.1ml of 0.1% trypan blue (BDH Chemicals Ltd.) in 0.85% saline was added to a cell monolayer and examined within four min. Cells were considered viable if the nuclear area failed to take up the dye. Populations were used that showed 80% + viability.

2.8. Attachment Tests

The following attachment tests were carried out:

(1) Opsonic Test:- Macrophages and trypanosomes allowed to interact in the direct presence of the test solution or serum.

(2) Macrophage Cytophilic Antibody Test:- Macrophages pre-treated with the test sera, then incubated with live trypanosomes.

(3) Opsonic Adherence Test:- Trypanosomes pre-incubated with the test sera and then allowed to interact with fresh macrophage monolayers.

2.8.1. Measurement of Opsonic Activity

0.2ml of the substance or serum to be tested plus 0.2ml of the washed trypanosome suspension at the optimum concentration of 1.0×10^8/ml were thoroughly mixed on a clean microscope slide. The flying coverslip from an incubated Leighton tube was removed and washed in either 0.85% physiological saline, modified from Locke (1901) (Brocklehurst 1973) or balanced salt solution (BSS) (Dresser & Greaves 1973), to remove superficial débris and unattached cells. It was then placed on the slide such that the trypanosome/serum suspension and the macrophage monolayer were in intimate contact. The slide was incubated in a humidity chamber in air at 37°C. for an optimum time of 15 min.

After incubation, the coverglass was removed, thoroughly washed in either physiological saline with Mg^{++} ions or BSS containing Mg^{++} and Ca^{++} ions to remove unattached trypanosomes, fixed in methanol for 30 sec and stained in a 10% Giemsa solution (BDH Chemicals Ltd.), pH 7.0 for 20 min. It was then mounted in Uvinert mountant (G.T. Gurr Ltd.) and subsequently examined under oil-immersion (x 1250 magnification).

The opsonic activity was quantified by systematically counting five hundred macrophages per random microscope-preparation. The number of trypanosomes attached or partially-engulfed was recorded. This was referred to as the Attachment Index.

2.8.2. Measurement of Macrophage Cytophilic Antibody Activity

0.2ml of the serum or substance to be tested was placed on a clean glass slide and a washed macrophage-seeded coverslip inverted on top. This was incubated in a humidity chamber in air at room temperature (approx. 22°C.) for 60 min. The coverglass was removed and the passively-sensitized cells were washed five times in either physiological saline or BSS. 0.2ml of the trypanosome-suspension (1.0×10^8/ml) was then placed on a further slide and the inverted coverslip placed on top. This was incubated at 37°C. for 15 min. The coverslip was then removed, washed, fixed in methanol and stained in 10% Giemsa solution and finally mounted in Uvinert mountant. Five hundred macrophages per slide were counted and the number of attached trypanosomes was recorded.

2.8.3. Measurement of Opsonic Adherence

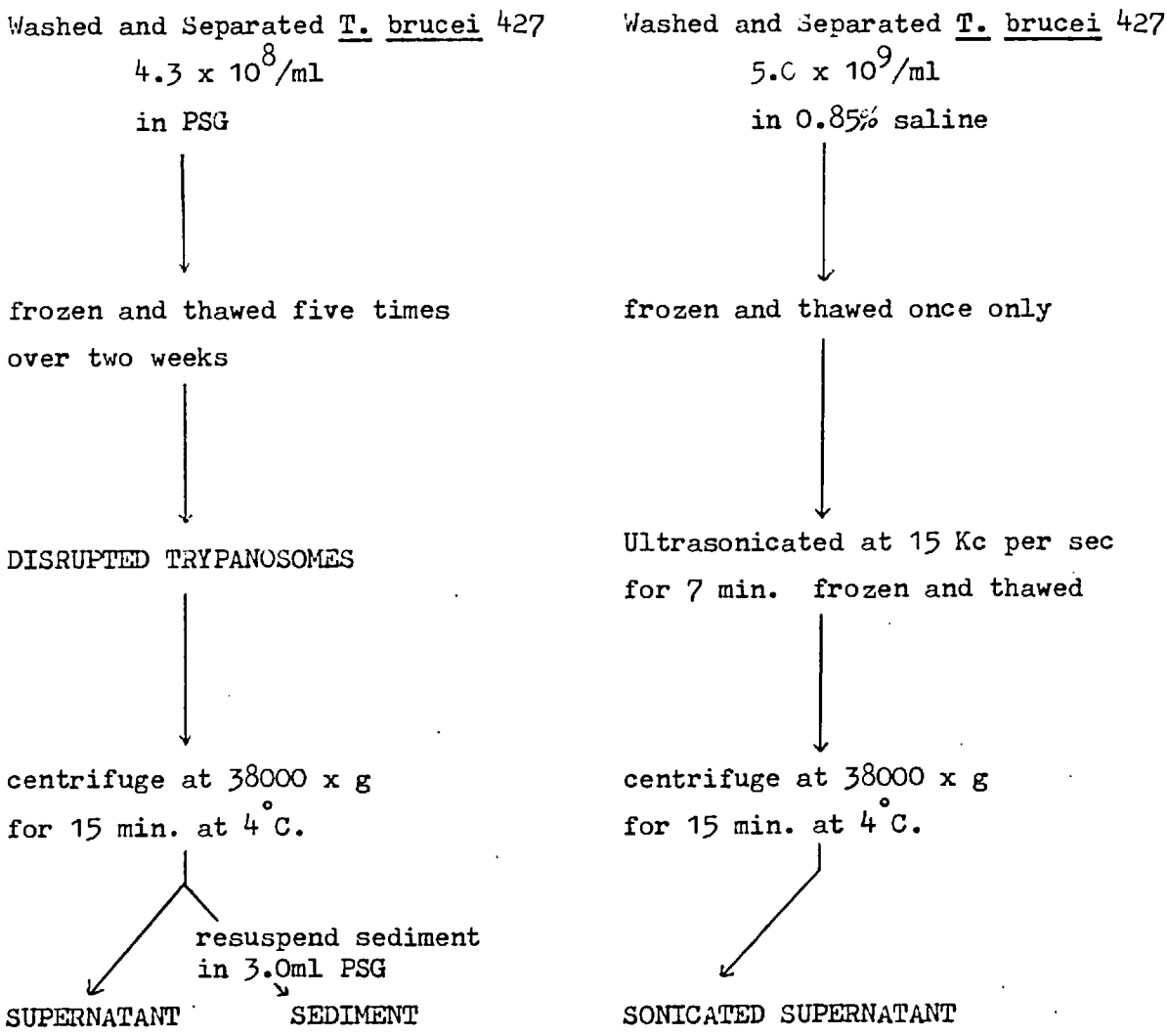
0.2ml of washed, trypanosome suspension (5.0×10^8/ml) in PSG and 0.2ml of the substance or serum to be tested were thoroughly mixed in plastic tubes. They were incubated in air at 37°C. for 30 min. and were either used immediately, or after incubation, were centrifuged at 1500 x g for 3 min. and the supernatant removed. 0.5ml of PSG were added, thoroughly agitated and the trypanosomes were re-washed by centrifugation a further three times. Finally the washed trypanosomes were re-suspended in 0.2ml of PSG. In both instances, with or without the washing procedure, trypanosome-motility was checked microscopically immediately before use.

0.2ml of the 'opsonized' or the 'opsonized and washed' trypanosome-suspension was placed on a microscope slide and the macrophage monolayer placed on top. Incubation at 37°C. for 15 min. was allowed to proceed, and the washed monolayer was fixed, stained and finally examined. The number of attached trypanosomes per 500 macrophages was again counted.

2.9. Passive Sensitization

(i) Preparation of Trypanosome Antigen

Trypanosome antigen, used in the production of immune complexes, was prepared as below:-



(ii) Cell Sensitization

0.5ml of the relevant trypanosome fraction and 0.5ml of serum were incubated together at 37°C. for 60 min. Macrophage monolayers, previously washed in BSS, were inverted on top of 0.2ml of the antigen-serum mixture and incubated in an humidity chamber in air at 37°C. for 30 min. After sensitization, the cell monolayers were washed five times in BSS and placed face-downwards on a slide to which had been added 0.2ml of live trypanosome suspension. The slides were re-incubated at 37°C. for 15 min. They were washed, fixed, stained in Giemsa and examined. The number of trypanosomes attached per 500 macrophages was recorded for each preparation.

2.10. Preparation of Serum Components

2.10.1. Gel Filtration

Hyperimmune, infected and uninfected rabbit sera were separated on the basis of molecular size, on a G200 Sephadex column (Pharmacia Ltd.), at 10°C. 2.0ml samples were used and their densities increased by the addition of 10% sucrose. The sample was applied to the top of the column under the buffer solution by means of a syringe. The buffer used was 0.1 M Tris-HCl in 1.0 M sodium chloride (pH 8.0); 3.0ml fractions were collected and the protein concentration was measured by reading, against the buffer, the U.V. absorbance at 280 nm using a Beckman 200 spectrophotemter. The fractions were pooled accordingly and dialysed for 18 hours at 4°C. against 1 l. of 0.15 M. sodium phosphate buffer (PBS), pH 7.1. The fractions were then freeze dried for 48 h. and reconstituted in the original sample volume. They were re-dialysed for a further 2 h. against 0.15 M PBS and the final samples were stored at -20°C. until used.

2.10.2. Preparation of Rabbit IgG

The technique adopted was a modification of the 'Batch Method' of Stanworth as described by Mostratos & Beswick (1969).

Two hundred g of dry, pre-swollen DEAE cellulose (Whatman DE 52) were thoroughly washed in 1.0 l. of distilled water, pH 6.0, in a Buchner funnel with a No.1 sintered glass sieve by means of gentle suction. It was washed through with 1.0 l. of PBS, pH 7.5, and equilibrated in 500 ml of the buffer for 12 h. at 4°C. After a further wash the cellulose was allowed to drain naturally, and 60g samples were used for each preparation.

One hundred ml of cooled PBS was poured into each of the cellulose fractions and 10.0 ml of hyperimmune or uninfected rabbit serum which had been previously dialysed for 12 hours against PBS at 4°C., was slowly added to the cellulose mixtures. The slurries were stirred and

allowed to stand for 3 h. at 4°C. with occassional stirring. A further 100ml of cold PBS was added to each funnel containing the serum/ cellulose slurry and the filtrate collected. Twenty five ml of buffer was then passed through and the filtrate similarly retained. The filtrates were combined and an equal volume of saturated ammonium sulphate was added. After thorough mixing and "vortexing", the precipitated globulin was separated by centrifugation at 17,500 x g for 15 min. at 4°C. The precipitate was dissolved in a minimum of 0.85% saline and coarsely filtered. The solution was then dialysed against 1.0 l. of 0.85% sodium chloride for 12 h. at 4°C. One ml aliquots were prepared and maintained at -20°C. until required.

2.10.3. Preparation of Properdin and Properdin-Depleted Sera

Properdin and properdin-depleted sera were prepared from uninfected and hyperimmune rabbit sera, by the modified technique of Pillemer et al (1956).

Two hundred and fifty mg of zymosan A prepared from S. cerevisiae yeast (Sigma) were used for the preparative method. Zymosan A is known to react satisfactorily with the C3 component of sera thus making C3-deficient serum (R3); to complex with properdin in serum at 17°C. in the presence of Ca^{++} and Mg^{++} ions; and to enable properdin to be suitably recovered from a properdin-zymosan (PZ) complex.

The zymosan, previously stored at 4°C., was evenly suspended in 20.0 ml of 0.15 M sodium chloride in a glass bottle and placed in a boiling water bath for 60 min. It was then centrifuged in an MSE -18 high-speed centrifuge at 2000 x g for 30 min. The supernatant was discarded, and the deposit was re-suspended in 4.0 ml of barbital-sodium buffer (BS), pH 7.4. This was maintained at 17°C. in a constant temperature bath. Ten ml of serum were also equilibrated at 17°C. for approximately 2 h. Two ml of the zymosan suspension were added to the serum and the mixture manually agitated every 10 min. for 60 min. The temperature of the water bath was carefully controlled. Temperatures below 16°C. could have resulted in an incomplete removal of properdin, whilst those above 18°C. cause an increased loss of C3 activity (Pillemer et al 1956).

After 60 min. the pH of the mixture was adjusted to 6.9 with 1.0 N hydrochloric acid, and then immediately centrifuged at 2000 x g for 30 min. at 1°C. The supernatant - properdin-depleted serum (RP) - was carefully decanted, made into 5.0 ml aliquots and stored at -20°C. until required.

The precipitate - PZ complex - was removed and made up into five individual 5.0 ml aliquots with the addition of 25.0 ml of PBS pH 6.0

at 1°C. The complexes were evenly suspended by shaking and vortexing. They were immediately centrifuged at 2000 x g for 30 min. at 1°C., and the supernatant washings were discarded. The washing step was repeated two additional times, spinning for 30 min. and 15 min.

The washed PZ complex was suspended in 18.0 ml of Michaelis buffer pH 7.4 and 7.0 ml of 2.0 M sodium chloride at 37°C. and stirred for 60 min. at this temperature. It was then centrifuged at 2000 x g for 60 min. at 1°C. and the precipitate discarded.

The properdin eluate supernatant was dialysed against 2.0 l. of distilled water for three days, with 24 hour changes of the water, at 2°C. After dialysis the pH of the solution was adjusted to 5.8 and allowed to stand for 90 min. at 1°C. This was followed by centrifugation at 2000 x g for 30 min. at 1°C. and the supernatant was discarded.

The crude properdin precipitate was extracted with 17.0 ml of BS pH 7.4, previously maintained at 1°C. This was centrifuged at 2000 x g for 30 min. at 1°C. The supernatant was retained and the precipitate was re-extracted with 8.0 ml of BS and again centrifuged at 2000 x g for 30 min. at 1°C. The precipitate was discarded and the supernatant added to the previous extract.

The final volume of extracted properdin in BS at a pH of 7.3, was made into 8.0 ml individual aliquots and maintained at - 20°C. until required.

2.10.4. Preparation of Complement-Deficient Sera

(i) Inactivation of Serum Complement

Rabbit and mouse sera were heated in a water bath at 56°C. for 30 min. All sera were tested within 5 h. of treatment.

(ii) Preparation of Complement "R" Reagents

(a) Sera Deficient in C1 and C2 Activity (R1 and R2)

0.5 ml of whole sera were heated in a water bath at 50°C. for 15 min (Mathews et al 1974). Thereafter maintained at -20°C. until required. A proportion of alternate pathway factor B may also have been destroyed by heating to this temperature (Reynolds et al 1975, Gallin et al 1975).

(b) Sera Deficient in C4 Activity (R4)

C4 was inactivated by ammonia treatment, which also destroys a proportion of C3 present (Lachmann et al 1973). 0.25 ml of a 0.15 N solution of ammonium hydroxide made from 14 N .880 ammonia in water was added to 1.0 ml of serum and incubated at room temperature (22°C.) for 90 min. It was then neutralized with 0.25 ml of 0.15 N hydrochloric acid, and stored at -20°C. until required.

(c) Sera Deficient in C3 and C4 Activity (R3 and R4)

These two components were inactivated by potassium thiocyanate treatment, which probably also inactivated C5. A 1.0 M solution of potassium thiocyanate was made up in distilled water and maintained at 4°C. One ml was slowly added to an equal volume of serum and gently vortexed throughout. This was kept at 4°C. for 18 h. and then dialysed against PBS, pH 7.1, for 2.5 hours and then against BSS for 12 hours at 4°C., to restore Ca^{++} and Mg^{++} ions (Mathews et al 1974). The serum was maintained at -20°C. until used.

(d) Sera Deficient in C3 Activity (R3)

C3 was inactivated by the use of cobra venom, courtesy of Mr. J.K. Lenahan, Silwood Park, Ascot. Fresh, crystalline cobra venom was collected from Naja nigricollis six hours before use. The crystals were dissolved in slightly warmed PBS, pH 7.2, to a 0.5% solution.

0.5 ml of this solution was added to 0.5 ml of serum and incubated at 37°C. for 30 min. It was then stored at -20°C. until used. A factor present in cobra venom, (CVF) in association with a β-globulin fraction of normal serum can form an enzyme capable of destroying C3 (Lachmann 1973). Crude cobra venom in buffer can have this effect (Mathews et al 1974), whether or not the various contaminative proteins have been removed by boiling (Davey & Asherson 1967). CVF is a functional analogue of C3b, which is not susceptible to C3b inactivater (KAF). It is consequently able to activate the alternate pathway without the presence of properdin. As the regulatory feed back is disturbed, so serum C3 is converted to C3b, thus causing a depletion of C3 (see Fig (33) section 3.9.1.).

2.11. Inactivation of Serum IgM

(i) Heat Inactivation

Sera were heated in a water bath at 65°C. for 30 min.

(ii) 2-Mercaptoethanol (2-ME) Inactivation

Mouse sera were inactivated by mixing equal volumes of sera and 0.1 M 2-ME in PSG, and incubating at 22°C. for 60 min. (Kindmark 1972). A control using PSG alone was also run.

2.12. Assays

2.12.1. Ouchterlony Double-Diffusion Test

One per cent agar (Difco Laboratories) in 0.1% glycine buffer, pH 7.2, containing 0.2% sodium azide as a bacteriostat, was prepared. Fifteen ml of the heated agar solution were poured into a sterile 9.0 cm. diameter glass Petri dish and allowed to set at 4°C.. The substances under test were added to the appropriate holes cut in the agar with a 9.0 mm diameter cork-borer. The plates were maintained at room temperature (22°C.) and read between 48 and 72 hours, examining for lines of precipitation.

2.12.2. Protein Measurement

Total protein concentration was measured with the Folin-phenol reagent after alkaline copper treatment, according to the method of Lowry et al (1951). Readings were made on a Beckman 200 spectrophotemeter at a wavelength of 627 mu.

2.12.3. Zymosan Assay for Properdin

The number of units of properdin present in the prepared samples was determined by the modified method of Pillemer et al (1956).

(i) Standardization of Zymosan

Two hundred and fifty mg. of boiled zymosan A were standardized in 4.0ml of barbitone buffer, pH 7.2 (Oxoid Ltd.).

(ii) Sensitization of Sheep Cells

Twenty five ml of sheep red cells in Alsever's solution (Glaxo Laboratories) were washed three times in barbitone buffer and re-suspended to a 4% suspension. An equal volume of a $^{1}/_{300}$ dilution of horse-haemolytic serum (Wellcome Reagents Ltd.), together with the red cell suspension were pre-incubated at 37°C. for 60 min., then quickly mixed together and re-incubated at the same temperature for a further 30 min. The indicator cells were used within 15 min.

(iii) Estimation of Properdin

0.1 ml of the standardized zymosan suspension plus 0.25 ml of RP was added to each of six, 400 by 100 mm round bottomed plastic tubes maintained at 4°C. To successive tubes were added increasing two fold dilutions of the test sample in barbitone buffer, starting with 0.24 ml. Barbitone buffer was added to each tube to a final volume of 0.75 ml. The tubes were covered, shaken gently and incubated at 37°C. for 60 min. shaking every 10 min. The tubes were centrifuged at 1500 x g for 10 min. and the supernatant from each tube retained, and the concentration of C3 present was determined.

For each supernatant, a series of two-fold dilutions from 1:1 to 1:16 were made in barbitone buffer, using 0.2 ml volumes. 0.01 ml of previously-prepared R3 was added to each tube and the volume made up to 0.5 ml with buffer. One ml of previously sensitized sheep cells were added to each tube and incubated at 37°C. for 30 min. The tubes were then centrifuged at 1500 x g for 5 min. The dilution which gave 50% haemolysis was determined by comparing each tube with a 50% haemolysis-standard prepared by adding a trace of saponin (Hopkin & Williams) to 0.5 ml of sensitized sheep cells in 1.0 ml of barbitone buffer.

Three control tests were also made: (a) in the absence of zymosan and properdin; (b) in the presence of zymosan but the absence of properdin and; (c) in the presence of whole serum.

The calculation of the number of units of C3 and of properdin present in each sample was determined according to the method of Pillemer et al (1956).

2.13. Absorption Studies

(a) Whole Trypanosomes

T. brucei 427 and S42 were separated on a DEAE cellulose column and re-suspended in PSG to an approximate concentration of 1.0×10^9/ml. One ml of trypanosome suspension was added to 1.0 ml of undiluted serum or previously-prepared rabbit IgG (see Section 2.10.2.), and thoroughly mixed. After incubation at 37°C. for 60 min. the mixture was centrifuged at 3800 x g for 15 min. and the supernatant retained. The procedure was carried out five times *in toto* and gave a final dilution of approximately $^1/_{32}$. The absorbed sera were finally decanted, made into 0.3 ml aliquots and stored at -20°C. until required.

(b) Disrupted Trypanosomes

T. brucei 427, 8.1×10^8/ml, separated and washed, were frozen at -20°C. for 24 h. They were maintained in PSG and allowed to thaw at room temperature (22°C.). They were then subjected to ultrasonic disintegration at 15 Kc per sec. for 7 min. at 4°C., in an MSE 150 Watt Disintegrator.

One ml of the trypanosome sonicate was mixed with 1.0 ml of previously prepared rabbit IgG and two absorptions at 37°C. for 60 min. were carried out. 0.3 ml aliquots of absorbed IgG were kept at -20°C. until used.

(c) Liver

0.5 g. (net weight) of mouse liver was homogenized in five times its volume of PSG and incubated with an equal volume of undiluted serum at 37°C. for 60 min. The mixture was centrifuged at 3800 x g for 30 min. at 4°C. and the supernatant retained and stored at -20°C. until used.

(d) Zymosan

Undiluted sera were depleted of properdin by absorption with a standardized suspension of zymosan at 17°C. (see Section 2.10.3.).

One ml. of a standardized suspension of zymosan, maintained at 23°C., was mixed with 1.0 ml. of previously prepared rabbit IgG and incubated in a water bath at 37°C. for 60 min. The mixture was centrifuged at 38000 x g for 15 min. and the supernatant retained. This was re-absorbed for a further 60 min. with 1.0 ml. of the zymosan suspension. Aliquots were stored at -20°C. until required.

A control was used for each absorption study, substituting PSG for the absorbative.

2.14. *In vivo* Phagocytosis

One ml. volumes of washed, separated *T. brucei* 427 were incubated at 23°C. for 30 min. with equal volumes of (a) PBG, (b) specific mouse hyperimmune *T. brucei* 427 serum diluted five times less than the agglutinating titre and (c) trypanosome supernatant (see Section 2.9.).

0.2 ml. volumes containing 6.1×10^6 live trypanosomes were then inoculated intraperitoneally into each of a series of CD-1 mice. These were left for varying periods of time before the peritoneal exudate of each individual was recovered. One ml. of the cell suspension from each mouse was collected in a siliconized tube on ice, mixed thoroughly and a sample was removed. The number of mononuclear cells, the total number of trypanosomes present and the number of trypanosomes which had been engulfed by or had attached to phagocytes, were recorded.

2.15. Surface Phagocytosis

A clean microscope slide was covered with the material to be tested and placed flat in the bottom of a 9.0 cm. glass Petri dish lined with Whatman No.1 filter paper soaked in TCM. 0.2 ml. of live T. brucei 427, 3.2 x 10^8/ml. was distributed evenly over the surface and mixed thoroughly. The dish was capped and incubated at 37°C. for 15 min. 0.2 ml. of mouse peritoneal cell suspension (2.0 x 10^6/ml.) was similarly administered and each preparation mixed and re-incubated at 37°C. for 15 min. After the second incubation, impression smears were taken on 9 x 35 mm. glass coverslips smeared with an albumin solution. The coverslips were fixed, stained in Giemsa and examined for attached or partially engulfed trypanosomes.

2.16. Cx-Reactive Protein

(i) Measurement

Levels of Cx-RP present in the sera of rabbits infected with T. brucei 427, and in drug-treated animals, were both qualitatively and quantitatively determined using the Hyland CR slide test kit (Travenol Laboratories Inc.). All sera were heat inactivated at 56°C. for 30 min. before testing.

(ii) Indomethacin Treatment

Rabbits infected with T. brucei 427 were orally dosed with 1.0 ml. of the anti-inflammatory drug, indomethacin BP (Merck, Sharp and Dohme Ltd.), in distilled water at a concentration of 5 mg./kg. body weight, at varying intervals post infection.

A non-infected control rabbit was similarly treated.

2.17. Chemotaxis

(i) Technique for Estimating Chemotaxis

Chemotaxis was measured *in vitro* using a modified type of Boyden-chamber.

A 2 ml. tuberculin syringe barrel (Sherwood Medical Industries Inc.), with its end sawn off was used. A hole large enough to admit the tip of a Pasteur pipette was made precisely 2.5 cm. from the sawn-off end. Two barrels were opposed, separated by a cellulose ester filter (Millipore Ltd.) of pore -size 8 μm, through which macrophages could actively migrate but could not drop passively. The join was then heat-sealed and the plungers placed in the opposite ends. The chambers were autoclaved and the join smeared with sterile vaseline immediately before use.

The lower compartment of the chamber was used for the test solutions, the upper compartment for the peritoneal cell suspension. The chambers were suspended vertically in a rack.

0.5 ml. of the test solution was administered using a sterile Pasteur pipette, and the plunger gently depressed until the solution was flush with the lower surface of the Millipore filter. No fluid was allowed to escape into the upper part of the container. The lower compartment was gently tapped to disperse any air bubbles which may have been present.

0.5 ml. of freshly-pooled peritoneal exudate cells from CD-1 mice at an approximate concentration of 5.0×10^6/ml. in cold TCM without a serum supplement, was similarly administered to the top half of the chamber. The plunger was not fully depressed, thus preventing increased pressure from forcing the solution through, or rupturing the filter.

The tests were incubated in moist air at 37°C. for an optimum time of 4 hours. Thereafter, they were removed, broken open and the filters removed with forceps. The filters were fixed and stained according

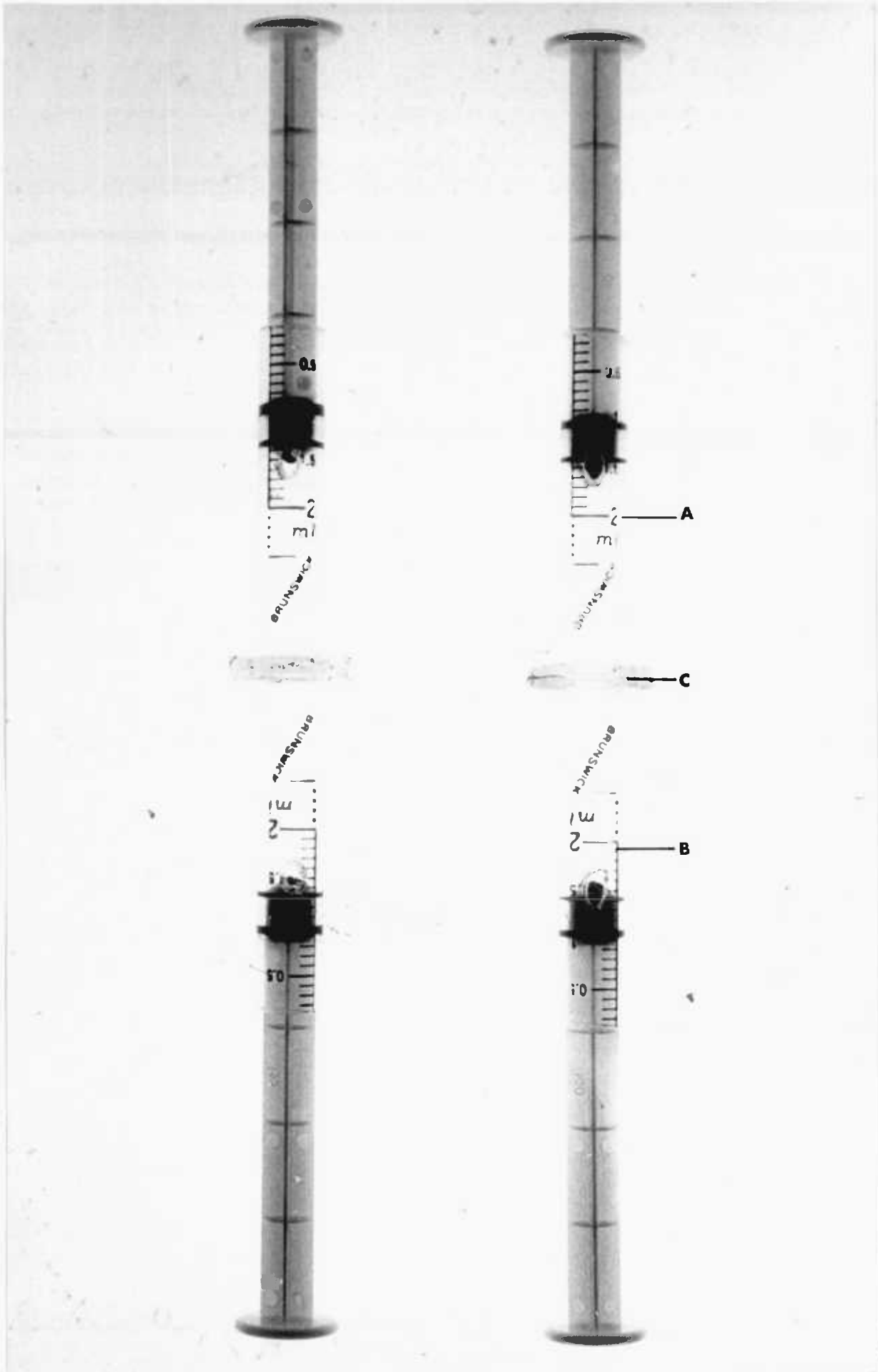


Plate D.

Plate D. Chemotaxis chambers.

A: Compartment A, containing mouse peritoneal exudate cells.

B: Compartment B, containing chemotactic test substance.

C: Millipore filter, 8μm diameter pore size.

to the technique of Boyden (1962a). The filters were finally reversed onto a microscope slide and examined in xylene. The membrane was thus rendered transparent and the cells could be visualised at all levels.

(ii) Assessment of Results

Morphologically recognizable macrophages adhering to the far side of the filter were counted. Care was taken to select representative areas of the membrane and within these areas, ten randomly-selected fields were scored and the mean count per field calculated. The magnification used was x 480. All results were adjusted to a standard Chemotactic Index (CI).

Symon et al (1972), indicated the great deal of variation present in the number and activity of migrating cells, variations in absolute cell and background counts, as well as other factors impossible to adequately control. They employed, therefore, a CI, using the formula:-

$$CI = \frac{\text{Test count} - \text{control count}}{\text{Casein control count} - \text{Gey's control count}} \times 100$$

The 'control count' represented either Gey's solution or plasma depending on whether a cytotaxin or cytotaxigen was being investigated. It was possible, therefore, to directly compare different experiments using different samples. Symon et al (loc cit), were nevertheless aware that the results obtained could at best be only semi-quantitative, since the casein control itself could fluctuate.

(iii) Test and Control Solutions

The diluent medium used for the tests was Gey's solution prepared as described by Dresser & Greaves (1973). Heparin was omitted. Penicillin (200 µ/ml.) and streptomycin (50 µg/ml.) were added. Gey's solution already contained Ca^{++} and Mg^{++}, considered essential for chemotactic responses (Becker 1975).

All tests included a positive and a negative control. Gey's solution which was not itself chemotactic was used as the negative control. A solution of casein (Hopkin and Williams) at an optimum concentration of 5mg/ml. in Gey's solution was used as the positive control (Symon et al 1972).

For those experiments involving serum or plasma, a background control was also used. Plasma was preferred to serum as the latter tended towards higher background counts.

2.18. Analysis of Results

Data were subjected to statistical analysis on the University of London Computer, CDC 6600.

Individual experiments were tested using Duncan's New Multiple Range Test, and the treatments grouped into homogenous subsets at a significance level of 0.5% (P = 0.005).

Correlation between grouped experiments was tested by means of an incomplete 3 - factor analysis of variance, with probability levels of 5% and 1% (P = 0.05; P = 0.01).

Correlation co-efficients between experimental results and infective parasitaemia were calculated using a linear regression analysis on a programmable WANG 720B.

PART 3. EXPERIMENTAL

3.1. Opsonization and Mouse Macrophages

3.1.1. Kinetics of Phagocytosis by Mouse Peritoneal Macrophages

(a) Effect of Organism Concentration

The opsonic test was carried out to determine the optimum macrophage:trypanosome ratio to be used for subsequent experiments.

T. brucei 427, collected from heavily infected (4+) rats, was used in conjunction with albino mouse peritoneal macrophages and whole hyperimmune 427 serum. Macrophage monolayers at an approximate seeding concentration of 1 x 10^6/ml., were washed in fresh BSS and incubated for 15 min. at 37°C. with a mixture of 0.2 ml. of serum and 0.2 ml. of trypanosome suspension. The latter was diluted to the required concentrations, in PSG.

The results are presented in Fig. (1). Peak values were obtained with organism : cell ratios greater than 100:1. Ratios between 100:1 and 1000:1 were significantly similar at a 0.5% level and represented trypanosome concentrations per ml. of between 1 x 10^8 and 1 x 10^9, respectively. Concentrations in excess of 1 x 10^9 per ml. gave rise, in the presence of hyperimmune serum, to an inordinately high number of large and irregularly-shaped agglutinins, which although attaching to macrophages en masse, made quantification unreliable.

(b) Effect of Incubation Time

The opsonic test was performed to find the optimum incubation time for the attachment of T. brucei 427 to peritoneal macrophages in the presence of hyperimmune serum.

Individual tests using 0.2 ml. of trypanosomes - 4.6 x 10^8/ml. and 0.2 ml. of whole hyperimmune 427 serum, were incubated with washed macrophage monolayers at 37°C. for varying periods up to 180 min.

The number of individual trypanosomes which had attached to, or had been partly engulfed by macrophages were counted, rather than those which had been totally phagocytosed. This was decided upon due to the difficulties of accurately quantifying morphologically-distinct trypanosomes within the body of the macrophage (Takayanagi et al 1974b), and also because, although adherence may well be considered to be the first step in engulfment (Takayanagi et al 1974a), many reports have indicated that these two processes are independent, each relying upon separate factors (Rabinovitch 1967, Chour & Kreier 1972).

The highest number of attachments was recorded after incubation between 15 and 30 min. (Fig (2)). Incubation for periods longer than 30 min. gave statistically similar results, suggesting that high site-saturation was rapidly reached, initially declined and thereafter stabilised. Endocytosed particles were visible between 15 and 60 min. incubation, indicating the rapidity of ingestion. Indeed it has been suggested that any ingestive mechanism lasting longer than 60 to 90 min. should not qualify as phagocytosis per se (Karnovsky et al 1970).

(c) Effect of Serum Concentration

The opsonic test was carried out using T. brucei 427 - 3.0×10^8/ml. - peritoneal macrophage monolayers and serial dilutions of hyperimmune serum.

Mouse hyperimmune T. brucei 427 serum was diluted with 0.85% physiological saline. 0.2 ml. was incubated with the trypanosome suspension and washed mouse macrophages for 15 min at 37°C. Dilutions lying between 1:80 and 1:10240, twice the agglutinating-antibody titre (Appendix 3), gave statistically comparable results, similar to those obtained with the negative controls. Undiluted and serum diluted 1:20 and 1:80 gave significantly higher values (Fig (3)).

Fig. 1.

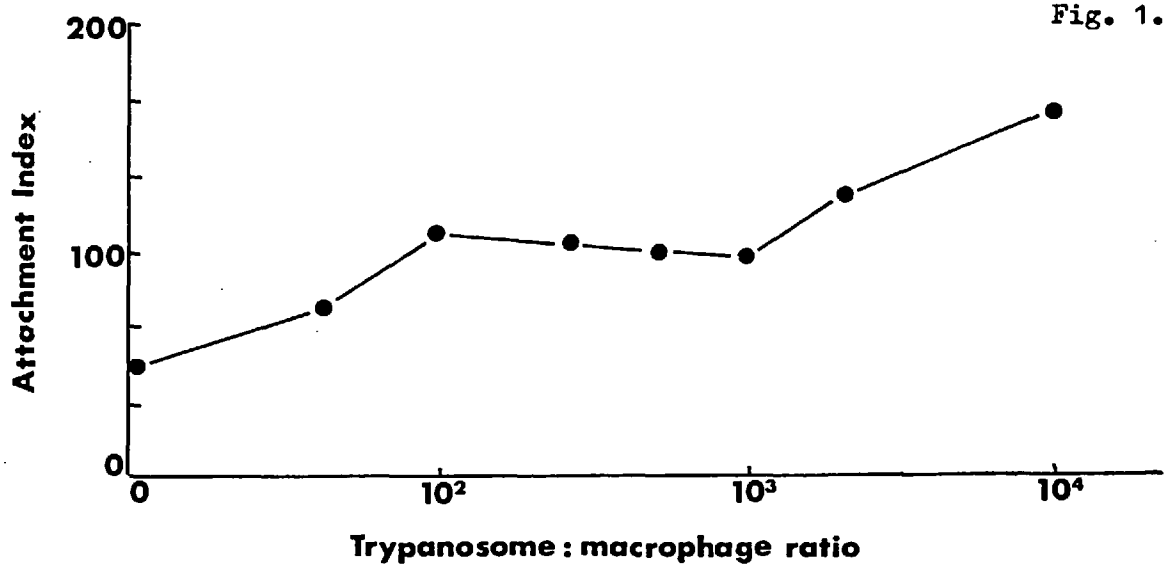


Fig. 2.

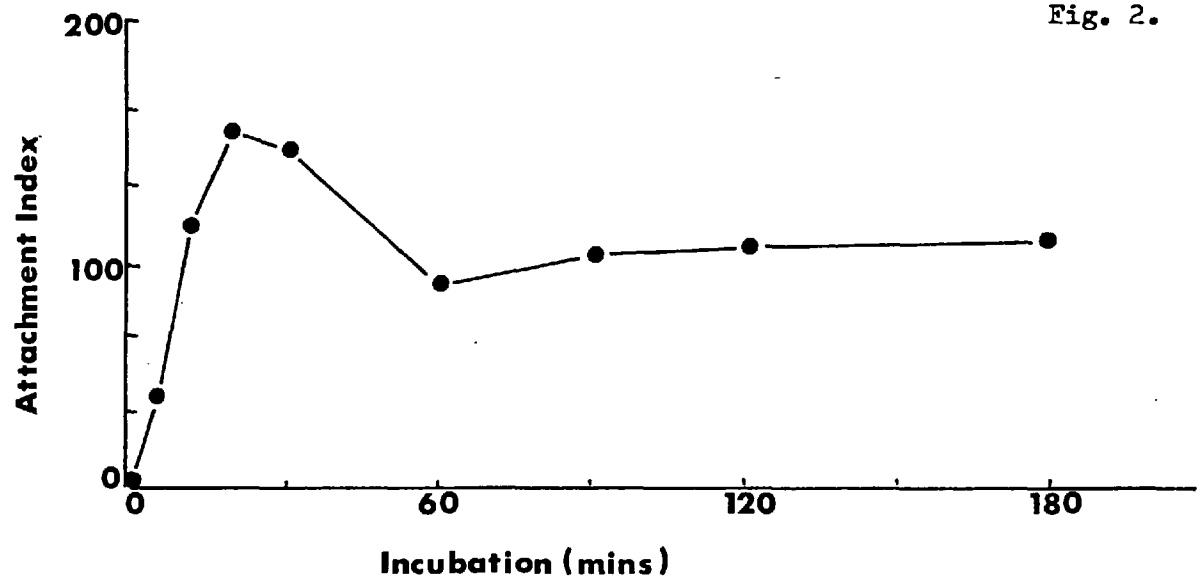


Fig. 3.

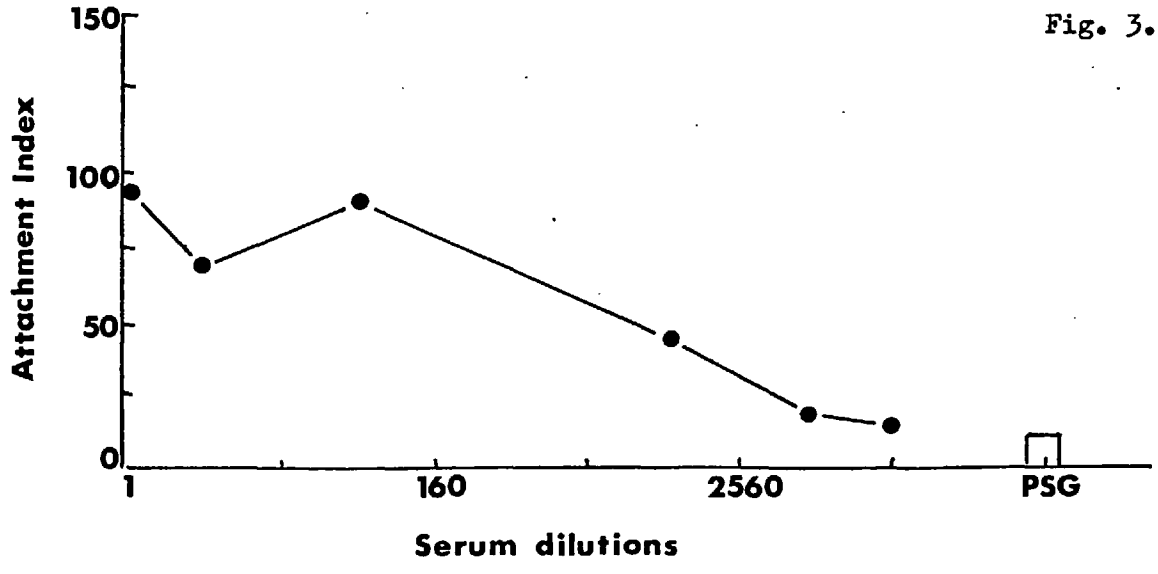


Fig. 1. Effect of parasite concentration on the attachment of T. brucei 427 to mouse peritoneal macrophages (opsonic test).

Fig. 2. Effect of incubation time on the attachment of T. brucei 427 to mouse peritoneal macrophages (opsonic test).

Fig. 3. Effect of serially diluted mouse hyperimmune 427 serum on the attachment of T. brucei 427 to mouse peritoneal macrophages (opsonic test).

For subsequent experiments, trypanosomes were used at an optimum concentration of between 1 and 5 x 10^8/ml.; serum was used undiluted, or more rarely at a dilution of 1:10; and the preferred incubation time was 15 min. For all treatments, unless otherwise specified, the final incubation temperature was 37°C. Temperatures above 40°C. caused the macrophages to degranulate and die (personal observations).

3.1.2. Macrophages and Erythrocytes

Macrophages have for long been recognised as efficient mediators in the removal of damaged and senescent erythrocytes from the circulation (Vernon-Roberts 1972). The attachment of red cells to macrophages has been shown not to necessarily require the presence of specific cell-sensitizing serum factors (Vaughan & Boyden 1964), and indeed chemically treated or damaged erythrocytes may well adhere in a serum-free medium (Rabinovitch 1967). The mechanism of attachment and subsequent ingestion of heterologous or injured homologous red cells in the absence of serum has been suggested as being primarily tactile rather than humoral (Stuart 1970). Although effective adherence may also be a property of a naturally-occurring cytophilic antibody (Vaughan 1965b).

The efficiency of albino mouse peritoneal macrophage monolayers which had been cultured in vitro for 24 h. to attach and engulf both homologous and heterologous erythrocytes in the presence of uninfected and infected sera was tested. This single experiment was initially established to confirm that the method of culture finally adopted was producing monolayers of active, viable macrophages, and that the conditions were suitable for monitoring the attachment of biological particles. The following tests were set up:-

1. A cytophilic test was performed using macrophage monolayers sensitized with freshly prepared uninfected albino mouse serum (NMS). The particles consisted of a suspension of sheep red blood cells in Alsever's solution (SRBC) (Wellcome Reagent Ltd.), washed three times in PBS and resuspended to a concentration of 1×10^8/ml. The final incubation was carried out at 25°C., as previous incubations at 37°C. resulted in the lysis of significant numbers of red cells.

2. As in test 1, but substituting mouse red blood cells (MRBC) at a concentration of 1 x 10^8/ml. in PBS, for SRBC.

3. An opsonic test was carried out using washed SRBC and NMS. Incubation was again at 25°C. for 15 min.

4. As in test 3, substituting MRBC for SRBC.

5. As in test 3, substituting uninfected mouse plasma for NMS.

6. As in test 5, substituting MRBC for SRBC.

7. An opsonic test was performed using SRBC and mouse hyperimmune T. brucei S42 serum.

8. As in test 7, substituting MRBC for SRBC.

9. An opsonic test using NMS and MRBC which had been previously washed and resuspended in PBS and maintained at 20°C. for 24 h.

10. An opsonic test using MRBC and serum from a heavily infected (4+) mouse infected 36 h. previously with T. brucei 427.

From the results in Fig. (4) it can be seen that significantly high numbers of sheep red cells attached to macrophages in the presence of both NMS and, to a lesser extent, hyperimmune S42 serum. High cytophilic attachment was evident, suggesting the possible presence of surface receptor sites capable of mediating the adherence of heterologous erythrocytes in the absence of specific serum factors.

Comparatively low values were obtained using homologous erythrocytes in conjunction with both NMS and plasma and with hyperimmune and infected sera. Similar results were seen with stored mouse erythrocytes and homologous serum. Attachment caused by sera appeared more effective than that by plasma.

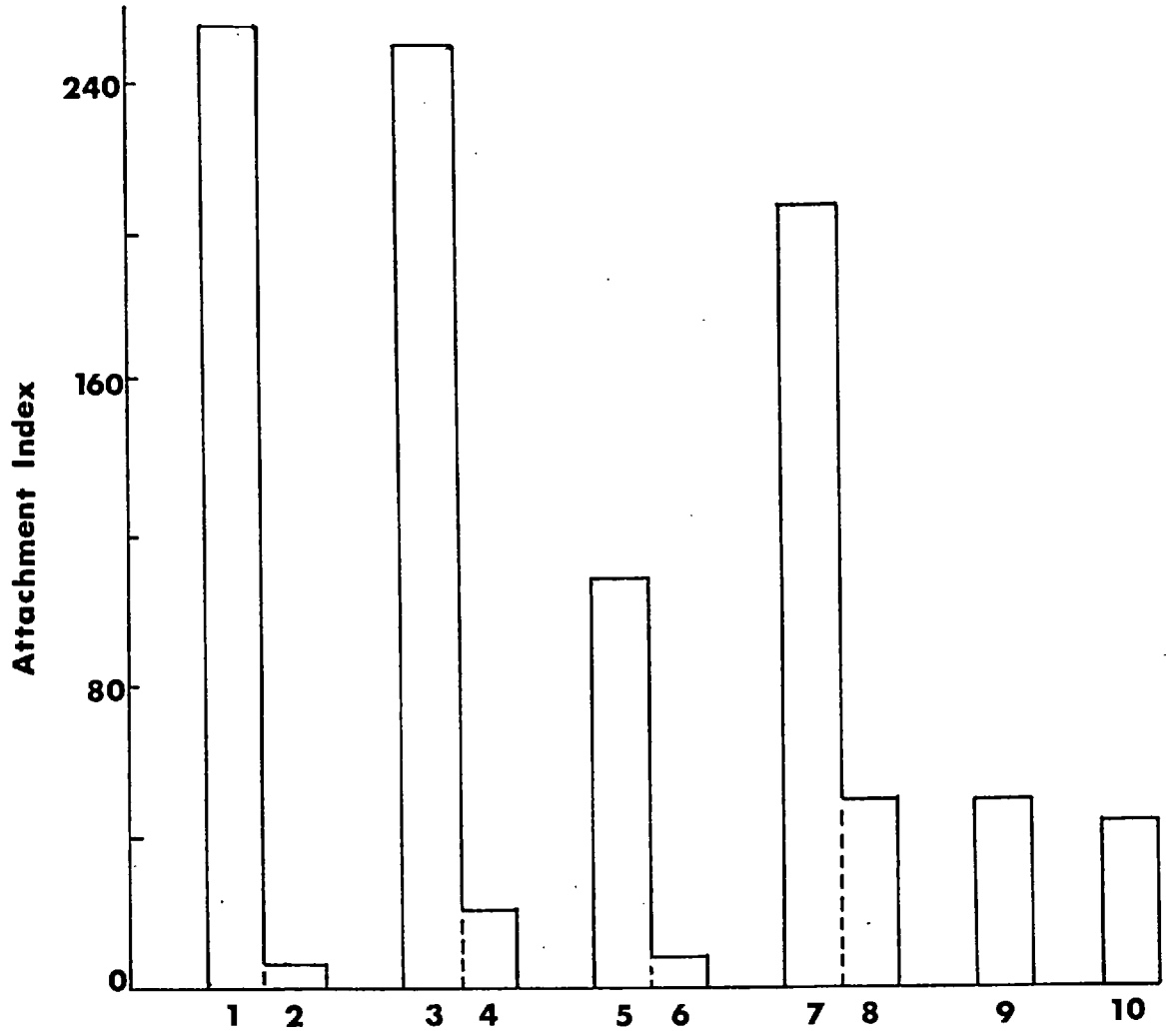


Fig. 4.

Fig. 4. Attachment of homologous and heterologous erythrocytes to cultured mouse peritoneal macrophages.

1. NMS plus SRBC (cytophilic test)
2. NMS plus MRBC (cytophilic test)
3. NMS plus SRBC (opsonic test)
4. NMS plus MRBC (opsonic test)
5. Mouse plasma plus SRBC (opsonic test)
6. Mouse plasma plus MRBC (opsonic test)
7. Mouse hyperimmune T. brucei S42 serum plus SRBC (opsonic test)
8. Mouse hyperimmune T. brucei S42 serum plus MRBC (opsonic test)
9. NMS plus stored MRBC (opsonic test)
10. T. brucei 427 infected mouse serum plus MRBC

A variety of factors could have influenced the results, thus high levels of natural antibodies against heterologous red cells have been indicated in calf serum and natural blood group isoagglutinins and heteroagglutinins may have partially promoted attachment.

In some of the heterologous tests (3 and 7), a small number of red cells were observed localized within individual macrophages. These were, however, relatively infrequent and in the minority, perhaps indicating that although conditions may have been suitable for attachment, they were not necessarily so for ingestion (Rabinovitch 1967, Vaughan & Boyden 1964).

Non-specific heterogenous serum factors appeared capable, therefore, of recognizing foreign macromolecules and effecting in vitro attachment to viable macrophages. It is possible that the mouse erythrocytes were not sufficiently modified by storage to be adsorbed, or that specific opsonic factors are required for the attachment and subsequent uptake of damaged or effete autochthonous cells (see Section 3.1.11). Lo Buglio et al (1967), have reported that red cells coated with non-specific IgG can bind to human monocytes, and despite not involving C', nor as a prelude to erythrophagocytosis, rapid morphological damage may occur.

The specific and non-specific factors produced in response to trypanosome inoculation did not appear to confer a greater potential for red cell attachment. Macrophages contain specific receptor sites for immune IgG, and it is probable that red cells bind, at least partially, by a cytophilic mechanism. It is possible, therefore, that hyperimmune serum may competitively displace cytophilic antibody, hence causing a reduction in the number of red cells which can attach.

It is also possible that the experimental temperature may have been too low for optimum attachment. A reduction in ambient temperature is known to decrease intracellular digestion (Cohn 1970), and it is

important in the attachment and dissociation of cytophilic antibody. The normal freedom of motion of surface membrane lipids may well be impaired following a depression of physiological temperature (Schroit & Gallily 1974). The observed lysis of red cells at 37°C. may have been a result of activated serum components or extracellular enzymic secretions from the macrophages themselves (Elsbach 1974).

3.1.3. Opsonization in an Homologous System

The opsonic test was used to determine the effect of immune sera obtained throughout infection, on the attachment of trypanosomes to mouse peritoneal macrophages. A series of tests was arranged as indicated below, (Table (1)).

Table (1)

Peritoneal Macrophages	Trypanosome Strain & Source		Sera
Albino mouse	427	mice	Albino mouse
Albino mouse	427	rats	Albino mouse
Albino mouse	S42	rats	Albino mouse
Albino mouse	427	rats	CBA mouse
CBA mouse	427	rats	CBA mouse
CBA mouse	427	rats	Albino mouse

Low, but measurable numbers of trypanosomes attached to mouse peritoneal macrophages in the presence of immune antisera. Fig. (5), shows the attachment indices using 427 trypanosomes and homologous antisera. In all instances a sharp increase was observed rising from control levels, with the activity increasing with both parasitaemia and days post infection. NMS, PSG and serum obtained from mice inoculated with FCA gave similar results to each other and to those of day 0 (serum taken 2 h. prior to infection). An enhanced attachment index was obtained using homologous hyperimmune serum.

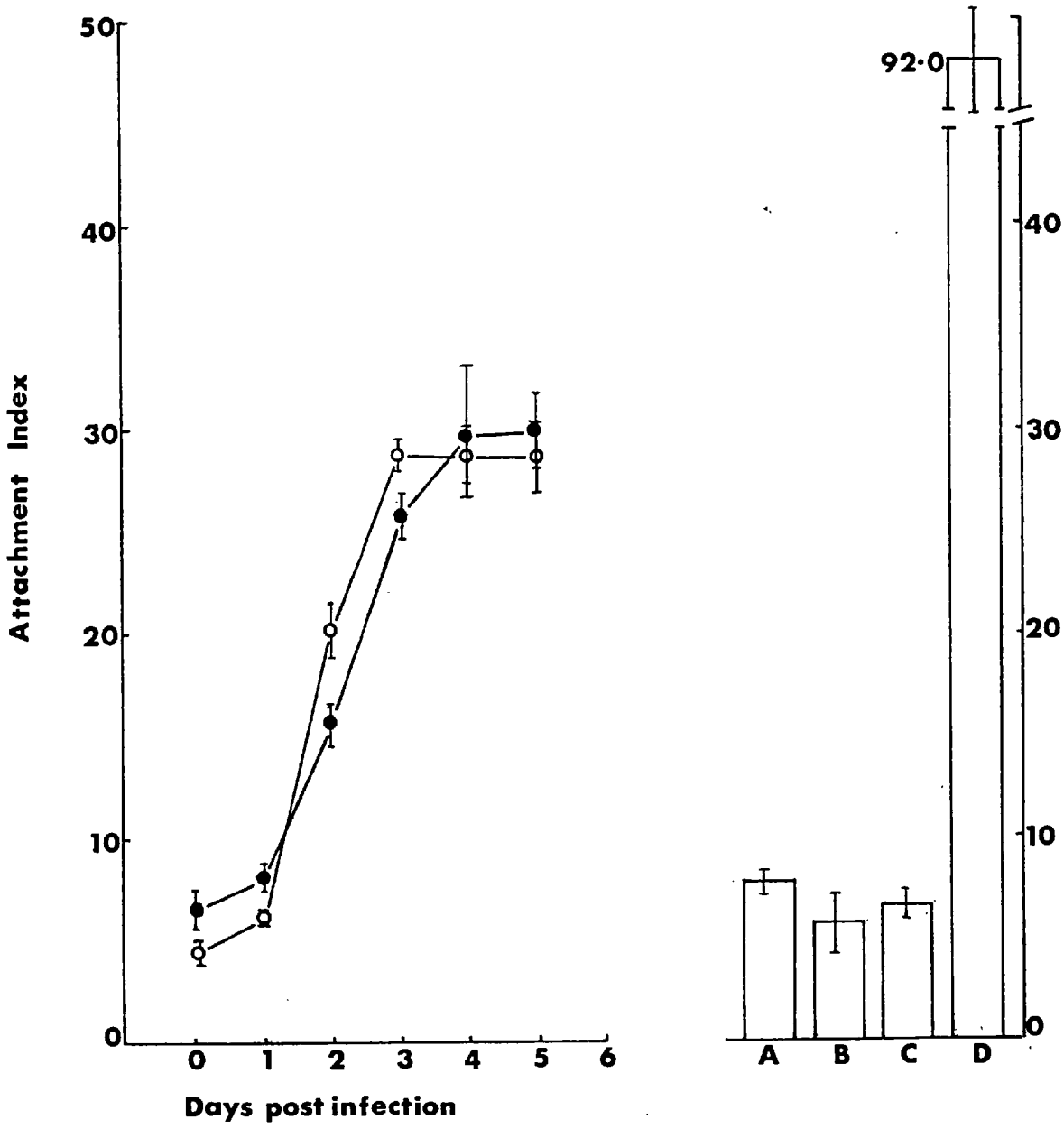

Attachment Index
50
40
30
20
10
0
0 1 2 3 4 5 6
Days post infection
92·0
40
30
20
10
0
A B C D

Fig. 5.

Fig. 5. Attachment of T. brucei 427 obtained from infected rat blood to cultured albino mouse peritoneal macrophages.
Effect of homologous sera (opsonic test).

●——● Mean (± SE) of 10 replicates - serum series A

o——o Mean (± SE) of 10 replicates - serum series B

A: PSG Mean (± SE) of 4 replicates

B: NMS Mean (± SE) of 4 replicates

C: Serum from mice immunized with FCA
Mean (± SE) of 4 replicates

D: Mouse hyperimmune T. brucei 427 serum
Mean (± SE) of 5 replicates

No significant differences were seen between the various tests employing 427 trypanosomes. Parasites obtained from heavily infected (4+) mice instead of from rats gave similar results, justifying the subsequent use of rats as the major trypanosome source. Similarly, both inbred CBA and random bred albino mice, with or without their homologous counterparts, gave compatible results; so albino mice were usually the preferred source for both peritoneal cells and sera. It has been reported that a higher proportion of damaged or physiologically variable peritoneal cells may be obtained from outbred mice than from inbred and F_1 hybrid animals (Baughn & Bonventre 1975). These authors also noted, however, variations between different inbred strains. Mice of a constant age were also used, partially through ease of handling and culture, but also because the efficiency of the phagocytic response is known to decrease as the animal gets older (Benacerraf et al 1957). Sera obtained from different infection series also gave similar results (Fig. (5)). For details of collection and estimated parasitaemia, see Appendix (2).

With the more chronic strain of trypanosome (S42) and its homologous antisera, there was again an initial rise from control values (Fig. (6)), similar to that seen with the 427 trypanosomes and antisera. After the first week post infection, the levels tended to fluctuate, with the overall values being higher than those obtained with the homologous 427 system, despite the lower parasitaemias present (Appendix 2). Mouse hyperimmune S42 serum gave an increased attachment index, approximately twenty times higher than the control values. Low results were obtained using hyperimmune 427 serum with S42 trypanosomes, suggesting that attachment may be partly strain-specific.

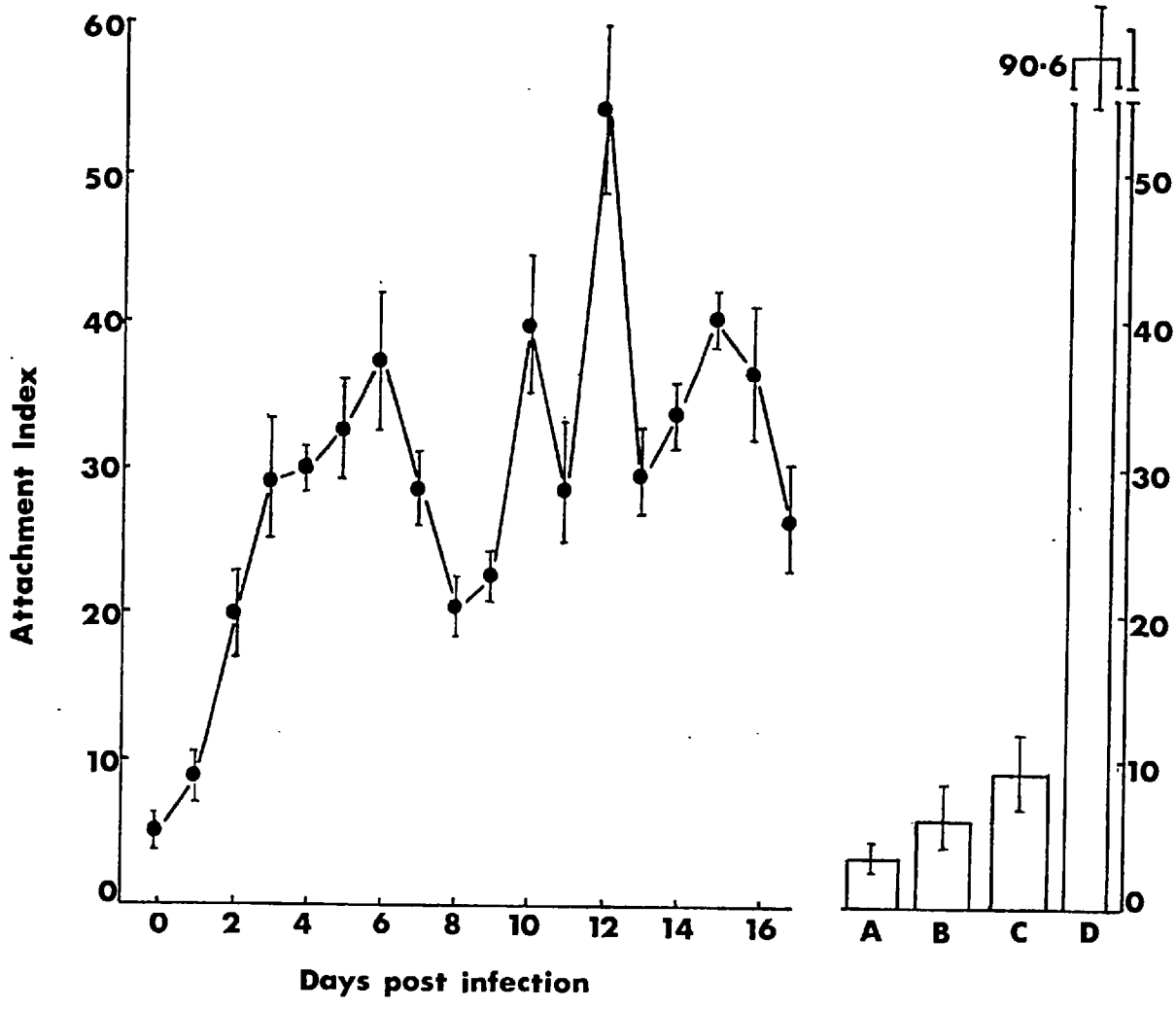

Attachment Index
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
Days post infection
90·6
A
B
C
D

Fig. 6.

Fig. 6. Attachment of *T. brucei* S42 obtained from infected rat blood to cultured mouse peritoneal macrophages.
Effect of homologous sera (opsonic test).

●——● Mean (± SE) of 8 replicates - serum series A'

A: PSG Mean (± SE) of 5 replicates

B: FCA Mean (± SE) of 5 replicates

C: Mouse hyperimmune *T. brucei* 427 serum
Mean (± SE) of 5 replicates

D: Mouse hyperimmune *T. brucei* S42 serum
Mean (± SE) of 5 replicates

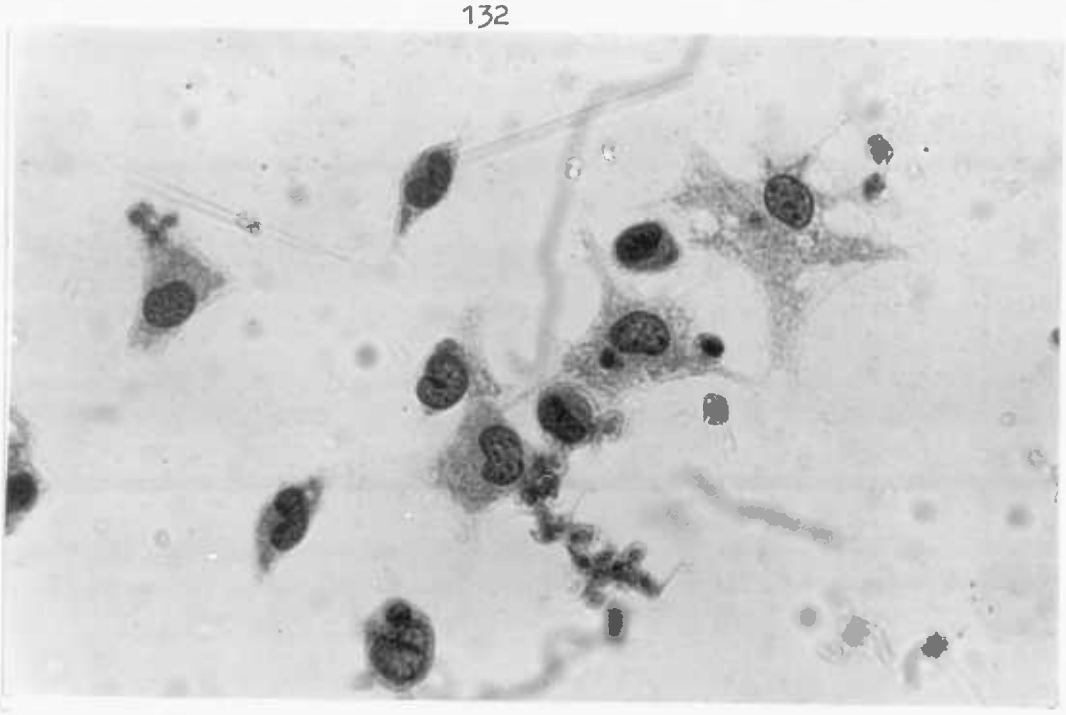


Plate E.

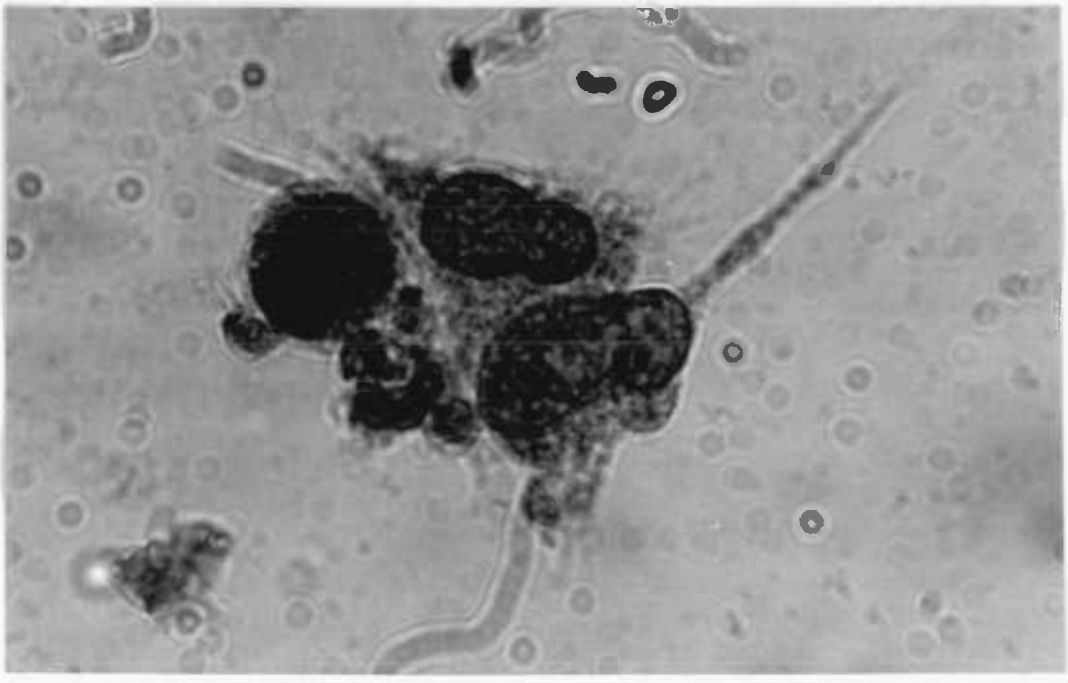


Plate F.

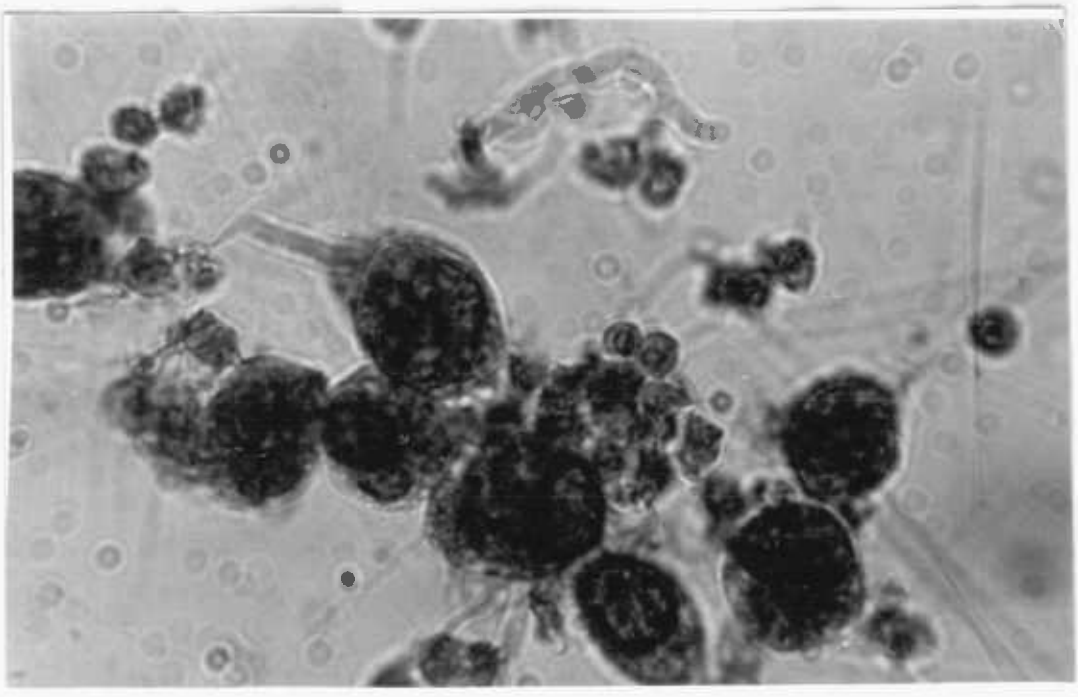


Plate G.

Plate E. Attachment of T. brucei 427 to cultured mouse peritoneal macrophages in the presence of mouse hyperimmune T. brucei 427 serum (x400 magnification).
A number of parasites may additionally be seen localised within the phagocytic cells.

Plate F.

Plate G. Attachment of T. brucei 427 to mouse macrophages in the presence of hyperimmune serum (x1000 magnification).
Note coalescence of macrophages and trypanosome agglutinins.

3.1.4. Variant Specificity within an Homologous System

It was considered possible that the serum-mediated attachment of trypanosomes to mouse macrophages might be dependent upon the variant surface antigens present within the trypanosome coat. If variant specific antibodies were needed for successful opsonization, then trypanosomes of a differing antigenic type appearing late in infection would not attach to macrophages in the presence of early immune sera.

Accordingly, parasites were isolated from individual mice initially infected with approximately 400 trypanosomes - T. brucei S42 - on days 4, 7 and 18 post infection. Each infection was subsequently raised in rats within 3 days and the opsonic test was performed using previously-prepared homologous immune sera from CD-1 mice (for further details concerning the preparation of isolates see Section 3.7.2).

Agglutinating antibody tests indicated indistinct antigenic differences between the three trypanosome isolates used; with only low titres present in the sera throughout infection. Antibodies were first detected eight days post infection (Appendix 3).

As indicated in Fig. (7), the trypanosome populations taken on each of the three occasions responded similarly in the presence of homologous antisera. The three tests showed, however, a very slight significant difference at the 1% level, although this appears primarily due to intrinsic variations within the test and not to variations in antibody response. No correlation was observed between the attachment index of each trypanosome isolate and its agglutinating antibody titre; indeed the highest initial response (circa 10% attachment) occurred with the day 18 population, despite the absence of detectable agglutinating antibody against this isolate, throughout the infection.

Although antigen differences appeared to exist between the days 4 and 18 isolates, these remained unclear and it is considered possible

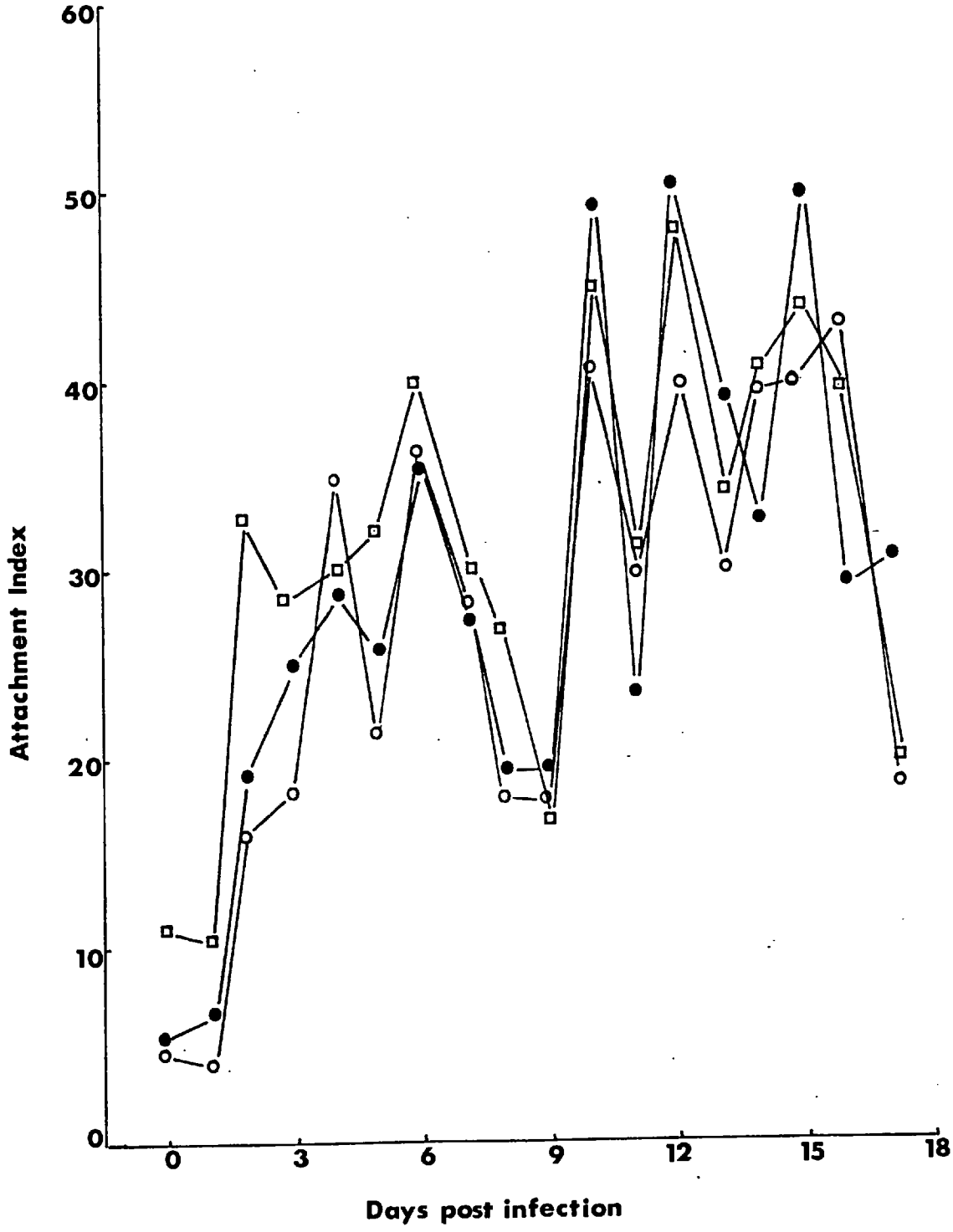

60
50
40
30
20
10
0
Attachment Index
0
3
6
9
12
15
18
Days post infection

Fig. 7.

Fig. 7. Attachment of T. brucei S42 isolated from mice on three occasions during infection, to mouse peritoneal macrophages in the presence of homologous sera (serum series A') (opsonic test).

●——● Isolated 4 days post infection

○——○ Isolated 7 days post infection

□——□ Isolated 18 days post infection

All results expressed are the mean (± SE) of 3 replicates.

that the trypanosome populations tested were not antigenically distinct. This may be partly a reflection of the selective method whereby different mice were used for the collection of sera and partly due to the possibility that the initial inoculum may have consisted of a mixed population.

3.1.5. Opsonization in an Heterologous System

In order to determine the specificity of opsonic attachment to mouse macrophages, an heterologous system comprising 427 trypanosomes and S42 antisera, was investigated.

As is evidenced from Fig. (8), generally low results, little different from control levels, were recorded throughout infection. The reaction showed a significant degree of strain specificity, with a low attachment index (2.5%) recorded even in the presence of heterologous hyperimmune serum. There was no correlation between the attachment index and parasitaemia (Appendix 2).

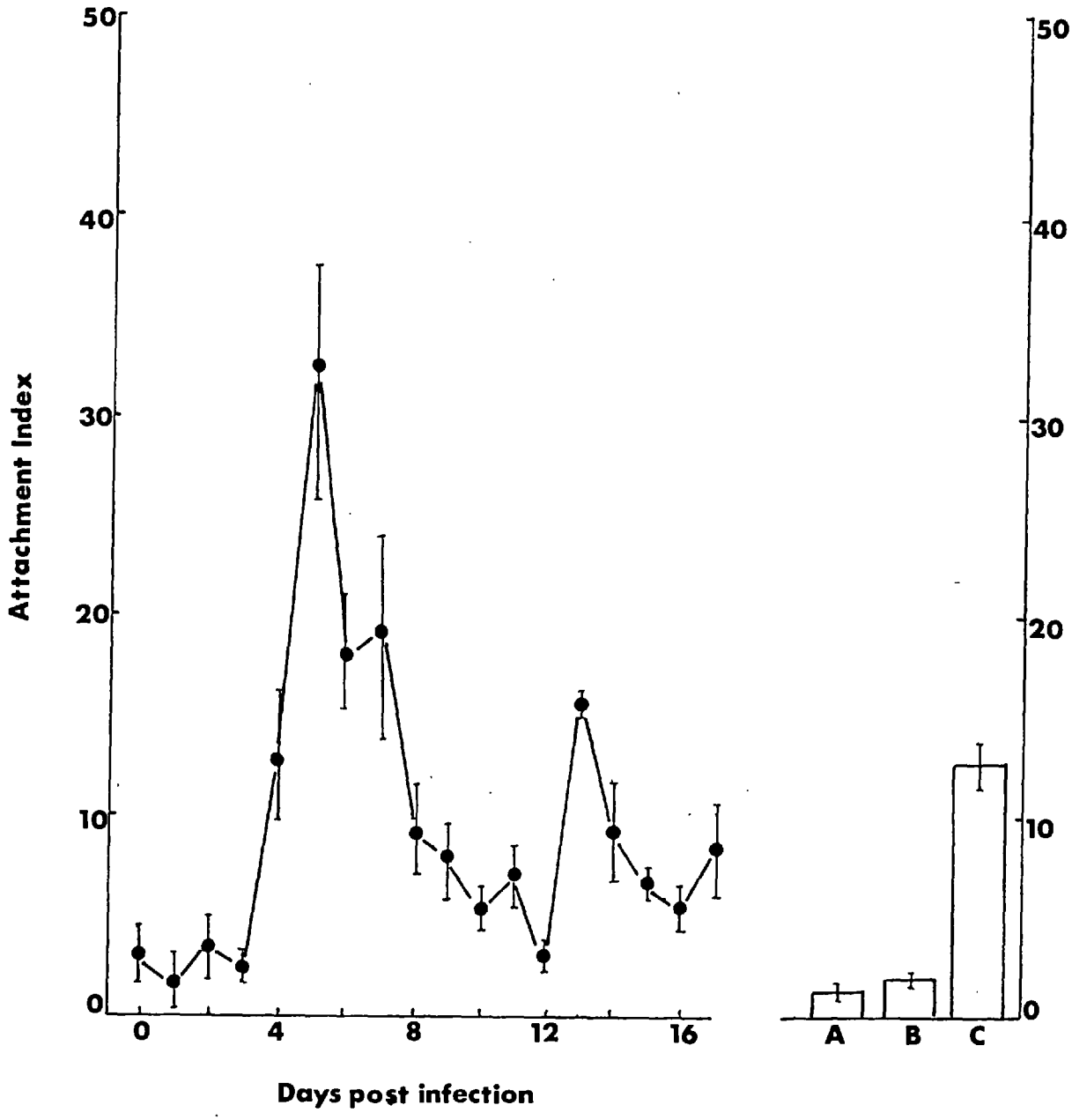


Fig. 8. Attachment of T. brucei 427 to mouse peritoneal macrophages. Effect of heterologous mouse sera (serum series A') (opsonic test).

A: PSG

B: NMS

C: Mouse hyperimmune T. brucei S42 serum

All results expressed are the mean (± SE) of 5 replicates.

3.1.6. Opsonization in Immune Mice

It was of interest to establish whether peritoneal macrophages from specifically-immune mice possessed an altered capacity to adsorb homologous trypanosomes in the presence of sera.

The opsonic test was carried out using T. brucei 427 and immune sera collected from albino mice infected with homologous trypanosomes. Homologous and heterologous hyperimmune sera, and hyperimmune serum absorbed with trypanosomes, were also tested.

Peritoneal macrophages were cultured from (a) normal, uninfected mice; (b) mice which had been inoculated intraperitoneally with whole blood containing T. brucei 427, three days previously and showed a 4+ parasitaemia; (c) five day infected mice similarly inoculated and showing a 4+ parasitaemia, and; (d) hyperimmune 427 mice.

High attachment indices were obtained using macrophages from immune mice (Fig. (9)). The figures from normal animals rose almost linearly from control levels and could be closely correlated with infective parasitaemia, whereas those from 3 and 5 day immune mice gave high, stable results, approximately ten times higher than uninfected control values - 26% attachment compared with 2%. The test sera had a slight but significant enhancing effect on the values obtained with the immune macrophage cultures, especially in the case of hyperimmune 427 serum, with attachment values of approximately 50%. Heterologous, absorbed homologous hyperimmune and uninfected sera all gave high attachment levels of about 20%, but which were, nevertheless, lower than those obtained with immune sera. The results using absorbed serum suggests that attachment is largely mediated by specific immune factors capable of being absorbed out of serum by live trypanosomes.

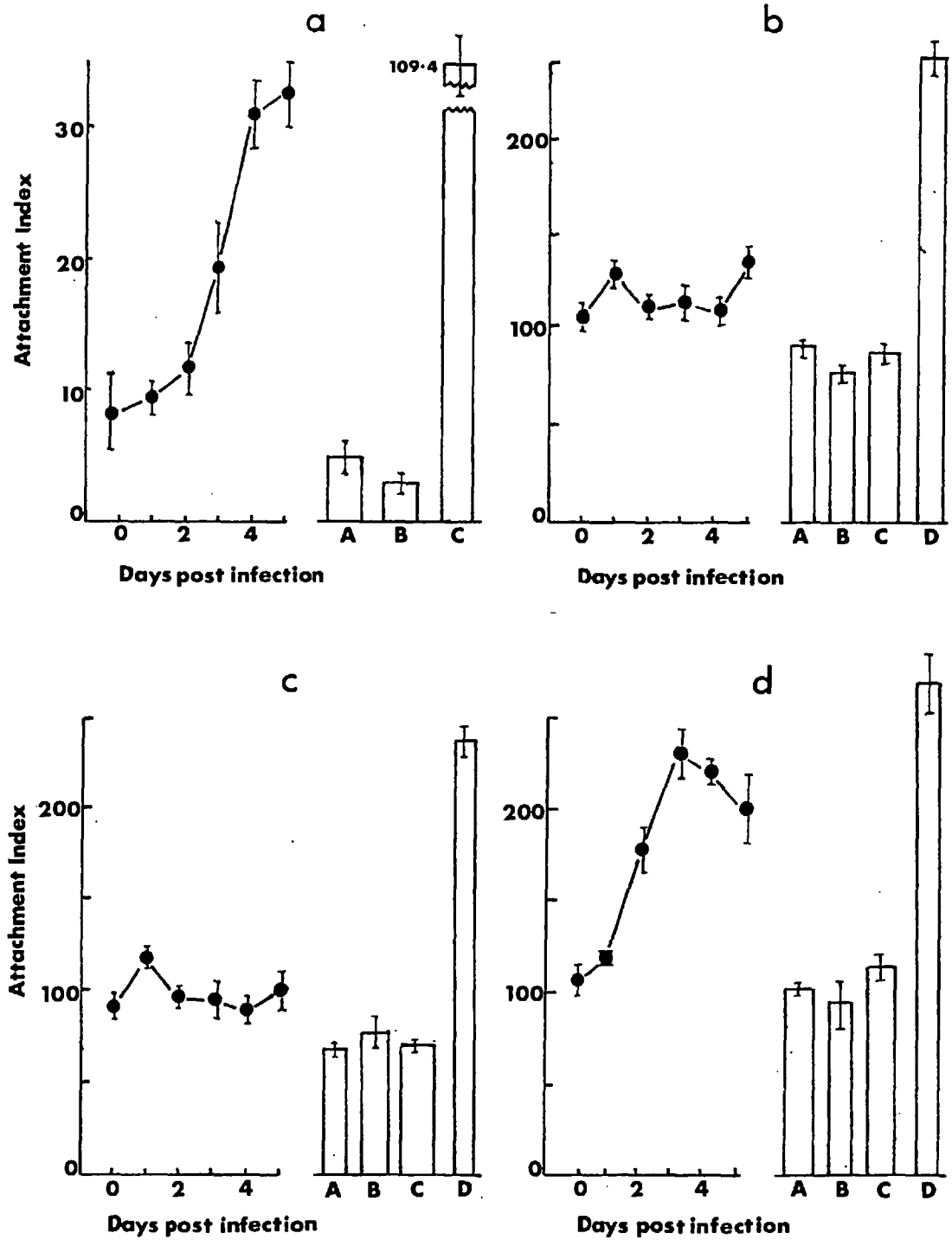
Highly significant differences existed between the responses obtained with monolayers from uninfected, day 3 and 5, and hyperimmune mice. The latter showed an initially high respone, similar to that

of the 3 and 5 day cultures, which gradually increased. In the presence of hyperimmune 427 serum over 50% of the macrophages counted had attached trypanosomes.

The high values obtained using specifically immune mice may have been due to the generation of trypanosome-specific antibody, capable of either indirectly activating the resident macrophage population, or of attaching cytophilically to the surface of individual mononuclear cells (cf: Kazar et al 1975). The amount of antibody produced by mice during a 3 or 5 day infection is small (Appendix 3), and it is known (Rabinovitch 1970), that a large quantity of specific antibody is required for effective cytophilia. Macrophage cytophilic antibody is also fairly easily eluted at 37°C., suggesting low attachment in both the peritoneal cavity of mice and after 24 h. in culture.

The intraperitoneal inoculation of large numbers of live trypanosomes, and of dead trypanosomes in FCA, could stimulate the cells in the peritoneum and act as effective inducing agents. Induced cells are often larger and more granular and show a greater surface-membrane activity. There exist fundamental differences in the amount and degree of discrimination possessed by activated and non-activated mouse peritoneal cells. Phagocytic mediation by Fc and C' receptors are altered and different Ig subclasses vary in importance (Bianco et al 1975). Further, phagocytes may themselves be altered directly by infection causing increases in both metabolism and enzyme content (Jenkin & Rowley 1963). Large numbers of monocytes could be recruited from the circulation and mature in the peritoneal cavity, elicited mononuclear cells are known to be a mixture of established peritoneal macrophages and much younger ones brought in from the blood (Karnovsky et al 1970).

In the case of hyperimmune mice, mycobacteria-induced necrosis could cause large-scale immigration and cell-activation. Trypanosome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Attachment Index
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100
200

Fig. 9.

Fig. 9. Attachment of T. brucei 427 to peritoneal macrophages cultured from non-infected, infected, and hyperimmune CD-1 mice.

Effect of homologous infected serum, homologous and heterologous hyperimmune sera and hyperimmune serum absorbed with live trypanosomes (opsonic test).

●———● Mean (± SE) of 5 replicates. Serum series C.

(a) Macrophages from normal, uninfected mice

A: PSG

B: NMS

C: Mouse hyperimmune T. brucei 427 serum

(b) Macrophages from 3 day infected mice

(c) Macrophages from 5 day infected mice

(d) Macrophages from hyperimmune 427 mice

A: NMS

B: Mouse hyperimmune T. brucei S42 serum

C: Absorbed mouse hyperimmune T. brucei 427 serum

D: Mouse hyperimmune T. brucei 427 serum

All results expressed are the mean (± SE) of 5 replicates.

antibody complexes could also act chemotactically, and high titres of agglutinating antibody might be present.

During the normal culturing of mononuclear cells, therefore, free opsonizing and/or cell-sensitizing antibody present within the peritoneal cavity, could be transferred along with the macrophages (Steinman & Cohn 1974; Emeis & Wisse 1975). In order to determine whether the increased activity observed with macrophages from hyperimmune mice was due to this possibility, the peritoneal exudate cells were thoroughly washed in culture medium before seeding. The attachment of T. brucei 427 was subsequently measured in the presence of NMS, infected, hyperimmune, and absorbed hyperimmune 427 sera.

Certain macrophage monolayers were treated with trypsin (B.D.H. Chemicals Ltd.) at a concentration of 1×10^{-5}% in TCM and iodoacetate (B.D.H. Chemicals Ltd.) at a concentration of 0.5mM in TCM, before testing. The former often has a slight enhancing effect on opsonization (Werb 1975), the latter an inhibiting effect, under normal conditions. Trypsin can also preferentially remove IgG molecules from passively sensitized macrophages (Hoy & Nelson 1959). The TCM in individual Leighton tubes containing macrophage monolayers, was aspirated and replaced with 1.0ml. of either trypsin or iodoacetate. The solutions were allowed to cover the monolayers and the tubes were re-incubated at 37°C. for 30 min. Control monolayers were similarly treated with fresh TCM alone. The macrophages were washed in BSS and tested normally. It has been suggested that the perfunctory washing of glass-adherent macrophage monolayers may not, necessarily, be sufficient to remove non-adherent cells or test particles (Baughn & Bonventre 1975). In all experiments, however, very few unattached trypanosomes were seen in the final preparation, and as the ratio of trypanosomes to macrophages was invariably greater than 100:1, it was considered that thorough washing in fresh saline was sufficient.

The results (Fig. (10)), indicated small but statistically insignificant differences between macrophages taken from hyperimmune mice and cultured direct, and those that had been thoroughly washed to remove weakly-sensitizing cell antibody. The slight differences were probably a reflection of the increased fragility of the macrophages following repeated, centrifugal washing.

Trypsinization of hyperimmune macrophages before the addition of sera and trypanosomes had no significant effect upon attachment. Iodoacetate, however, had a profound inhibitory effect in the presence of hyperimmune 427 serum, reducing attachment five fold.

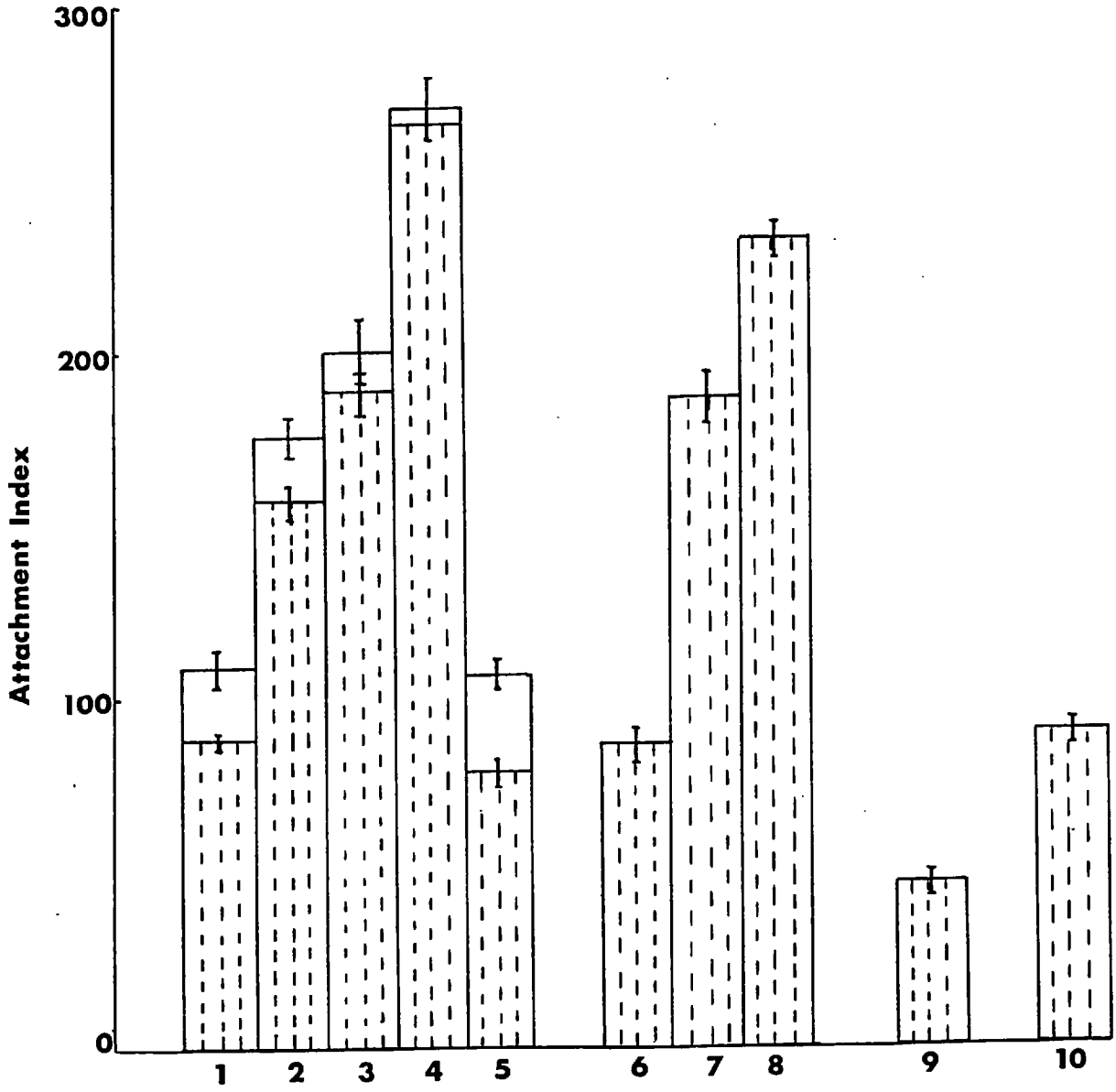


Fig. 10.

Fig. 10. Attachment of T. brucei 427 to peritoneal macrophages from hyperimmune mice, (a) cultured direct, and (b) washed in TCM before seeding (opsonic test).

cultured direct

washed before seeding

1. NMS
2. T. brucei 427 - 2 day infection - serum series C
3. T. brucei 427 - 5 day infection - serum series C
4. Mouse hyperimmune T. brucei 427 serum
5. Absorbed mouse hyperimmune T. brucei 427 serum
6. NMS
7. Day 5 infection serum
8. Hyperimmune T. brucei 427 serum

} macrophage monolayers pre-treated with 1×10^{-5}% trypsin in TCM

9. Hyperimmune T. brucei 427 serum — macrophage monolayers pre-treated with 0.5mM iodoacetate in TCM
10. PSG

All results expressed are the mean ($\pm$ SE) of 5 replicates.

3.1.7. Effect of Complement

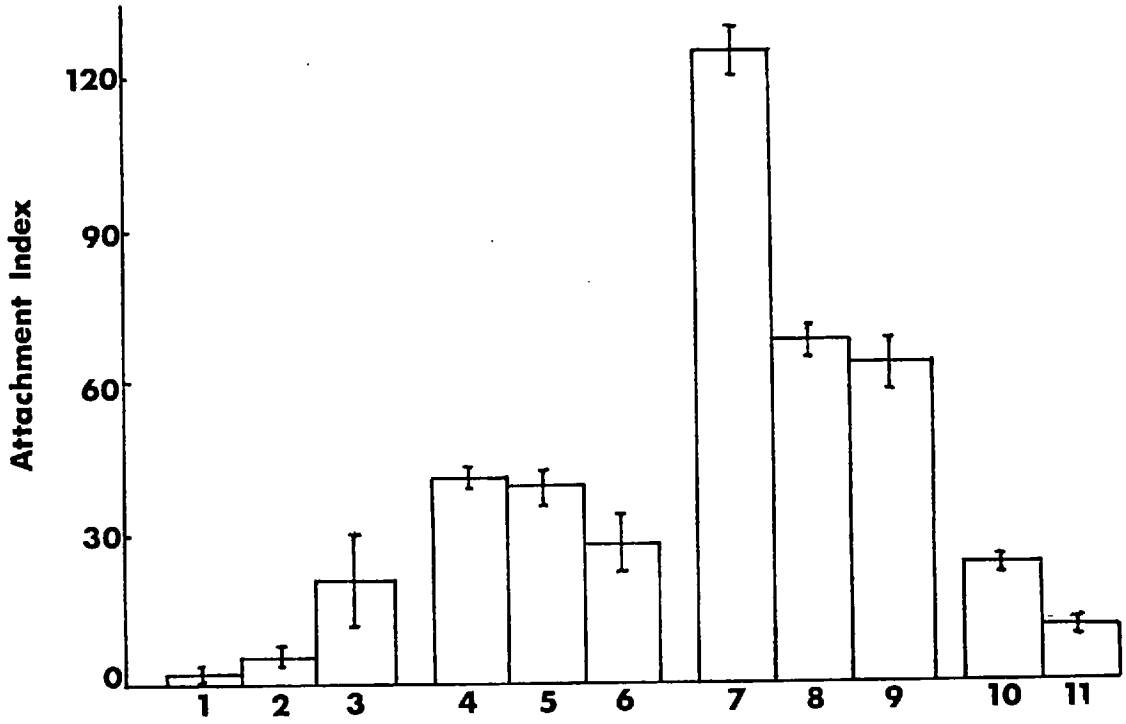
The opsonic test was performed in order to determine the effect of heat-inactivated sera in mediating the attachment of T. brucei 427 to normal mouse peritoneal macrophages.

Pooled sera from T. brucei 427-infected mice and hyperimmune sera were tested. The source of artificially high levels of C' was reconstituted guinea pig serum (Wellcome Reagents Ltd.), at a physiological dilution of 1:10, it was either used alone or was added to an equal volume of previously inactivated serum.

Fig. (11), indicates the low opsonic values obtained using pooled sera (days 0 and 1, days 5 and 6). Heat inactivation alone and heat inactivation followed by the addition of guinea pig serum had no effect, all treatments showing statistical similarity. The enhanced opsonic activity expressed by hyperimmune 427 serum, was markedly reduced following heat treatment, but was not restored upon the addition of guinea pig serum. Guinea pig serum itself, as a non-specific C' source, had no enhancing effect either alone or in conjunction with heat inactivated sera, suggesting that the opsonic integrity of immune mouse serum depends largely upon specific serum factors.

Heating sera to 56°C. for 30 min. would be expected to affect serum substances other than complement. Different C' components themselves vary in their heat lability; non-specific serum antibodies and other globulins could be denatured or altered by such treatment, and the site specificity of immune substances accordingly affected. Heating to 56°C., might cause the partial aggregation of γ globulin or albumin and globulin. These macromolecular complexes could reduce in vitro opsonization simply by removing available free antibody.

Similar results were obtained when sera from mice infected with T. brucei S42 were tested (Fig. (12)). Heat-inactivated day 15 immune serum tested homologously, showed reduced opsonic activity which remained unaltered upon the addition of guinea pig serum.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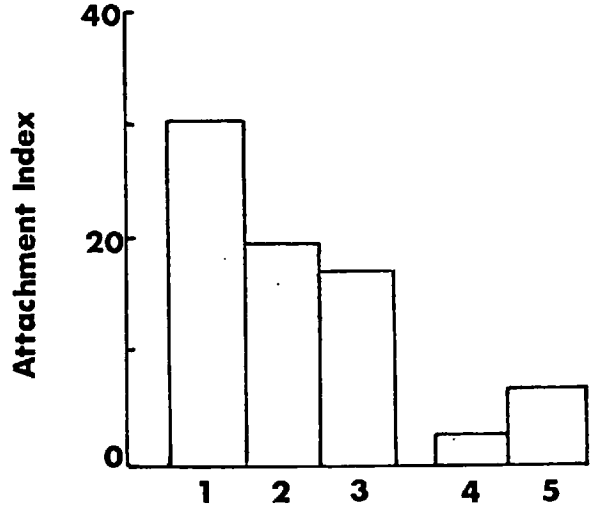
11

Fig. 11.


Attachment Index
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5

Fig. 12.

Fig. 11. Attachment of T. brucei 427 to mouse peritoneal macrophages (opsonic test).
Effect of homologous heat-inactivated sera (56°C for 30 min) (serum series B).

1. Pooled sera days 0 + 1
2. Pooled sera days 0 + 1 - heat inactivated
3. Pooled sera days 0 + 1 - heat inactivated - plus guinea pig serum

4. Pooled sera days 5 + 6
5. Pooled sera days 5 + 6 - heat inactivated
6. Pooled sera days 5 + 6 - heat inactivated - plus guinea pig serum

7. Mouse hyperimmune T. brucei 427 serum
8. Mouse hyperimmune T. brucei 427 serum - heat inactivated
9. Mouse hyperimmune T. brucei 427 serum - heat inactivated - plus guinea pig serum

10. Guinea pig serum (1/10 dilution)
11. Guinea pig serum - heat inactivated

All results expressed are the mean ($\pm$ SE.) of 5 replicates.

Fig. 12. Attachment of *T. brucei* S42 to mouse peritoneal macrophages (opsonic test).
Effect of homologous heat-inactivated immune serum (56°C for 30 min) (serum series A').

1. Day 15 immune serum
2. Day 15 immune serum - heat inactivated
3. Day 15 immune serum - heat inactivated - plus guinea pig serum
4. Guinea pig serum (1/10 dilution)
5. Guinea pig serum - heat inactivated

3.1.8. Effect of Complement and IgM

Pooled immune and hyperimmune 427 sera were treated with 0.1M 2-mercaptoethanol, and hyperimmune serum was, in addition, heated to 65°C. for 30 min.

The results in Fig. (13), indicate that mercaptoethanol treatment of immune sera did not affect their opsonic activities. A slight reduction was noted with hyperimmune serum, suggesting that IgM antibodies play a small, although hardly significant role in opsonization. This was further supported by the finding that heat-treatment, designed to destroy both IgM and C' activity, had no effect on the number of attachments recorded. Indeed, the addition of guinea pig serum to heat-treated hyperimmune sera resulted in a further fall in opsonic activity. It is possible that guinea pig serum may inhibit opsonization either by competitively coating available receptor sites or affecting serum components, rendering them ineffective at mediating attachment. Volanakis & Kaplan (1974), indicated that selectivity for human as opposed to guinea pig C' could occur in sera containing rheumatoid factor (RF) and aggregated IgG, and for CRP binding. They suggest limitations on either the binding capacity or on the structural specificity of C' (most especially C1q) between different species.

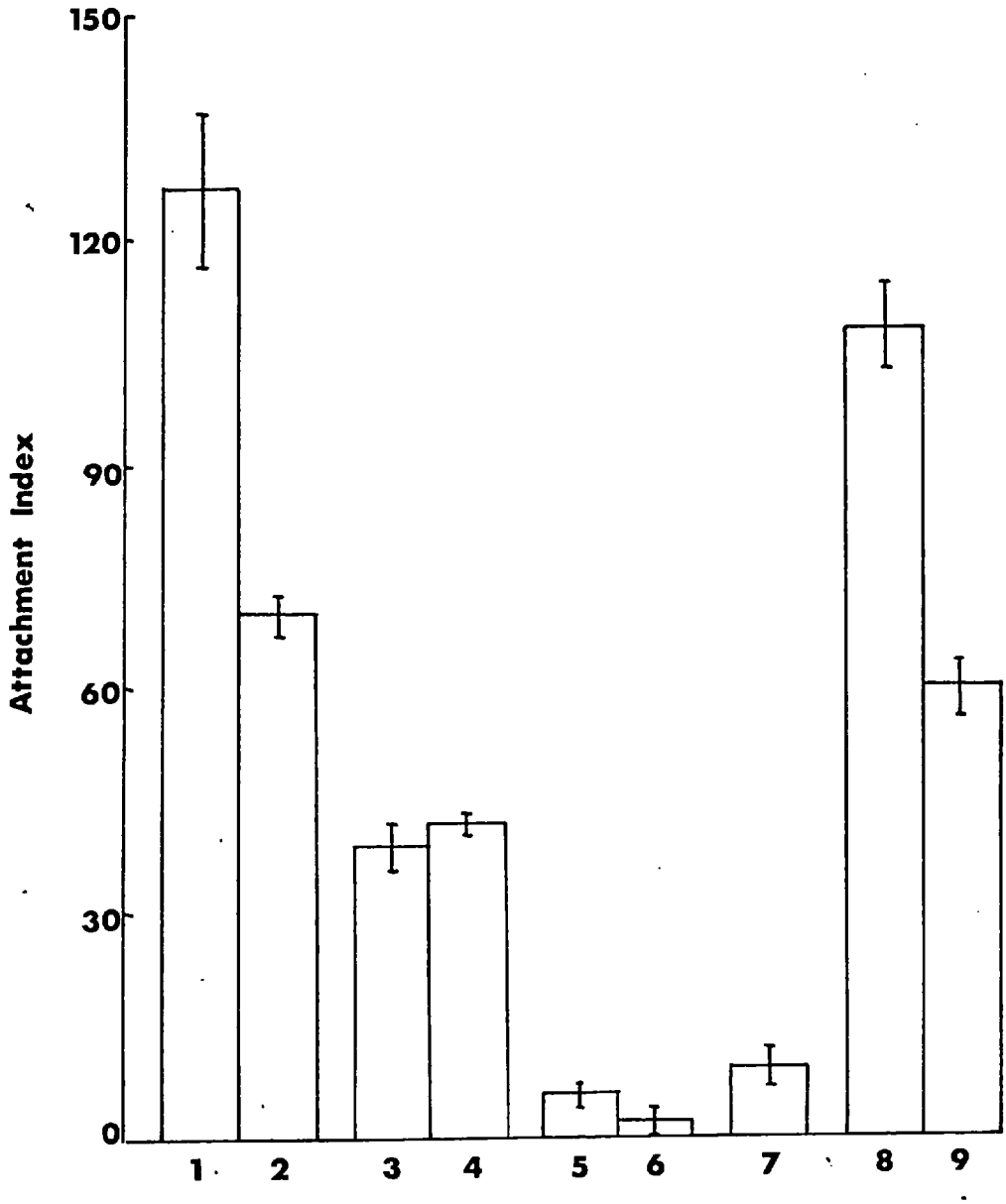

Fig. 13.

Fig. 13. Attachment of T. brucei 427 to mouse peritoneal macrophages (opsonic test).
Effect of heat inactivation (65°C for 30 min) and mercaptoethanol treatment of immune (serum series B), and hyperimmune sera.

1. Mouse hyperimmune T. brucei 427 serum
2. Mouse hyperimmune T. brucei 427 serum - incubated with 0.1M 2-mercaptoethanol

3. Pooled sera days 5 and 6
4. Pooled sera days 5 and 6 - incubated with 0.1M 2-mercaptoethanol

5. Pooled sera days 0 and 1
6. Pooled sera days 0 and 1 - incubated with 0.1M 2-mercaptoethanol

7. 0.1M 2-mercaptoethanol

8. Mouse hyperimmune T. brucei 427 serum - heat inactivated
9. Mouse hyperimmune T. brucei 427 serum - heat inactivated - addition of guinea pig serum (1/10 dilution)

All results expressed are the mean ($\pm$ SE) of 5 replicates.

3.1.9. Effect of Drugs and Chemicals

Numerous substances including many naturally-occurring compounds, surfactants and pharmacological agents have been shown to elicit in cells a variety of metabolic reactions which are often indistinguishable from those occurring during phagocytosis. A series of both pharmacologically and physiologically-active agents have been implicated in general inflammatory reactions, capable of affecting vascular and capillary permeability, cell emigration and tissue and cellular homeostasis (Elsbach 1974). Trypanosome infections caused by brucei sub-group organisms are themselves characterized by gross inflammation and intense tissue necrosis associated with widespread physiological disturbances to the host. Large numbers of active mononuclear cells are typically seen in extravascular tissues and necrotic fluids (Goodwin 1970). Several pharmacologically-active substances have been directly implicated in these chronic inflammatory responses and they and their tissue products may be largely responsible for the mediation of large scale vascular and metabolic changes so typical of chronic trypanosomiasis.

Various drugs and chemicals which were either known to be important mediators in inflammatory responses, had been reported to considerably enhance the attachment and phagocytosis of particulate antigen, or were believed effective in altering normal cellular metabolism, were investigated.

The effect of these substances, either alone or in conjunction with serum proteins, on the direct in vitro attachment of trypanosomes to mononuclear phagocytes, was considered.

The opsonic test was performed using mouse peritoneal macrophages and strain 427 trypanosomes. Compounds were tested either alone or with 10% NMS or 10% hyperimmune 427 serum (Table (2)). All dilutions were prepared in sterile glass bottles using TCM. If necessary, the pH was adjusted to 8.0 by the addition of 4.4% sterile sodium bicarbonate

solution (Wellcome Reagents Ltd.). The drugs Berenil and Mel B were either used neat or dissolved in distilled water, according to the manufacturers' instructions. For the test involving trypain and specific trypsin inhibitor, prior incubation of the macrophage monolayer with 1.0 ml. of the trypsin solution was carried out for 30 min. at 37°C. The treated cells were then washed five times in 0.85% physiological saline and re-incubated with 1.0 ml. of the trypsin inhibitor for 5 min. at 37°C. All test samples were initially incubated at 37°C. for 15 min. and then allowed to adjust to room temperature (22°C.), before use. Macrophage monolayers were washed in 0.85% physiological saline before testing.

The TCM, in which the chemicals were dissolved, contained available Ca^{++} and Mg^{++} ions (in the form $CaCl_2$ and $MgSO_4$ $7H_2O$). These two factors have been considered necessary for both cell spreading (Cohn 1975) and attachment and phagocytosis (Mudd et al 1934), although the cytophilic role of Ca^{++} ions remains equivocal (Davey & Asherson, 1967) and Hirsch & Strauss (1964), have indicated that these ions may not necessarily be a pre-requisite for successful opsonization - other factors such as a hypotonic environment may be more important (Hirsch 1974). Nevertheless, most monolayer washings were made in BSS (containing Ca^{++} and Mg^{++}), with a few in physiological saline (containing Ca^{++}) (see Appendix I).

Table (2)

Chemical	Concentration	Alone	Hyper-Immune	NMS
HISTAMINE (BDH Chemicals Ltd.)	0.2% w/v	+	+	+
5-HYDROXY TRYPTAMINE (BDH Chemicals Ltd.)	0.2% w/v	+	+	+
TRYPSIN (BDH Chemicals Ltd.)	0.2% w/v	+	+	+
TRYPSIN (BDH Chemicals Ltd.)	10µg/ml	+	+	
F.C.A. (Difco)	Neat	+	+	+
DETERGENT (Lab-Brite BHC)	0.01ml/ml	+	+	
BERENIL (Diminazene Aceturate) (Hoescht)	0.175mg/ml	+	+	
MEL B (Melasen Oxide/Bal) (May & Baker)	0.075mg/ml	+	+	
D-BIOTIN (Vit H) (BDH Chemicals Ltd.)	0.15µg/ml	+	+	
ADRENALIN (BDH Chemicals Ltd.)	0.01µg/ml	+	+	
BRADYKININ (Sandoz Products Ltd.)	0.01µg/ml	+	+	
ACETYL CHOLINE CHLORIDE (BDH Chemicals Ltd.)	0.01µg/ml	+	+	
ZYMOSAN A (Sigma)	10µg/ml	+	+	
TRYPSIN INHIBITOR (Soya bean) (BDH Chemicals Ltd.)	10µg/ml	+	+	
TRYPSIN + TRYPSIN INHIBITOR	-	+	+	
ATP (Sigma)	0.4mg/ml	+		
DEXTRAN (BDH Chemicals Ltd.)	0.2% w/v	+		

Table (2) - (Cont.)

Chemical	Concentration	Alone	Hyper-Immune	NMS
CASEIN (Hopkin & Williams)	5mg/ml	+		
SODIUM AZIDE (BDH Chemicals Ltd.)	1%		+	
IODOACETATE (BDH Chemicals Ltd.)	0.5mM		+	
FORMALDEHYDE (BDH Chemicals Ltd.)	0.1%		+	

The results in Fig. (14) indicate that chemicals alone were not usually sufficient to initiate widespread attachment of trypanosomes to mouse peritoneal macrophages. Low, control values of approximately 3% were expressed using histamine, detergent, casein, ATP and FCA. Slightly higher levels of about 10% attachment were obtained with trypsin, at both concentrations, and dextran. It is known that dextran is readily phagocytosed *in vivo* (Bowers 1970), and is capable of increasing the phagocytic index (K) of rats (Stiffel *et al* 1970) and inducing pinocytosis (Cohn 1970). Large increases of around 30% were seen with 5-HT. The trypanocidal drugs Berenil and Mel B gave about 10% attachment, as did adrenalin. Biotin, acetylcholine, zymosan and the vasodilator bradykinin, however, gave lower values, but which were, nevertheless, usually higher than the control levels obtained with PSG or NMS. A certain amount of non-specific site competition may be involved, especially in the case of zymosan. Zymosan particles, themselves, can by phagocytosed and could partially block macrophage receptor sites (Crowder *et al* 1969). Macrophages pretreated with trypsin, followed by trypsin inhibitor, showed over 10% attachment, significantly higher than that obtained when macrophages and trypanosomes were incubated with trypsin alone.

Upon mixing with 10% NMS prior to testing, the opsonic activities of both histamine and trypsin increased three and four fold respectively. Values for FCA remained low, although this may well have been a reflection of its general immiscibility. Many adjuvants are considered to be surface active molecules. FCA may affect phospholipid metabolism and be cytotoxic towards macrophages (Munder *et al* 1970). Again, a high attachment index was recorded using 5-HT in association with NMS.

In the presence of 10% hyperimmune 427 serum, increased values were obtained for nearly all treatments. In most instances, however, these were similar to hyperimmune control levels (*circa* 20% attachment); significantly higher results being obtained with Berenil, biotin and 5-HT.

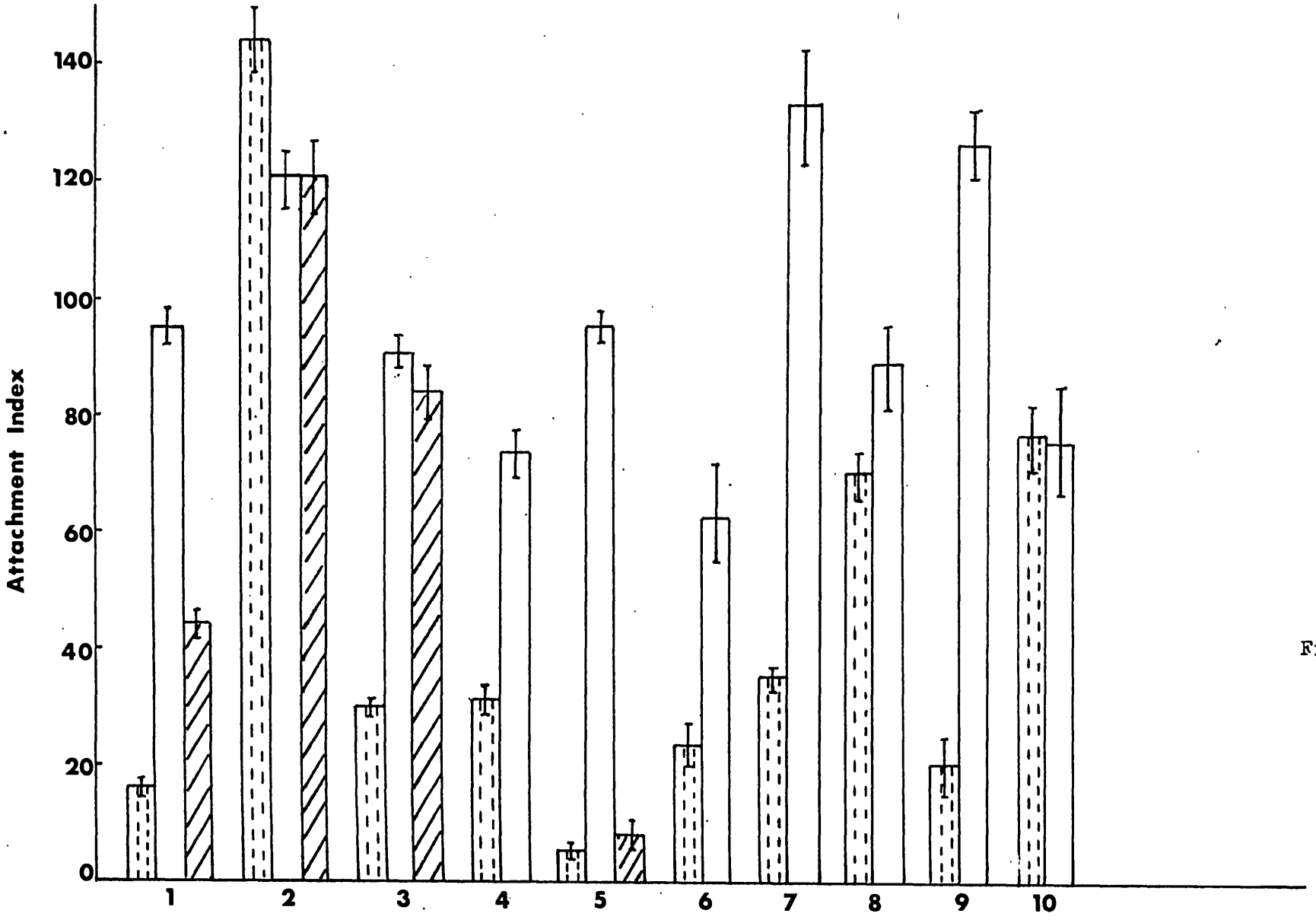

Fig.14. /

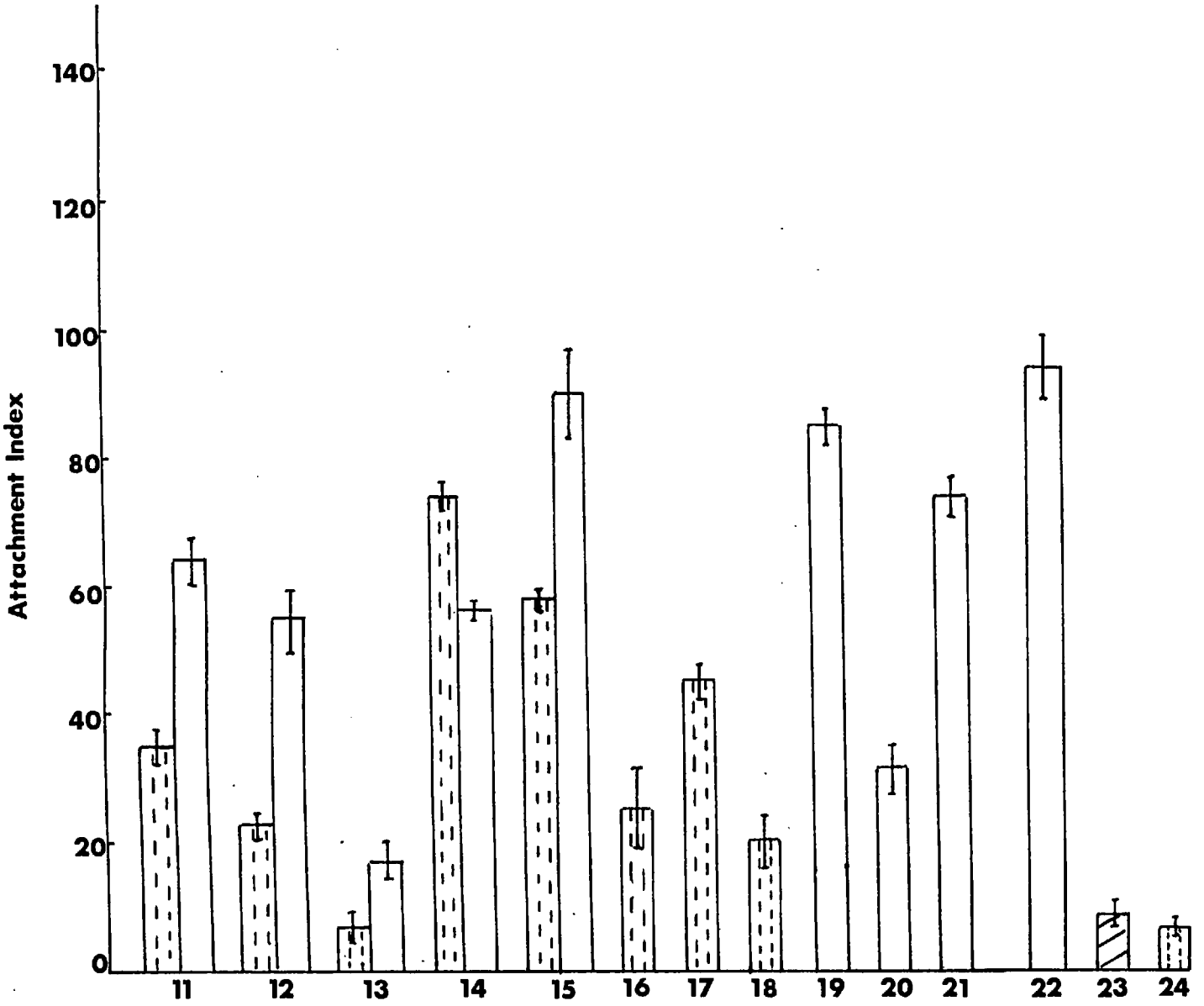
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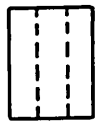
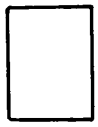
Fig. 14. (contd.)

Fig. 14. Attachment of T. brucei 427 to mouse peritoneal macrophages (opsonic test).

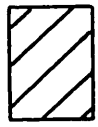
Effect of chemicals alone and in conjunction with NMS and hyperimmune 427 serum.



Test substance alone



Test substance plus 10% mouse hyperimmune T. brucei 427 serum



Test substance plus 10% NMS

1. Histamine
2. 5-HT
3. Trypsin (0.2%)
4. Trypsin (10µg/ml)
5. FCA
6. Detergent
7. Berenil
8. Mel B
9. D - Biotin
10. Adrenalin
11. Bradykinin
12. Acetyl choline chloride
13. Zymosan A
14. Trypsin inhibitor
15. Trypsin plus trypsin inhibitor
16. ATP
17. Dextran
18. Casein
19. Sodium azide
20. Iodoacetate
21. Formaldehyde
22. Mouse hyperimmune serum
23. NMS
24. PSG

All results expressed are the mean ($\pm$ SE) of 5 replicates.

Low values, invariably showing less than 10% attachment, were recorded with iodoacetate, acetyl choline and zymosan. The reaction of zymosan with hyperimmune serum may well have caused the fixation of large quantities of C3 (Henson & Cochrane 1969), which could have reduced the opsonic efficiency of the immune serum.

Most of the chemicals at the concentrations and for the period of time they were employed, appeared to have no significant effects on the morphological appearance of the cultured macrophages or on the motility of the trypanosomes. Detergent, however, at a variety of concentrations, caused a marked reduction in trypanosome activity, and even at concentrations of 1×10^{-5}ml/ml, could well have been toxic. The glycolytic inhibitor Mel B had no directly observable effects on either macrophages or trypanosomes, but when Berenil was used, approximately 20% of the macrophages observed were vacuolated and some partially disrupted. Trypanosome motility was markedly reduced.

These results initially indicated that 5-hydroxytryptamine (5-HT) either alone or in conjunction with NMS or hyperimmune 427 serum, was capable of significantly enhancing attachment. 5-HT has previously been reported as enhancing both macrophage phagocytosis (Northover 1961), and that of Tetrahymena pyriformis (Csaba & Lantos 1973). Different concentrations of 5-HT in TCM were therefore prepared and their efficacy tested to determine the optima.

The results (Fig. (15)), indicate slow but progressive decreases in activity for all treatments, from concentrations of 2×10^{-1} to 2×10^{-5} w/v. The values for 5-HT alone and with NMS, show close similarity, suggesting that natural serum factors are not needed for opsonization. The significantly higher levels obtained with hyperimmune 427 serum may reflect both the enhancing effect of the chemical and the intrinsic opsonic activity of the serum.

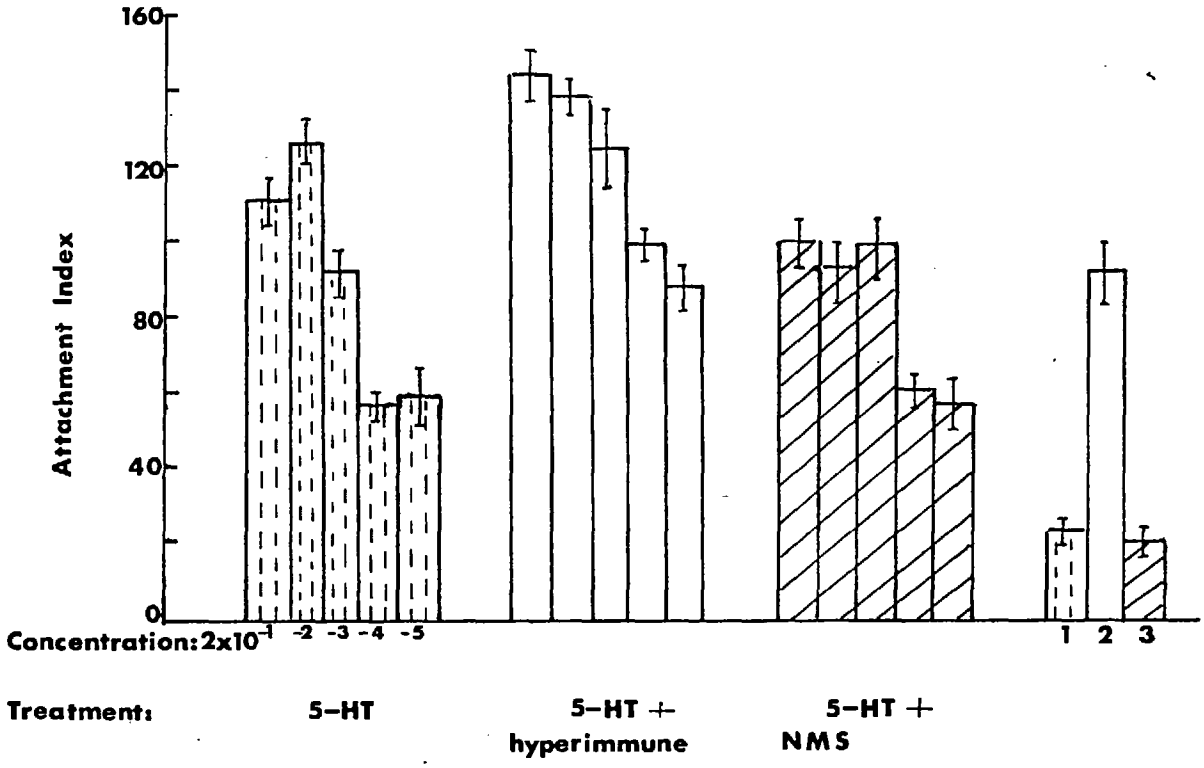


Fig. 15. Attachment of T. brucei 427 to mouse peritoneal macrophages (opsonic test).

Effect of different concentrations of 5-HT alone and in conjunction with NMS and hyperimmune sera.

1. PSG
2. Mouse hyperimmune T. brucei 427 serum
3. NMS

All results expressed are the mean (± SE) of 5 replicates.

3.1.10. Effect of Absorbed Sera

Previous experiments (3.1.6.) had indicated that the opsonic activity of hyperimmune 427 serum could be absorbed out by live, homologous trypanosomes. The effect, therefore, of non-homologous parasites and normal mouse tissue was determined.

The opsonic test using T. brucei 427 and mouse peritoneal macrophages was performed using hyperimmune S42 and 427 sera absorbed with homologous and heterologous trypanosomes, homogenized mouse liver in buffer, and PSG.

The results are presented in table (3). The opsonic activity expressed by sera absorbed with homologous and heterologous trypanosomes was markedly reduced, suggesting that trypanosomes are capable of adsorbing specific serum factors responsible for opsonization. Although hyperimmune 427 serum lost its opsonic ability when absorbed with heterologous (S42) trypanosomes, it was not able to enhance the attachment of live S42 trypanosomes when mixed in the presence of macrophage monolayers (section 3.1.5.). This could be a reflection of incubation time or competing factors perhaps modifying cytophilic attachment, hence effecting a degree of phagocytic discrimation.

Hyperimmune serum absorbed with normal liver homogenate similarly lost its opsonic ability, indicating that the effective serum factors may not be trypanosome-specific. Alternatively, non-specific-cytophilic absorption by mouse erythrocytes or specific absorption by liver phagocytes could have occurred.

SERUM	ABSORBED WITH	ATTACHMENT INDEX[1]	STD. DEV.[2]
Mouse hyperimmune 427	P.S.G.	108.6	14.1
Mouse hyperimmune 427	*T. brucei* 427	24.0	5.6
Mouse hyperimmune 427	*T. brucei* S42	21.8	4.5
Mouse hyperimmune 427	Liver homogenate	21.6	5.0
Mouse hyperimmune S42	P.S.G.	11.8	3.1
Mouse hyperimmune S42	*T. brucei* S42	30.6	7.1

TABLE 3: Absorption of hyperimmune sera with homologous and heterologous trypanosomes and tissue homogenate Opsonic test using *T. brucei* 427 and murine macrophages.

1. Mean of 5 replicates
2. Standard Deviation

3.1.11. Effect of Foreign Particles in the Blood Clotting Mechanism

Due to the reports that particle recognition may be mediated via interdediaries of the fibrinolytic system (Wilkins & Myers 1966, Wilkins 1971), the possibility of normal serum proteins coating the trypanosome surface and modifying site recognition, was investigated.

The opsonic test using T. brucei 427 and mouse peritoneal macrophages was used and the following treatments undertaken.

1. It was considered that during the normal separation and washing of trypanosomes prior to experimentation, any naturally-adhering host serum proteins would be removed (cf. Rowley 1966). Although both trypanosomes and macrophages may be subsequently sensitized with normal, immune and hyperimmune sera in vitro, the initial in vivo possession of non-specific serum components could act concomitantly or be an essential pre-requisite for recognition. Accordingly, 427 trypanosomes were removed by cardiac puncture from heavily infected (4+) rats, the whole blood was placed in heparinized tubes and centrifuged at 1500 x g for 5 min. Part of the buffy layer containing the concentrated trypanosomes was removed and re-centrifuged at less than 300 x g for 3 min. The trypanosomes present in the supernatant were removed and resuspended at a concentration of 1.0×10^8/ml. in the original rat plasma. A number of erythrocytes, white blood cells and platelets were present in the final suspension, but these could only have been satisfactorily removed by further washings. The trypanosomes were used immediately, their motility being checked microscopically before hand.

2. Trypanosomes were collected as in treatment 1. Instead of using heparinized tubes, the blood was collected in plastic tubes containing ethylenediaminetetraacetic acid dipotassium salt (E.D.T.A.) 2 mg/ml.

3. Trypanosomes were collected in EDTA and separated on a DEAE cellulose column. They were eluted with one washing of PSG, approx. 75 ml. and then concentrated in the same wash of PSG. The trypanosomes were thus effectively freed from blood cells, but not from strongly-adherent serum proteins. Ten per cent NMS was added and the live suspension used immediately.

4. Trypanosomes which had been collected in heparinized tubes, were separated, washed and concentrated in PSG. Ten per cent NMS was added immediately before use.

5. A suspension of separated, washed trypanosomes in PSG was added in a 1:1 ratio to sonicated rabbit fibrin. The fibrin, at a concentration of 30 mg/ml. in saline/EACA (E-aminocaproic acid) had been previously ultrasonicated at 20,000 c/s for between 5 and 15 min. at 4°C. EACA at a concentration of 50mg/100 ml. saline was used to prevent fibrinolysis (Facer 1974).

6. 0.2% w/v trypsin (Gurr) in TCM was added to a suspension of washed trypanosomes and maintained at 37°C. for 30 min. before use.

7. As in treatment 5, plus the addition of 10% NMS.

8. As in treatment 6, plus whole normal mouse plasma added in a 1:1 ratio.

9. One ml. of separated, but unwashed trypanosomes, previously collected in EDTA, was mixed with an equal volume of thrombin (Leo Pharmaceutical Co. Ltd.) at 50U/ml. in 0.5M calcium chloride and incubated at 37°C. for 30 min.

10. Thrombin and rabbit fibrin were incubated at 37°C. for 30 min. and to this was added washed and separated trypanosomes which had been collected in heparinized tubes. Ten per cent NMS was added immediately before use.

11. Trypanosomes were separated from heparinized blood, washed and concentrated in PSG.

The results are shown in Fig. (16). Unwashed, unseparated trypanosomes collected in heparinized tubes gave fairly low values for attachment (circa 6%), whereas those which were collected in EDTA tubes gave higher results. Similarly enhanced values were seen using partially washed trypanosomes in EDTA mixed with NMS, but reduced attachment was again observed with separated trypanosomes, either alone or with the supplement of NMS. This suggests that either heparinization (treatments 1, 4 and 11) reduces, although not necessarily eliminates, the opsonic activity of natural serum proteins coating the trypanosome surface, or that EDTA non-specifically enhances opsonization. The presence of even such small amounts of EDTA would chelate divalent ions, indicating that these may not be essential for non-specific attachment. Nevertheless, enhanced values were obtained in the presence of $CaCl_2$, (treatments 9 & 10) (see below). It is possible that the highly acidic heparin molecule can modify surface charges present on particles. The indication that treatments 1 and 2 were only slightly different and that the addition of NMS did not materially increase the attachment of washed and separated trypanosomes (treatment 4), suggests that host protein coating may be involved. The enhanced values obtained using trypanosomes collected in EDTA suggest that the critical factor for recognition in the absence of immune sera may well be the functional integrity of a surface coat, perhaps consisting of fibrin or fibrinogen. Adsorption of the latter to trypanosome surfaces has been indicated (Facer 1974). It is possible that negatively charged trypanosomes (Goodwin 1970) are initially coated with fibrin-like substances upon entering the circulation and can be recognised in the presence of natural serum antibodies. This recognition system gives low opsonic values and may well be modified by

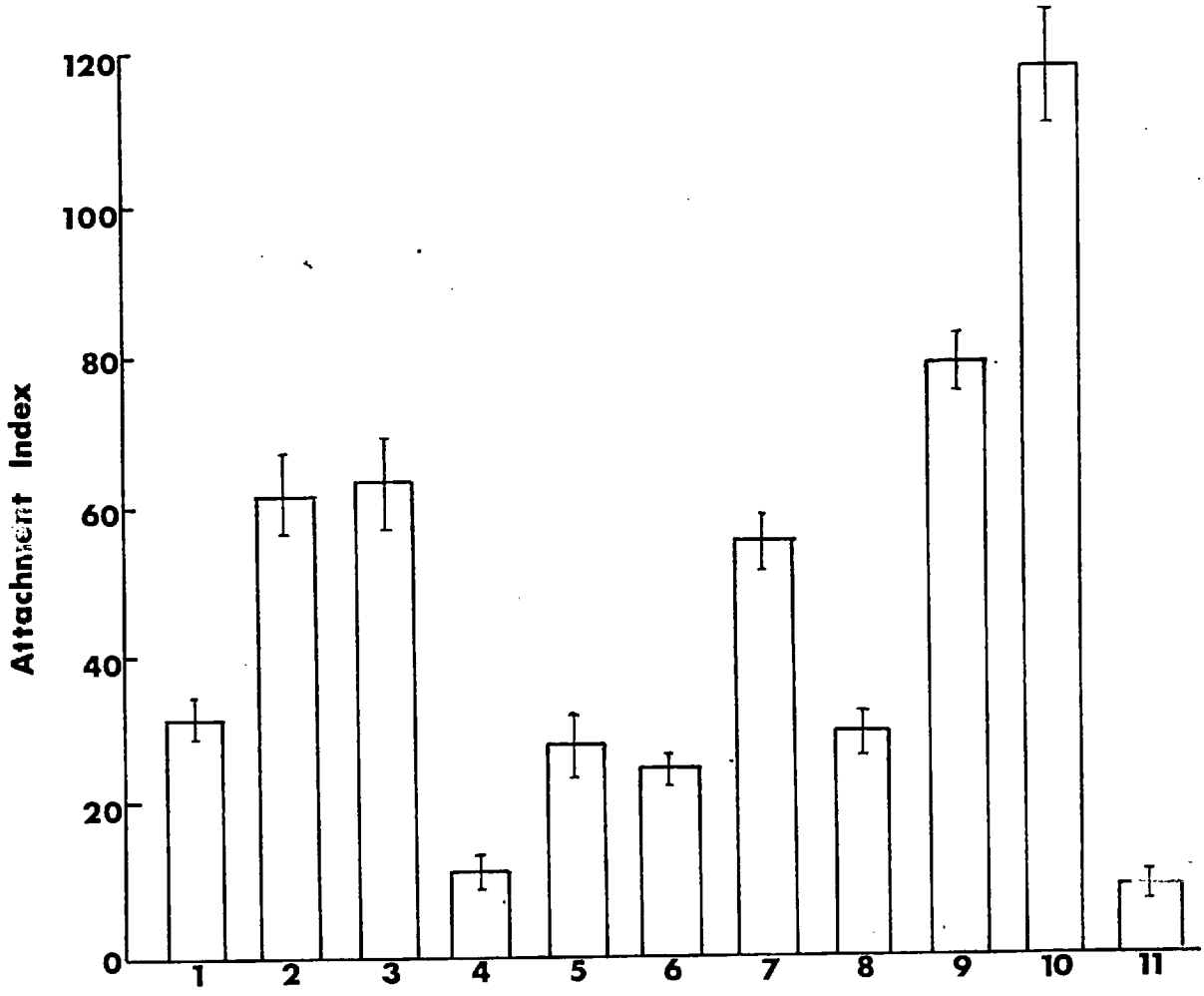


Fig. 16.

Fig. 16. Attachment of T. brucei 427 to mouse peritoneal macrophages (opsonic test).

Effect of foreign particles in the blood clotting mechanism.

1. Unwashed, unseparated trypanosomes collected in heparinized tubes.
2. Unwashed, unseparated trypanosomes collected in EDTA tubes.
3. Unwashed trypanosomes collected in EDTA tubes, plus 10% NMS.
4. Washed, separated trypanosomes, plus 10% NMS.
5. Washed, separated trypanosomes, plus sonicated rabbit fibrin in saline/EACA.
6. Washed, separated trypanosomes, plus 0.2% trypsin in TCM.
7. Washed, separated trypanosomes, plus rabbit fibrin, plus 10% NMS.
8. Washed, separated trypanosomes, plus trypsin, plus normal mouse plasma.
9. Unwashed trypanosomes collected in EDTA tubes, plus thrombin.
10. Washed, separated trypanosomes, plus pre-incubated thrombin and rabbit fibrin.
11. Washed, separated trypanosomes.

All results expressed are the mean ($\pm$ SE) of 5 replicates.

the appearance of specific immune factors produced during the course of infection. Early observations (quoted in Stuart 1970) indicated that droplets of India Ink injected into the bloodstream of frogs became coated with a fibrin-like substance, and were rapidly phagocytosed by fixed macrophages. This form of reaction would cause an increased stickiness; particles would non-specifically aggregate initiating further coagulation and increased phagocytosis. Particles could also be trapped on and within clots and on tissue surfaces, perhaps facilitating surface-phagocytic uptake. Curran & Clark (1964), have suggested that fibrin coating within the rat peritoneum may initiate phagocytosis and that the in vivo fibroblastic response may be secondary to and controlled by the phagocytic response.

Low attachment values were, however, obtained when sonicated rabbit fibrin was mixed with washed and separated trypanosomes (treatment 5). This may have been due to the absence of non-specific serum factors such as natural antibodies, for when 10% NMS was added, the opsonic activity increased (treatment 7). The preparative methods for treatments 2 and 3 could easily have caused trypanosomes to retain surface coverings of both fibrin and natural antibody.

Trypsination of separated trypanosomes would be expected to modify the surface coat. As has been previously recorded (section 3.1.9.), the addition of trypsin to macrophage-trypanosome suspensions causes a slight, though not appreciable increase in opsonic activity. Little change was observed, however, following prior incubation of trypanosomes with trypsin, either alone or in conjunction with normal mouse plasma (treatments 6 and 8). It is probable that plasma coagulation processes had already been initiated during collection (by the introduction of glass pipettes etc.). The reduced values were probably due either to the necessity of intact trypanosomes, or more likely, due to trypsin digestion of the various plasma components.

Much higher values were recorded when unwashed trypanosomes were mixed with thrombin (treatment 9) and when washed and separated trypanosomes were mixed with pre-incubated thrombin and rabbit fibrin plus NMS (treatment 10). Calcium ions present in the thrombin solution and required for efficient coagulation could also have assisted in initiating attachment.

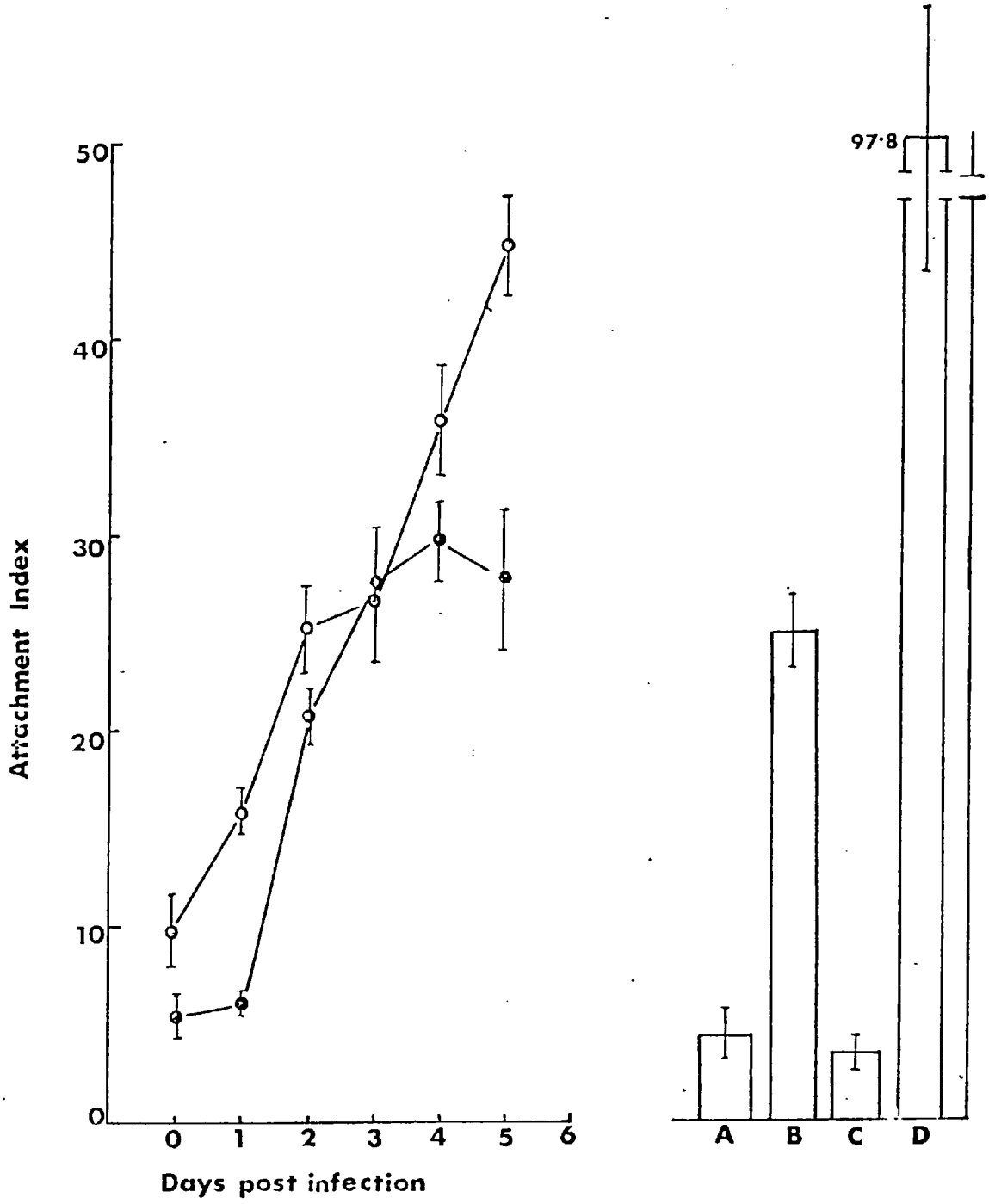
3.1.12. Opsonization and Mouse Alveolar Macrophages

The biochemical and functional heterogencity of peritoneal and alveolar macrophages has been widely investigated (Sorkin et al 1970). The enzyme composition of lysosomes vary considerably, thus rabbit alveolar macrophages have been shown to contain high concentrations of acid phosphatase, aminopeptidase and lysozyme (Vernon-Roberts 1972); antigen handling similarly varies and the speed and efficiency of phagocytosis differ. Alveolar macrophages appear more dependent upon oxidative energy for phagocytic metabolism than do peritoneal cells and have correspondingly altered respiratory rates and demands (Karnovsky et al 1970). Indeed the 'macrophage' family of cells have been shown to exhibit such wide divergences in metabolic response to phagocytosis, depending upon species and history that doubts may be cast upon the reliability of many in vivo and in vitro comparisons (Karnovsky et al 1970).

Nevertheless, a comparison was made between the relative effectiveness of mouse peritoneal and alveolar macrophages on the attachment of trypanosomes in the presence of immune sera. The opsonic test using 427 trypanosomes, infected and hyperimmune 427 sera was performed.

The alveolar macrophage monolayers tended to be uneven, the cells themselves were smaller, more rounded and less inclined to spread on glass than were peritoneal macrophages. They also appeared less granular. The alveolar cells from several mice were initially pooled before culturing. The presence of contaminative lymphocytes from genetically heterogenous animals could have been partially responsible for some of the observed differences. It would be expected, however, that non-adherent damaged cells would be removed by washing.

The results are shown in Fig. (17). As with peritoneal cells, a sharp almost linear response between attachment and days post infection was obtained. Levels rose from control values on day 0 to reach a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Attachment Index
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5
6
Days post infection
97·8
A
B
C
D

Fig. 17.

Fig. 17. Attachment of T. brucei 427 to mouse alveolar macrophages (opsonic test).

Effect of homologous mouse sera (serum series B).

●——● Attachment to mouse peritoneal macrophages. Mean (± SE) of 10 replicates.

o——o Attachment to mouse alveolar macrophages. Mean (± SE) of 5 replicates.

A: PSG

B: Hyperimmune T. brucei S42 serum

C: Absorbed hyperimmune T. brucei 427 serum

D: Hyperimmune T. brucei 427 serum

maximum of 9% attachment by day 5, and could be correlated with parasitaemia. The results obtained during the later half of the infection were significantly higher than those obtained from peritoneal cells. Attachment was still, however, comparatively low, less than 10% recorded. Absorbed hyperimmune 427 serum gave low control values, shereas heterologous hyperimmune serum gave significantly higher results, similar to those obtained 2 and 3 days post infection.

The essentially minor differences observed between the two cell types are probably, therefore, a reflection of different culture techniques and physiological optima. Just as normal and induced peritoneal cells appear physiologically and functionally distinct, so alveolar macrophages differ both morphologically and metabolically from peritoneal cells. Despite the obvious shortcomings of direct comparison the results nevertheless suggest that the attachment of trypanosomes to either cell type is primarily dependent upon mediation by serum factors. Phagocytic cells, in general, tend to respond non-specifically during phagocytic processes, with any selective discrimination being effected by serum (Blanden 1968).

3.2. Opsonic Adherence to Trypanosomes

3.2.1. Effect of Chemicals and Sera

Previous experiments indicated that when trypanosomes and immune sera were mixed in the presence of mouse macrophage monolayers, a low but nevertheless significant amount of attachment occurred. It has been further suggested that this enhanced adherence was primarily due to the adsorption onto the trypanosome surface of specific opsonizing substances capable of mediating subsequent recognition. Nevertheless, the possible role played by true cytophilic antibody could not be eliminated, as serum substances were invariably present to both opsonize trypanosomes and sensitize the macrophage cell surface. Although many people have used the term 'cytophilic antibody' synonymously with the macrophage Fc receptor (see Cohn 1975, Kloetzal & Deane 1970), and despite the fact that the adherence of monomeric IgG may be relatively loose - with affinities similar to those expressed by most rheumatoid factors (Arend & Mannik 1975) - a valid distinction can still be drawn between cell sensitizing antibody or soluble complexes which can show multiple attachment to multiple receptor sites, and opsonizing antibody which, binding via its Fab portion can subsequently interact with macrophage membrane receptors through its Fc piece (Steinman & Cohn 1974, Portis & Coe 1975). Indeed a mosaic of attachment sites may be envisaged - IgG site, Fc recepter site etc., each one distinct and independent.(Allison 1974).

The effect of incubating trypanosomes with known chemicals and specifically immune sera prior to macrophage presentation was therefore determined.

The opsonic adherence test was performed using normal mouse peritoneal macrophage monolayers washed in BSS, and 427 trypanosomes. The latter were either initially sensitized with the chemical or serum to be tested and used immediately, or were sensitized and then thoroughly washed to remove excess, non-attached material. Hence attachment could be effected only by true opsonizing agents specifically capable of coating the trypanosome surface. All washings were made in PSG pH 8.0,

as it has been indicated that loss of the trypanosome surface coat may be caused by a drop in pH (Allsopp et al 1971), although conversely, Taylor et al (1974) have suggested that the surface coat ultrastructure is little effected by saline washes.

The following tests were set up (Table (4)). Dilutions were made in TCM and adjusted to pH 8.0 where necessary.

Table (4)

Chemical	Alone	Hyperimmune	NMS
HISTAMINE	+		+
5-HT	+	+	+
TRYPSIN	+	+	+
DETERGENT	+		
ATP	+		
DEXTRAN	+		
CASEIN	+		
SODIUM AZIDE		+	
IODOACETATE	+	+	
FORMALDEHYDE		+	

The results are presented in Fig. (18). In each case, where appropriate, the results obtained with the opsonized and the 'opsonized and washed' preparations are compared with those obtained from the opsonic test.

None of the chemicals, apart from the detergent, had any visible effects on the trypanosomes. Similarly, the macrophage monolayers appeared normal after testing there being no abnormal granulation and/or vacuolation. As previously indicated, detergent markedly reduced

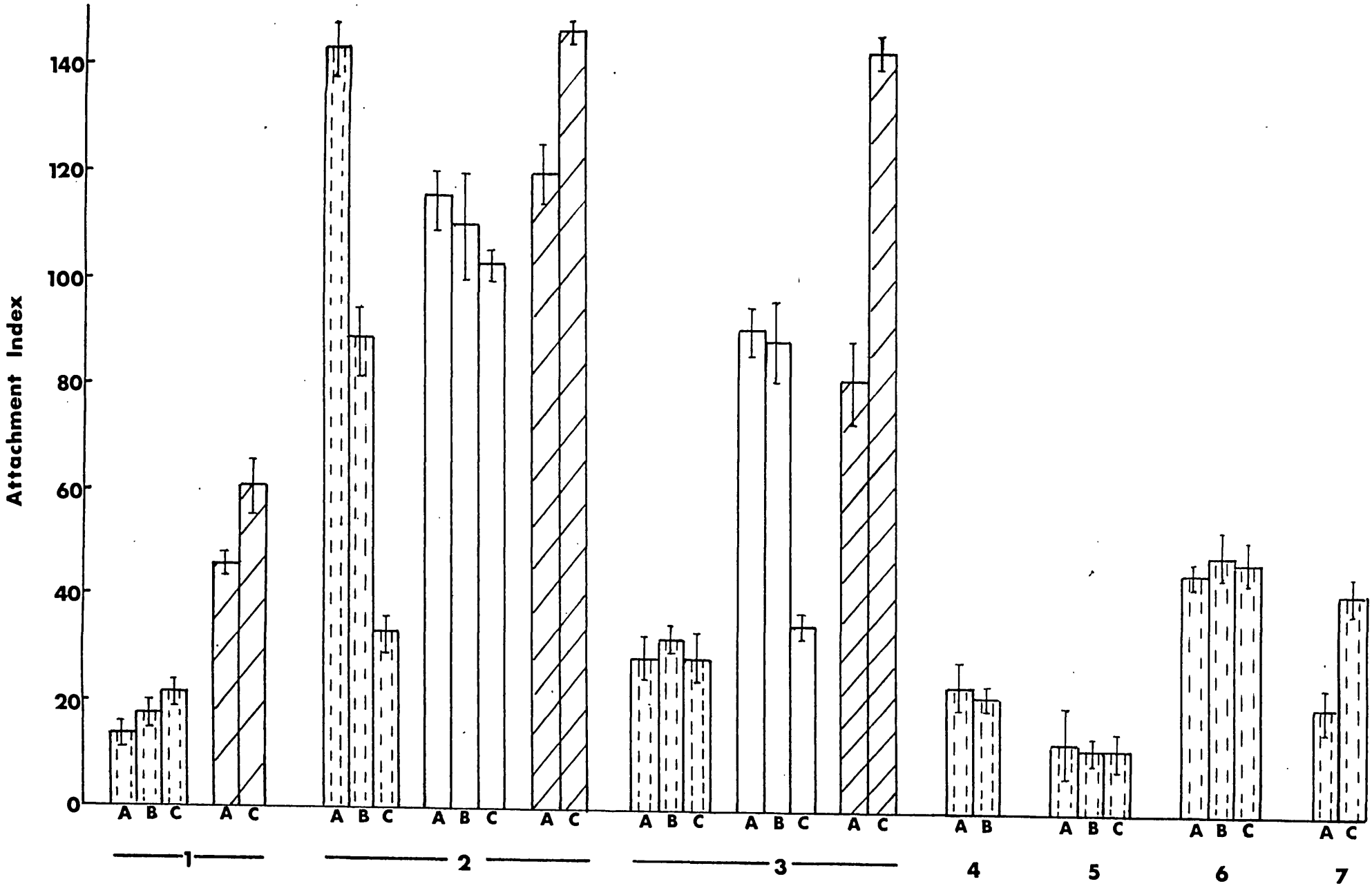
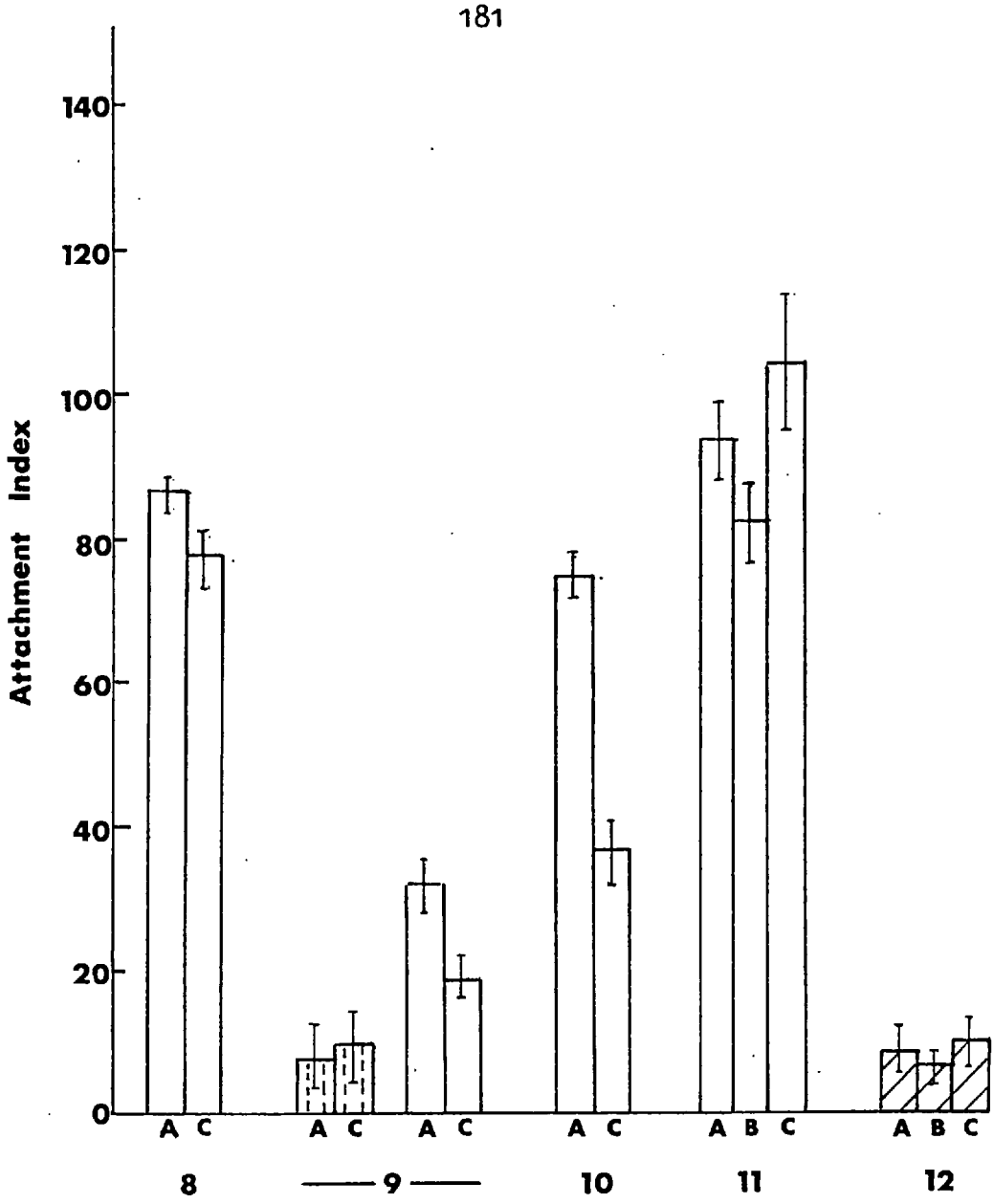


Fig. 18./


Attachment Index
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120
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80
60
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A C
A C
A C
A C
A B C
A B C
8
9
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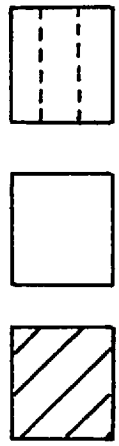
11
12

Fig. 18. (contd.)

Fig. 18. Attachment of T. brucei 427 to mouse peritoneal macrophages. Effect of trypanosome pre-incubation with chemicals and sera (opsonic adherence test).



Test substance alone

Test substance plus 10% mouse hyperimmune T. brucei 427 serum

Test substance plus 10% NMS

A: Opsonic test

B: Pre-incubation with trypanosomes

C: Pre-incubation with trypanosomes, followed by washing in PSG (opsonic adherence test)

1. Histamine
2. 5-HT
3. Trypsin
4. Detergent
5. ATP
6. Dextran
7. Casein
8. Sodium azide
9. Iodoacetate
10. Formaldehyde
11. Mouse hyperimmune T. brucei 427 serum
12. NMS

All results expressed are the mean ($\pm$ SE) of 5 replicates.

trypanosome mobility, and it was found impossible to satisfactorily wash the individual parasites, hence no accurate results could be obtained.

When the following substances were mixed with a suspension of live trypanosomes in the presence of a macrophage monolayer, or when they were used to pre-opsonize trypanosomes before presentation, the attachment indices obtained were essentially similar:- trypsin, dextran, detergent, histamine and ATP. All values were low, usually less than 10% attachment being recorded. Similar results were obtained following trypanosome preopsonization and washing. The very low results suggest that aspecific, non-immunological attachment may occur to a slight extent, or alternatively that the small quantities of non specific calf serum present in the macrophage culture medium may act opsonically. Foetal calf serum has been reported to cause macrophage stimulation through cytophilic mediation (Mackaness 1970), and more adult bovine serum may contain antibodies capable of reacting with antigenic determinants on the surface of erythrocytes and mouse macrophages (Cohn 1970). Calf serum may also cause lysosomal proliferation and stimulate hydrolase production.

NMS alone does not function opsonically to any appreciable extent. Homologous hyperimmune serum, however, causes a significant increase, with maximum attachment values of around 20%. The results suggest that surface adsorption of specific serum factors by trypanosomes may be the major mechanism in attachment.

When trypsin and hyperimmune serum were mixed and used for pre-opsonization, high attachment values (18%) were obtained before washing, but fell to control levels (trypsin alone, 7%), after washing. The high initial results may be a reflection solely of the activity of hyperimmune serum, which may either weakly opsonize trypanosome surface sites altered by trypsonization, or excess may attach cytophilically, either alone or

in the form of soluble complexes, to macrophages. When trypsin and NMS were used to sensitize trypanosomes and subsequently washed off, high attachment values were recorded (29%) - significantly higher than with hyperimmune serum alone (21%), and also greater than the recorded effect of trypsin and NMS using the opsonic test (17%). It is suggested that trypsin at high concentrations is capable of altering and/or partially removing the variant surface coat (Wright & Hales 1970). Hyperimmune antibodies may form insoluble complexes with particulate material and be removed upon washing, or trypsin digestion may inactivate specific antibodies, modifying or destroying the Fab, or more likely, the Fc portion, thus affecting subsequent binding to macrophage receptors. The non-specific antibodies in NMS may have been capable of attaching to the newly exposed surface determinants following trypsin treatment. It has been previously recorded, however, that normal mouse plasma and trypsin when used to preopsonize trypanosomes, did not materially increase opsonic activity (section 3.1.11.). This could be related to trypsin digestion and the formation of fibrin-like complexes which could competitively inhibit non-immunological trypanosome attachment.

It may be speculated, therefore, that for non-immunologic uptake of trypanosomes in vivo, the presence of a fibrinoid coating on trypanosomes is required. If this fibrinoid layer and the parasite surface coat are removed, complexes are produced which can inhibit subsequent trypanosome attachment. Similarly if trypsinization is carried out in the presence of hyperimmune serum, either specific antibodies are denatured or insoluble complexes are formed which are either partially removed in washing or are selectively phagocytosed, thus blocking available macrophage receptor sites. If trypanosome surface coat is removed in the presence of non specific antibody, large insoluble complexes are not formed, but non-immunologic recognition is effected and some soluble complexes may be generated.

When 5-HT was mixed with trypanosomes and macrophages, or pre-incubated with trypanosomes and then introduced to phagocytic cells, high levels of attachment were recorded (23% and 18% respectively). If, however, the pre-opsonized trypanosomes were thoroughly washed before testing, the levels fell significantly to around 6% attachment. This suggests that 5-HT is not capable of specific opsonization but may be able to sensitise macrophages. It has been suggested that lipid receptors for 5-HT may be present on macrophages (Davey & Asherson 1967), and interestingly, the functional characteristic of cytophilic receptor sites may be phospholipid or phospholipoprotein. High values of about 20% attachment were obtained for all treatments involving hyperimmune serum and 5-HT. This may be primarily a result of the effects of immune sera on opsonization. Nevertheless, high values were similarly recorded with 5-HT and NMS. It is possible, therefore, that 5-HT may affect trypanosome surface sites allowing a degree of non-specific opsonization, perhaps slightly analogous to the effects of trypsin.

The non-specific surface active substances dextran and detergent gave slight increases in attachment, probably in the case of the latter due to its toxic nature and consequent effect on the trypanosome surface. Interestingly, normal mouse serum contains a high level of free fatty acid which could have surface-tension effects on particles in vivo. It also accumulates as lipid droplets in macrophages (Cohn 1970). Many surface active agents can depress cellular respiration (Elsbach 1974).

Again low values were recorded for histamine in both the opsonic (3%) and opsonic adherence (4%) tests. Increased levels were seen with mixtures of histamine and both immune and non-immune sera, again, however, more a reflection of the effect of serum than of the chemical itself.

The opsonic adherence test using casein gave attachment levels twice as high as those recorded with the opsonic test (9% and 4% respectively).

Casein itself does not appear an efficient opsonin, although its known chemotactic role could affect random macrophage migration *in vitro*. It has, however, been reported as inhibiting phagocytosis by polymorphs, perhaps by adsorption onto the cell surface (Russel & Reiter 1975).

A very low attachment index was recorded using iodoacetate, either alone or in conjunction with hyperimmune serum for all tests, suggesting it is an effective inhibitor of attachment. Sodium azide did not appear to affect the opsonic ability of hyperimmune serum whereas formaldehyde solution caused a significant reduction in its activity.

3.3. Macrophage Cytophilic Antibody in Mice

3.3.1. Cytophilic Antibodies in Mice Sera

The previous experiments indicated that trypanosome surface antigens were partly susceptible to both sera and chemical treatment and could be modified, thus affecting attachment. It was therefore investigated whether macrophage specific antibodies were present in sera from mice with trypanosomiasis, what effect these had on mediating trypanosome adherence and what was the chemical nature of the macrophage receptors themselves.

Cytophilic antibody activity was measured using 427 trypanosomes, peritoneal macrophages from uninfected and hyperimmune mice, and various chemicals and prepared sera.

(a) Effect of Immune and Hyperimmune Sera

Sera were obtained from mice infected with T. brucei 427 on days 1, 3 and 5 post infection. Mouse and rabbit hyperimmune 427 sera were also used and the effect of heat inactivation of day 5 infected and mouse hyperimmune serum on cytophilic attachment was tested.

The results are presented in Fig. (19). There was a small but significant increase in macrophage cytophilic antibody activity from sera taken during the course of infection. Low control values recorded with day 1 serum rose approximately 3 fold by day 5. The values for days 3 and 5 were statistically similar. Enhanced cytophilic levels were similarly obtained using homologous hyperimmune serum, being about seven times higher than control levels using NMS, but nevertheless still only representing approximately 15% cell positivity.

It is possible that the mice repeatedly immunized with dead trypanosomes and FCA produced a high concentration of IgG_2 antibodies (Del Guerico et al 1969), which are believed to be effective in cytophilia. Animals immunized with live antigen in saline may synthesize predominantly IgG_1. The differences in effect may also, however, be a

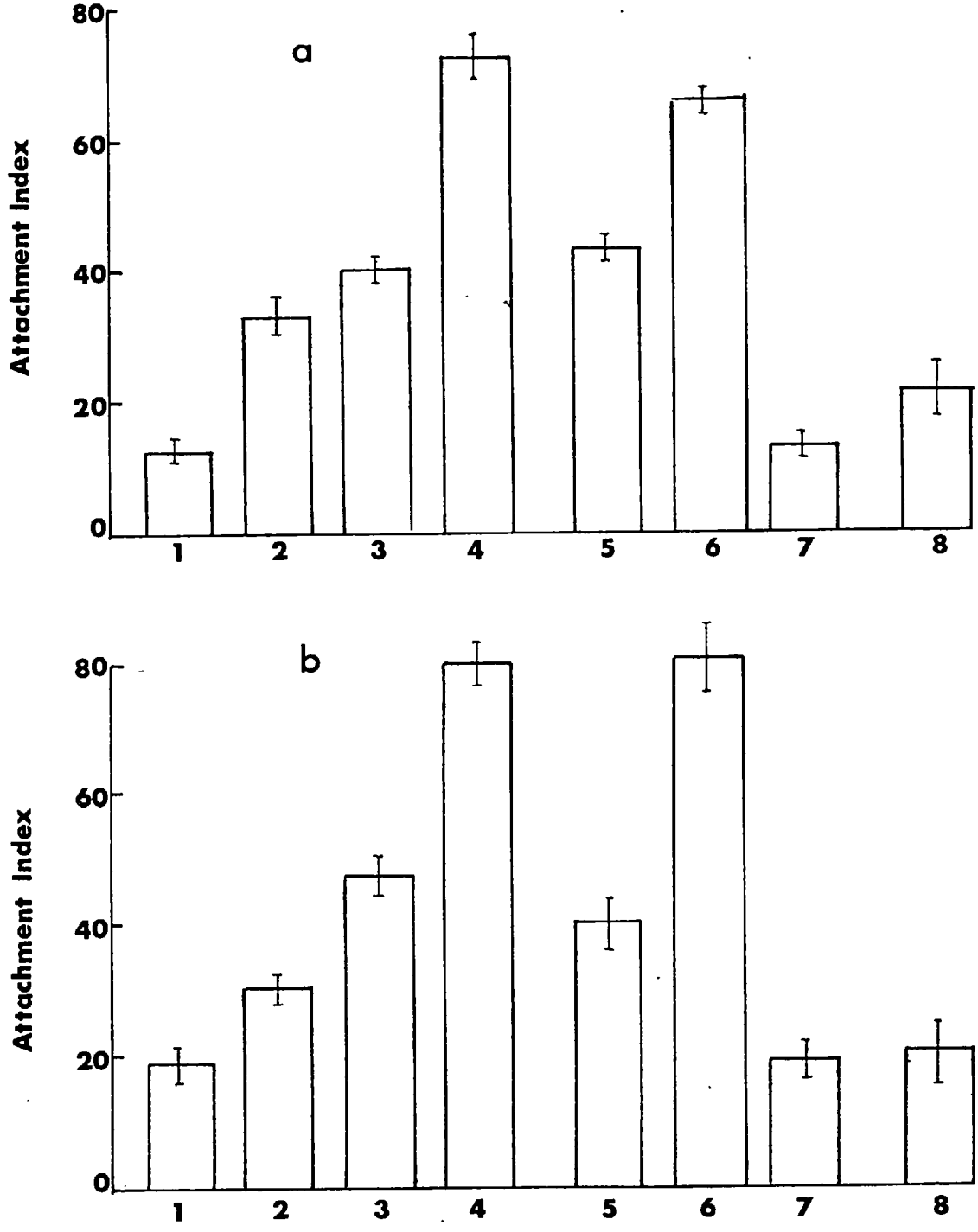


Fig. 19.

Fig. 19. Attachment of T. brucei 427 to mouse peritoneal macrophages (cytophilic antibody test).

Effect of homologous immune (serum series C) and hyperimmune sera.

(a) macrophages obtained from uninfected mice

(b) macrophages obtained from hyperimmune mice

1. NMS
2. 3 day infected serum
3. 5 day infected serum
4. Mouse hyperimmune T. brucei 427 serum
5. 5 day infected serum - heat inactivated (56°C)
6. Mouse hyperimmune serum - heat inactivated (56°C)
7. Guinea pig serum (1/10 dilution)
8. Rabbit hyperimmune T. brucei 427 serum

All results expressed are the mean ($\pm$ SE) of 5 replicates.

reflection of time. Thus only low levels of antibody were produced during an acute infection, with higher and more specific quantities present in hyperimmune serum. All mouse hyperimmune sera tested gave agglutination titres of $^{1}/_{5,120}$. No agglutination titres were found in sera from acutely infected animals (T. brucei 427), and only low titres in sera from mice infected with the more chronic strain (T. brucei S42) (Appendix (3)). Only small, subagglutinating amounts of sera are required for effective opsonization, slightly higher amounts for cytophilic attachment. Indeed, high serum dilutions which may no longer show in vitro bacterial agglutinating power, may still be capable of producing a marked effect in vivo (Stiffel et al 1970).

Heterologous rabbit hyperimmune serum gave low results with little cross reaction, suggesting a degree of species specificity. Heat inactivation of both day 5 infected and mouse hyperimmune sera had no effect on cytophilic attachment indices, neither did normal guinea pig serum at a 1:10 dilution, indicating that normal C' mediation was not important, adherence presumably being a result of heat-stable factors in immune sera. Caution needs exercising, however, when assessing results involving mouse C'systems. The test system itself is particularly unsatisfactory as mice tend to possess low and abnormal concentrations of various factors, most notably C3 (Raffel 1961, Rowley 1966). In order for usual pathway activation to be effective, antigen-antibody-C' complexes generate sequential C' components, with C3 usually representing the site-specific factor. Membrane bound cytophilic antibody can nevertheless bind C' under appropriate conditions. Contact sensitivity to oxazolone in mice, for instance, may well be mediated by specific cytophilic antibodies ($7SIgG_2a/7SIgM$) attaching via trypsin sensitive macrophage receptors (Askenase & Hayden 1974).

When the same tests were performed using peritoneal cells from hyperimmune mice, similar results were obtained for all treatments. As previously indicated, little or no natural cytophilic attachment was present, some elution may have occurred, however, during incubation at 37°C. (Berken & Benacerraf 1966). It is possible, therefore, that non-specifically activated macrophages still require the discriminating effects of specific opsonizing serum. It needs emphasizing, however, that if the reaction temperature is reduced below that of the physiological level, then the method of attachment may vary (Huber & Fudenberg 1968). Liew (1971), has indicated that the maximum binding of γ_2 to peritoneal cells occurs at 0°C. or 20°C. This was reduced by 75% at 37°C., due he suggested, to digestion and elimination.

(b) Effect of Chemicals and Sera

In order to investigate the susceptibility of the macrophage receptor sites, the effects of various enzymes and chemicals known either to effect opsonic attachment or to specifically react with macrophages were tested in conjunction with undiluted mouse hyperimmune 427 serum or NMS.

All enzymes and chemicals to be tested were dissolved in TCM and whole or halved coverslip preparations containing macrophage monolayers were exposed to the chemicals at the concentrations indicated. The overlying TCM was removed from individual Leighton tubes and replaced with 1.0ml. of the test solution. The coverslips were re-incubated at 37°C. for 30 min. All slides were removed and washed three times in BSS and the monolayers thereafter incubated normally for 60 min. at 22°C. with either sensitizing serum or PSG. Subsequent washings were made in physiological saline.

The following chemicals were tested in conjunction with 10% hyperimmune 427 serum, using both uninfected mouse peritoneal macrophages

and monolayers from hyperimmune animals:- trypsin (0.2% w/v); sodium azide (1% w/v), iodoacetate (0.5mM and 0.05mM) and formaldehyde (10% and 0.1%).

The results of the cytophilic test are shown in Fig. (20). A summary of the comparative effects of chemical treatment on cytophilic, opsonic and opsonic adherence activities, using normal mouse peritoneal macrophages and live T. brucei 427 are given in table (5).

Pre-incubation of the macrophage monolayers with 0.2% trypsin caused a significant two-fold reduction in the cytophilic antibody activity of hyperimmune serum when tested with normal, uninfected macrophages. This fall in activity was markedly reduced, yet still significant, when macrophages from hyperimmune animals were used. Trypsin may, therefore, at these high concentrations and for the long incubation time, be partially digesting certain of the macrophage receptor sites, thus preventing optimum cell-sensitization by hyperimmune serum factors. It suggests that a proportion, at least, of the effective sites may be proteinaceous. Interestingly, cultured mouse fibroblasts which are trypsinized invariably show widescale modifications of surface antigen (Molinari & Platt 1975). From table (5), it appears that prior trypsinization of the phagocyte or pre-opsonization of the parasite with trypsin and hyperimmune serum can markedly reduce attachment, yet no changes are seen when trypanosomes and trypsin-treated sera are mixed with macrophages. This may well be due to variations in incubation time. Attachment appears largely due to opsonization of intact trypanosomes, and can still be effected by trypsin-treated macrophages. It has been suggested that the macrophage binding sites for IgG are enhanced following proteolysis (Werb 1975).

Sodium azide, which is known to inhibit energy yielding metabolic pathways and thus affect engulfment, caused a slight loss of cytophilic activity of hyperimmune serum when tested with both normal and hyperimmune

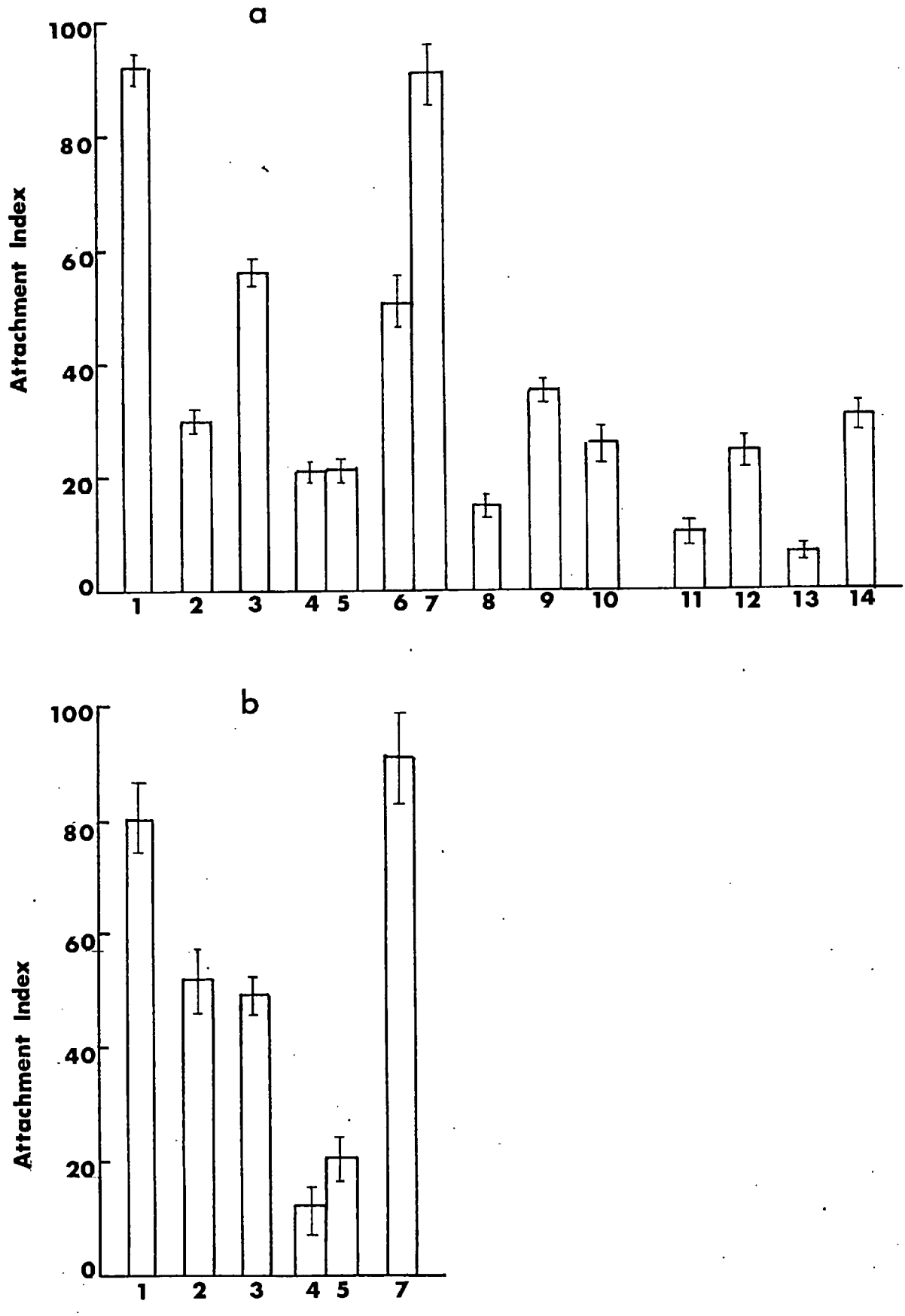

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Fig. 20.

Fig. 20. Attachment of T. brucei 427 to mouse peritoneal macrophages (cytophilic antibody test).

Effect of chemical treatment on NMS and hyperimmune sera.

(a) macrophages obtained from uninfected mice

(b) macrophages obtained from hyperimmune mice

1. Mouse hyperimmune T. brucei 427 serum
2. Trypsin
3. Sodium azide
4. Iodoacetate (0.5mM)
5. Iodoacetate (0.05mM)
6. Formaldehyde (0.1%)
7. Formaldehyde (10%)
8. PCMB
9. Fluorescein isothiocyanate
10. Sodium nitrite
11. NMS
12. PCMB
13. Fluorescein isothiocyanate
14. Sodium nitrite

All results expressed are the mean ($\pm$ SE) of 5 replicates.

TREATMENT	ATTACHMENT INDICES		
	Opsonic Test	Opsonic Adherence Test	Cytophilic Antibody Test
Mouse hyperimmune 427	84.8	103.0	73.2
Trypsin + hyperimmune 427[1]	91.6	36.6	28.2
Sodium azide + hyperimmune 427	86.8	76.8	56.0
Iodoacetate + hyperimmune 427	31.6	19.8	21.0
Formaldehyde + hyperimmune 427	74.2	34.0	50.6
N.M.S.	9.8	18.2	10.0

TABLE 5: Comparisons between Opsonic, Opsonic Adherence and Macrophage Cytophilic Antibody Activities, using T. brucei 427 and murine macrophages.

1. Hyperimmune serum used at a 10% concentration

macrophages. This suggests that attachment alone may not be totally energy dependent, although endocytosis-proper may well cause an increase in respiration and oxygen uptake (Baldridge & Gerard 1933), but cf: (Karnovsky et al 1970). Sodium azide does not appear to materially affect trypanosome opsonization.

Iodoacetate, at both concentrations, gave significant reductions in cytophilic activity for both normal and hyperimmune macrophages. The effective surface sites therefore may partially consist of free sulphydryl groups (-SH), which are destroyed by iodoacetate treatment, phagocytosis can thus be inhibited (Perkins 1970). Iodoacetate also blocks the Embden-Meyerhof pathway (Brogan 1966), and may consequently affect trypanosome opsonization, low values being recorded for all treatments. It is known to inhibit amino acid transport in T. brucei (Voorheis 1971).

Formaldehyde, which can react with both free amino and sulphydryl groups, caused a slight but significant reduction in the cytophilic activity of hyperimmune serum when used at a concentration of 10%, but not at 0.1%. Davey & Asherson (1967), noted that the guinea pig cytophilic receptor to SRBC was resistant to 0.4% w/v formaldehyde. Low results were also obtained for the opsonic adherence test but not for the opsonic, suggesting that pre-incubation of the chemical with either trypanosomes or macrophages can modify structural sites.

The cytophilic activities of hyperimmune 427 serum and NMS were further tested using normal mouse macrophages which had been pretreated with the following chemicals: p-chloromercurybenzoate (PCMB) (BDH Chemicals Ltd.) (0.5mM); fluorescein isothiocyanate (FIT) (BDH Chemicals Ltd.) (500 g/ml.), and sodium nitrite (Hopkin & Williams Ltd.) (50mM). PSG was used as the non serum control.

The results are shown in Fig. (20). Low cytophilic activities were recorded for all treatments involving preincubation with the three chemicals tested.

It is possible, therefore, that the macrophage receptors are sensitive to both iodoacetate and PCMB - both reacting with free -SH groups, and slightly less so to formaldehyde and FIT - both capable of reacting with amino groups. This, together with the results obtained with trypsin, suggest that amino groups are important in site specificity, but not as critical as sulphydryl groups. The oxidising agent, sodium nitrite, may also destroy site activity. Normal hydrogen peroxide production by macrophages may thus have an effect. Perhaps via an H_2O_2-myeleoperoxidase type system (Klebanoff - pathway) although the evidence suggests that only small amounts (if any) are produced by either cultured or fresh mouse cells (Karnovsky et al 1970). It also needs considering that numerous chemicals etc. which are used to inhibit specific pathways also act on other biochemical reactions and cellular functions not necessarily related to the primary mode of action. For example, iodoacetate not only inhibits glycolysis but also interferes with K^+ and Na^+ transport, causes cell swelling and reduces lipase activity (Elsbach 1974).

3.4. Passive Sensitization of Macrophages

Cross (1973), has indicated that if T. brucei are disintegrated and centrifuged at 20,000 x g, a non-sedimented protein fraction, which is absent from culture forms, is produced. This is believed to represent a proportion of surface coat material. In intact parasites, this layer may be separated from the plasma membrane following cell rupture or, as previously indicated, enzymic digestion. Accordingly, the effect of passively sensitizing cultured mouse peritoneal macrophages with specific antigen-antibody complexes made from this soluble fraction and immune sera, on the subsequent attachment of whole, live T. brucei 427 was examined.

Table (6), summarizes the results of several experiments using mouse or rabbit antisera as the antibody source and 'trypanosome supernatant' (section 2.13) as the antigen source. In one experiment, the macrophage monolayer was incubated with 1.0 ml. of a 0.2% w/v solution of trypsin in TCM for 1 h. at 37°C., and subsequently washed three times, before normal cell-sensitization was carried out.

The results indicate that pre-sensitization with trypanosome antigen-antibody complexes was effective in the attachment of comparatively large numbers of trypanosomes to sites on the macrophage surface. Complex sensitization involving mouse hyperimmune serum gave values over three times higher than those obtained when hyperimmune serum was tested cytophilically, and NMS complexes gave up to 7.5% attachment, compared to a mere 2.5% in the cytophilic test. Complexes produced with homologous hyperimmune serum gave higher results than those using heterologous serum - 45% and 16% respectively, suggesting a degree of species specificity. There was also a significant difference between the values obtained using homologous hyperimmune, 4 day infected mouse serum and NMS.

The role of C' in sensitization was investigated to determine whether a mechanism similar to IA was responsible. It has been indicated

PASSIVE SENSITIZATION

TABLE 6

ANTIGEN	ANTISERUM	OTHER TREATMENT	ATTACHMENT INDEX	STD. DEV.
Supernatant	Mouse hyperimmune 427	0	225.7	15.0
Supernatant	Rabbit hyperimmune 427	0	79.0	11.3
Supernatant	NMS	0	37.2	3.9
Supernatant	T. brucei 427[4]			
	Day 4 infected	0	105.7	13.4
Supernatant	T. brucei 427[4] Day 2 infected	0	39.0[1]	1.4
Supernatant	Mouse hyperimmune 427	Cells suspended in 0.2% trypsin before sensitization	239.0	20.8
Supernatant	Mouse hyperimmune 427	0.01M 2-mercaptoethanol treatment of antiserum	171.5[1]	27.6
Supernatant	Mouse hyperimmune 427	Antiserum heat inactivated at 56°C for 30 min	210.2	12.2
Supernatant	Mouse hyperimmune 427	Antiserum heat inactivated plus the addition of guinea pig serum[2]	219.2	30.9

TABLE 6 - contd.

PASSIVE SENSITIZATION

ANTIGEN	ANTISERUM	OTHER TREATMENT	ATTACHMENT INDEX	STD. DEV.
Supernatant	Guinea Pig serum (C')	0	59.7	11.9
0	Mouse hyperimmune 427	0	73.0[3]	9.8
0	T. brucei 427[4]			
	Day 1 infected	0	14.5[3]	8.5

TABLE 6: Sensitization of murine macrophages by antigen-antibody complexes and cytophilic antibody. Using T. brucei 427.

1. mean of 2 replicates, all other results are means of 4 replicates.
2. 0.1 ml, used as a source of C'.
3. Cytophilic Antibody Test.
4. see Appendix

by Uhr (1965), that C' might be important in the sensitization of guinea pig lymphocytes, but not macrophages by bacterial antigen-antibody complexes. In the system investigated, heat inactivation of hyperimmune serum did not affect the high values obtained. Normal guinea pig serum, used as a known source of C', gave low results comparable to control levels.

Wellek et al (1975), demonstrated that guinea pig peritoneal macrophages possessed receptors capable of mediating both the attachment and ingestion of C3b and C3d-coated immune complexes; and it is known that macrophage C3 receptors can be destroyed by trypainization. Prior treatment of the macrophage monolayers with trypsin, however, had no adverse effect on subsequent trypanosome attachment. This suggested that the specific receptor sites involved in this attachment were not proteinaceous, and that a mechanism dissimilar to IA, not mediated via the trypsin sensitive C3 surface receptors, was operative. Huber & Holm (1975), have indicated that macrophage receptor sites to the Fc-fragment of IgG are, unlike those for the activated third component of C', resistant to trypsin digestion.

Mercaptoethanol-treatment of hyperimmune 427 serum caused a reduction in activity of about 25%, suggesting that a proportion of complexes which normally develop involve 19S IgM.

Table (7), shows the results of similar experiments using 'trypanosome sediment' and 'sonicated supernatant' (section 2.13.). 'Sediment', 'supernatant' and 'sonicated supernatant' were used for cell-sensitization in both the absence and presence of specific sera. The effects of absorbed sera and of trypsin and 5-HT were examined and the sensitization of live trypanosomes with immune complexes was investigated.

Low, statistically similar, control values were recorded for all treatments in the absence of serum except when 'sediment' and 'sonicated supernatant' had been incubated at 37°C. for 60 min., and when macrophages

TABLE 7

PASSIVE SENSITIZATION

ANTIGEN	ANTISERUM	OTHER TREATMENT	ATTACHMENT INDEX[1]	STD. DEV.
Supernatant	0	0	21.4	3.6
Sonicated supernatant	0	0	30.6	3.0
Sediment	0	0	26.0	5.1
Sonicated supernatant plus sediment	0	0	79.0	9.3
Supernatant plus sediment	0	0	46.2	5.8
Sonicated supernatant	Mouse hyperimmune 427	0	378.8	20.0
Sediment	Mouse hyperimmune 427	0	59.0	9.4
Sediment	NMS	0	35.6	5.3
Sediment	Mouse hyperimmune 427 absorbed[2]	0	13.0	6.8
Supernatant	Mouse hyperimmune 427 absorbed	0	30.2	8.3
Sediment	0	Cells suspended in 0.02% trypsin before sensitization	38.0	5.9

TABLE 7 - contd.

PASSIVE SENSITIZATION

ANTIGEN	ANTISERUM	OTHER TREATMENT	ATTACHMENT[1] INDEX	STD.DEV.
Sediment	Mouse hyperimmune 427	Cells suspended in 0.02% trypsin before sensitization	78.4	11.0
Sonicated supernatant	0	Cells suspended in 0.02% trypsin before sensitization	40.6	6.1
Sonicated supernatant	Mouse hyperimmune 427	Cells suspended in 0.02% trypsin before sensitization	432.4	39.4
Sonicated supernatant	Mouse hyperimmune 427	Cells suspended in 0.02% 5-HT before sensitization	351.4	21.1
Sediment	0	Cells suspended in 0.02% 5-HT before sensitization	90.2	9.0
Disrupted trypanosomes	Mouse hyperimmune 427	0	135.6	14.0
Disrupted trypanosomes	NMS	0	30.6	9.0
Supernatant	Mouse hyperimmune 427	Trypanosomes "sensitized" with antigen-antibody complex	191.6	12.5
Sediment	Mouse hyperimmune 427	Trypanosomes "sensitized" with antigen-antibody complex	94.8	6.8

TABLE 7: Macrophage sensitization using antigen-antibody complexes prepared from T. brucei 427 supernatant, sonicated supernatant and sediment.

1. mean of 5 replicates

2. Hyperimmune serum absorbed with live, homologous trypanosomes.

had been pre-treated with 5-HT. These two treatments gave about 15% attachment.

The test involving 'sonicated supernatant' and mouse hyperimmune serum gave similar, slightly higher results to those obtained before - circa 75% attachment. NMS and absorbed hyperimmune serum gave low values as did treatments involving hyperimmune sera and trypanosome 'sediment'. Trypsin (0.02% w/v), again did not affect activity, and neither did pre-treatment with 5-HT (0.02% w/v). Intermediate values (approx. 25%), were found using whole homogenates of disrupted trypanosomes as the antigenic source and hyperimmune serum. Treatment of trypanosomes with supernatant and hyperimmune sera also enhanced attachment, this was less marked when trypanosome 'sediment' was used.

A useful comparison can be made between these results and those obtained with the opsonic and macrophage cytophilic antibody tests (Fig. (21)). The number of trypanosomes which attached in the presence of antigen-antibody complexes was far higher than when immune antibodies alone were tested opsonically or cytophilically. Trypsinization of the macrophage monolayer which would modify proteinaceous C^1-receptor sites only had a deleterious effect in the case of cytophilic antibody attachment, suggesting that C' factors may be involved in cytophilic attachment of immune sera. Heat inactivation of sera prior to testing, however, caused no appreciable changes in opsonic, cytophilic or 'complex' attachment. It is possible, therefore, that trypsinization destroys specific receptor sites other than those for C', or that macrophages themselves are capable of producing the necessary C' components needed to assist the cytophilic attachment of trypanosomes. Nevertheless, although the macrophage receptor site appears different, antigen-antibody complexes do possess some, although not all, of the effects of cytophilic antibody; viz: an increased cell sensitization resulting in an enhanced attachment of trypanosomes.

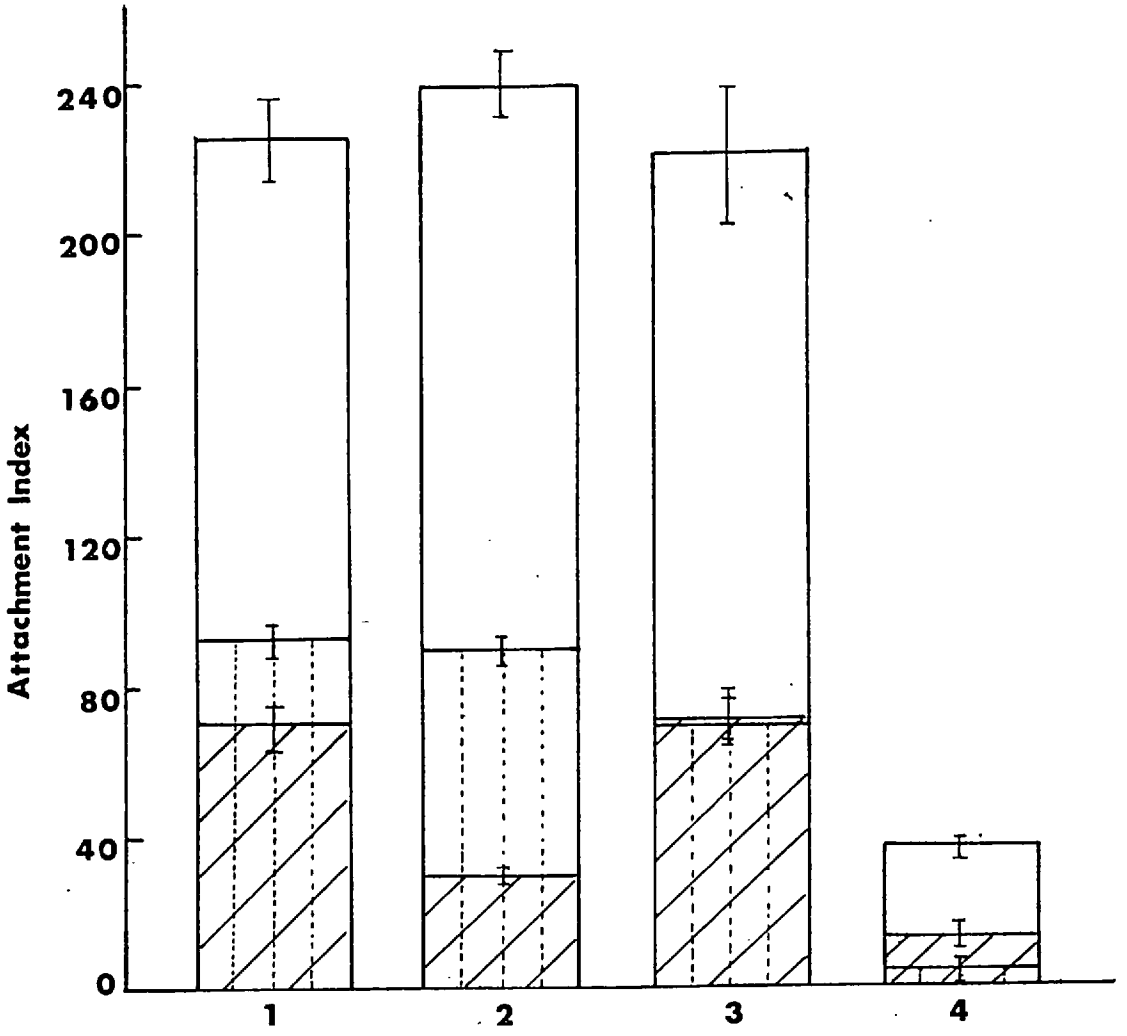
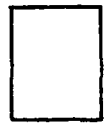
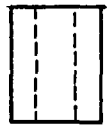


Fig. 21.

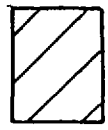
Fig. 21. Attachment of T. brucei 427 to mouse peritoneal macrophages. A comparison of opsonic, cytophilic antibody activity and passive sensitization of macrophages.



Passive sensitization using soluble immune complexes (mean (± SE) of 4 replicates)



Opsonic test (mean (± SE) of 5 replicates)



Cytophilic antibody test (mean (± SE) of 5 replicates)

1. Mouse hyperimmune T. brucei 427 serum
2. Mouse hyperimmune T. brucei 427 serum plus prior trypsinization of monolayer
3. Mouse hyperimmune T. brucei 427 serum, heat inactivated (56°C)
4. NMS

3.5. Peritoneal Cell Activity *in vivo*

Any conclusions obtained from the results using mouse peritoneal macrophages, trypanosomes and specific sera, have necessarily suffered from being drawn from *in vitro* experiments. The problems of *in vivo* experimentation are necessarily more complex and more difficult to rigidly define. Nevertheless, an attempt was made to assess the role of *in vivo* phagocytosis of trypanosomes and to correlate this, if possible, with the observed *in vitro* results. The intraperitoneal inoculation of a known number of parasites at times intervals before washing out the peritoneum was adjudged to give an indication of both the extent and rate of the normal phagocytic response of CD-1 mice.

In the first experiment, mice were inoculated with *T. brucei* in PSG and peritoneal washouts made at irregular intervals up to 22 h. The results are shown in table (8) and graphically represented in Fig. (22a & b). The number of cells recovered remained fairly constant throughout the experiment, suggesting that within the time period considered, little immigration or emigration from the peritoneal cavity occurred. It was found that approximately 60% of the cells could be morphologically distinguished as mononuclear, samples were removed after 1 and 180 min. and fixed and stained. It has been recorded that the mouse peritoneal cavity generally contains between 70% and 75% mononuclear phagocytes, about one third of which may superficially resemble medium to large lymphocytes (Daems & Brederoo 1970).

The precentage-inoculum recovered was determined from the formula: $^{Y}/_{X} \times 100$, where X represented the total number of organisms inoculated (6.1×10^{6}) and Y, the total number recovered. This value remained fairly constant throughout the experiment at approximately 85%.

The precentage-phagocytosis was obtained from the formula $\frac{100(T-S)}{T}$ where T was the number of trypanosomes present in the peritoneal cavity at each time interval, and S the number present in the supernatant

TABLE 8

Time after Injection (mins)	No. of Peritoneal Cells/ml x 10^4	No. of Trypanosomes/ml x 10^4	No. of Trypanosomes attached/ engulfed /ml x 10^4	% Inoculum Recovered	Log % Inoculum Recovered	% Phago-cytosis	Log % Phago-cytosis
A: Trypanosomes inoculated without previous treatment. Inoculum: 6.1×10^6 parasites							
0	115	0	0	0	-	0	-
1	154	525	55	86.0	1.9345	10.5	1.0203
2	108	490	37	80.3	1.9047	7.5	0.8779
5	150	508	112	83.3	1.9206	22.0	1.3434
30	182	501	101	82.1	1.9143	20.2	1.3045
45	127	469	128	76.9	1.8859	27.3	1.4360
90	112	600	150	98.5	1.9934	25.0	1.3979
180	184	480	85	78.7	1.8960	17.7	1.2482
255	150	530	115	86.9	1.9390	21.7	1.3365
1320 (22h)	155	0	0	0	-	0	-
B: Trypanosomes pre-opsonized with mouse hyperimmune 427 serum. Inoculum 6.1×10^6 parasites							
0	155	0	0	0	-	0	-
5	120	580	20	95.1	1.9781	34.5	1.5376
15	90	560	80	91.8	1.9628	14.3	1.1546
25	110	480	120	78.7	1.8958	25.0	1.3979
35	80	400	120	65.6	1.8167	30.0	1.4771
45	125	280	180	45.9	1.6618	64.3	1.8080
60	135	300	140	49.2	1.6918	46.7	1.6690
90	100	380	140	62.3	1.7944	38.8	1.5663
120	175	220	100	36.1	1.5570	26.3	1.4202

TABLE 8 - contd.

Time after Injection (mins)	No. of Peritoneal Cells/ml s 10^4	No. of Trypanosomes/ ml x 10^4	No. of Trypanosomes attached/ engulfed /ml x 10^4	% Inoculum Recovered	Log% Inoculum Recovered	% Phago-cytosis	Log% Phago-cytosis
C: Trypanosomes pre-treated with T. brucei 427 'supernatant'. Inoculum: 6.1 x 10^6 parasites							
2	110	600	120	98.4	1.9929	20.0	1.3010
10	150	580	240	95.1	1.9781	41.4	1.6167
20	95	360	60	59.0	1.7710	16.7	1.2217
30	105	440	80	72.1	1.8581	18.2	1.2596
40	160	380	100	62.3	1.7944	26.3	1.4202
50	95	360	60	59.0	1.7710	16.7	1.2217
65	95	240	40	39.3	1.5948	16.7	1.2217
95	75	240	20	39.3	1.5948	8.3	0.9206
125	100	180	40	29.5	1.4699	22.2	1.3468

TABLE 8: Survival and recovery of T. brucei 427 after inoculation into the peritoneum of normal mice

(i.e. not attached or engulfed) (Whitby & Rowley 1959). This value rose rapidly during the first few minutes from low values of about 10% to peak levels of around 25%. After the initial rise, the values tended to remain fairly constant with slight fluctuations.

Quantifiably, the technique was unreliable as errors in inoculation, washing and counting could occur. The high percentage-inoculum recovered indicates that, up to 255 min., few trypanosomes escaped the confines of the peritoneum. A proportion were attached to or endocytosed by phagocytes, but had not been taken into the general circulation. After 22 h. no trypanosomes could be found, either free or attached to phagocytes. The rapid initial uptake of trypanosomes soon levelled off to average values of 20%, gradually falling with time. This initial uptake may be of damaged or partly lysed individuals, the vast majority of parasites obviously escaping non-specific phagocytic recognition, as is evidensed by the low numbers of attached or engulfed trypanosomes found at the various time intervals. The fact that the precentage-inoculum recovered remained reasonably constant throughout, suggests that the results for phagocytic uptake may be fairly accurate. Although comparatively low values were recorded, in vivo phagocytic uptake appeared more efficient than that observed in vitro, although, of course, a multitude of inter-related, unknown factors could be involved. Nevertheless, no in vitro system has yet been designed that can approach the intra-peritoneal rate of phagocytosis (Rowley 1966).

The experiment was repeated using trypanosomes pre-incubated with mouse hyperimmune 427 serum or 'trypanosome supernatant' (section 2.13.). When the former was used, the precentage-inoculum recovered decreased significantly from initial values of 95% after 5 min. to 36% after 120 min. (Fig. (22b)). The number of peritoneal cells per ml. remained fairly constant, again about 60% mononuclears. The percentage phagocytosis also rapidly increased to 35% within 5 min., reaching 64% after 45 min., and

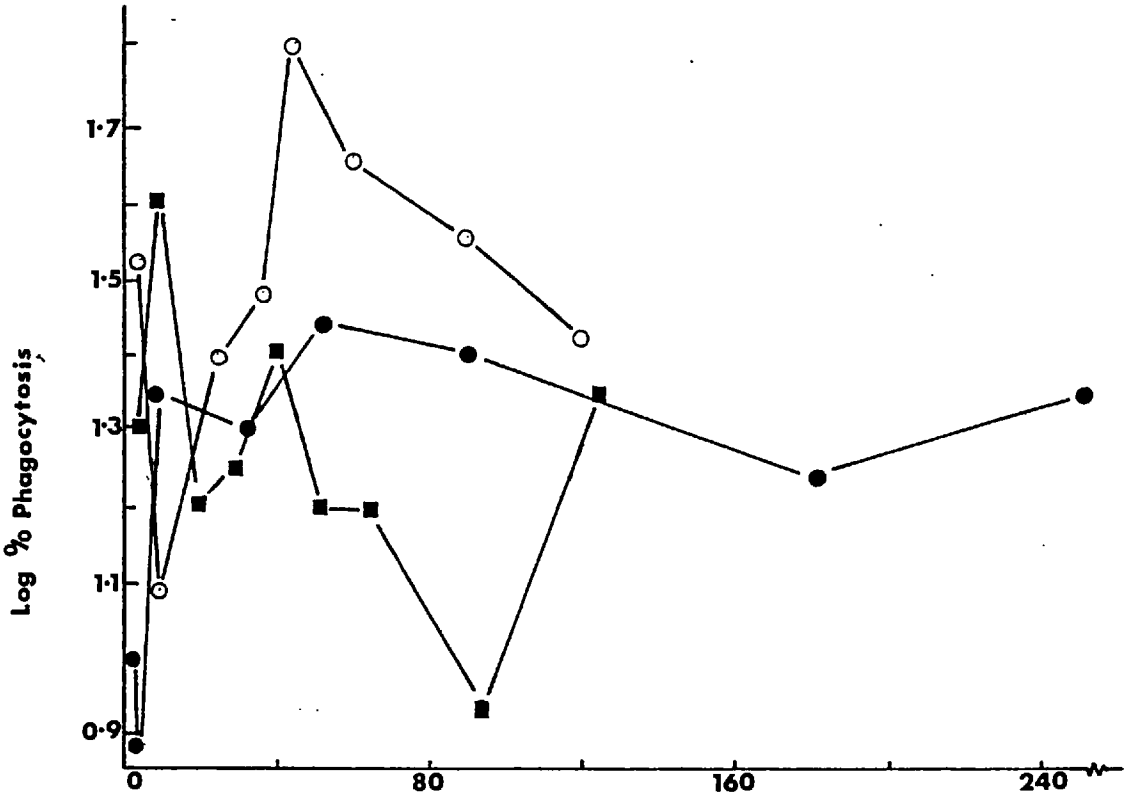


Fig. 22a.

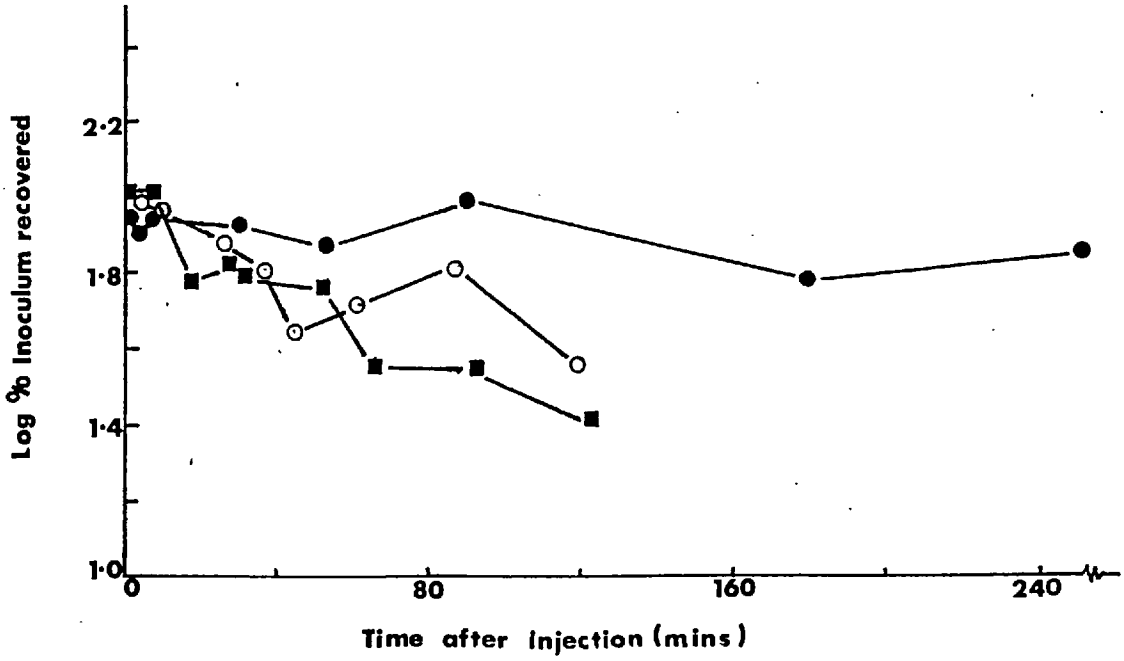


Fig. 22b.

Fig. 22a. Phagocytosis of T. brucei 427 in the peritoneum of normal CD-1 mice.

Fig. 22b. Recovery of T. brucei 427 from the peritoneum of CD-1 mice.

●——● trypanosomes inoculated without serum.

○——○ trypanosomes pre-opsonized with mouse hyperimmune T. brucei 427 serum.

■——■ trypanosomes pre-treated with T. brucei 427 "supernatant".

thereafter fell quite sharply (Fig. (22a)). Despite the greater *in vivo* uptake generated by pre-opsonization, at least 50% of the trypanosome inoculum still, however, managed to escape the phagocytic response.

Trypanosomes in conjunction with 'supernatant' again showed a decrease in percentage recovered with time, with a 50% increase in rate over that obtained following serum treatment (4.1:2.7) (Fig. (22b)). Yet the percentage of phagocytosed parasites remained fairly low, slightly below those of control lvels (Fig. (22a)). It is suggested, therefore, that the presence of soluble trypanosomal antigen or immune serum effects the removal of trypanosomes from the peritoneum of normal mice and that, in the case of serum, this is partially due to increased phagocytosis. Trypanosomes alone remain for significant periods within the peritoneal cavity, whereas those in association with antigen do not do so, yet neither are they engulfed above control levels. It further suggests that normal peritoneal fluid does not contain sufficient opsonin for effective phagocytosis. A proportion of activity may have been due to the numbers of polymorphonuclear cells present in the peritoneal lavage; no attempt being made to restrict readings to morphologically identifiable macrophages. There was no evidence to indicate a preferential migration of mononuclear cells into the peritoneum following the introduction of parasites, over the relatively short periods considered.

3.6. Surface Phagocytosis

Following the reports of Wood et al (1946), and Wood (1960), that the phagocytosis of encapsulated bacteria could be achieved in the absence of antibody, provided a suitable substrate was present; it was decided to investigate this phenomenon in in vitro studies using trypanosomes.

Trypanosomiasis as typified by brucei sub-group organisms is a chronic inflammatory disease. One of the most striking changes which occurs in inflammation is the apparent 'stickiness' of leucocytes which can adhere to endothelia. Under normal conditions they can move freely, but following injury they tend to attach to the walls of the body cavity beneath the area of injury or elsewhere. The same effect can be obtained by the intravenous injection of bradykinin (Ebert & Grant 1974). Goodwin (1970, 1971), has observed the in vivo engulfment of trypanosomes by mononuclear cells, which appeared stuck to or trapped in the capillaries or damaged vessels of chronically infected rabbits. Curran & Clark (1964), have indicated that phagocytes are capable of recognizing and adhering to microscopically rough surfaces in the peritoneal cavity of rats.

The following surfaces were accordingly tested:-

1. Clean glass microscope slide.
2. Single layer of aluminium foil.
3. Layer of mucus obtained from the buccal cavity of a freshly killed CD-1 mouse.
4. Single layer of Whatman No.1 filter paper soaked in TCM.
5. Fibrin clot obtained from T. brucei 427-infected rat blood, spread flat on a microscope slide.
6. Albumin solution (BDH Chemicals Ltd.) on a glass surface.
7. Freshly excised mouse lung from an uninfected animal, used within 30 min. of dissection, thoroughly washed and maintained in TCM.

8. Freshly excised mouse liver, similarly treated.
9. Freshly excised mouse spleen, similarly treated.
10. Freshly excised mouse gastric mucosa, similarly treated.
11. Mouse lung boiled in 5 ml. TCM for 30 min.
12. Boiled mouse liver.
13. Boiled mouse spleen.
14. Boiled mouse gastric mucosa.
15. Fibrin clot + 10% mouse hyperimmune 427 serum.
16. Mouse gastric mucosa + 10% hyperimmune serum.
17. Boiled mouse liver + 10% hyperimmune serum.

All tissues were cut with a scalpel and the exposed surface used for the test. The gastric mucosa was spread flat. The hyperimmune serum (diluted in TCM) was applied to the tissue surface before the trypanosomes.

Due to the necessity of using fresh peritoneal cell suspensions instead of macrophage monolayers, and of making selective impression smears onto glass coverslips, it was found unreliable to attempt a count of a fixed number of cells, determine the number of attached trypanosomes and thus make direct comparisons between treatments. The concentration of available trypanosomes and peritoneal cells varied considerably, as did the surface areas and absorbancies of the test surfaces. Consequently, an estimate was made of the average number of attached trypanosomes per phagocyte. Provided the initial sample size was large enough (in excess of 200 cells counted), comparisons should be valid.

The results are presented in Fig. (23). For nearly all preparations, the number of attached trypanosomes per macrophage was high. This was, however, partially a reflection of the technique. Large numbers of unattached trypanosomes were found on most smears, and it was found impossible to differentiate between *in situ* fixation and genuine receptor-

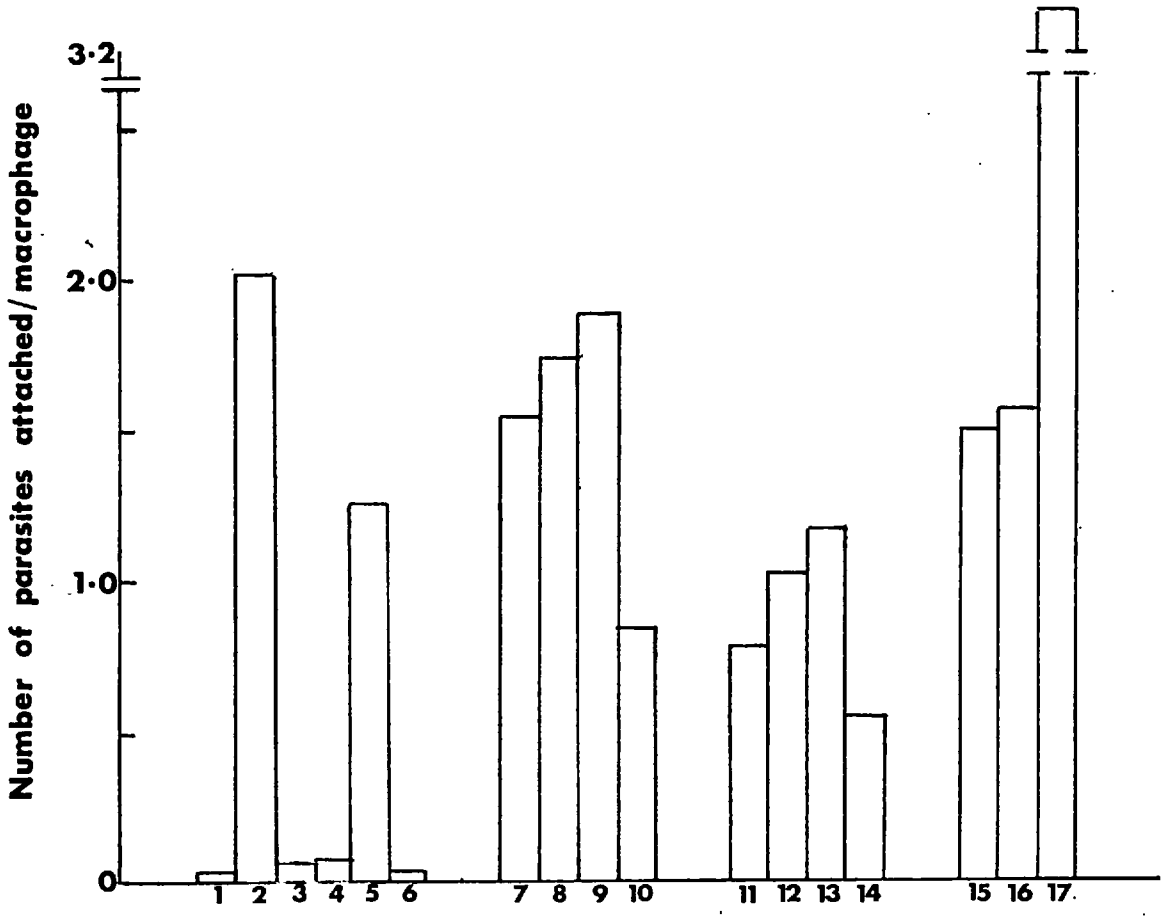

Number of parasites attached/macrophage
3·2
2·0
1·0
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Fig. 23.

Fig. 23. Attachment of T. brucei 427 to mouse peritoneal exudate cells. The phagocytic properties of various surfaces.

1. Glass
2. Aluminium foil
3. Mucus
4. Filter paper
5. Fibrin clot
6. Albumin
7. Lung
8. Liver
9. Spleen
10. Gastric mucosa
11. Boiled lung
12. Boiled liver
13. Boiled spleen
14. Boiled gastric mucosa
15. Fibrin clot plus 10% mouse hyperimmune T. brucei 427 serum
16. Gastric mucosa plus hyperimmune serum
17. Boiled liver plus hyperimmune serum

mediated adherence. As a result, no direct comparisons can be made between these results and those obtained using coventional macrophage monolayers. The peritoneal cells recovered were usually rounded, only occassional ones showing extension. A proportion would have been expected to have penetrated the various surfaces tested, although natural membrane penetration in vitro may not be common (Ambrose & Easty 1973).

High values were obtained from the fresh tissue surfaces (lung, liver and spleen), but not so from the gastric mucosa (presumably because of cell damage caused by the acid (Wood et al 1946)). After boiling the tissues, the recorded values, although still comparatively high, were significantly reduced. This may have been due to morphological alterations to the tissue surface or, more likely, to destruction of natural opsonizing tissue antibodies which may have been originally present. It has previously been suggested that homogenized normal mouse liver can absorb out a proportion of the opsonizing activity of hyperimmune serum (section 3.1.10). Few attachments were recorded on glass surfaces, mucus, filter paper or albumin, but more on aluminium foil and the layer of fibrin. Inert surfaces, therefore, appear capable of supporting significant activity in the absence of sera, although this is dependent upon the correct physical property - perhaps related to the size of both the phagocytic cell and the trypanosome. The three treatments involving hyperimmune serum gave high results, presumably largely dependent upon the opsonic ability of immune sera.

3.7. Opsonization and Rabbit Macrophages

3.7.1. Opsonization in an Homologous System

The opsonic activity of sera obtained from infected rabbits was tested using cultured peritoneal macrophages from uninfected rabbits and 427 trypanosomes from heavily infected rats.

The induced rabbit macrophages were morphologically different from the unstimulated cells obtained from the peritoneal cavities of mice. The rabbit cells were larger and more diffuse, often containing endocytosed droplets of paraffin; they also possessed a higher number of dense bodies. They were as capable of attaching and spreading on glass as mouse cells, despite being cultured for only 4 or 5 h. They would also be expected to have an increased number and/or avidity of receptors (Franz et al 1975).

Immune sera were collected from three rabbits; S447, S448 and S449. They were each inoculated by the subcutaneous (s/c), intramuscular (i/m) and intravenous (i/v) routes respectively with between 2 and 6 x 10^8 T. brucei 427 in PSG. Sera were collected twice weekly from day 0 until 7 weeks post infection. Rabbits S447 and S449 died on days 55 and 54 respectively. They both showed typical symptoms, S449 showing high blood parasitaemia throughout the infection. Rabbit S448 showed no external symptoms, and was aparasitaemic after 35 days of infection. It remained asymptomatic and aparasitaemic twelve weeks post infection. For further details on the course of infection, see section 3.10. Details of the parasitaemia, sera collected and the agglutinating antibody titres for each rabbit are given in Appendices 2 & 3. Immune and hyperimmune sera were further tested for the presence of γ globulin and anti-trypanosome antibodies by the Ouchterlony double diffusion method (Appendix 3). Serum was also collected from an uninfected control rabbit (S522) at weekly intervals for six weeks.

The results of the opsonic test are given in Fig. (24). The opsonic activity of sera from each of the three infected rabbits showed a progressive rise from control levels to reach an optimum value between about 10 and 25 days post infection. Thereafter the values tended to level out. They remained approximately eight times higher than those obtained from uninfected control sera (S522). The latter values were nevertheless some ten times higher than those recorded from NMS in an homologous system. It is known that a higher level of natural opsonin is present in rabbits than in mice (Stiffel et al 1970). Very high results,-130% attachment, or trypanosome:macrophage ratios of 1.3:1 - were recorded with homologous rabbit hyperimmune 427 serum, obtained by the repeated inoculation of rabbit S454 with dead trypanosomes and FCA. Low values (3.5%), were obtained using sera from rabbit S456 repeatedly inoculated with FCA alone.

The results for the three infected rabbits were statistically different at a 5% level. This may have been a reflection of both the intrinsic variability of the test and of the animals used. Rabbit S447 showed a rapid increase, approaching 100% attachment after 12 days, with high levels remaining for the rest of the infection. This was closely correlated with the level of agglutinating antibody. Similarly high titres were obtained with sera from rabbits S448 and S449, yet in both instances, the recorded attachment indices (optima of 70% and 80% respectively), were less than those from rabbit S447. A possible result of differences in infection due to different inoculation routes and genetic differences between outbred animals.

The high values obtained in vitro suggest that the attachment and subsequent phagocytosis of trypanosomes by rabbit macrophages in the presence of highly immune sera may well be an effective defence mechanism during initial infection.

Fig. 24.

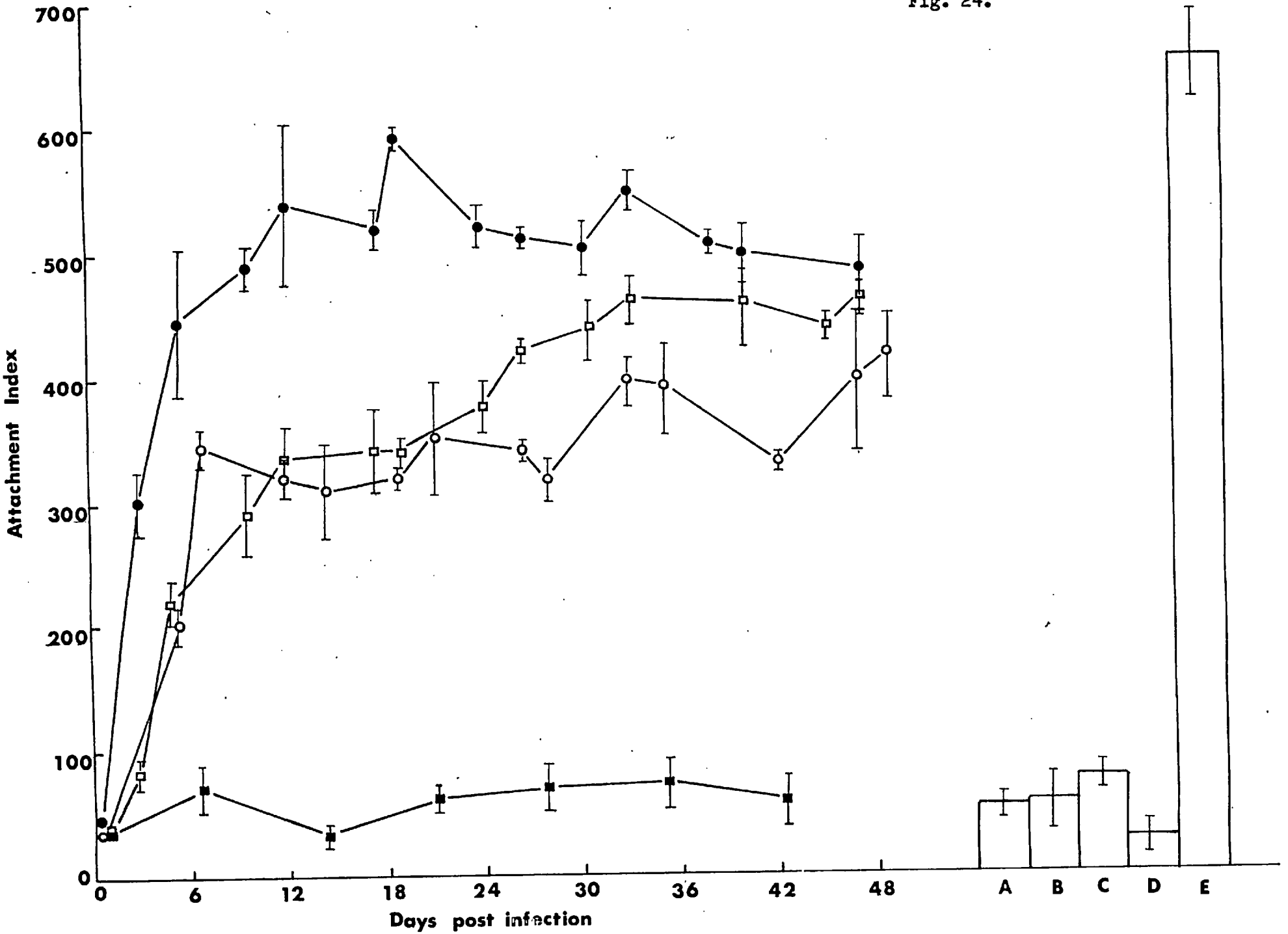

700
600
500
400
300
200
100
0
Attachment Index
0
6
12
18
24
30
36
42
48
Days post infection
A
B
C
D
E

Fig. 24. Attachment of T. brucei 427 to rabbit peritoneal macrophages (opsonic test).

The effect of homologous rabbit sera.

●———● rabbit S447 - infected s/c.

o———o rabbit S448 - infected i/m.

□———□ rabbit S449 - infected i/v.

■———■ rabbit S522 - non-infected control.

A: TCM

B: PSG

C: 0.85% saline

D: FCA (rabbit S456)

E: Rabbit hyperimmune T. brucei 427 (rabbit S454)

All results expressed are the mean ($\pm$ SE) of 5 replicates.

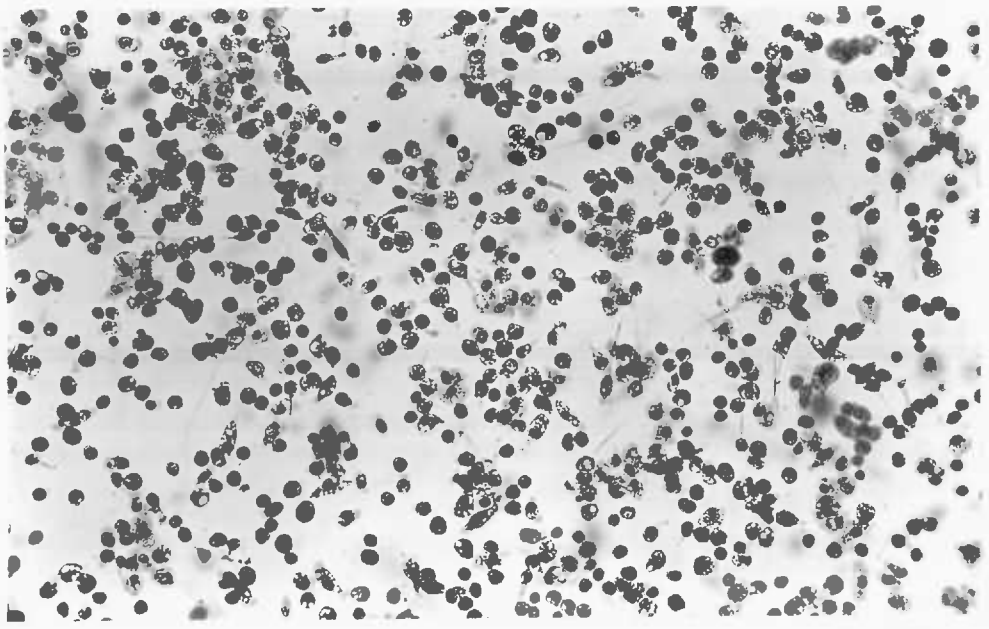


Plate H.

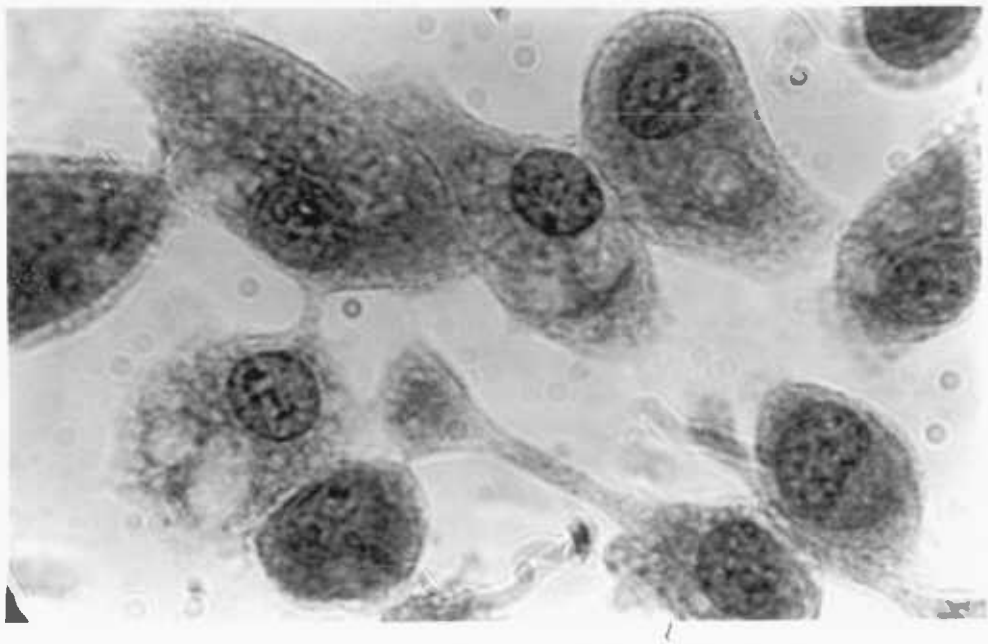


Plate I.

Plate H. Rabbit peritoneal macrophages obtained from an oil-induced animal, cultured for four hours. Fixed and stained in Giemsa solution (x250 magnification). Note heterogenous shape and size of cells and the number of cytoplasmic extensions.

Plate I. Cultured rabbit peritoneal macrophages (x1000 magnification). Note vacuolar appearance, long cytoplasmic extensions and cytoplasmic bridges.

Plate J.

Plate K.

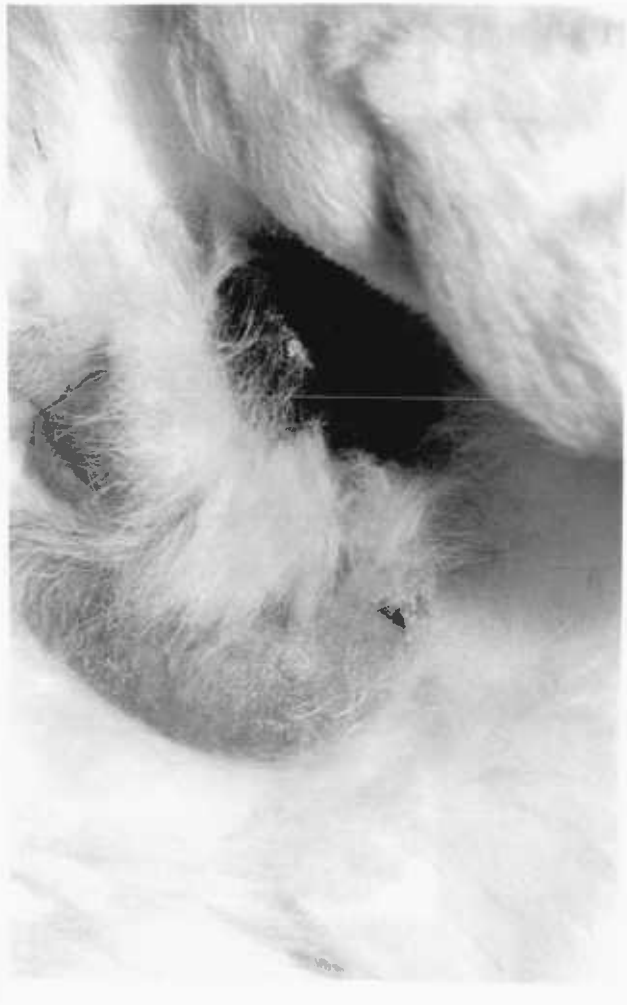


Plate L.

Plate J. Uninfected control rabbit.

Plate K. Rabbit infected with T. brucei 427. 36 days post infection. Note typical oedema around head and face.

Plate L. Rabbit infected with T. brucei 427. Necrotic lesions around genitalia.

3.7.2. Variant Specificity within an Homologous System

A close relationship between opsonic activity and antibody titre has been demonstrated. It was not apparent from the results obtained whether this activity was variant specific. This possibility was, therefore, tested.

(a) The opsonic test was performed using peritoneal macrophage monolayers prepared weekly from uninfected, induced rabbits and sera from rabbits S447 and S468. Both animals had been infected s/c with T. brucei 427, and the infections ran normal courses (Appendix 2). Sera were collected biweekly.

The normal passage infection of T. brucei 427 in 4+ infected mice was raised in albino rats and the trypanosomes subsequently washed and separated. Most of the parasites were used as the day 0 source for testing. A proportion (5.0×10^8) were injected s/c into rabbit S513. Approximately 2 ml. of blood was removed from the marginal ear vein at weekly intervals (days 7, 14, 21 and 28) and inoculated intraperitoneally into CD-1 mice, using heparin as the anticoagulant. Within 3 days, the heavily infected mice were sacrificed and the trypanosomes sub-inoculated into rats. These were bled within a further 3 days, the trypanosomes were washed and separated and used as the source of particulate antigen for each opsonic test. The complete results can be found in Appendix (3), and are graphically represented in Figs. (25a) and (25b).

(b) The opsonic test using both homologous and antologous sera was also carried out. Induced macrophages were cultured at weekly intervals. Immune sera were obtained from rabbits S531 and S532, s/c inoculated with 5.6×10^8 T. brucei 427. For details of the infections see Appendix (2).

Rabbit S531 was also used as the source of variant trypanosome populations. The day 0 trypanosomes were again raised in passage mice. S531 was bled at weekly intervals (days 7, 14, 21, 28, 35, 42 and 49) and mice were inoculated. Within 3 days the resultant infections were re-passaged and 2 days later the mice were sacrificed. The trypanosomes in whole blood were rapidly frozen in liquid nitrogen (-70°C.) in capillary tubes, using 7.5% glycerol as a preservative. Eight separate trypanosome stabilates were thus prepared. These were individually thawed and inoculated into CD-1 mice. After a maximum of 3 days, albino rats were infected and bled 2 or 3 days later. The washed, separated trypanosomes were used as the source of particulate antigen throughout. The results are given in Appendix (3) and graphically in Figs. (25c) and (25d).

(c) It was conceivable that the original strain of trypanosome - T. brucei 427 - consisted of a mixed population, hence masking any antigenic variation which may have been present. Consequently, a cloned strain, T. brucei Etat 4, was also tested.

Oil-induced macrophages were obtained from uninfected rabbits. Rabbit S576 was used as the source for both specific antisera and for trypanosome populations. T. brucei Etat 4 ran a chronic course in rabbit S576, with symptoms very similar to those normally expressed by T. brucei 427. Sera were again prepared at biweekly intervals from day 1 until day 33. The rabbit died 40 days post-infection (Appendix (2)).

Day 0 stabilate (basic antigenic type) was prepared from the initially infected mice. Rabbit S576 was s/c infected with 8.3×10^7 trypanosomes in whole mouse blood, and thereafter the infection was raised in CD -1 mice at 7 day intervals (days 7, 14, 21 and 28). Stabilates were prepared as before and maintained at -70°C. until required. The results are presented in Appendix (3) and in Fig. (25e).

The antibody agglutinating test was also carried out for each stabilate population against the whole range of autologous sera collected, in order to determine the number of distinct populations present (Appendix (3)).

The weekly values for rabbits S468 (Fig. (25b)) and S576 (Fig. (25e)) were not significantly different at a 5% probability level, whereas those for rabbits S447, S531 and S532 were. The opsonic activity of each serum series tested against the various populations showed a progressive increase from low levels to attachment levels around 100%. In many instances optimum values with variant populations were not seen until late in infection. These did not, however, show a sequential progression; thus a population isolated 49 days post infection could exhibit a more rapid and earlier increase than one isolated after 14 days (S532, Fig. (25d)). The agglutination test for S576 sera indicated at least 4 separate trypanosome populations, yet the opsonic test could not distinguish between them. Trypanosomes isolated 28 days post infection were capable of being maximally opsonized with day 19 serum. No obvious differences were seen between autologous and homologous sera or between T. brucei Etat 4 and T. brucei 427 populations. It appears, therefore, that the opsonins present in immune rabbit sera are not primarily variant specific.

The rate of increase and amount of activity appears dependent upon the quantity of antibody present, and is thus related to the duration of infection. Consequently, S468, which had an infection lasting 79 days, did not show maximum attachment for any population tested, until about 35 days post infection, whereas S532, which died after 50 days, showed maximum attachment after about 25 days. In each case, linear increases were observed, varying considerably in slope. Thus S447 day 0 population reached a maximum after about 10 days, yet the day 14 population did not do so until 30 days post infection.

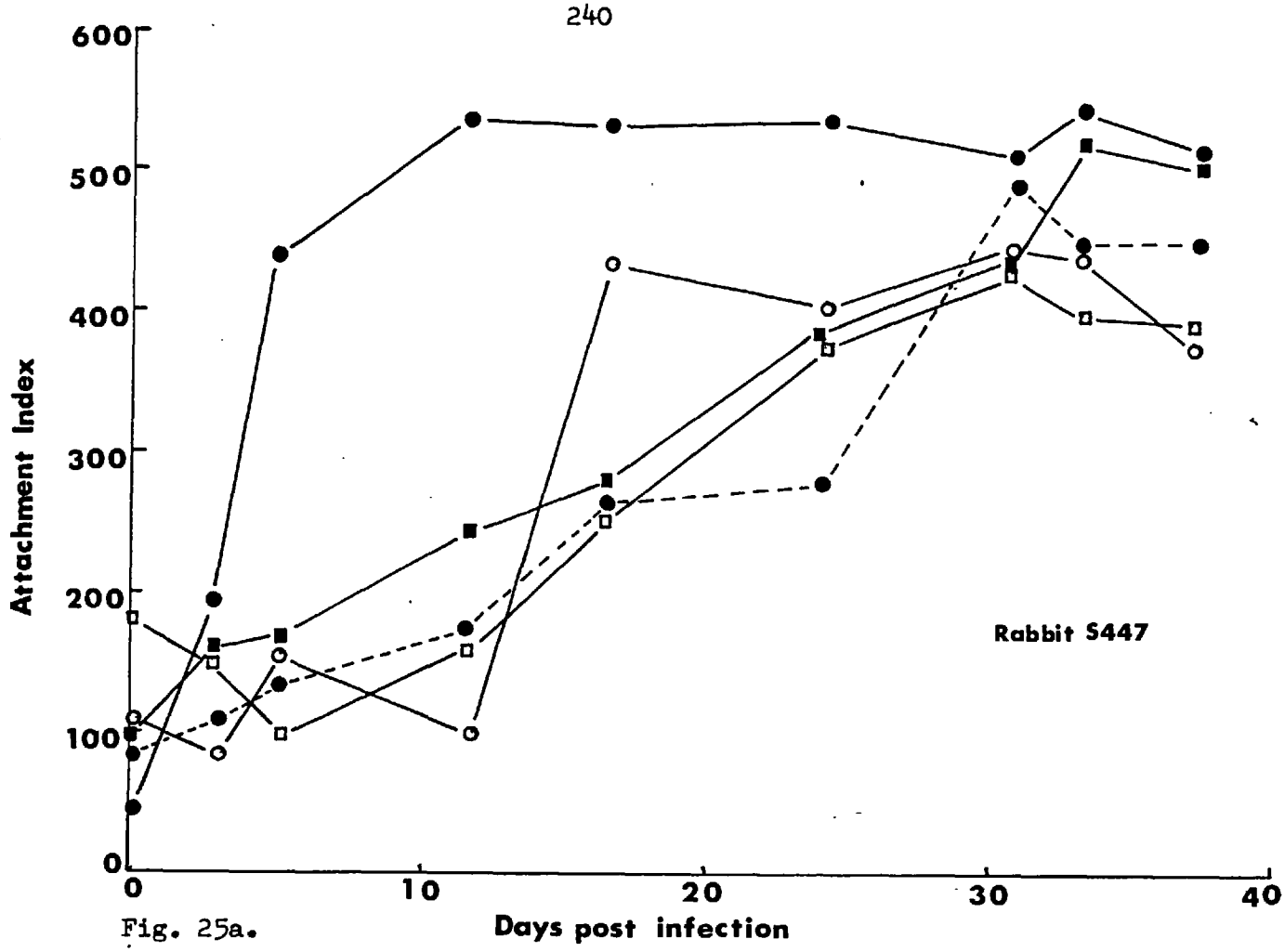


Fig. 25a.

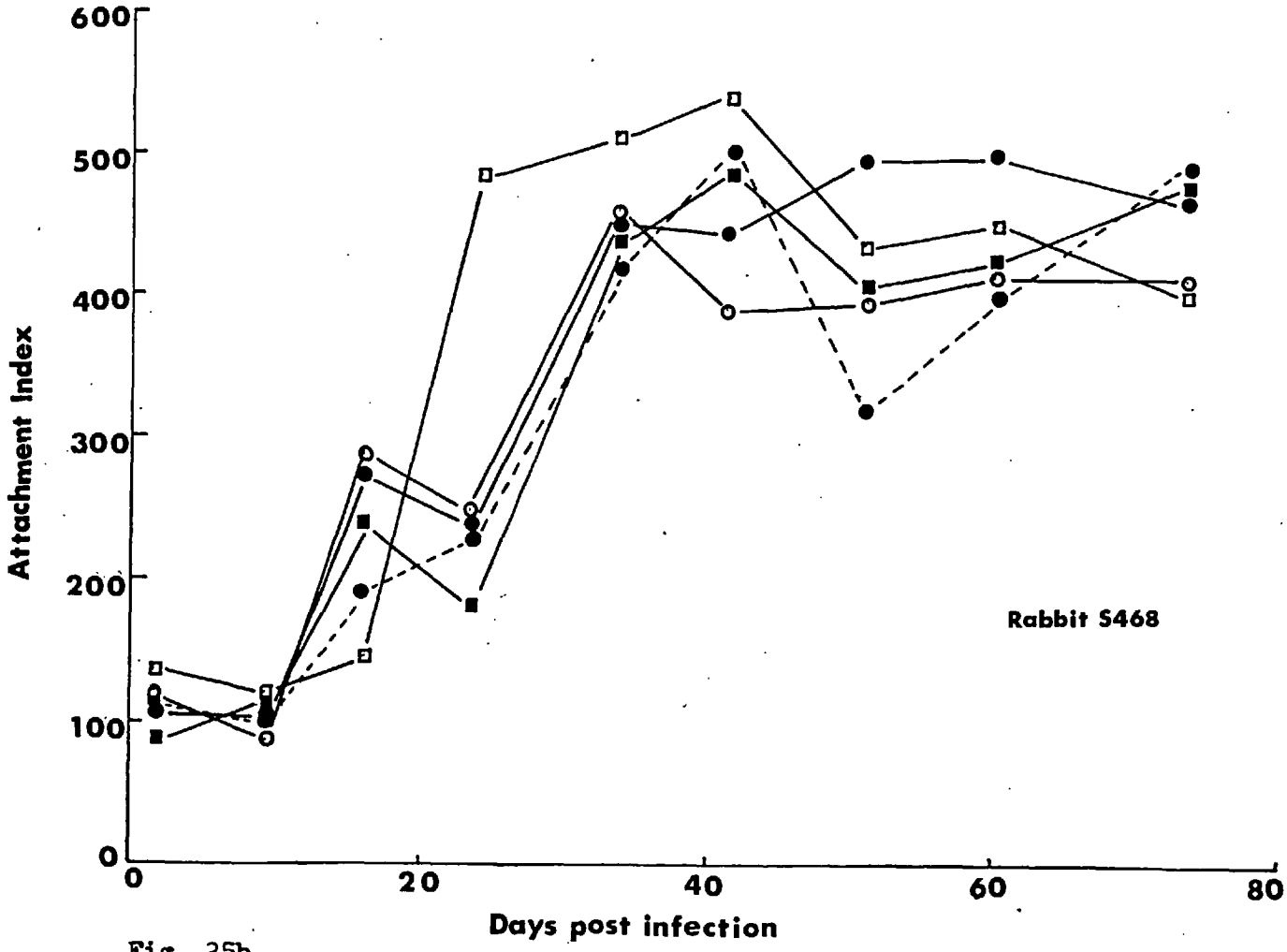


Fig. 25b.

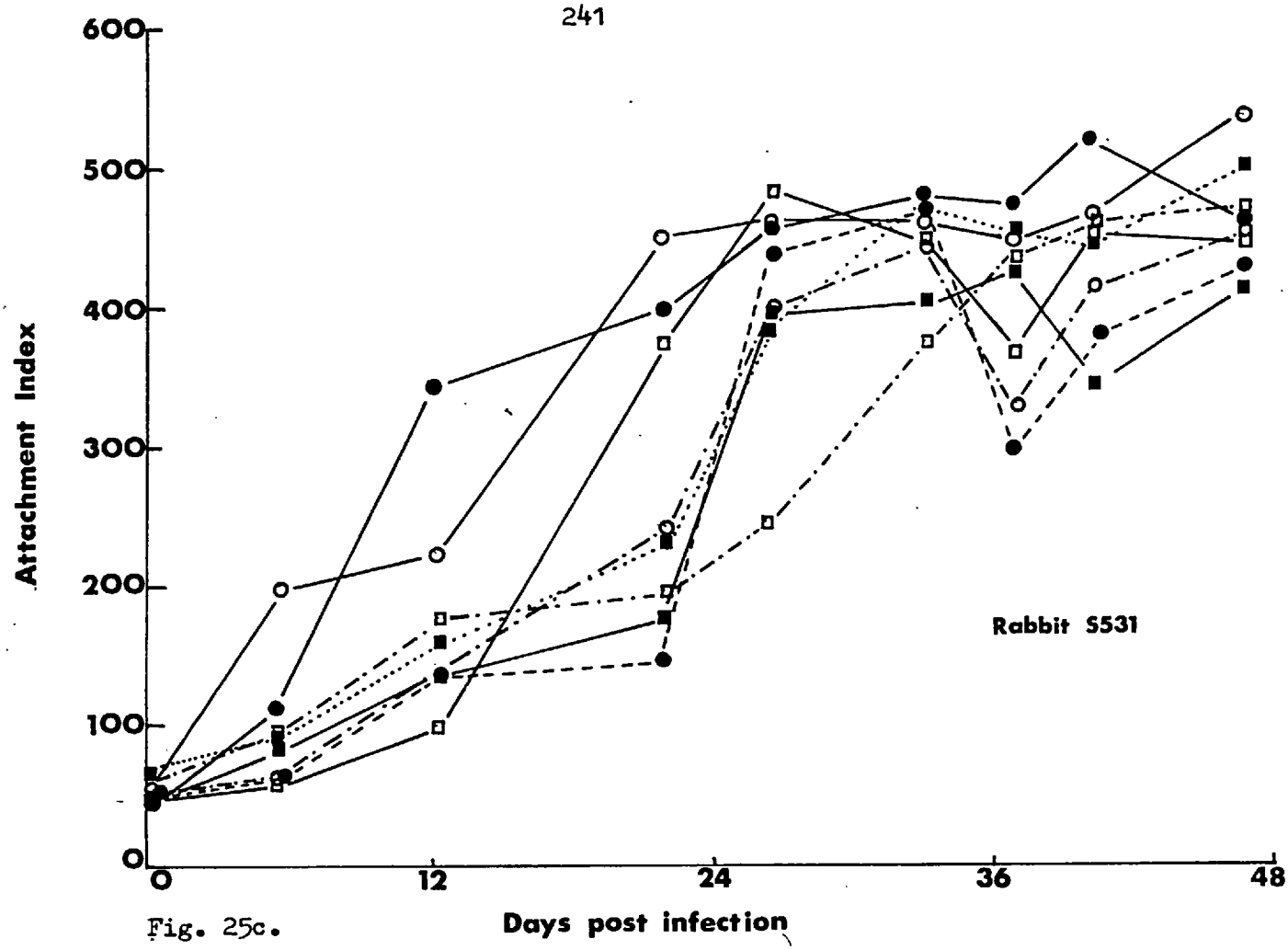


Fig. 25c.

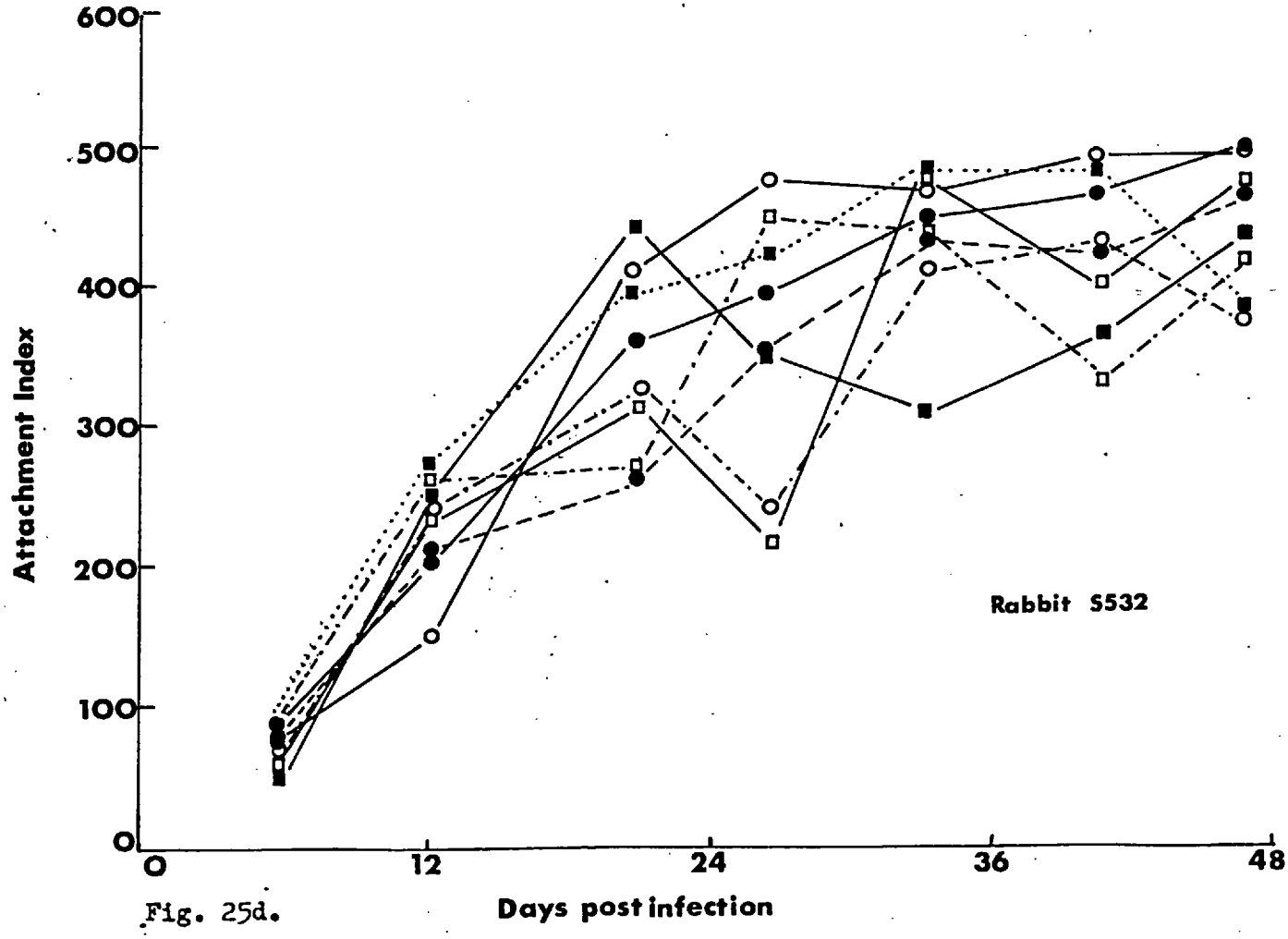


Fig. 25d.

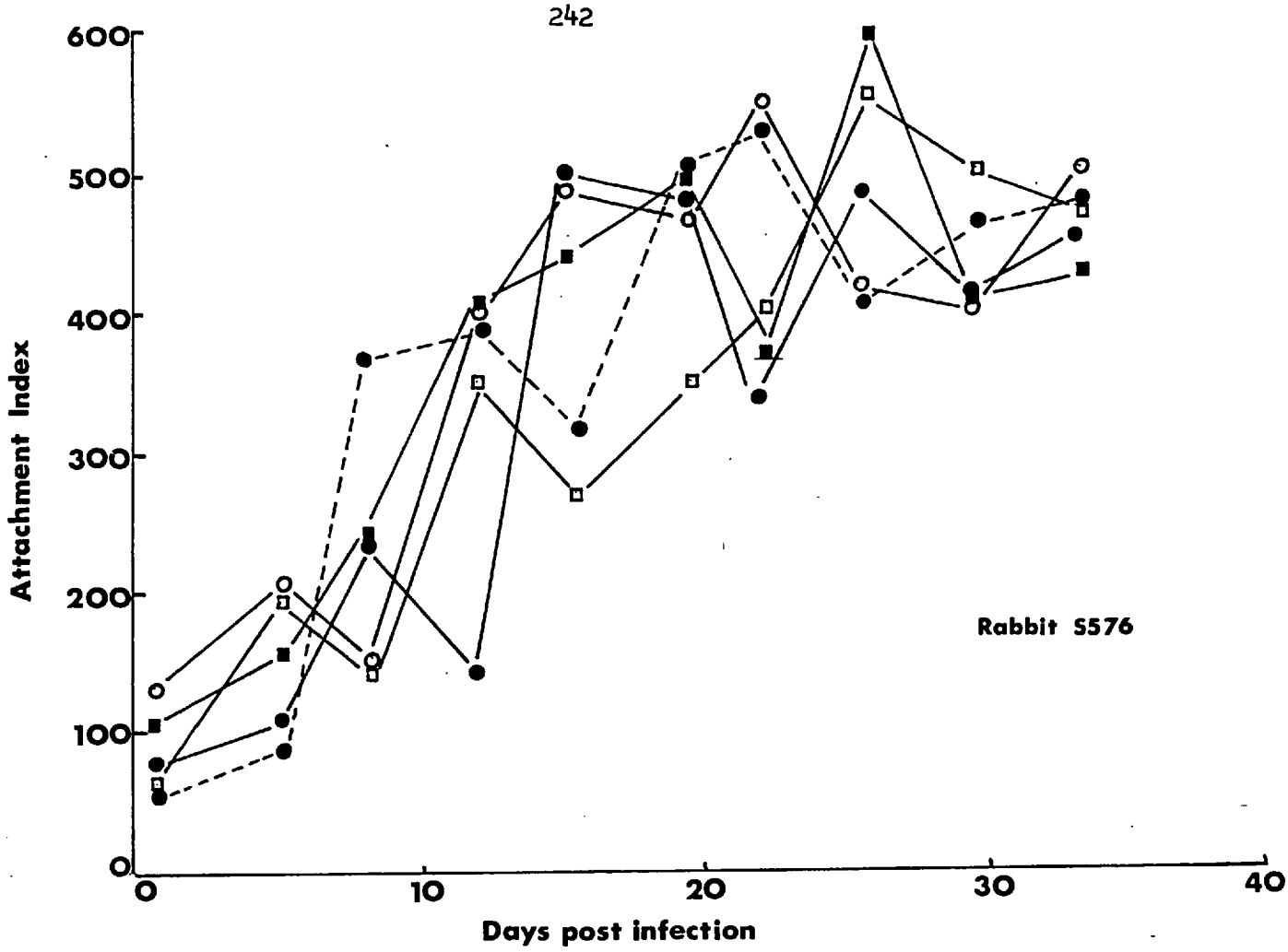

600
500
400
300
200
100
0
Attachment Index
0
10
20
30
40
Days post infection
Rabbit S576

Fig. 25e.

Figs. 25a-e. Attachment of T. brucei to rabbit peritoneal macrophages (opsonic test).
Effect of antigenic variants.

a. Rabbit S447 infected s/c with T. brucei 427 } variants raised
b. Rabbit S468 infected s/c with T. brucei 427 } in rabbit S513

c. Rabbit S531 infected s/c with T. brucei 427 } variants raised
d. Rabbit S532 infected s/c with T. brucei 427 } in rabbit S531

e. Rabbit S576 infected s/c with T. brucei Etat 4 — variants raised in rabbit S576

Variants isolated on days post infection:

●——● day 0 (basic antigen)
○——○ day 7
□——□ day 14
■——■ day 21
●----● day 28
○-·-·-○ day 35
□-·-·-□ day 42
■·····■ day 49

All results expressed are the mean (± SE) of 3 replicates (see Appendix 3)

Plate M.

Plate M. Rabbit infected with T. brucei Etat 4. 28 days post infection.

Note intense oedema of head and necrotic involvement of eyes and nares.

3.7.3. Opsonization in an Heterologous System

To investigate the degree of specificity, induced peritoneal macrophages from a rabbit were tested for their capacity to attach T. brucei 427 in the presence of antisera raised against an infection of T. brucei S42 in mice (Series B) (Appendix (2)).

The results of the opsonic test are given in Fig. (26) and may be compared with the same heterologous test, but involving peritoneal macrophages from normal mice (section 3.1.5.). A higher number of trypanosomes attached to rabbit macrophages; the attachment index remained initially stable and rose during the latter half of the infection, to reach a maximum value of 40%, 16 days post infection. Sera collected during the first week gave values of approximately 17% attachment, suggesting that a proportion (approx. 42%) of the observed activity was not due to specific opsonization. This may have been due to the activated nature of the rabbit macrophages, the phagocytic potential of which was non-specifically enhanced through oil-induction.

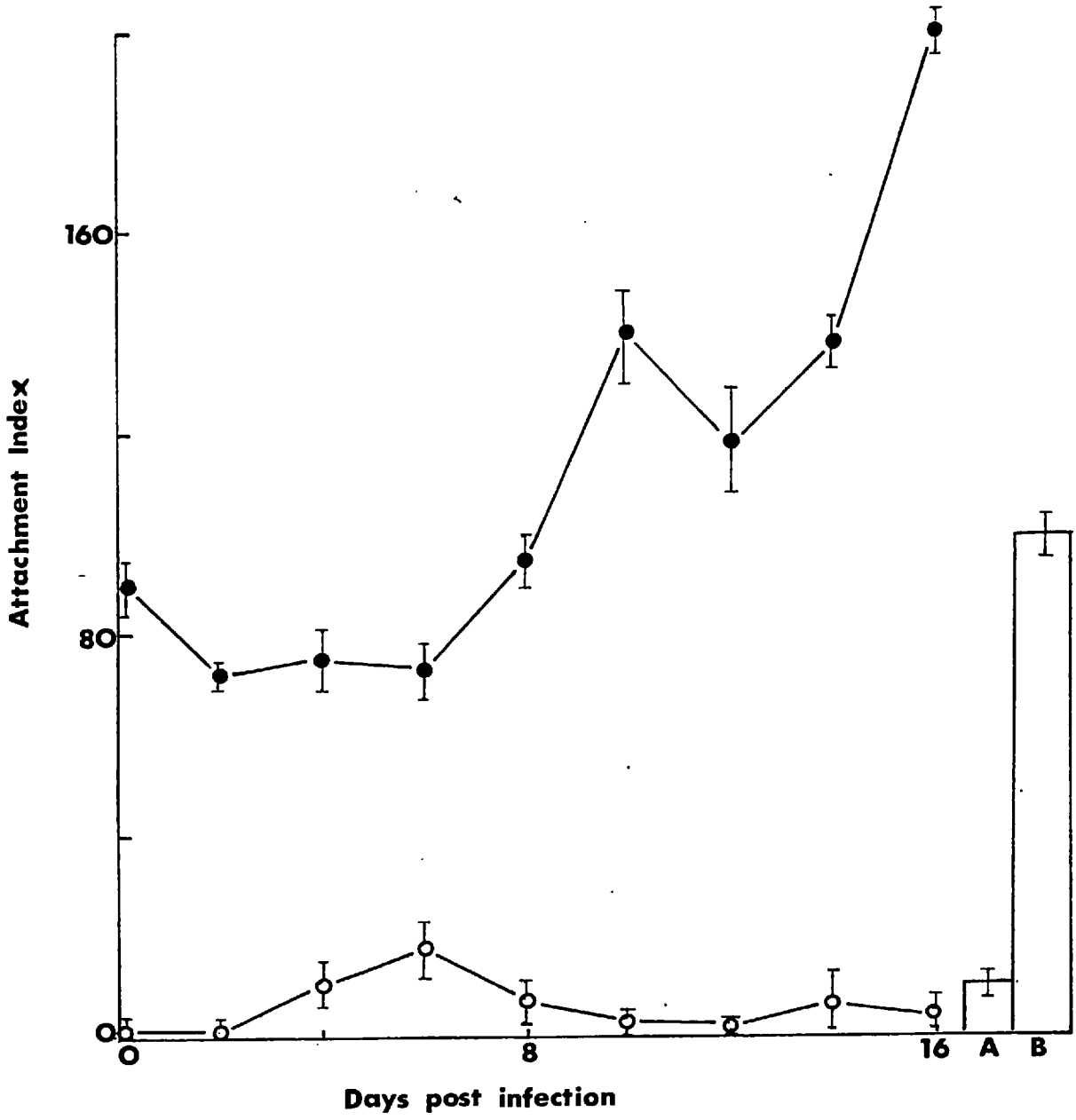


Fig. 26.

Fig. 26. Attachment of T. brucei 427 to rabbit and mouse peritoneal macrophages (opsonic test).
Effect of heterologous immune sera.

o——o mouse peritoneal macrophages. T. brucei S42 serum (serum series B')

A: mouse hyperimmune T. brucei S42 serum

●——● rabbit peritoneal macrophages. T. brucei S42 serum (serum series B')

B: mouse hyperimmune T. brucei S42 serum

All results expressed are the mean (± SE) of 5 replicates.

3.7.4. Effect of Chemicals

A selection of chemicals which had been previously shown to affect the attachment of trypanosomes to mouse peritoneal macrophages either through opsonic or cytophilic adherence, were tested using rabbit macrophages (see section 3.1.9.).

The opsonic test was carried out using induced peritoneal macrophages, T. brucei 427 and the following chemicals:- trypsin (0.2% w/v); sodium azide (1% w/v); iodoacetate (0.05mM) and 5-HT (0.2% w/v).

All chemicals were dissolved in TCM and adjusted, if necessary, to pH 8.0. Sera from rabbit S447 taken on days 0, 10, 31 and 47 post infection, and rabbit hyperimmune 427 serum were added to each treatment at a concentration of 10%. The mixtures were incubated at 37°C. for 15 min. and used at R.T.

The results in Fig. (22), indicate that trypsin in conjunction with immune and hyperimmune sera caused a reduction in opsonic activity. This was most marked with hyperimmune serum, a decrease of over 60% being recorded. The value for day 0 serum was increased about four fold. All results were statistically similar at a 0.5% level.

Similar results were obtained using sodium azide. A large fall was noted with immune sera, but again a slight enhancement with day 0 serum. The effect of iodoacetate was less pronounced, except in the case of hyperimmune serum, but similar to that of sodium azide. Thus suggesting that selective immune recepter sites, most probably on the macrophage surface, are partially destroyed by these agents. It is also possible that the parasites or immune antibodies are affected.

Consistently high opsonic values of around 100% were recorded with 5-HT in conjunction with all sera tested, perhaps indicating the sensitizing role of 5-HT.

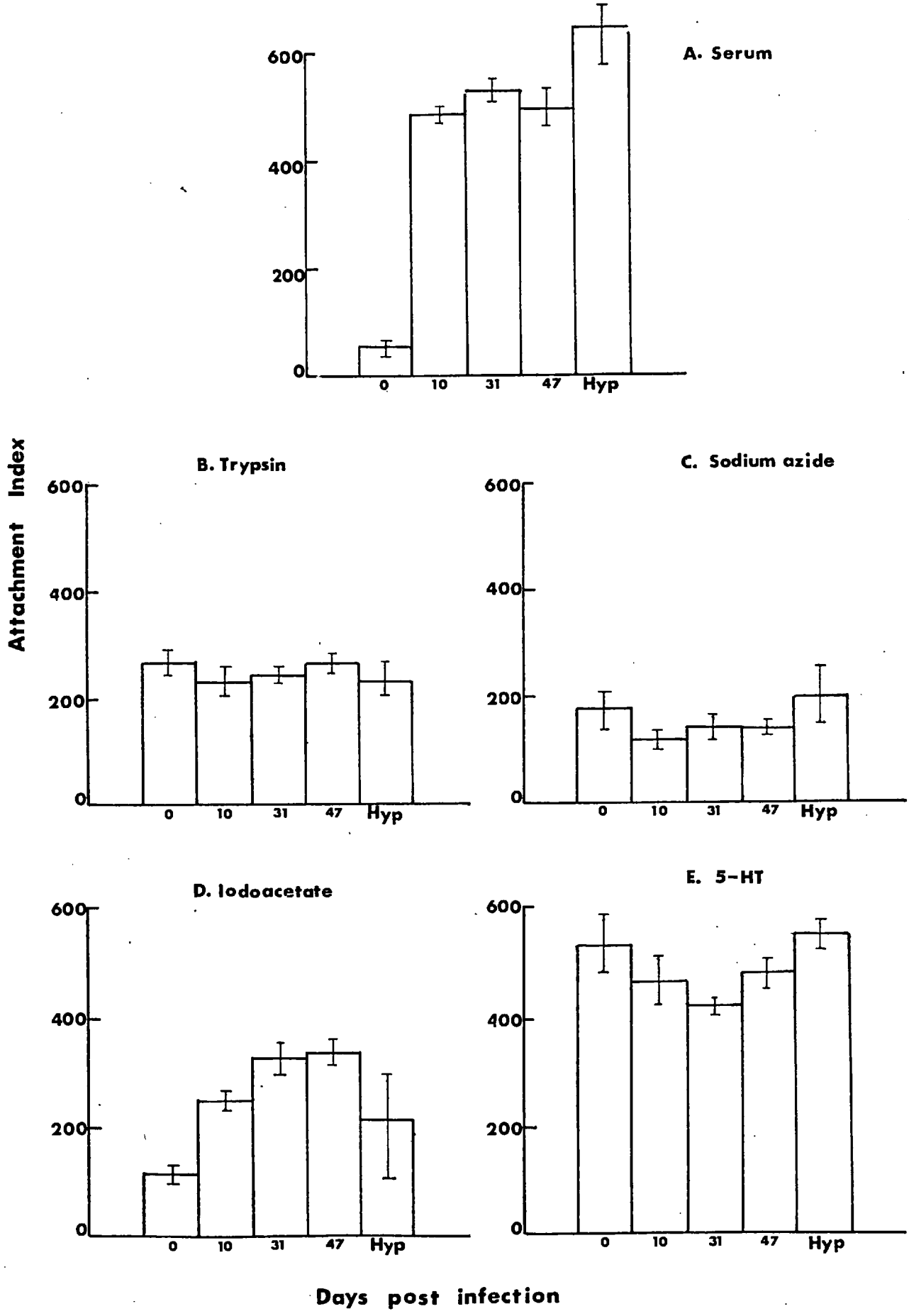

A. Serum
B. Trypsin
C. Sodium azide
D. Iodoacetate
E. 5-HT
Attachment Index
600
400
200
0
0
10
31
47
Hyp
Days post infection

Fig. 27.

Fig. 27. Attachment of T. brucei 427 to rabbit peritoneal macrophages (opsonic test).

Effect of chemical treatment of immune and hyperimmune sera.

Infected sera obtained from rabbit S447.

Mouse hyperimmune T. brucei 427 serum (Hyp) obtained from rabbit S454.

All results expressed are the mean ($\pm$ SE) of 5 replicates.

3.7.5. Opsonic Activity of Fractionated Rabbit Sera

Figs. (28), (29), (30) show the effect of gel filtration on the opsonic activities of fractionated NRS, hyperimmune 427 and infected serum taken from rabbit S468, 56 days post infection.

The opsonic test using rabbit macrophages and T. brucei 427 was carried out for each sample. Low, statistically-similar results were obtained from each fraction of NRS, indicating a general lack of activity. The infected serum, however, showed a major peak of activity corresponding to fraction III, suggesting that IgG is the effective opsonin. The values for fractions I, II, IV and V were statistically similar at a 0.5% probability level. A similar picture was seen using fractionated hyperimmune serum. The major peak again corresponded to low MW γ globulin-IgG. Some significant activity was, however, expressed by fractions II and IV. Despite the high levels of IgM present in the infected serum it did not materially enhance attachment.

Fig. (30) also indicates the effect of fractionated hyperimmune serum tested with T. brucei 427 and cultured mouse peritoneal macrophages. Low values were obtained with this heterologous system, but with nevertheless a slight significant peak of activity corresponding to the IgG fraction. The results further suggest that serum-mediated attachment may be largely species-specific.

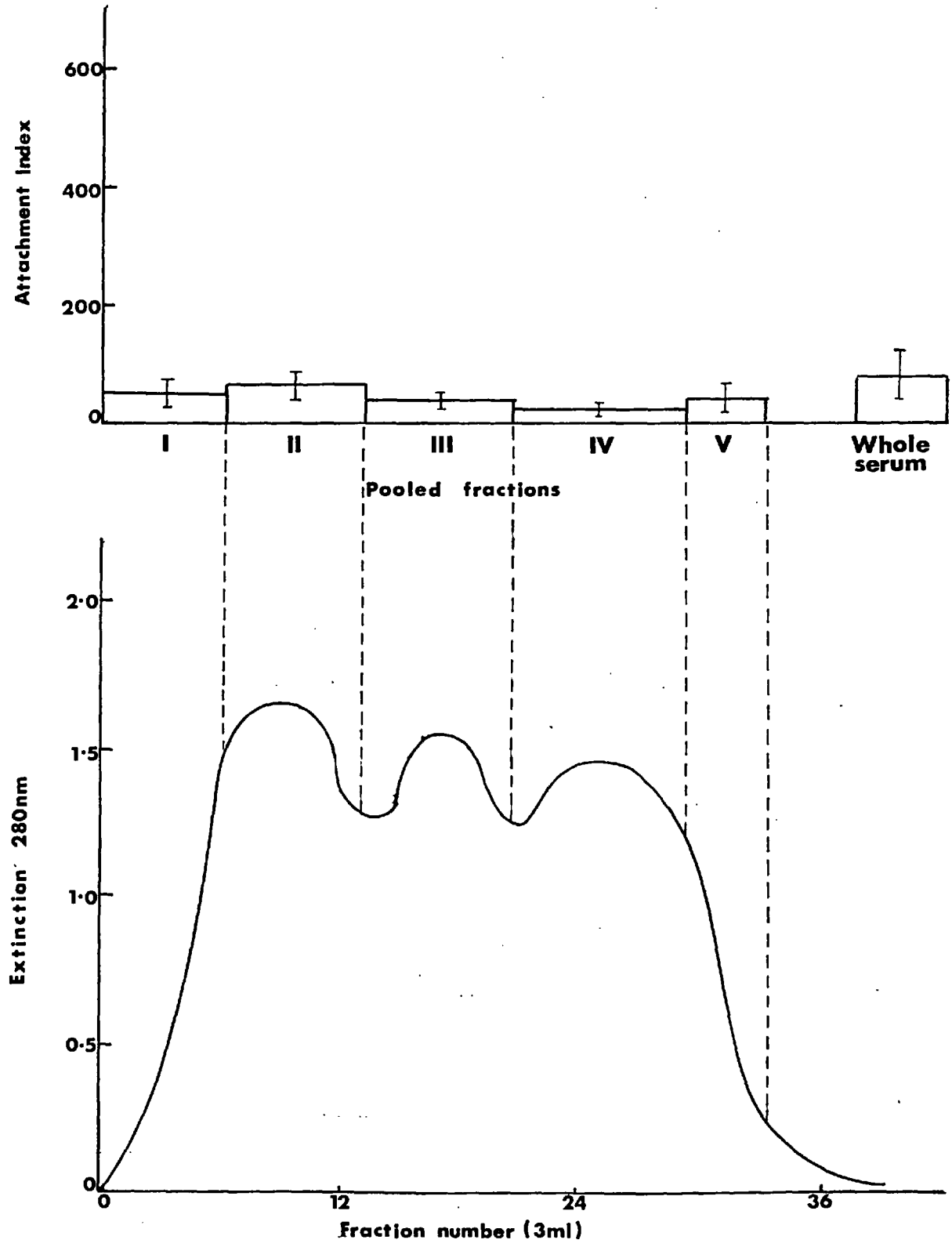

Attachment index
600
400
200
0
I
II
III
IV
V
Whole serum
Pooled fractions
Extinction 280nm
2·0
1·5
1·0
0·5
0
0
12
24
36
Fraction number (3ml)

Fig. 28.

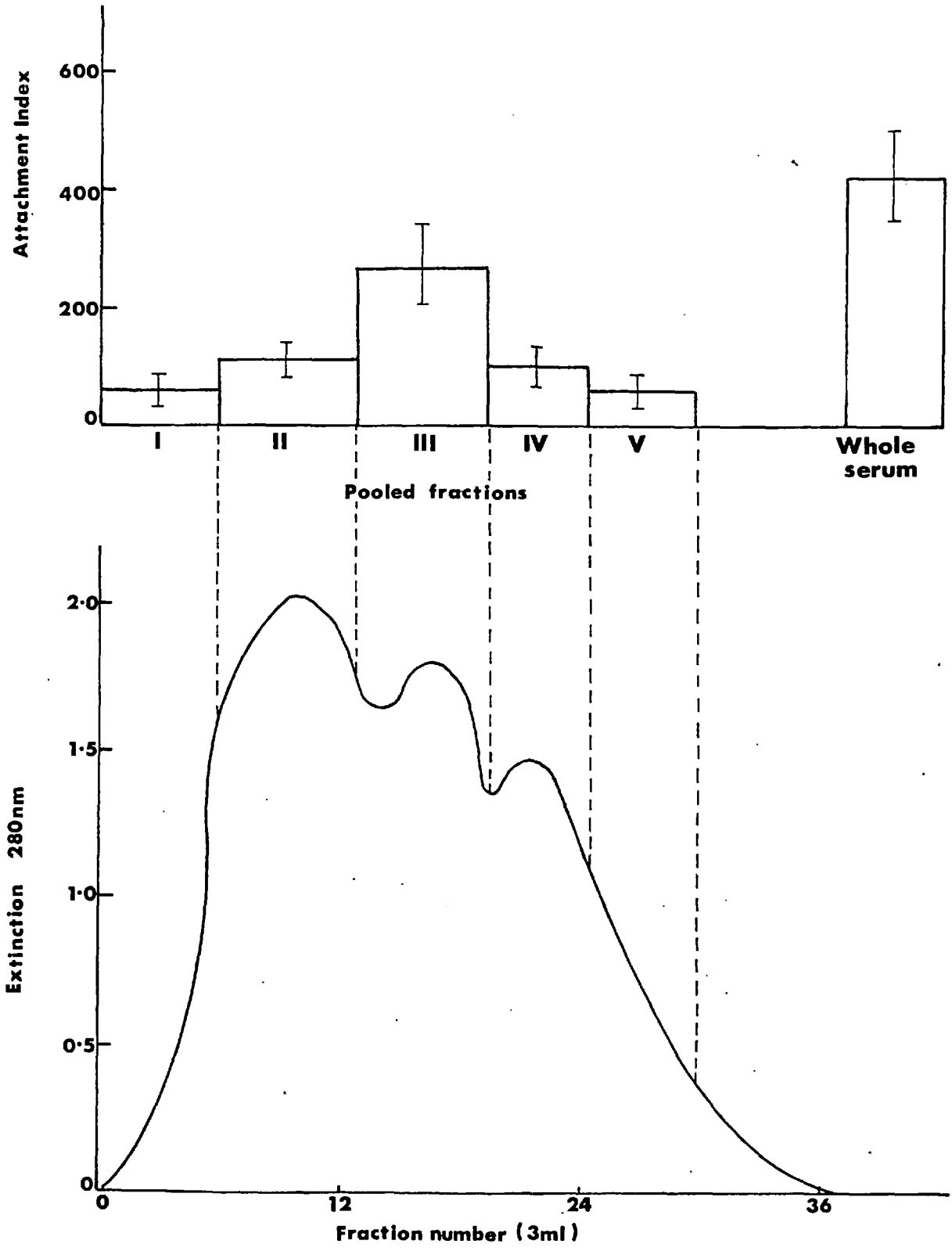

Attachment Index
600
400
200
0
I
II
III
IV
V
Whole serum
Pooled fractions
Extinction 280nm
2·0
1·5
1·0
0·5
0
0
12
24
36
Fraction number (3ml)

Fig. 29.

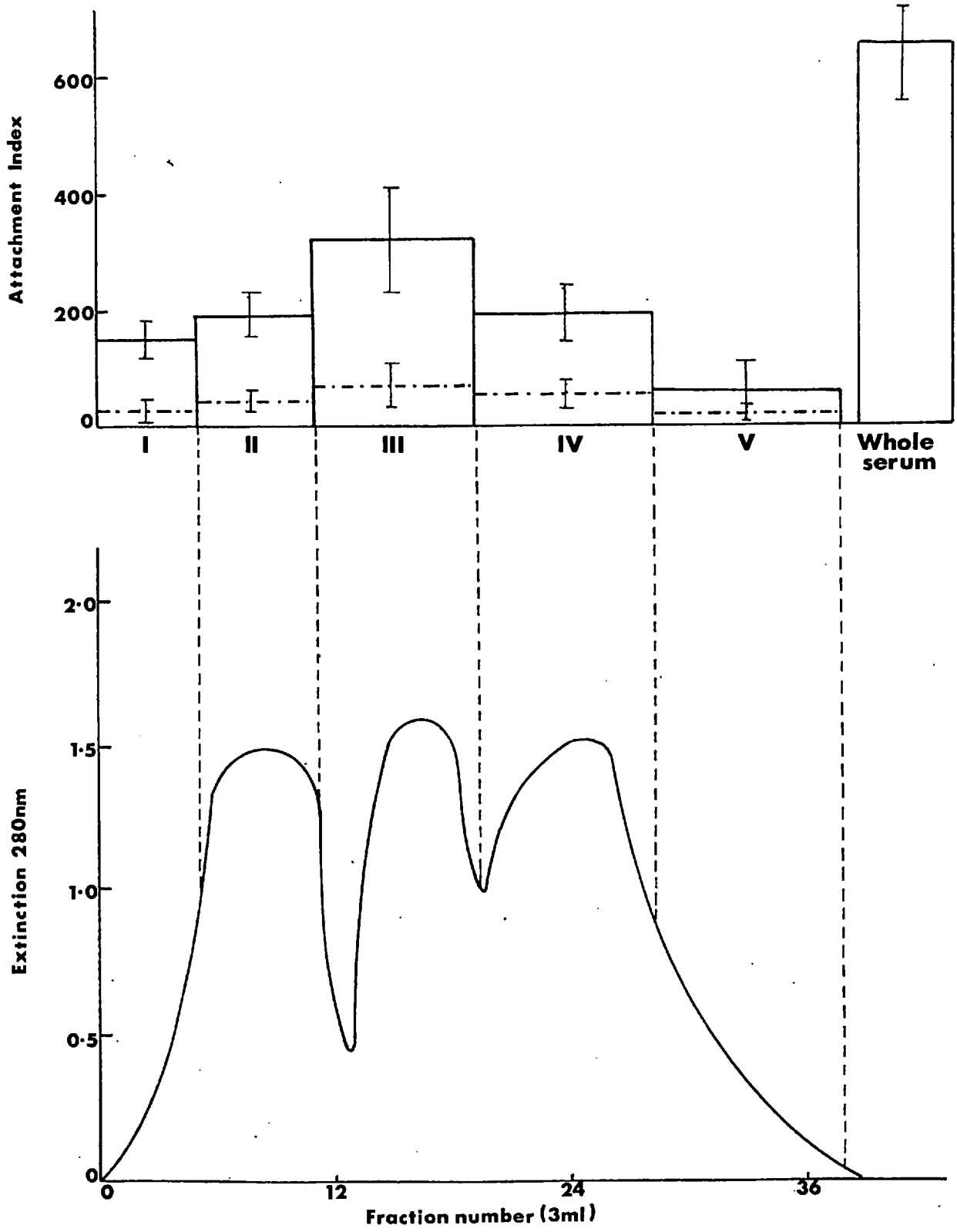

Attachment Index
600
400
200
0
I
II
III
IV
V
Whole serum
Extinction 280nm
2·0
1·5
1·0
0·5
0
0
12
24
36
Fraction number (3ml)

Fig. 30.

Separation of opsonic activity on G200 Sephadex.

Fig. 28. Normal rabbit serum (opsonic test)

Fig. 29. Infected rabbit serum (S468 - 56 days post infection)
(opsonic test)

Fig. 30. Rabbit hyperimmune T. brucei 427 serum (opsonic test)

——— Rabbit peritoneal macrophages (homologous)

-.-.-.-. Mouse peritoneal macrophages (heterologous)

3.8. Macrophage Cytophilic Antibody Activity in Rabbits

3.8.1. Cytophilic Antibodies Present in Rabbit Sera

The cytophilic antibody activity of infected sera from rabbits S447, S448 and S449 was measured, using induced peritoneal macrophages and T. brucei 427.

The results are presented in Fig. (31). Cytophilic antibody activity was present in the immune sera of all rabbits tested. It increased gradually from initial low levels and reached a maximum - approximately 50% attachment - after about 2 weeks post infection. At no stage did the cytophilic antibody activity approach that of opsonic activity, which gave attachment values of around 100%. Again the rise was associated with increased levels of serum antibody.

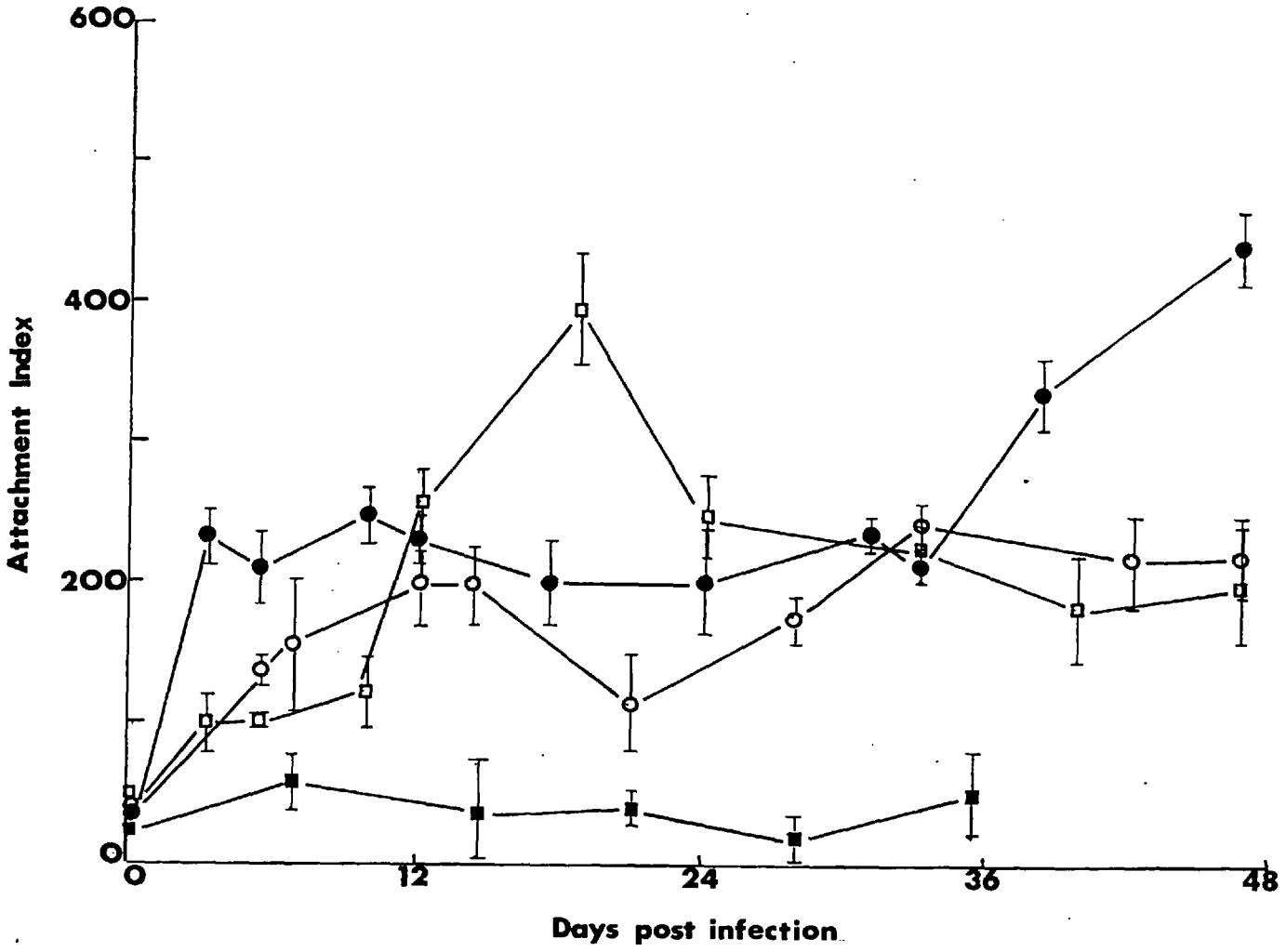


Fig. 31. Attachment of T. brucei 427 to rabbit peritoneal macrophages (cytophilic antibody test).

Effect of homologous rabbit sera.

●——● rabbit S447 - infected s/c

○——○ rabbit S448 - infected i/m

□——□ rabbit S449 - infected i/v

■——■ rabbit S522 - non-infected control

All results expressed are the mean (± SE) of 5 replicates.

3.8.2. Cytophilic Antibodies and Variant Specificity

The macrophage cytophilic antibody test was carried out using rabbit macrophages, sera from rabbit S531 and trypanosomes isolated from the same animal on days 0, 28 and 42 post infection.

The results (Fig. (32)), were not significantly different at a 1% probability level, suggesting that the cytophilic antibody activity observed during infection is not variant specific.

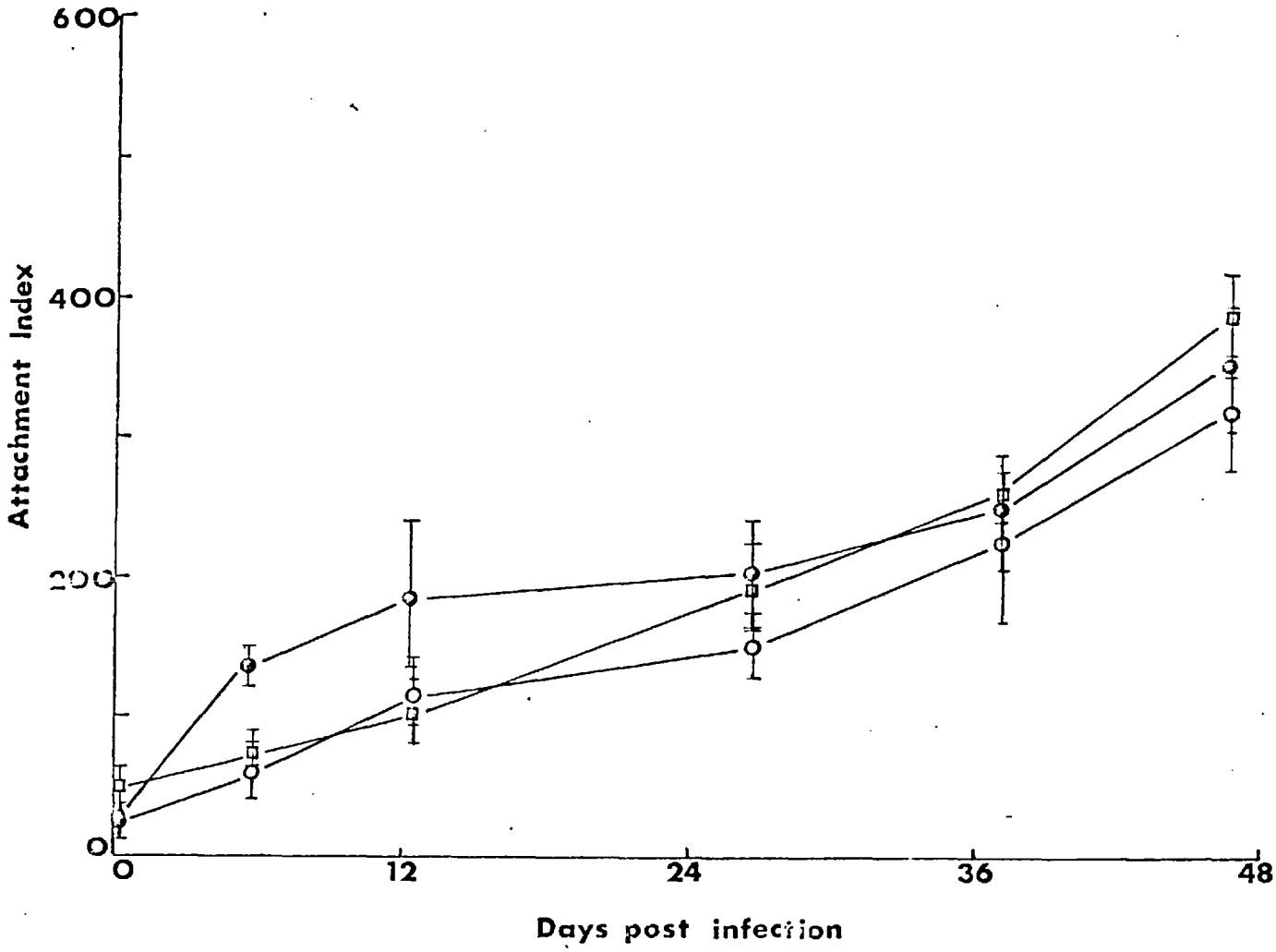


Fig. 32. Attachment of T. brucei 427 to rabbit peritoneal macrophages (cytophilic antibody test).

Effect of antigenic variants.

Sera and variant populations from rabbit S531.

Variants isolated on days post infection:

●——● day 0 (basic antigen)

o——o day 28

□——□ day 42

All results expressed are the mean (± SE) of 5 replicates.

3.9.1. Effect of Rabbit Complement Components

Investigations were undertaken to determine the effects of rabbit C' and selected C' components on the attachment of trypanosomes to rabbit macrophages. The relative importance of specific and non specific antibodies, the classic and the alternate C' pathways were studied utilizing the opsonic adherence and the macrophage cytophilic antibody tests.

Oil-induced peritoneal macrophages were obtained from uninfected rabbits and used in conjunction with T. brucei 427 and rabbit hyperimmune 427 serum which had been selectively depleted of its C' components (section 2.9.4.). Freeze dried, purified rabbit C' prepared from pooled rabbit sera was reconstituted with diluent (Miles Laboratories Inc.).

Both attachment tests were performed normally. Any secondary incubations with purified rabbit C' were carried out at 37°C. for 30 min. All washings were made in BSS.

The arrangement of selective inactivation of the hyperimmune serum was as follows:-

S = sensitized site on trypanosome or macrophage surface

A = C'-fixing antibody

C' = Complement

1-9 = Individual Complement Components.

(a) SAC' —classic→ 1, 4, 2, 3, 5 - 9
SAC' —alternate→ 3, 5 - 9
Uninterrupted classic and alternate C' pathways

(b) SAC' → (1, 4, 2) 3, 5 - 9
SAC' → 3, 5 - 9
Interrupted classic pathway by (R_1 & R_2) and R_4 treatment (in parenthesis)

(c) SAC' → (1, 4, 2, 3) 5 - 9
SAC' → (3) 5 - 9
Interrupted classic and alternate pathways by R_3 treatment (in parenthesis)

(After Mathews et al 1974).

For further elucidation of the pathways involved and the means of selective inactivation see section 2.9.4. and Fig. (33).

Ideally the individual treatments should have been assayed against standardised preparations of purified components, hence establishing the efficiency and, more importantly, the selectivity of the various preparative methods. For conclusive and effective separation of the classic and alternate pathways the definite removal of the SAC142 complex needs to be made, and further investigations would need to establish this. The possible use of animals genetically deficient in specific selected C' components, could also be considered. These reservations, therefore, need to be borne in mind when considering presented conclusions regarding the respective roles of both pathways in mediating attachment.

Nevertheless, the cumulative effects of the various reagents were assessed and table (9) shows these comparisons. It needs emphasising that reciprocal treatments are not necessarily additative and that the methods of activation of the two C' systems are not identical. Opsonization by means of the classic system is totally dependent upon antibody; it may enhance but not be essential for opsonic activity mediated by properdin (Stossel *et al* 1973). The alternate pathway can also be activated by endotoxin (Alper *et al* 1975) and polysaccharides in the absence of antibody (Stossel *et al* 1973). Nevertheless in the present *in vitro* system it has, for the convenience of comparison, been assumed that for both the classic and alternate pathways an antibody-antigen-C' reaction is of prime importance.

The complete results are presented in Fig. (34). Those for the opsonic adherence test indicated high attachment values for hyperimmune serum, which were reduced following incubation with rabbit C'. This may have been due to saturation with C' molecules causing either allostearic changes in previously established Ig molecules, or full activation of the

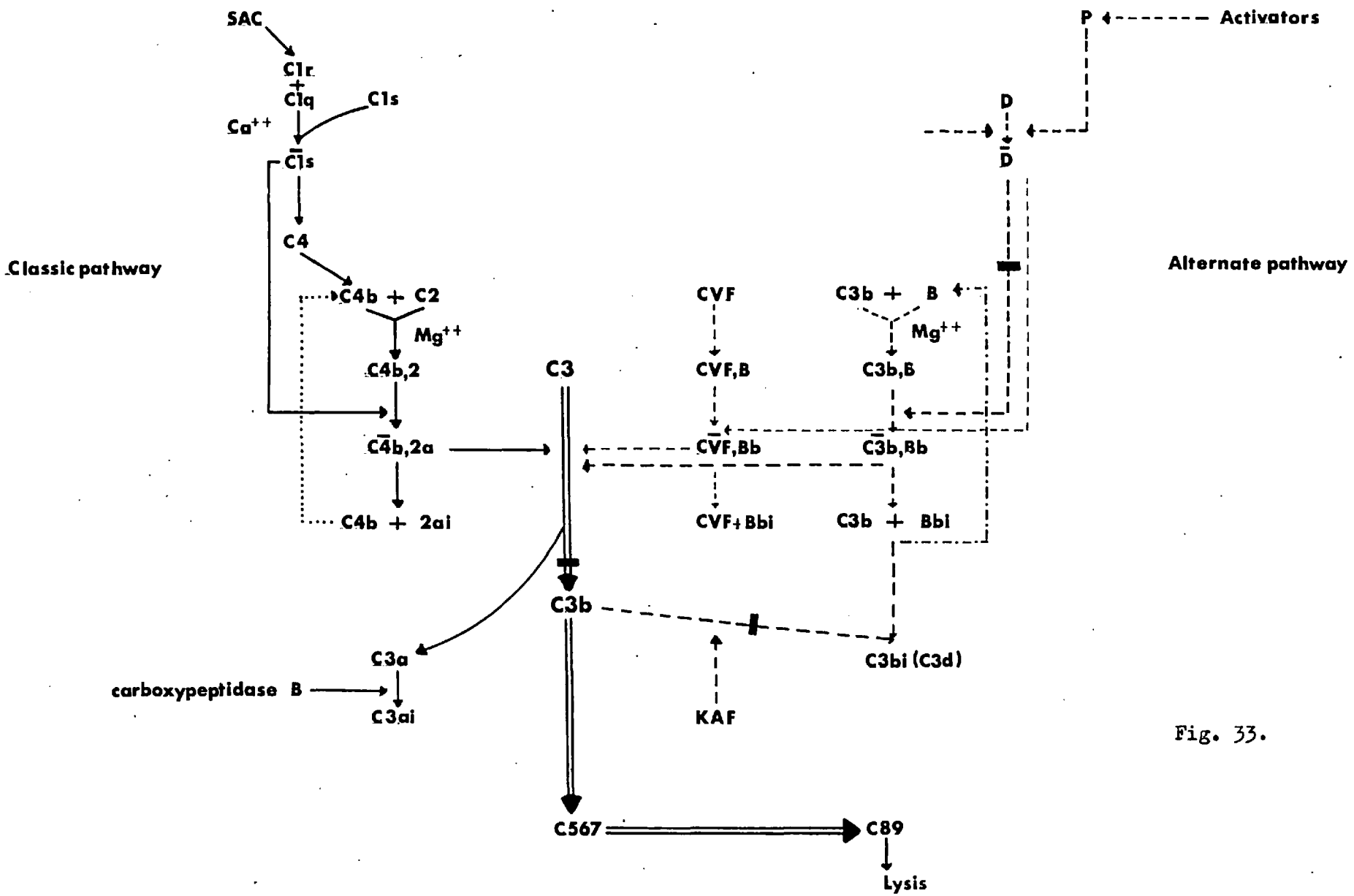

SAC
C1r
+
C1q
C1s
Ca++
C̄1s
C4
Classic pathway
C4b + C2
Mg++
C4b,2
C4̄b,2a
C4b + 2ai
P
Activators
D
D̄
Alternate pathway
CVF
C3b + B
Mg++
CVF,B
C3b,B
C3
CV̄F,Bb
C3̄b,Bb
CVF+Bbi
C3b + Bbi
C3b
C3a
carboxypeptidase B
C3ai
KAF
C3bi (C3d)
C567
C89
Lysis

Fig. 33.

Fig. 33. Schematic representation of the classic and alternate complement pathways showing the C3b feedback and the cobra venom cycle. (After Halbwachs et al 1975).

————	Classic pathway
- - - - - - - -	Alternate pathway
- - - - - - - - -	Cobra venom cycle
■■■■	Possible sites of KAF action (Alper et al 1975)
$\overline{C4b}$,2a	C3 convertase
$\overline{C3b}$,Bb	Alternate pathway convertase
KAF	C3b inactivator
CVF	Cobra venom factor
B; D	Factors B and D in alternate pathway
P	Properdin

For details of Complement terminology see Lachmann (1975)

C3b	IA
	lysis
	opsonization
C3d	specific binding sites
	opsonization
	no IA
	no lysis
	may provoke immunoconglutinin production

	PERCENTAGE ATTACHMENT[1]	
	Opsonic Adherence	Cytophilic Antibody
Hyperimmune serum	100	100
Antibody[2]	41.3	59.9
Classic C' Pathway	51.1	10.7
Alternate C' Pathway	7.5	29.4
Antibody + Classic[3]	92.5	70.6
Antibody + Alternate	48.8	89.3

TABLE 9: Comparative effects of antibody and complement via the classic and alternate pathways on the attachment of T. brucei 427 to rabbit macrophages

1. All results worked as a percentage of that obtained using rabbit hyperimmune 427 serum.
2. Attachment mediated by immune factors only, not requiring C' fixation.
3. Representing antibody-mediated and antibody-C' mediated attachment.

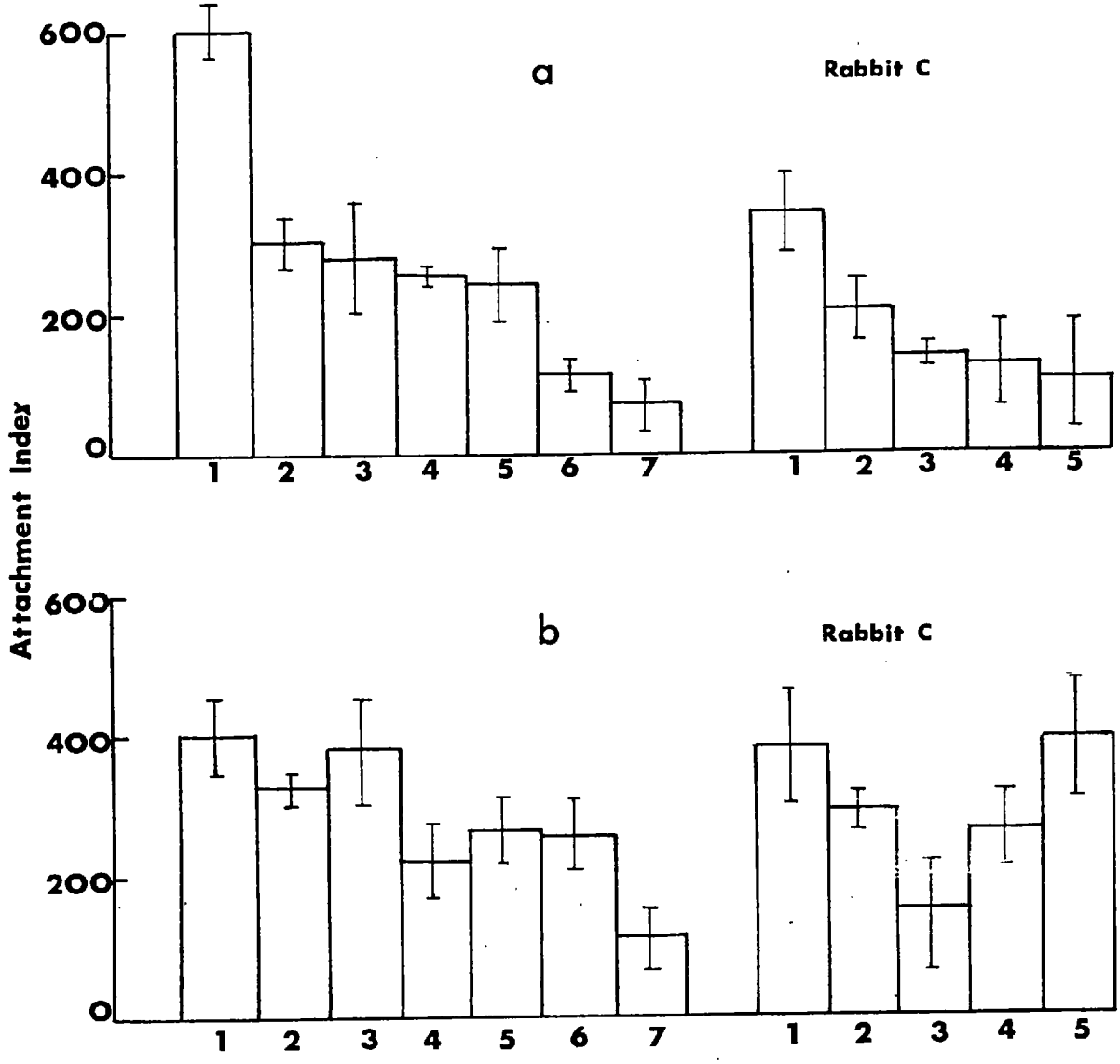

Attachment Index
600
400
200
0
a
Rabbit C
1 2 3 4 5 6 7
1 2 3 4 5
b
Rabbit C
1 2 3 4 5 6 7
1 2 3 4 5

Fig. 34.

Fig. 34. Attachment of T. brucei 427 to rabbit peritoneal macrophages. Effect of decomplemented rabbit sera.

a. Opsonic adherence test

b. Cytophilic antibody test

1. Rabbit hyperimmune T. brucei 427 serum
2. Hyperimmune serum, depleted of C' components C1 and C2 (R1 & R2)
3. R4
4. (R3 & R4)
5. R3
6. Rabbit C'
7. NRS

All results expressed are the mean ($\pm$ SE) of 3 replicates.

C' pathway. Non-specifically aggregated rabbit IgG and its Fab fragment can initiate C' activation via the alternate pathway, and there appear to be additional C' reactive sites (other than those located in the Fc region) in the Ig's of different species (Stanworth & Turner 1973). In addition, antigen-antibody complexes can activate late C' components beginning at C3, attributable to an activating site located in the $F(ab^1)_2$ region of the molecule (Sandberg et al 1971).

Trypanosome opsonization was largely effected by antibody. However, C' fixation by both pathways also occurred with seemingly the classic system predominating. This is typical in situations of antibody excess (Stossel et al 1973). The addition of rabbit C' to previously-depleted sera did not, however, restore maximum activity. It is possible that pre-incubation of trypanosomes with the C3-depleted sera could have directly affected the plasma membrane of the parasites, due to the phospholipase present in cobra venom (Godfrey & Taylor 1969). The presence of plasma should, however, have had an ameliorating effect (Godfrey et al 1970).

High cytophilic antibody activity was recorded using hyperimmune serum and a proportion of non specific attachment with C'. Again antibody appeared to be of major importance, with C' fixation via the alternate system perhaps slightly more effective than that by the classic pathway. The subsequent addition of purified rabbit C', suggested a possible change from alternate to classic fixation. It suggests, therefore, that a form of IA may be involved in cytophilic attachment. This would necessitate the probable formation of SAC1423 (classic pathway) with the bound C3 $\longrightarrow$ C3b carrying the reactant. An inordinately large amount of C4 could also give a similar reaction. C3b interacts with KAF and normal IA becomes reduced. It is possible that prolonged incubation in KAF-rich solutions such as whole serum or rabbit C', may reduce the amount of effective IA, hence explaining the reductions in activity seen when

classically depleted sera are re-incubated with full C' components. In the presence of insufficient KAF, most C3 would be in the form C3b. The alternate pathway would be inhibited via both C3b and factor D. It is also possible that alternate pathway fragments may inhibit the classic pathway (Gallin et al 1975).

Previous results using mouse macrophages, homologous serum and washed T. brucei 427 originally indicated the equivocal and confusing nature of C' interactions (sections 3.1.7. and 3.4.). The heat inactivation of normal and infected mouse serum had little effect on opsonic activity, whereas that for hyperimmune serum did. The restoration of C' in the form of a 1:10 dilution of purified guinea pig serum did not restore the opsonic activities to previous levels. Infected sera taken from a chronic infection in mice gave similar results to those seen with hyperimmune 427 serum. Heat inactivation of both immune and hyperimmune sera appeared to have no effect on cytophilic attachment; whereas, destruction of C' receptor sites on the macrophage surface, did.

The more detailed studies using the rabbit system have indicated a similarly confused system. Like the studies of Klesius et al (1973), on the percentage of phagocytic activity observed with group B streptococci in man, an intricate relationship appears to exist between non-C' opsonizing factors (antibodies) and C', via both the classic and alternate pathways. Activation of both pathways has been reported as enhancing a variety of factors including IA, erythrophagocytosis, chemotaxis and bacterial ingestion, and may be very important in non specific host resistance, (Alper et al 1975). Nagle et al (1974), have detected components of both pathways in the glomerular deposits of monkeys infected with T. rhodesiense.

3.9.2. Effect of Rabbit Complement Components Supplemented with IgG

Rabbit hyperimmune 427 serum was selectively depleted of properdin and both the classic and alternate C' pathways; whilst NRS was depleted of properdin. The activity of these treated sera were examined on their own and in the presence of previously prepared IgG from both normal and hyperimmune rabbit sera.

The IgG samples were tested for the presence of γ globulin and for anti-trypanosomal antibodies by the Ouchterlony double diffusion test. Both samples contained γ globulin and the immune IgG gave one distinct line against a suspension of T. brucei which had been previously disrupted by freeze-thawing and lysed in distilled water (see Appendix (3)).

The opsonic adherence test was performed using rabbit macrophages and T. brucei 427. The results are given in Fig. (35) and again the comparative effects of the several pathways with and without normal or immune IgG are presented in Table (10). It needs emphasizing, however, that although the parts played by individual pathways can be assessed, they are not examined in isolation. The synergistic effects are obviously of great importance and the inter-relationships between them are probably more complex than may appear by simple delineation.

The results of the opsonic adherence test indicate that properdin-depletion of hyperimmune serum reduced activity, suggesting the slight importance of C' fixation via the alternate pathway. The actual role of properdin itself has not been fully resolved and neither it nor the complete pathway have been satisfactorily categorized (Nelson 1974). Thus the inactivation of properdin may not itself prevent the alternate pathway from functioning, and indeed, properdin may act independently.

Normal IgG (16.0 mg/ml total protein) did not enhance attachment, whereas immune IgG (36.6 mg/ml), gave high opsonic values, but less than that seen with hyperimmune serum.

	Addition (3)	% Attachment Opsonic Adherence
Hyperimmune Serum		100
Antibody		44.7
Classic C' Pathway		47.0
Alternate C' Pathway		8.3
Antibody + Classic		91.7
Antibody + Alternate		53.0
Hyperimmune Serum	Normal IgG(1)	100
Antibody	Normal IgG	13.6
Classic C' Pathway	Normal IgG	26.0
Alternate C' Pathway	Normal IgG	60.3
Antibody + Classic	Normal IgG	39.7
Antibody + Alternate	Normal IgG	74.0
Hyperimmune Serum	Immune IgG(2)	100
Antibody	Immune IgG	30.0
Classic C' Pathway	Immune IgG	50.8
Alternate C' Pathway	Immune IgG	19.1
Antibody + Classic	Immune IgG	80.8
Antibody + Alternate	Immune IgG	49.2

TABLE 10: Comparative effects of antibody and complement via the classic and alternate pathways on the attachment of T. brucei 427 to rabbit macrophages in the presence of excess normal and immune IgG.

1. Concentration of 16.0 mg/ml total protein
2. Concentration of 36.6 mg/ml total protein
3. Attachment tests carried out in the presence of normal and immune IgG.

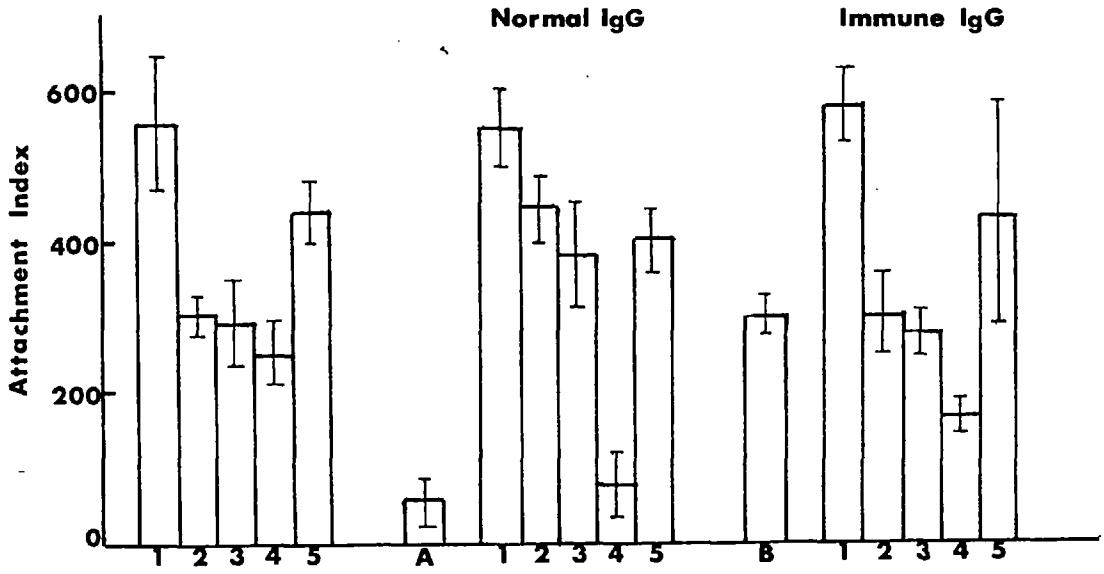


Fig. 35. Attachment of T. brucei 427 to rabbit peritoneal macrophages.(opsonic adherence test).
Effect of rabbit C' components supplemented with normal and immune IgG.

A: Normal IgG

B: Immune IgG

1. Rabbit hyperimmune T. brucei 427 serum

2. (R1 & R2)

3. R4

4. R3

5. Hyperimmune serum depleted of properdin (RP)

All results expressed are the mean ($\pm$ SE) of 3 replicates.

The presence of both normal and immune IgG molecules profoundly affected opsonization. Under normal conditions, antibody and classic pathway components were most effective, but when normal IgG was present in excess, the C' systems, especially the alternate pathway, assumed proportionately more significance, because, it is suggested, free IgG was blocking available macrophage receptor sites to immune IgG. Excess immune IgG similarly reduced the contribution of antibody, but preferentially enhanced that of the classic C' pathway.

It appears, therefore, that free IgG is generally inhibitory. Huber et al (1969), found that antigen-IgG complexes could bind to monocytes in the absence of C' and that this reaction was strongly inhibited by even low concentrations of free IgG, but could be overcome by a small number of bound C3 molecules per cell (Huber et al 1968).

These results suggest, therefore, that free normal IgG is less effective in inhibiting the attachment of antigen-antibody-C' complexes than of antigen-antibody complexes. Thus, the greatest reduction in attachment in the presence of free IgG was seen with R3 hyperimmune serum. In this treatment, all C' activity had been effectively removed, with antigenic attachment solely mediated by specific antibody. In all the other treatments, C3 activity should have been present, generated either through the classic or alternate pathways. Consequently, a proportion at least of attachment could be mediated by antigen-antibody-C' complexes, thus excess free IgG in the system was less effective in depressing attachment. Indeed, trypanosome adherence was increased with free IgG. These results may be seen as both a reflection of the different components involved in opsonization and of the specificity of C'-depletion.

3.9.3. Effect of Rabbit IgG

Investigations were made to determine the contribution of IgG antibodies to the opsonic and cytophilic attachment of T. brucei 427 to rabbit peritoneal macrophages.

Hyperimmune rabbit serum, normal and immune IgG were absorbed with homologous and heterologous strains of trypanosome. Normal and hyperimmune sera were also absorbed with zymosan A at 17°C.

The results of the opsonic adherence and cytophilic antibody tests are presented in Table (11). The high opsonic activity of hyperimmune serum can be absorbed out by live trypanosomes and to a slight extent with zymosan, thus preferentially removing part, at least, of the alternate pathway. Poor activity was expressed by normal IgG, whilst immune IgG showed greater opsonic ability which again could be largely removed following absorption with homologous trypanosomes. Opsonic activity could be restored by immune but not normal IgG. The results suggest that antibodies, more specifically, immune IgG are important in mediating opsonic attachment of trypanosomes.

Very similar results were obtained for the cytophilic test, with again immune IgG antibodies showing the major activity. Zymosan-absorbed hyperimmune serum, however, showed a marked decrease in cytophilic ability, suggesting that antibody-C' reception is also important, and perhaps independent of immune IgG surface sites.

			ATTACHMENT INDEX[1]	
			Opsonic Adherence	Cytophilic Antibody
HYPERIMMUNE 427			570.6(97.3)[2]	395.3(62.3)
	absorbed with	1. Homologous 427 trypanosomes	75.0(32.9)	33.0(11.3)
		a. plus normal IgG	64.0(41.1)	147.0(44.0)
		b. plus immune IgG	376.3(70.3)	398.3(48.9)
		c. plus absorbed immune IgG[3]	90.0(22.3)	96.7(15.5)
		2. Heterologous S42 trypanosomes	102.3(38.8)	26.0(6.2)
		a. plus normal IgG	44.7(5.7)	194.7(87.0)
		3. Zymosan (RP)	425.0(47.7)	171.3(26.5)
NORMAL RABBIT SERUM			69.7(23.4)	87.0(13.0)
	absorbed with	1. Zymosan (RP)	44.3(17.9)	79.3(14.0)
NORMAL RABBIT IgG			55.7(28.0)	44.7(16.5)
	absorbed with	1. T. brucei 427	52.0(12.8)	38.3(21.0)
		2. T. brucei S42	51.3(41.5)	24.3(11.9)
IMMUNE RABBIT IgG			290.3(49.9)	614.0(31.6)
	absorbed with	1. Homologous 427 trypanosomes	76.7(7.1)	144.7(18.5)

TABLE 11: Comparative effects of normal and immune IgG on the attachment of T. brucei 427 to rabbit macrophages

1. mean of 3 replicates
2. standard deviations in parentheses
3. absorbed with live T. brucei 427

3.9.4. Effect of Normal IgG on Antibody and Antibody-C Complexes

Investigations were undertaken to determine the effect of normal rabbit IgG on the attachment of T. brucei 427 preopsonized with antibody, C', and antibody-C' complexes, to rabbit macrophages.

It has been established by Lay & Nussensweig (1968), that IgG can inhibit the phagocytosis of erythrocyte-antibody complexes but not of erythrocyte-antibody-C' complexes. From this it was concluded that erythrocyte-antibody-C' complexes bind to receptor sites other than those for IgG. Bjornson & Michael (1974a) found that normal IgG could inhibit the phagocytosis of Pseudomonas aeruginosa preopsonized with immune IgG antibodies, but had no effect on the uptake of bacteria which had been preopsonized with immune IgG and non-specific serum. They concluded, therefore, that normal IgG could facilitate the interaction of C' with the leucocyte cell membrane.

Trypanosomes were sensitized with antibody and C' alone, and attachment was measured in the presence of excess normal IgG. Pretreatment was also performed using antibody-C' mixtures, with subsequent attachment similarly measured with normal IgG in the test medium.

The results are presented in Table (12). Trypanosomes which had been preopsonized with either rabbit C' or immune IgG alone showed a significant reduction in attachment in the presence of free normal IgG. Whereas those parasites which had been pre-treated with hyperimmune serum, a mixture of immune IgG and rabbit C', or immune IgG and hyperimmune serum, did not show this reduction.

These results further suggest that free IgG can inhibit the adherence of trypanosome antigen-antibody complexes, but not of trypanosome antigen-antibody-C' complexes. Indeed an enhanced attachment was observed with the latter, perhaps suggesting a slight stimulating mechanism.

TREATMENT	ADDITION[3]	FACTORS PRESENT			ATTACHMENT INDEX[1] Opsonic Adherence	
		Hyperimmune Serum	Immune IgG	Complement	Mean	Difference
P.S.G.		-	-	-	26.7(21.2)[2]	
P.S.G.	NORMAL IgG	-	-	-	61.3(12.0)	+ 34.6
Hyperimmune 427		+	-	+	534.3(97.0)	
Hyperimmune 427	NORMAL IgG	+	-	+	553.0(148.7)	+ 18.7
Rabbit C'		-	-	+	176.0(60.0)	
Rabbit C'	NORMAL IgG	-	-	+	72.7(16.8)	-103.3
Immune IgG		-	+	-	340.7(49.1)	
Immune IgG	NORMAL IgG	-	+	-	298.0(43.5)	- 42.7
Hyperimmune 427 Immune IgG		+	+	+	464.0(65.5)	
Hyperimmune 427 Immune IgG	NORMAL IgG	+	+	+	603.0(157.0)	+139.0
Immune IgG Rabbit C'		-	+	+	322.3(30.8)	
Immune IgG Rabbit C'	NORMAL IgG	-	+	+	337.7(68.5)	+15.4

TABLE 12: Effect of normal IgG on antibody and antibody-complement complexes affecting the attachment of T. brucei 427 to rabbit macrophages.

1. mean of 3 replicates
2. standard deviation
3. Pre-treated macrophages and trypanosomes tested in the presence of normal IgG

3.9.5. Effect of Properdin

Both normal and hyperimmune rabbit sera were selectively depleted of properdin by their absorption at 17°C. with zymoson A. To these absorbed sera were added previously prepared normal and immune IgG and hyperimmune serum. Normal and hyperimmune properdin, prepared from their respective rabbit sera, were used in conjunction with hyperimmune serum absorbed with homologous trypanosomes, and properdin-depleted normal and hyperimmune sera.

The opsonic adherence and cytophilic antibody tests using T. brucei 427 and cultured rabbit macrophages were carried out. The results are given in table (13).

Preopsonization of trypanosomes with hyperimmune RP caused a small reduction in attachment, suggesting that properdin may be of slight importance. Normal IgG did not enhance the low opsonic activity of normal RP, whereas immune IgG enhanced the activity of both normal and hyperimmune RP. This suggests that immune IgG molecules are more important than properdin in trypanosome opsonization. Hyperimmune P (33.3 units/ml.) did not restore the activity of absorbed hyperimmune serum, and normal P (8.3 units/ml.) did not affect normal RP. These results suggest that although properdin, whether from normal or immune sera, confers slight opsonic activity, the major contributory factor in the specific opsonization of trypanosomes lies in the immune IgG antibody fraction.

Properdin appeared more important in mediation via cytophilic attachment. Thus hyperimmune RP caused a very significant reduction in activity. Normal IgG did not enhance the activity of normal RP whereas immune IgG did to a certain extent. Furthermore immune IgG was capable of increasing the effectiveness of hyperimmune RP and hyperimmune serum restored full efficiency to hyperimmune RP. These results suggest that immune IgG alone is not sufficient for maximum cytophilic activity.

TREATMENT		FACTORS PRESENT			ATTACHMENT INDEX[1]	
		Properdin	Normal IgG	Immune IgG	Opsonic Adherence	Cytophilic Antibody
N.R.S.		+	+	-	52.7 (9.6)[2]	79.7(18.5)
Normal R.P.[3]		-	+	-	45.7 (6.1)	70.3(20.4)
Hyperimmune 427		+	-	+	540.7(111.9)	371.3(46.5)
Hyperimmune R.P.[4]		-	-	+	441.0 (75.6)	177.0(15.4)
Normal R.P.	Normal IgG	-	+	-	70.0 (34.4)	44.7(11.9)
Normal R.P.	Immune IgG	-	+	+	494.3 (87.9)	194.7(23.7)
Hyperimmune R.P.	Immune IgG	-	-	+	574.3(67.5)	365.0(44.3)
Normal R.P.	Hyperimmune 427	+	+	+	408.7(90.6)	393.7(36.4)
Absorbed hyperimmune 427[5]		-?	-	-?	73.7 (9.5)	38.0 (9.0)
Absorbed hyperimmune 427	Hyperimmune R.P.	+	-	-?	56.0 (42.1)	36.0(18.7)
Normal R.P.	Normal P	+	+	-	49.3 (31.8)	46.7(12.5)
Hyperimmune R.P.	Hyperimmune P	+	-	+	356.7 (55.1)	273.7(46.9)

TABLE 13: Effect of Properdin on the attachment of T. brucei 427 to rabbit macrophages

1. mean of 3 replicates
2. standard deviation
3. Properdin-depleted NRS
4. Properdin-depleted rabbit hyperimmune 427 serum
5. Absorbed with live T. brucei 427

3.9.6. Effect of Trypsin

Lay & Nussensweig (1968) and Bjornson & Michael (1974b), have proposed that leucocyte surface receptors for C' are trypsin-sensitive, whereas the IgG receptors on phagocytic cells are trypsin-insensitive. The effect of IgG alone, in combination with C', and C' alone were investigated in order to determine whether discrete receptor sites were present on the macrophage surface. T. brucei 427 were preopsonized with PSG, immune IgG, hyperimmune serum, rabbit C', a mixture of hyperimmune serum plus immune IgG and rabbit C' plus immune IgG, and were tested in the presence and absence of normal IgG to attach to trypsinized and normal rabbit macrophages. Macrophage monolayers were incubated with 1.0 ml. of a 0.2% w/v trypsin solution for 60 min. at 37°C. and then with 1.0 ml. of soyabean trypsin-inhibitor (0.2% w/v in TCM) for 10 min. at 37°C. Control macrophages were similarly treated, using TCM for both incubations. All monolayers were further washed in 1.0 ml. of TCM immediately before use. The results are presented in Table (14) and Fig. (36).

When trypanosomes were preopsonized with immune IgG alone, the presence of excess free normal IgG in the test system caused a slight reduction in attachment to the macrophage surface. This may be due, it has been suggested, to competition for available IgG receptor sites. Trypanosomes which had been pretreated with antibody-C' complexes (immune IgG plus C'; immune IgG plus hyperimmune surum), showed no reduction, instead a slight increase in attachment in the presence of free IgG was recorded. Hyperimmune serum, alone, however, showed a slight reduction. Nevertheless, it is suggested that antigen-antibody-C' complexes do not compete with free IgG for surface sites, but are able to attach to different, discrete receptors.

Bjornson & Michael (1974b), working on the attachment and phagocytosis of P. aeruginosa in the presence of human serum components by polymorphs

TREATMENT	ADDITION[3]	TRYPSINIZATION	OPSONIC ADHERENCE[1] Mean[2]	Difference
P.S.G.			15.3 (7.4)	
P.S.G.		Trypsin	23.7(17.6)	+ 8.4
P.S.G.	Normal IgG		63.0(14.4)	
P.S.G.	Normal IgG	Trypsin	54.7(11.1)	- 8.3
Immune IgG			393.7(22.1)	
Immune IgG		Trypsin	442.3(47.2)	+48.6
Immune IgG	Normal IgG		340.7(29.9)	
Immune IgG	Normal IgG	Trypsin	398.7(36.0)	+58.0
Hyperimmune 427			573.7(90.2)	
Hyperimmune 427		Trypsin	537.7(190.8)	-36.0
Hyperimmune 427	Normal IgG		514.3(51.0)	
Hyperimmune 427	Normal IgG	Trypsin	573.3(63.7)	+59.0
Hyperimmune 427 - Immune IgG			471.3(142.5)	
Hyperimmune 427 - Immune IgG		Trypsin	572.0(170.1)	+100.7
Hyperimmune 427 - Immune IgG	Normal IgG		596.3(80.8)	
Hyperimmune 427 - Immune IgG	Normal IgG	Trypsin	579.3(94.2)	-17.0
Rabbit Complement			225.3(42.7)	
Rabbit Complment		Trypsin	117.3(15.6)	-108.0
Rabbit Complement	Normal IgG		97.7(39.7)	
Rabbit Complement	Normal IgG	Trypsin	44.3(16.0)	- 53.4
Rabbit Complement - Immune IgG			322.3(30.8)	
Rabbit Complement - Immune IgG		Trypsin	240.9(79.4)	-81.4
Rabbit Complement - Immune IgG	Normal IgG		382.7(78.4)	
Rabbit Complement - Immune IgG	Normal IgG	Trypsin	286.7(97.5)	-96.0

TABLE 14: Effect of macrophage trypsinization on the attachment of pre-opsonized T. brucei 427 to rabbit peritoneal cells.

1. mean of 3 replicates
2. standard deviation
3. pretreated trypanosomes tested in the presence of normal IgG.

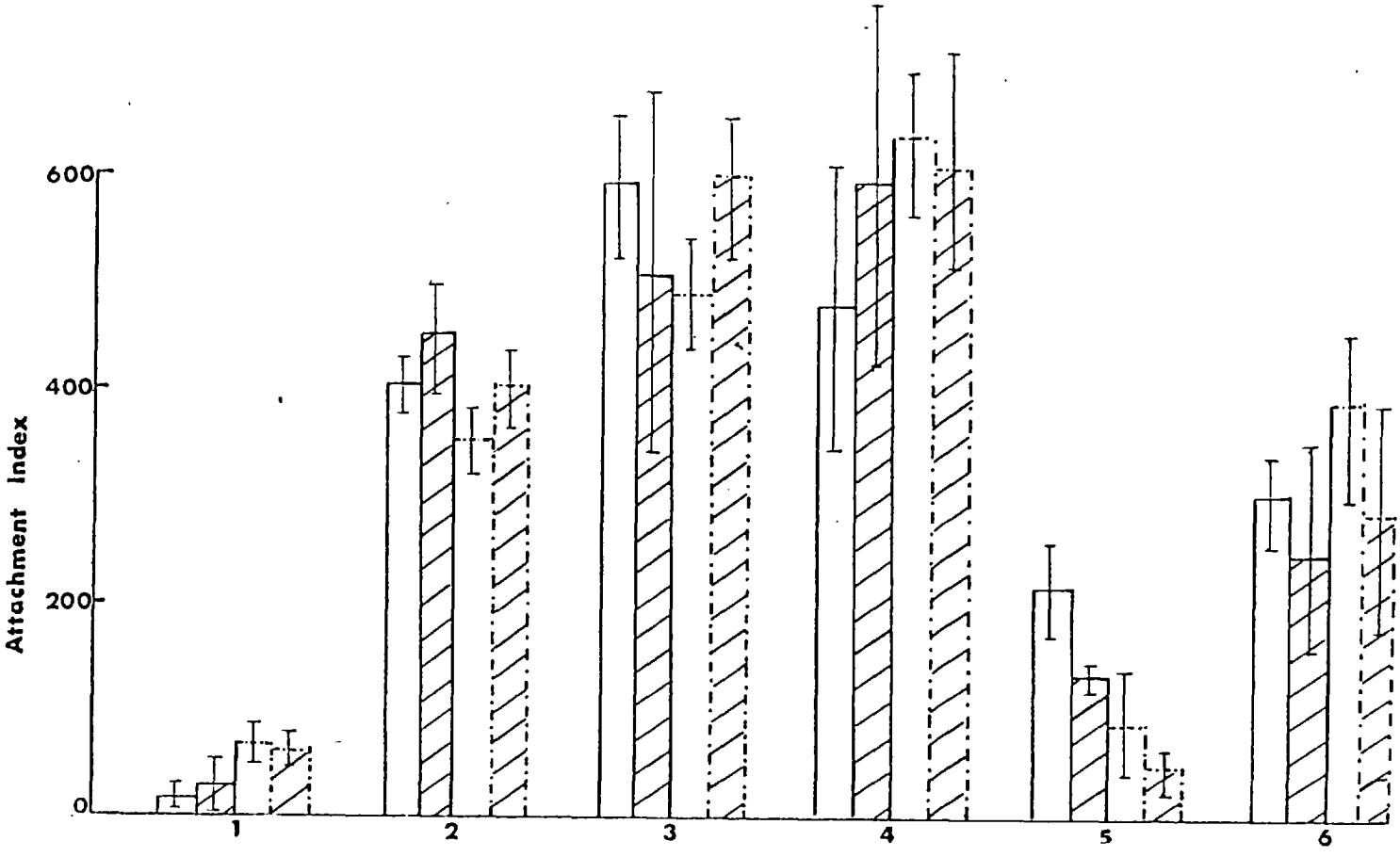


Fig. 36. Attachment of T. brucei 427 to rabbit peritoneal macrophages (opsonic adherence test).

Effect of macrophage trypsinization.

Trypsin treatment of macrophages

Excess normal IgG

1. PSG
2. Immune IgG
3. Rabbit hyperimmune T. brucei 427 serum
4. Hyperimmune serum plus immune IgG
5. Rabbit C'
6. Rabbit C' plus immune IgG

All results expressed are the mean (± SE) of 3 replicates.

suggested that trypsinization could destroy the surface receptors for C' and for C'-complex mediated reactions. Thus leucocyte trypsinization resulted in a decreased phagocytosis of bacteria preopsonized with IgG antibodies and C'.

In the experimental system, trypsinization had no deleterious effect on attachment via immune IgG alone, in fact it increased it somewhat. Trypsin treatment did, however, alter the adherence mediated by hyperimmune serum alone and immune IgG plus rabbit C', suggesting attachment is due to trypsin-sensitive C' receptors on the macrophage surface. Immune IgG plus hyperimmune serum, although capable of forming antibody-C' complexes with trypanosome antigen and not being inhibited in the presence of free IgG, nevertheless gave an increased attachment value following macrophage trypsinization. It is possible that two distinct methods of adherence may occur depending upon the specificity of the available surface sites. Attachment via immune IgG receptors may occur in conditions of antibody excess, or when C'3 sites are not available. Alternatively, other complex sites may exist, resistant to trypsin treatment or other factors - such as different Ig's or subclasses - present in hyperimmune serum, may be capable of effecting attachment.

Huber et al (1968), indicated that human monocytes and macrophages contained IgG receptors capable of functioning in vitro in the absence of C' and that there were separate receptors for antigen-antibody-C' complexes involving either IgG or IgM. There were no discrete sites, however, for IgM alone. The authors suggested that the two receptors (antibody and antibody-C' complex) acted co-operatively, with the C3 receptor compensating for the inefficiency of IgM and for the inhibition of immune IgG by free IgG. The IgG receptor, conversely, could compensate for the lower C' binding capacity of IgG to IgM. A similar mechanism may exist for the attachment of trypanosomes. IgM alone is relatively inefficient in mediating adherence, yet is effective in C' fixation.

3.9.7. Effect of Absorbed Sera

In an attempt to further elucidate the nature of the receptor and its activity, normal and immune IgG were absorbed with trypanosome antigen or zymosan. Macrophage monolayers were trypsinized as before and the attachment of trypanosomes preopsonized with the IgG preparations to the pretreated macrophages was determined. The results are given in Fig. (37).

Macrophage trypainization had no effect on the opsonic activity of PSG, normal or immune IgG. The absorption of normal IgG with live parasites, sonicated trypanosome or zymosan, had no effect.

The activity of immune IgG was, however, removed following absorption with live trypanosomes, and, to a limited extent with the sonicate, but not with zymosan (which at the temperature used, would have preferentially removed any residual C3). Macrophage trypsinization had no effect.

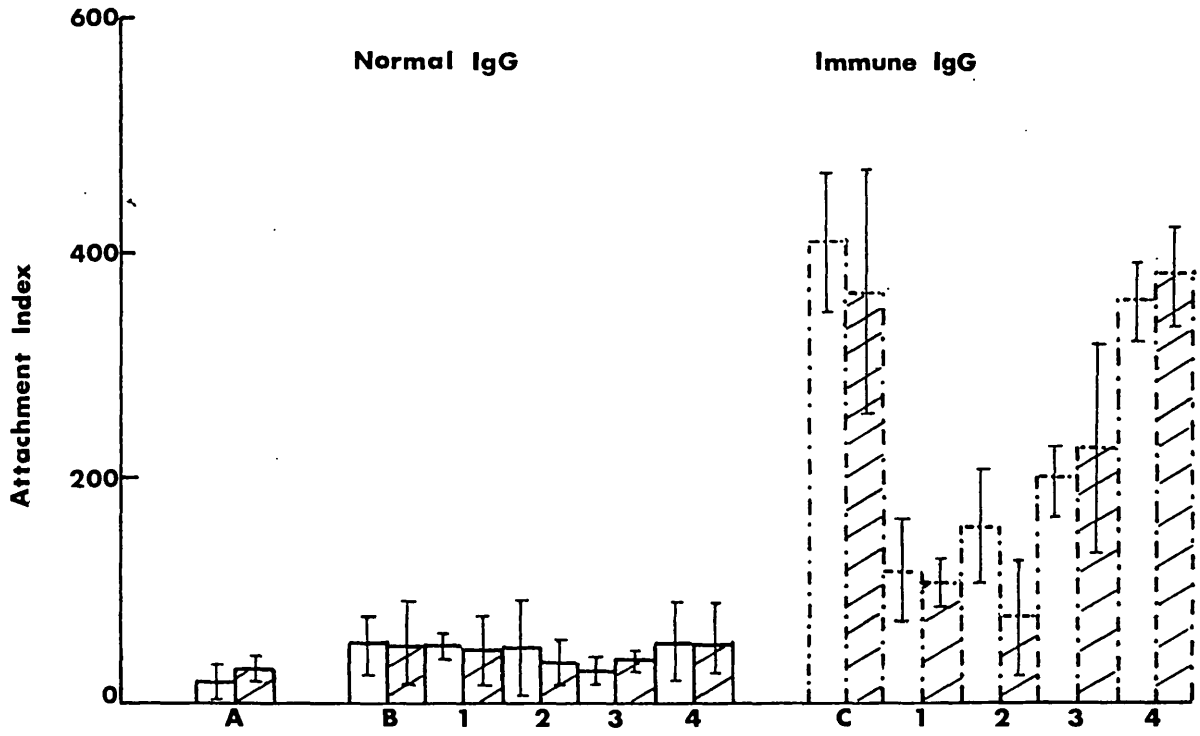


Fig. 37. Attachment of T. brucei 427 to rabbit peritoneal macrophages (opsonic adherence test).

Effect of absorbed normal and immune IgG and trypsinized macrophages.

Trypsin treatment of macrophages

Excess normal IgG

Excess immune IgG

A: PSG

B: Normal IgG

C: Immune IgG

Absorbed with:

1. live T. brucei 427
2. live T. brucei S42
3. sonicated suspension of T. brucei 427
4. zymosam A (37 C)

3.9.8. Effect of Rabbit IgM

The IgM fraction of immune rabbit serum was tested for its opsonizing and cytophilic roles in the attachment of T. brucei 427 to peritoneal macrophages.

Immune serum from rabbit S468 was taken 56 days post infection and separated on a G200 Sephadex column. Those fractions corresponding to the major 19S IgM peak were pooled, freeze dried and dialysed. The separated fraction was tested by agar diffusion for the presence of rabbit γ globulin and anti-trypanosome antibodies (Appendix (3). γ globulin was present, but only a faint precipitin line was evident when tested against disrupted trypanosomes. The band was close to the 'serum' well suggesting it was mainly macroglobulin. It is considered that a high propertion of activity was lost during preparation.

The IgM was tested for its ability to attach trypanosomes and was subsequently added to both trypanosomes and macrophages which had been pretreated with hyperimmune serum, hyperimmune serum absorbed with homologous trypanosomes or hyperimmune R3. The results are given in Fig. (38).

IgM was not effective in specifically opsonizing trypanosomes. When used as a supplement to absorbed or C3-depleted sera it slightly enhanced attachment, but this was only just significant at a 0.5% probability level.

The results for the cytophilic test are only marginally different at a 1% probability level (variance ratio of 7.96 to 7.50), to those of the opsonic adherence test. Again insignificant activity was recorded using IgM alone, and it had no effect on absorbed or C3 depleted sera.

19S IgM has, however, been reported as capable of attaching cytophilically to macrophages (Portis & Coe 1975). 7S IgM and C'-fixing 7s IgM may do likewise. Nevertheless, despite the high levels of

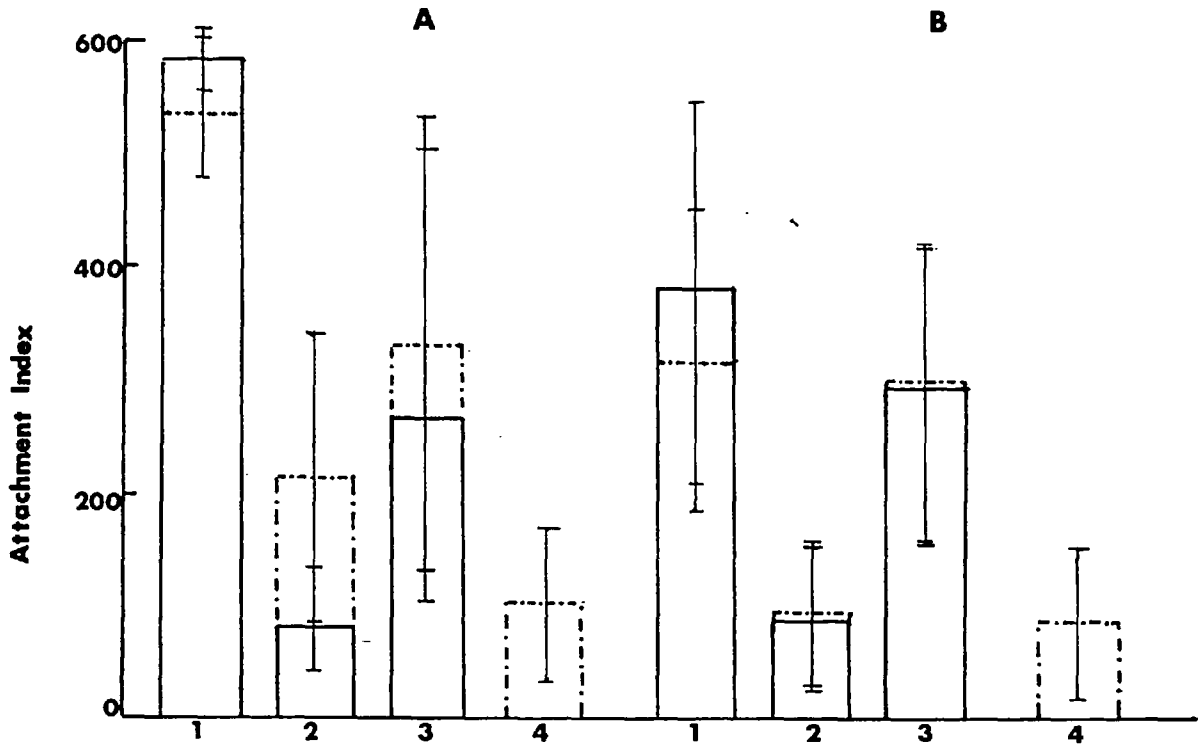


Fig. 38. Attachment of T. brucei 427 to rabbit peritoneal macrophages. Effect of rabbit IgM - prepared from rabbit S468, 56 days post infection.

A: Opsonic adherence test

B: Cytophilic antibody test

—— without supplementary IgM

------ with supplementary IgM

1. rabbit hyperimmune T. brucei 427 serum
2. hyperimmune serum absorbrd with live T. brucei 427
3. R3
4. IgM alone

All results expressed are the mean ($\pm$ SE) of 3 replicates.

macroglobulin produced during infection, its high agglutinating ability and its capacity for C' fixation, IgM does not appear to mediate the attachment of trypanosomes to rabbit macrophages (vide supra).

Summary

The results presented in sections 3.7; 3.8 and 3.9, suggest that immune rabbit sera produced during chronic trypanosome infections and artificially-prepared hyperimmune sera are both effective in mediating the attachment of significant numbers of trypanosomes to rabbit peritoneal macrophages. The highest levels have been recorded in the presence of hyperimmune sera, the activity of which may be absorbed out by live trypanosomes. During infection both the specific trypanosome opsonic and the macrophage cytophilic antibody activities of sera increase and tend to reach and optimum value (of approximately 100% in the former case) after 2 or 3 weeks. The cytophilic response remains lower and usually more linear than the opsonic activity. Increased values first appear several days after the rise in agglutinating antibody. Low attachment levels, only, are recorded in the absence of specifically immune sera, although non-specific antibodies present in homologous normal serum may have a slight effect.

Trypanosome-attachment, either through specific parasite opsonizing antibodies or cytophilic antibodies does not appear to be mediated by variant-specific antibodies. There is no direct correlation between adherence and the appearance of variant subpopulations during infection. Attachment may be largely dependent upon the numbers of trypanosomes present at any one stage relative to the quantity and specificity of immune IgG antibody produced in response.

It is considered that a limiting factor to attachment, may be the number of available macrophage surface sites. Under the experimental conditions, trypanosomal opsonization appears a more effective mechanism than cytophilic attachment, although variations in requirements exist for the two processes.

Trypanosome opsonization itself is largely dependent upon antibodies, most significantly, immune IgG. Attachment may be partially mediated

through the activation of specific complement components. The classical pathway appears more effective, although alternate pathway involvement may certainly contribute, most especially in the presence of excess normal IgG. Properdin itself has some slight benefit. Differences in attachment and combination are seen with the development of complexes, with or without complement, suggesting various different receptor sites present either on the trypanosome surface, or, more likely, on the macrophage.

Complement components assume a greater importance in cytophilic attachment, although most mediation is still as a direct result of antibody. Both the classical and alternate pathways, however, may be selectively involved.

Trypanosomes pre-opsonized with immune IgG are still able to attach to macrophages after trypsinization, suggesting that antibody mediation is largely via trypsin-resistant receptor sites on the phagocyte surface. Trypsin treatment can reduce attachment with C' components, but does not affect the attachment in the presence of Ag-Ab complexes. Trypsinized trypanosomes, however, appear less capable of adsorbing specific serum opsonins, suggesting that antigenic receptor sites are either destroyed or that surface coat material is removed and large insoluble complexes are formed between available IgG specific antigenic sites and opsonizing antibodies.

Separated IgM has very little effect on attachment, despite the large amounts of variant specific IgM produced during infection. Some slight activity is noted, and it may be of importance in C' fixation.

3.10. Cx-Reactive Protein

Thomasson et al (1973), detected Cx-RP in the sera of rabbits infected with T. congolense. It rose rapidly from initial levels one week post infection to peak concentrations after three weeks, thereafter slowly declining. The authors suggested that heightened levels of Cx-RP could prove to be of value in the early diagnosis of trypanosomiasis, and further considered it could be involved in early-host immunity. Pure human CRP has been shown to increase the phagocytic activity of neutrophils (Ganrot & Kindmark 1969).

Cx-RP levels were therefore monitored in rabbits infected with T. brucei 427, and any changes related to the observed disease symptoms and to the phagocytosis-promoting effects of the tested sera.

Rabbits S447, S448 and S449 were inoculated with live trypanosomes as previously described (section 3.3.1.), and bled twice weekly, up to 7 weeks post infection. All sera samples were heat inactivated and initially tested for the presence or absence of Cx-RP. Those that gave a positive response were again tested to give a Cx-reactive titre.

Serum was taken from a sham-inoculated control animal, S453, at five, weekly intervals and from rabbits S454, S459 and S456, inoculated respectively with disrupted trypanosomes and FCA; disrupted trypanosomes alone, and FCA alone (see sections 2.4.1. and 3.3.1.). None of the control animals showed any disease symptoms and no trypanosomes were detected in the blood. Those inoculated with FCA (S454, S456), developed localized necrotic lesions and an abscess formed at the inoculation-site.

Cx-RP levels were detected in the sera of two of three infected rabbits examined (S447 and S448), and rose during infection. None of the control rabbits showed any increase in Cx-RP, suggesting that its elevation was a host response towards the tissue damage caused by the trypanosome infection. Although FCA can induce localized inflammation (Dumonde & Glynn 1962), this was not detected in elevated Cx-RP levels.

Rabbit S447, showed typical disease symptoms, with low blood parasitaemia present throughout infection (Appendix (2.)); and lesions around the head and fore-limbs. Cx-RP levels rose after 5 days infection and rapidly reached a maximum by day 17. Thereafter they declined slowly until the death of the animal on day 54 (Fig. (39a)). Cx-RP was detected early on in the course of the disease, before any external symptoms became evident, with peak values between days 14 and 28. The first signs of oedema and erythema occurred on day 18 and thereafter became more severe. Parasites were first recorded on day 24, by which time exudative dermatitis had developed. By the fourth week (day 31), small skin lesions had formed on the limbs and around the head; the eyes were swollen and prurulent rhinitis had set in. The animal lost weight rapidly after day 35. As the overall tissue necrosis became progressively more severe, so the levels of detectable Cx-RP slowly declined. They had not, however, fallen to control levels by day 47. This "premature" disappearance of Cx-RP is supposedly uncharacteristic of CRP formation in infectious diseases (Thomasson et al 1973).

Rabbit S448 developed an asymptomatic reaction to the disease. Parasitaemia remained very low, first recorded 33 days post infection. The animal became aparasitaemic after the fifth week of infection. No parasites were detectable in microscopic thick films, and two mice, each intraperitoneally inoculated with 2.0 ml. of whole rabbit blood, remained uninfected. No external disease symptoms were evident, and no Cx-RP was detected at any stage.

Rabbit S449 had an uncharacteristically high blood parasitaemia throughout infection. Parasites were first detected on day 3 and the maximum recorded parasitaemia was on day 17 (2.0×10^5/ml.). The animal died 54 days post infection. There was very little tissue involvement, a slight facial oedema by day 15 but no necrosis, dermatitis or lesions developed. Cx-RP was first detected at a very low titre (1:8) by day 10,

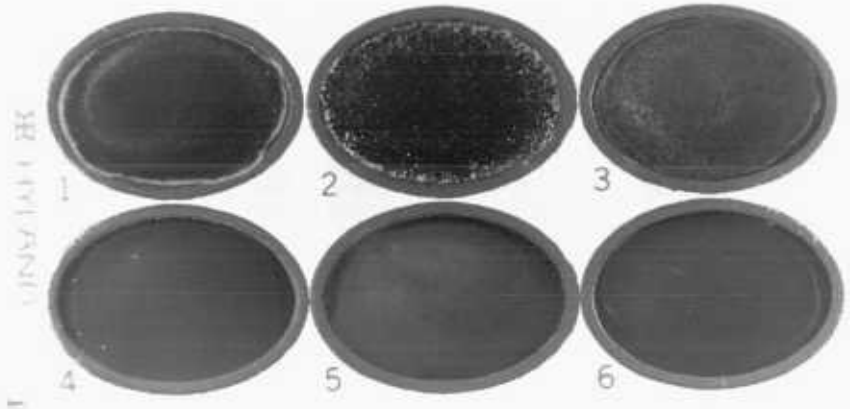


Plate N.

Plate N. Cx-RP levels in the serum of rabbit S447 infected with T. brucei 427. Test response 40 days post infection.

Oval numbers:

1. whole serum diluted 1:8 with buffer (+ve latex agglutination)
2. diluted 1:16 (+ve latex agglutination)
4. diluted 1:32 (+ve latex agglutination)
5. diluted 1:64 (-ve latex agglutination)
3. positive control
6. negative control

Titre taken at 1:32.

and remained at this level up to 47 days post infection.

Increases in Cx-RP in the sera of infected rabbits appeared to be a response to the inflammatory symptoms produced during infection. Cx-RP first became apparent during the acute stage of the disease and rapidly reached a peak, often before any external symptoms were visible. It thereafter declined as tissue necrosis became more severe. It did not appear to be of value in the early diagnosis of trypanosomiasis, although it may represent a good index of tissue response to injury. The recorded levels could not be correlated with parasitaemia, the production of specific antibody or the opsonic power of immune rabbit sera. The levels of Cx-RP are probably dependent upon the course of infection and reflect both the severity and longevity of the various temporal disease symptoms (Dutton 1955).

Goodwin (1974), suggested that anti-inflammatory drugs which could dampen down successive allergic reactions, might be of use in treating trypanosomiasis. The effect of the anti-inflammatory drug, indomethacin, on the course of T. brucei 427 infections in rabbits was investigated, and related to the pathological conditions produced, and the levels of Cx-RP present in the serum.

Oral dosage of 5 mg./kg. body weight was carried out daily. High dosage in humans can cause vomiting, diarrhoea and gastro-intestinal bleeding. Indomethacin is commonly used in the treatment of musculo-skeletal disorders such as osteo and rheumatoid arthritis and in the treatment of acute gout.

The following régimes were undertaken:-

Rabbit No.	Inoculation *T. brucei* 427	Inclusive Dosage; Days Post Infection Start	Stop
S 594	8.2×10^3	8	11
S 595	8.2×10^3	15	18
S 596	8.2×10^3	22	25
S 569	5.0×10^3	35	39
S 556	3.0×10^3	51	57
S 533	0.85% Saline	51	57

Each rabbit was bled at weekly intervals and at daily intervals during the course of drug treatment, 18 h. after each administration. The Cx-RP titres for each animal are graphically represented in Fig. (39b & c). For details of the parasitaemia, see Appendix (2).

Fig. (39b) shows the Cx-RP levels obtained with rabbits S556 and S533. S556 showed a response similar to that obtained with S447. Trypanosomes were first evident by day 8 and the disease ran a typical fulminating course thereafter with regular waves of parasitaemia. No external symptoms were evident until day 36, when facial oedema and testicular swelling were observed. Thereafter the symptoms became progressively more severe, until by day 50 there was widespread oedema of the face and the beginnings of necrosis. Five days into drug treatment (day 55), a slight reduction in both oedema and inflammation was observed. The necrosis, however, remained unaffected and the animal died 58 days post infection, having lost 0.6kg. in weight. Cx-RP was first detected on day 22 and rose rapidly to peak levels (titre of 1:64) by day 36 - the first day any external symptoms were noticed. Thereafter, the values

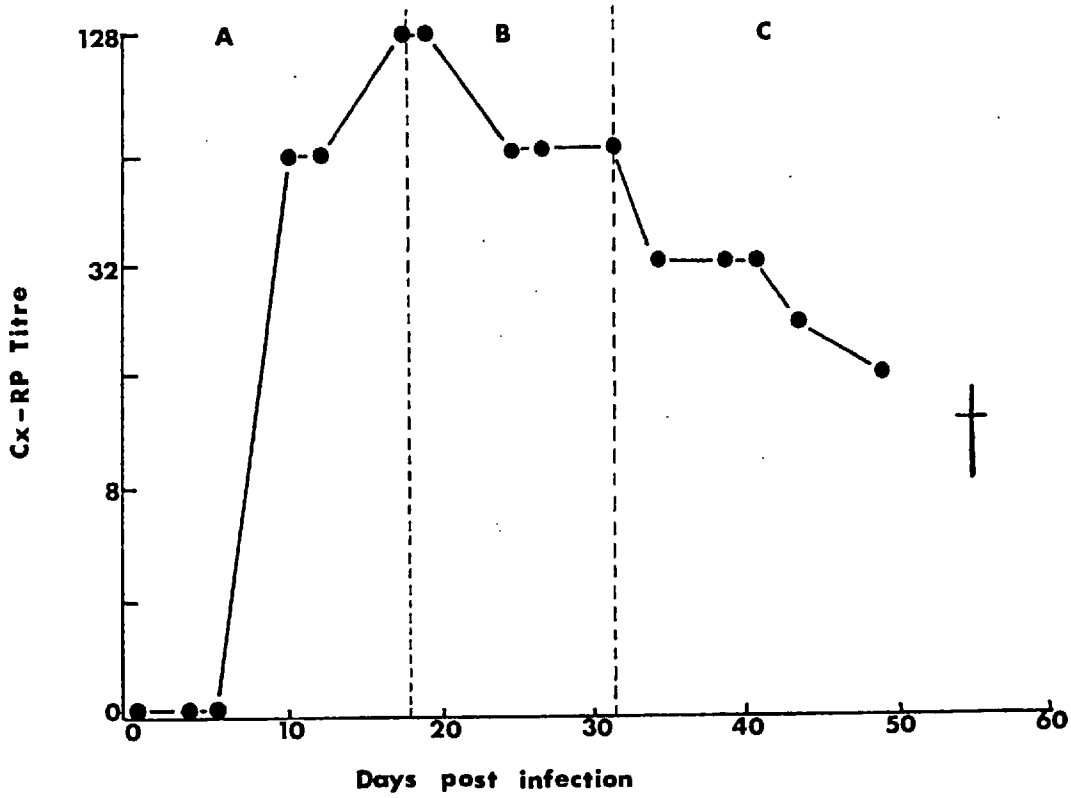


Fig. 39a.

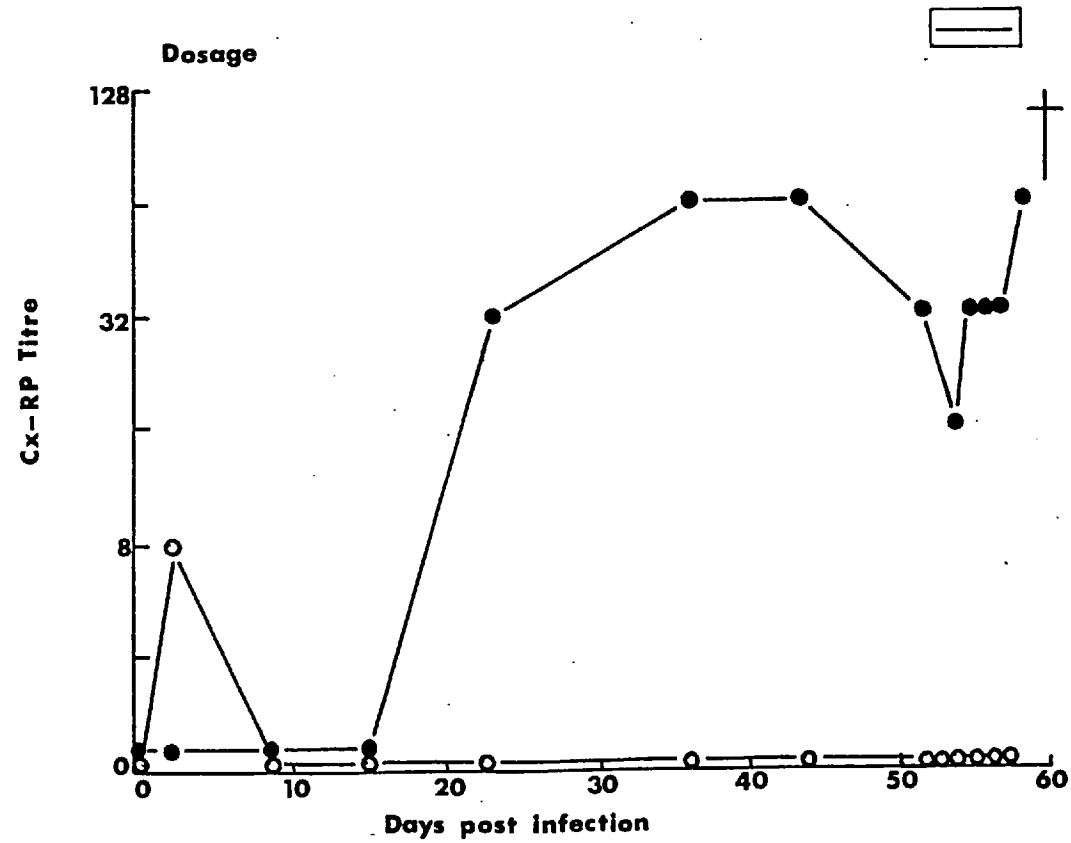


Fig. 39b.

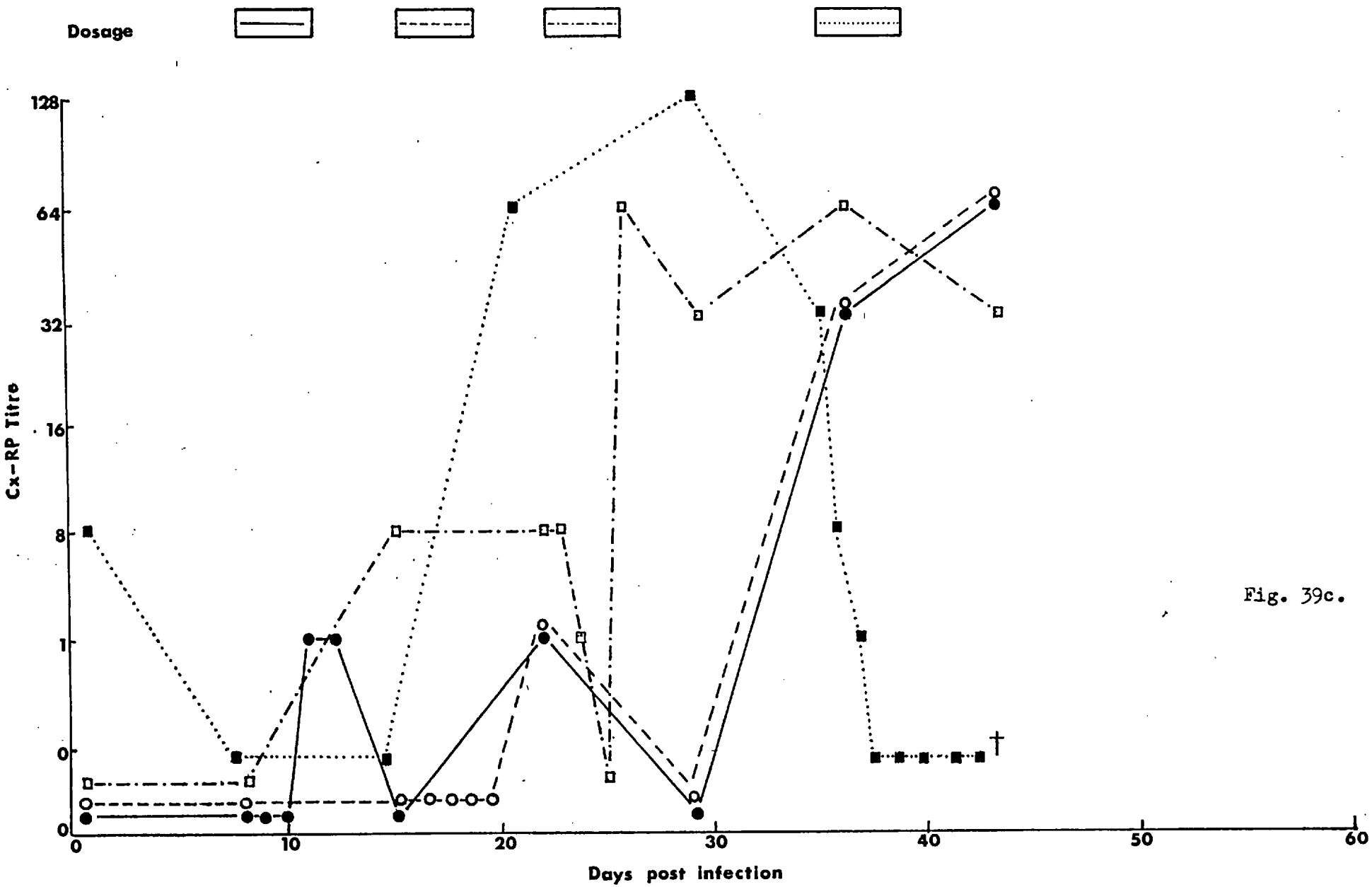

Dosage
128
64
32
16
8
1
0
0
Cx-RP Titre
0
10
20
30
40
50
60
Days post infection
†

Fig. 39c.

Fig. 39. Levels of CxRP in the sera of rabbits infected with T. brucei 427. Effects of indomethacin treatment.

(a) ●——● rabbit S447 infected s/c

A: no disease symptoms,

B: severe oedema and erosive exudative dermatitis,

C: skin lesions and progressive necrosis.

(b) ▭ oral dosage of indomethacin (5mg/kg body weight) inclusive days for each rabbit.

●——● rabbit S556 infected s/c dosage days 51-57

o——o rabbit S533 non-infected control

(c) ●——● rabbit S594 - dosage days 8-11

o- - -o rabbit S595 - dosage days 15-18

□-.-.-□ rabbit S596 - dosage days 22-25

■·····■ rabbit S569 - dosage days 35-39

gradually declined and by day 52, a titre of 1:16 was recorded. Drug treatment, however, caused a secondary increase in Cx-RP levels, reaching a final titre of 1:64 by day 57.

The control rabbit S533 gained 0.7kg. in weight. No Cx-RP was detected in sera collected except 1 day after the sham inoculation, when a titre of 1:8 was recorded. This probably represented a local inflammatory reaction either to the initial inoculation or to the bleeding regime.

Fig. (39c) shows the Cx-RP levels for the remaining drug-treated infected rabbits.

Rabbit S594 showed no symptoms until day 15. Thereafter oedema became more severe until by day 36 there was heavy involvement of the eyes and nostrils. Cx-RP levels remained low with titres of 1:1 on days 11, 12 and 22, until day 36 when a titre of 1:32 was recorded, rising to 1:64 by day 43.

A very similar response was obtained with rabbit S595. A slight oedema was present by day 15, but was markedly reduced following drug treatment. Between days 29 and 36, however, a great deal of facial inflammation developed. Cx-RP levels remained depressed, however, until 36 days post infection; a titre of 1:64 was recorded on day 43.

Rabbit S596 showed heavy facial oedema and inflammation by day 22. During and for 5 days after the cessation of drug-treatment, the symptoms became less severe. By day 36 they had reverted and were very heavy 43 days post infection. Cx-RP levels began to rise by day 15, but fell dramatically during treatment. Immediately afterwards the levels rose, reaching a titre of 1:64 by day 26. Fairly high levels were maintained with a recorded titre of 1:32 by day 43.

Rabbit S569 showed oedematous and necrotic symptoms after 22 days of infection which remained static during drug treatment. Thereafter,

it got progressively more severe, the animal dying on day 43. Cx-RP levels initially fell from a titre of 1:8 on day 0 (2 h. before inoculation), thereafter the values rose to reach a peak titre of 1:128 on day 28 and then fell as tissue involvement became more marked. Drug treatment between days 35 and 39 resulted in an extremely rapid decrease in Cx-RP levels which could not be detected from day 38 onwards.

With typical fulminating infections of T. brucei 427 in rabbits, therefore, serum levels of Cx-RP showed an initial rise, reached a peak and declined slowly as the tissue symptoms became more severe. Treatment with an anti-inflammatory drug early on in the course of infection retarded both the subsequent development of external symptoms and the initial rise in Cx-RP levels. If treatment was effected early enough, the levels remained low for several weeks after dosage was stopped, and only rose when a severe inflammatory reaction was visible. If treatment was carried out during the initial rise in Cx-RP levels, it was rapidly depressed, but immediately upon cessation, the levels increased to relatively high titres, even before external symptoms became evident. Drug treatment after the initial Cx-RP peak caused a rapid fall in activity which thereafter remained low. Treatment during the terminal part of the infection caused an increase in Cx-RP levels, which could have been due to local inflammation or gastric bleeding etc.

Indomethacin treatment had no effect on blood parasitaemia and appeared not to markedly alter the course of the disease. Anti-inflammatory treatment may, however, be of use in conjunction with trypanocidal agents.

3.11. Chemotaxis

Chemotaxis has been implicated in a variety of biological processes including cellular organization, inflammation, and the recognition of foreign matter. The response of normal mouse peritoneal exudate cells to chemotactic stimuli in vitro was investigated.

The full results of the effects of the various substances are given in Appendix (3.) and a summary presented in Tables (15; 16; 17). Casein at a concentration of 5mg./ml. (pH 7.5) in Gey's solution gave the most consistently high response. In many instances the casein did not easily dissolve so was either warmed to 37°C., or a small amount of 0.1 N sodium hydroxide was added and the pH brought back to 7.5 by the dropwise addition of 0.1 N hydrochloric acid. Excess casein and casesin in Gey's which had been stored for 24 h. at 4°C., exerted markedly reduced but still significant effects on the test cells. Gey's solution either alone or with the addition of penicillin (200 units/ml.) and streptomycin (100µg/ml.) was not chemotactic, neither was the TCM in which the peritoneal cells were suspended. Peritoneal cells are known to vary considerably from one day to the next in their ability to respond chemotactically (Ward et al 1970). For this reason, individual experiments using the same pool of exudate cells are presented in Appendix (3).

A series of negative controls were tested whereby both compartments of the chemotaxis chamber contained the substance under test. Compartment A consisted of a 1:1 mixture of peritoneal cell suspension in TCM and the test solution in Gey's, while compartment B contained the test solution alone. There was, consequently, no unidirectional stimulus, with any cellular responses being solely a result of altered migration and activity. Generally low indices were obtained for each of the four test substances. Gey's solution alone, however, gave a slightly enhanced value. ATP, which had been reported to both increase the

1. CONTROLS

(a) Positive Controls

For testing chemotactic response of murine peritoneal cells in TCM

TEST	MEAN C.I.(1)
Gey's solution	0
5 mg/ml casein in Gey's	100
Excess casein in Gey's	42.62
Stored casein in Gey's	27.0
T.C.M.	3.6

1. Chemotactic Index

(b) Negative Controls

For testing random migration of murine peritoneal cells

TEST	MEAN C.I.
Gey's solution	24.59
Casein in Gey's	9.83
A.T.P. in Gey's	1.1
T. brucei 427 supernatant	-5.8

2. EXPERIMENTS

(a) Mouse Tissue

TEST	MEAN C.I.
Muscle	2.4
Muscle + trypsin	7.5
Liver	6.5
Liver + trypsin	-13.1

(b) Chemicals

TEST	NORMAL MOUSE PLASMA	MEAN C.I.
Histamine	-	7.69
A.T.P.	-	24.98
Trypsin	+	143.8
5-HT	+	12.3

TABLE 15: Chemotaxis Summary

c. Sera/Plasma

TEST	Casein	Gey's	Heat Inactivated	Inactivated + C' restored	MEAN C.I.
N.M.S.(1)	-	+	-	-	20.5
N.M.S.	+	-	-	-	89.87
N.M.S.	-	+	+	-	-4.4
N.M.S.	+	-	+	-	8.7
N.M.S.	-	+	+	+	-15.5
N.M.P.(2)	-	-	-	-	6.9
N.M.P.	-	+	-	-	16.4
N.R.S.(3)	-	-	-	-	9.6
N.R.S.	-	-	+	-	-4.9
N.G.P.S.(4)	-	+	-	-	1.1
N.G.P.S.	+	-	-	-	132.4
N.C.S.(5)	-	-	+	+	20.49
Mouse Hyperimmune 427 Serum	-	-	-	-	7.2
Mouse Hyperimmune 427 Serum	-	-	+	-	4.5

TABLE 16: Chemotactic effects of homologous and heterologous sera and plasma on murine peritoneal cells.

1. normal mouse serum
2. normal mouse plasma
3. normal rabbit serum
4. normal guinea pig serum
5. normal calf serum.

d. Trypanosomes

TEST	N.M.P.	N.M.P. heat inact.	N.G.P.S.	N.G.P.S. heat inact.	Hyperimmune 427 Serum	Hyperimmune 427 Serum heat inact.	MEAN C.I.
Living *T. brucei* 427	+	-	-	-	-	-	29.6
Living *T. brucei* 427	-	+	-	-	-	-	23.0
Living *T. brucei* 427	-	-	+	-	-	-	13.4
Living *T. brucei* 427	-	-	-	+	-	-	29.2
Living *T. brucei* 427	-	-	-	-	+	-	4.1
Living *T. brucei* 427	-	-	-	-	-	+	4.3
Living *T. brucei* 427	-	-	-	-	-	-	1.1
T. brucei supernatant	+	-	-	-	-	-	28.9
T. brucei supernatant	-	-	-	-	+	-	40.9
T. brucei supernatant	-	-	-	-	-	+	57.2
T. brucei supernatant	-	-	-	-	-	-	5.6
T. brucei sediment	+	-	-	-	-	-	-5.8
T. brucei sediment	-	-	-	-	+	-	-3.3
T. brucei disrupted	+	-	-	-	-	-	-3.3
T. brucei heat killed	+	-	-	-	-	-	34.1
T. brucei heat killed	-	-	-	-	+	-	61.2

TABLE 17: Effect of trypanosomes and trypanosome-antibody complexes on the chemotaxis of murine peritoneal cells

effective metabolism and alter the speed and moving of macrophages (Senda et al 1975), perhaps by directly affecting pseudopodal activity (Cohn 1970), was used at a concentration of 0.33 mg./ml. Comparisons of the values obtained in the two control tests suggested that a true chemotactic response of peritoneal cells was being measured under the experimental conditions, furthermore, it has been shown that a chemotactic agent is capable of slowly diffusing through a filter membrane into the cell compartment, thereby establishing a gradient (Sorkin et al 1970).

In vivo cellular migration towards damaged tissues or areas of inflammation has been recorded, although this has invariably been associated with the presence of serum factors (Ramsey & Grant 1974), with little or no activity seen with normal or partially digested autologous tissues in the absence of serum. The chemotactic effects of mouse tissue were investigated. Small fragments of freshly dissected autologous muscle (extenser cruris), and liver tissue were crudely homogenized in 2.0 ml. of TCM and either allowed to autolyse aseptically for 24 h. at 37°C., or were partly digested by the addition of 1.0 ml. of 0.2% w/v trypsin in TCM, again for 24 h. at 37°C. None of the treated tissues generated any chemotactic activity, although trypsin is known to act as a cytotaxigen, splitting various C' components. Trypsin at concentrations of 2 mg./ml., however, may cause some reductions in migration (Wilkinson 1974a).

A selection of chemicals which had been previously shown to affect macrophage-trypanosome attachment were similarly tested. Histamine and 5-HT are both potent mediators of inflammation. Rat mast cells, which are rich in histamine, are known to produce an eosinophilotactic factor when stimulated, which is released in conjunction with histamine. There appears to be an association between eosinophil migration and lesions involving vasoactive substances, the latter may assist exudation and

cellular emigration *in vivo*, but do not themselves appear to be chemotactic. Peritoneal exudate cells did not show any chemotactic response towards histamine alone (0.2 mg./ml. in Gey's) or towards 5-HT (0.2% w/v) in the presence of 10% normal mouse plasma (NMP).

Sorkin *et al* (1970) have shown that none of the mediators of allergic reactions (histamine, 5-HT etc.) shows any true chemotactic activity. Trypsin and plasmin induce the formation of kinins which again appear to have no effect, although kallikrein may be an endogenous cytotaxin for neutrophils (Wilkinson 1974b).

Wilkinson (*loc cit*), found that the addition of ATP to chemotactic chambers did modestly increase the migration of human neutrophils, although he was unable to determine whether it affected random or directional migration. It could assist the migration of cells to other cytotaxins, even if not active itself. Some activity using ATP alone (0.33 mg./ml.) was observed in the test system, but could well have been due to the increased stimulation of peritoneal cells.

Wilkinson (*loc cit*) further reported that neutrophil chemotactic activity could be generated by the action of trypsin on serum C' and other proteins. The components C3 and C5 and their smaller split derivatives have been largely implicated. Certainly very high chemotactic activity was produced with trypsin in conjunction with NMP.

Conflicting reports have been made concerning the relative effects of serum and plasma from a variety of animals on the chemotactic response of macrophages and eosinophils. Some chemotactic factors may be generated during blood clotting (Sorkin *et al* 1970), other investigations involving the role of immune complexes suggest plasma as being necessary. Certainly in the control experiments using mouse peritoneal cells, more chemotactic activity was produced using NMS than NMP. For this reason, background controls using NMP were preferred. Significant chemotaxis

was produced with 10% NMS alone (20.5%), but this fell following heat inactivation at 56°C. for 30 min. (-4.4%). This was not, however, restored by the further addition of 10% guinea pig serum. High activity was generated using 10% NMS and casein, and this again fell following heat treatment. Low values (8.7%) were recorded despite the presence of casein in the test medium. It is possible that casein molecules had complexed one with another and reduced chemotactic activity by presumably decreasing the availability of surface hydrophobic sites.

Little chemotactic activity was generated by NMP either undiluted or at a 10% concentration in Gey's solution. Similarly, whole rabbit serum gave low values which were even further depressed following heat treatment. Normal guinea pig serum gave low results, which were enhanced in the presence of casein. Inactivated calf serum to which 10% guinea pig serum had been added gave results comparable to NMS in Gey's - approx. 20%. Hyperimmune 427 serum either alone or heat inactivated gave low results.

The effects of trypanosomes and trypanosome fractions were similarly tested in the presence and absence of both NMS and specific hyperimmune 427 serum.

Washed, living T. brucei 427 in PSG - 2.5×10^9/ml. - were not able to generate significant activity either alone, in conjunction with untreated or heat-inactivated hyperimmune serum, or with guinea pig serum. Higher values were recorded using fresh NMP and inactivated NMP plus guinea pig serum.

Significantly enhanced values were obtained using T. brucei 'supernatant' mixed with 10% hyperimmune serum, either fresh or heat inactivated. Lower values were recorded with NMP and negative control levels with 'supernatant' alone. T. brucei 'sediment', either with NMP or hyperimmune serum, did not generate any response, and neither did

disrupted trypanosomes plus NMP. High levels were obtained using 10% hyperimmune serum and heat-killed trypanosomes (a washed suspension heated at 70°C. for 30 min.).

The results suggest, therefore, that trypanosome antigen-antibody complexes may be important in the in vitro chemotactic response of mouse peritoneal exudate cells. The complexes presumably act as cytotaxigens and appear capable of generating cytotaxins from both normal and heat-treated sera. In addition to heat-labile split components of C', heat-stable factors, perhaps including serum kallikrein, may be generated by the formation of these complexes. Further experiments need to be undertaken, initially to investigate the properties of potential cytotaxins generated in fresh sera upon incubation with pre-formed complexes, thus leading to their subsequent isolation and eventual identification.

PART 4. DISCUSSION

A series of investigations have been made on the *in vitro* phagocytosis of trypanosomes in animals infected with acute and chronic strains of *T. brucei*. Particular emphasis has been placed on the attachment phase and on the various specific and non-specific factors capable of mediating adherence.

The results indicated that sera taken from acutely infected mice show relatively little opsonic activity. The course of infection was so short that comparatively little antibody was effectively generated, although lymphoid activity would have commenced from the moment of antigen introduction. No trypanosome agglutinating antibody was detected and the time course was such as to preclude the formation of variant serotypes. The pathology of the infection was typical of very acute disease, with no external clinical symptoms evident except terminal dyspnea and convulsions (Losos & Ikede 1972); the parasitaemia was abnormally high. The natural defence system in mice, therefore, appears hopelessly inefficient at regulating or even attempting to control the infection. Nevertheless, a definite increase in attachment throughout infection was observed *in vitro* and the experiments using artificially prepared hyperimmune serum and soluble antigen-antibody complexes, indicated the importance of immune antibodies, even at sub-detectable concentrations. With the chronic infection in mice, slightly higher attachment indices were recorded, and although the antibody titre remained low throughout infection an initial degree of regulation was apparent. A certain amount of recorded attachment may have been due to spontaneous, non-immunological clumping (Davies *et al* 1975).

In the chronically-infected rabbits, a high antibody response was present throughout the course of the disease and a large number of individual parasites and trypanosome-agglutinins attached to the cultured macrophages. The levels for both opsonic and cytophilic attachment tended

to rise rapidly for the first two weeks and thereafter began to stabilize, reaching an optimum value of around 100% attachment. This appeared to correspond to the first in vivo antigenic peak. In all instances, however, high agglutinating antibody titres were recorded several days before the high attachment indices were seen. It is suggested, therefore, that the production of specific antibody, lysis of the first serotype and the generation of antigen-antibody complexes are interrelated events concerned with the initial establishment of in vivo phagocytic activity. As the successive parasitaemic waves occur, so the relative quantities of antigen and antibody vary. Correspondingly, a number of stages are reached when optimum proportions would permit complex formation. Circulating antibodies which have the capacity to bind to macrophages either directly, or after complexing, could temporarily modify the reaction between phagocytes and trypanosomes in a regulatory manner; enhancing uptake when specific antigen-antibody complexes of high affinity are formed in regions of antigen excess, and depressing uptake when excess free antibody is present. This was observed in the in vitro experiments using free IgG molecules to block immune IgG-mediated attachment. The effect of differences in globulin concentration between the tissue fluid and serum in T. brucei-infected rabbits (Goodwin & Guy 1973), may be reflected in the efficiency of uptake. Interestingly, the lower M.W. IgG molecules are more likely to be initially present in tissue fluid than the larger, often membrane resistant, IgM; and T. brucei is a tissue parasite (Goodwin 1974).

The relative effects of trypanosome opsonizing and macrophage cytophilic antibody may be considered. Cytophilic sensitization of cell surfaces was greatly enhanced by the interaction with antigen, whether particulate or soluble. In the majority of experiments, however, similarities were seen between the opsonic and cytophilic activities, suggesting that in both cases, attachment was being mediated by the same reaction.

It is obviously difficult to effectively delineate between the processes involved, especially as even small amounts of eluted cytophilic material can directly opsonize particles (Askenase & Hayden 1974). Even if it is considered that the major role of cytophilic antibody is to act as an opsonin, a distinction may nevertheless be drawn between direct (pure cytophilic) and indirect (opsonic) antibody; the former being capable of attaching to macrophage receptor sites in the absence of antigen, whereas the latter may require prior complexing with either soluble or particulate antigen before surface adherence. It is possible that cytophilic antibody may either exist as a distinct population or bind differently and thus be more resistant to elution, as well as triggering important and distinctive cellular processes when antigen is bound. As was observed, the adherence promoting components present in hyperimmune sera may well have been largely removed by cytophilic absorption following incubation with homogenized mouse liver, certainly IgG receptors have been identified on the surfaces of human liver macrophages (Huber et al 1969).

Natural cytophilic antibodies are presumed to exist in mouse serum (Askenase & Hayden loc cit), although their function remains largely unknown. They may facilitate macrophage-lymphocyte interactions and 7S IgM, known to coat macrophages, may be an effective surface antibody for lymphocytes. It is possible that a proportion of the monovalent IgM produced during late trypanosomiasis may act similarly. This may, indeed, represent an abnormal synthesis and correspondingly affect lymphocyte-lymphocyte and lymphocyte-macrophage interactions.

Cytophilic antibody may have an important additional function - whereby it can be transported by migratory macrophages into sites of gross inflammation in extravascular areas, where the normal antibody concentration would be markedly reduced. If eluted, this could contribute to the local pathology and possibly cause the preferential accumulation of specific antibody subclasses.

The nature and effects of the various cytophilic agents need more elucidation. The experimental evidence strongly suggested that IgG molecules either alone (Tizard & Soltys 1971b), or, more effectively, those capable of fixing C', were of overiding importance in chronic trypanosome infections in rabbits. Both the classic and alternate pathways were implicated, although the results were necessarily inconclusive. IgG_1 can fix C' via the alternate pathway (Leslie & Cohen 1974a) and IgG_2 and indeed IgM fix C' classically (Berken & Benacerraf 1966). Nevertheless, separate receptors may well be involved and, as previously indicated, the ability to fix C' and attach cytophilically could be more co-operative than dependent upon each other.

Cytophilic antibody activity, itself, can, according to species, be a property of both IgG_1 and IgG_2 molecules and, in conditions of antibody excess, spatial competition may be important in limiting attachment (Dissanayake & Hay 1975). The experimental results indicated that the activity of hyperimmune mouse serum could be absorbed out by heterologous trypanosomes, yet, paradoxically, these same trypanosomes did not attach to mouse macrophage monolayers. This may have been due to differences in the site determinants on the macrophage surface; similar suggestions have been advanced by Portis & Coe (1975), dealing with hamster cytophilic antibody.

Takayanagi et al (1974b), indicated that homologous antiserum enhanced the attachment of T. gambiense to rat macrophages, but that C' components were not involved. This contrasts with the findings of Lange & Lysenko (1960), and Kloetzal & Deane (1970), working with T. lewisi infections. The present results have shown that cytophilic attachment to mouse macrophages was not reduced following the heat-treatment of hyperimmune serum; however, prior trypsinization of macrophages (known to inactivate C' receptors, but not affecting Ig Fc

receptor sites), markedly reduced adherence. The possibility has already been advanced that C' components synthesized by viable macrophages may be of importance. Heat treatment of mouse hyperimmune serum reduced trypanosome attachment as measured by the opsonic test. It is possible, therefore, that three (or more) separate methods of attachment to macrophages may occur. One, involving pre-formed antigen-antibody-C' complexes which can subsequently adhere to surface sites; the second involving antigen-antibody complexes similarly attaching; and the third involving pre-fixed antibody with or without macrophage-generated C', which could occur in heat inactivated serum. The important point is that the antibodies involved may represent different classes, varying in their susceptibility to heat treatment and in their ability to fix C'.

Both serum C' and intact C' receptor sites were important, although not essential, for serum-mediated attachment of trypanosomes to rabbit macrophages. In both the rabbit and mouse systems, however, C' was ineffective in serum lacking agglutinating antibodies. It is probable that there exist different requirements for adherence mediated by mouse and rabbit sera. Serum-mediated attachment was more effective in an homologous system involving rabbit macrophages and for maximum efficiency, may well have depended upon the presence of an intact serum C' system.

Takayanagi et al (1974b), similarly suggested that the opsonic and agglutinating antibody present in infected rats, were identical, and further that its effect was largely confined to the anterior part of the parasite, most especially the free flagellum. As has been indicated in the present studies, opsonic activity appeared after the initial rise in agglutinating antibody and unlike the observations of Takayanagi et al (loc cit), was not observed in the macroglobulin fraction. Further, despite agglutinating antibody being recorded from both the micro- and

macroglobulin fractions (Takayanagi & Enriquez 1973), the Japanese authors were unable to detect activity in the 7S fraction.

Anterior attachment of T. brucei to macrophages has been observed (W.H. Lumsden, pers comm), and Vickerman (1974), has further indicated that the trypanosomatid flagellum can form junctional complexes of the desmosome or hemi-desmosome type with the substratum or other flagellates. This "increased adhesiveness" may permit initial contact with phagocytic cells and be of some importance in agglutination. Experimental observations did not, however, reveal a proponderance of flagellar attachment, nor were agglutinated trypanosomes necessarily attached through their flagella as has been observed in T. lewisi infections (Lange & Lysenko 1960).

It has been largely assumed throughout, that both opsonic and cytophilic binding has been due to antibody factors. The serum-separation studies have confirmed this, but a proportion of attachment may nevertheless be as a result of non-Ig components. β_2-microglobulin, for example, a membrane protein produced by many nucleated cells and structurally similar to the constant C_H3 region of the IgG heavy chain, may bind to macrophages and mediate attachment (Anderson et al 1975). Certainly it can bind to lymphocytes and this appears distinct from Fc reception. Lalezari et al (1974), have demonstrated a spontaneous macrophage-red cell adherence different to both non specific attachment and antibody or antibody-C' mediated opsonization.

Loor & Roelants (1974), observed that a proportion of antigen could remain on or near the macrophage surface without being endocytosed - despite the cross-linkage of surface membrane components and the formation and redistribution of microprecipitates into polar caps. This type of binding appears independent of antibody, and again, not via the C_H3 or C_H2-complematary regions of the Fc receptor.

In those experiments involving excess free IgG, it was possible to competetively block the attachment of trypanosomes to immune IgG receptor sites. Occlusion of the reactive sites would have saturated the Fc receptors, with perhaps a similar situation occurring *in vivo*, maybe partially due to the blocking effects of excess IgM. A proportion of the observed attachment *in vitro*, as well as *in vivo* adherence in unimmunized animals could have been due to these "unsaturated sites", with perhaps the degree of antigen aggregation being important. It is possible that the observed stickiness in inflammatory states may be similarly related. It would be interesting to see the relationship, if any, between particulate sticking and the adherence of macrophages to damaged vessels or, indeed, to other surfaces, and its effects on surface phagocytic activity.

The macrophage receptors have been shown to be sensitive to reagents acting with free -SH groups, oxidising agents and, to a lesser extent, with those acting with free NH_2 and -SH groups. The binding site on the antibody itself may be independent of iodoacetate (Howard & Benacerraf 1966), nevertheless trypanosome opsonization was inhibited by iodoacetate, if not affecting the antibody, then perhaps modifying the available antigenic sites containing -SH groups, or perhaps disrupting metabolic processes such as amino acid transport. Certain of the inhibiting substances tested, even if not acting through a direct metabolic pathway, could have prevented surface attachment to macrophage sites. Chemical inhibitors may cross-link antigen receptors, thus preventing translocational diffusion by the membrane. Capping would be impaired with attachment and phagocytosis correspondingly reduced.

The chronic nature of *brucei* sub group trypanosomiasis would cause macrophage activation, both Fc ingestion and C'-mediated attachment would be increased (Bianco *et al* 1975), and activated macrophages would be more efficient in removing damaged red cells, hence contributing to

the observed anaemia. There appears to be a functional difference between activated and non-activated receptor sites. This may be due to cytoplasmic linkage alterations and/or variations in concentrations of receptor molecules with a threshold importance. Inflammatory agents and chemicals may be capable of functionally altering receptor sites. Thus 5-HT was able to cause quite appreciable changes in binding potential to mouse macrophages either alone or in the presence of hyperimmune serum. This, and certain of the other chemicals tested may have affected surface charges or perhaps altered the activity of membrane bound cyclic AMP.

Similarly, trypsin was capable of altering macrophage receptor sites and affecting free antibody and other serum components. Modification of the trypsin-sensitive amino acid sequences of Ig molecules may disrupt attachment, although specific receptor binding may be more important via carbohydrate fractions, than through peptide sequences (Portis & Coe 1975).

Macrophage trypsinization was capable of destroying C' receptor sites but not Fc receptors mediating Ig attachment. It is possible that antigenic structures at different levels within the cell membrane are affected according to concentration and incubation time. Certainly this would explain the lack of effect of trypsin in the opsonic test involving mouse macrophages, but its depressing activity in both the cytophilic and opsonic adherence tests.

Kossard & Nelson (1968), have reported an increased sensitization of mouse macrophages by hyperimmune serum following trypsinization. This was not observed in the present studies using mouse hyperimmune serum to trypanosomes. In the rabbit, however, prior trypsinization of cultured macrophages increased the effect of immune IgG. This may have been due to the removal of any natural cytophilic antibodies, or perhaps more likely, by removing masking, enzyme-sensitive receptors which could

themselves have possessed cytophilic antibodies of a completely different type or specificity.

It is difficult to say whether the induced macrophages produced in rabbits were activated in the fullest sense. As indicated, a proportion at least of the activity was considered to be due to non specific hyper-activity and not necessarily to adsorbed cytophilic antibody. Although this would more closely reflect in vivo conditions, it would not enable a satisfactory delineation between the different discriminatory processes except on a direct comparative basis. Valid comparisons between the roles of mouse and rabbit macrophages would be similarly affected. Perez & Terry (1973), have also indicated a similar problem involving monkey peritoneal cells, whereby any method used to obtain sufficient numbers could not ensure their continued normality. It has not yet been established how similar the induction of cells due to intraperitoneal injections is to activation as seen in chronic infections and delayed hypersensitivity reactions (Bianco et al 1975). Reikvam et al (1975), for example, stimulated mouse peritoneal cells in vitro using calf serum and in vivo using a Toxoplasma infection. Both populations showed enhanced lysosomal activities, yet the killing ability of the former was inferior to that of the latter. One mechanism around this problem would have been to use peritoneal cells from chronically infected animals throughout. Some initial experiments were performed but the results obtained were erratic and inconclusive and have not been presented. The number of viable macrophages obtained from the infected peritoneum was low and contaminated with both red cells and surprisingly, lymphocyte-like cells.

It is possible that macrophages exist in a variety of states or levels of activation (Bianco et al 1975). Thus activation may be achieved by a number of different methods - artificial inducing agents,

specific arming factors, or sensitized lymphoid cells. It has been suggested that the functional criterion for activation may be the phagocytic integrity of the C3b receptor (Bianco et al loc cit). Variations may also be due to maturation differences. Cooper & Houston (1964), indicating that both triglycerides and lipopolysaccharides can induce cell hyperactivity, suggested that within each population of cells only a proportion are actively phagocytic at any one time.

Immune complexes are capable of binding to specific receptor sites more effectively than monomeric antibody and can, by antigen exchange, confer an enhanced antigen binding capacity (Leslie & Cohen 1974b). The experimental results have confirmed this observation in trypanosome infections. Complex-formation greatly enhanced attachment, especially in the case of mouse macrophages specifically pre-sensitized with soluble trypanosome antigen-antibody complexes. Uhr (1965), demonstrated enhanced bacterial phagocytosis due to passive sensitization by complexes. These were partly endocytosed, suggesting a series of combining sites present on each complex, probably by an alteration in antibody configuration following antigenic contact, perhaps exposing Fab binding sites permitting attachment and ingestion. A similar mechanism may operate in trypanosome-attachment. It is not clear, however, whether the sites involved are the same as those for cytophilic antibody attachment, although it is suggested that they may be distinct. A large number of separate specific feceptors have been identified on the surfaces of both lymphoid and mononuclear cells - with obvious importance in effecting discrimination (Walker 1974). Indeed the requirements between cell types may be a partial reflection of cellular function. As previously indicated, the method of presentation and the complexed or conjugated state of the bound antigen is probably paramount in determining its fate (Feldman & Pollock 1974). The exact mechanism of particulate attachment following passive sensitization may be due to non-specific

macrophage activation, the availability of further free combining sites present on the attached complex, or the dissociation of soluble antigen from antibody following complex-adherence.

Takayanagi et al (1975), have indicated, however, that the addition of T. gambiense supernatant to rat macrophages blocked the attachment of whole trypanosomes. They suggested competition between immune complexes and opsonized whole trypanosomes. However, the macrophages were neither presensitized with antibody, soluble antigen or complexes and both cytophilic and opsonic attachment were measured. Nevertheless, this situation appears analogous to the site saturation due to excess free rabbit IgG previously described, and perhaps enables a distinction to be drawn between direct competition for available surface sites and the passive sensitization of receptors specific for performed complexes.

The problems concerned with being explicit about the quantitiy and quality of macrophage receptor sites are obvious. The heterogencity of receptors, nature of complexes or aggregated Ig and the sensitivity of the method of detection, all being of importance.

During chronic trypanosomasis, the levels of Cx-RP present in the sera of rabbits increased markedly and in most instances appeared to give a good indication of the pending inflammation. Cx-RP values rose before any external symptoms were apparent and declined gradually once necrosis and degenerative lesions had developed.

Ree (1971), detected raised levels of CRP in Gambian Africans with P. falciparum malaria. No correlation was noted with the level of any Ig, although the intensity of parasitaemia did appear to be a determinant of the presence or absence of CRP in the blood. This was not found in the present studies - T. brucei being a tissue parasite - although profitable investigations might be made using haematic trypanosome infections.

Ree (loc cit), agreed that the use of detectable CRP could not be used as a presumptive diagnostic test for malaria or any other disease. Alterations in serum CRP do not appear to have been looked at in human trypanosome infections, but if present, its value in effective diagnosis of specific infection would be limited, although raised CRP levels are indicative of general "illness". Perhaps initial screening of susceptible sleeping sickness patients using a CRP test allied to a parasite-specific serological technique would be useful in presumptive diagnosis.

The direct testing of purified CxRP or CRP on the phagocytosis of trypanosomes might be rewarding, as Ganrot & Kindmark (1969), have indicated its effeciency in promoting the uptake of bacteria by human neutrophils. CRP molecules may bind to the surface structures of encapsulated bacteria, rendering them liable to phagocytosis - a similar analogy may exist with trypanosomes. CRP may also play a part in C' activation (Volanakis & Kaplan 1974).

The effects of, and the role of phagocytic activity in experimental chronic trypanosomiasis may perhaps be visualized as follows. Following the initial infection and the rise of the first parasitaemic peak consisting of the basic antigenic type, large amounts of macroglobulin are produced. This contains a proportion of variant specific IgM, as well as smaller amounts of IgG. Agglutination, neutralization and lysis of the first serotype occurs, releasing large amounts of parasite debris into the tissue spaces and the circulation. These would include both soluble and insoluble common antigens. Mononuclear and polymorphonuclear cells already present within the inflammatory areas could non-specifically phagocytose both these insoluble particles and whole parasites. Due, however, to the low number of phagocytes, their unactivated state and the paucity of what appears to be specific opsonic

antibody, this control method remains largely inefficient. It may be compared with an initial non specific defence mechanism whereby whole trypanosomes are removed from the circulation. It has been suggested that dead or mechanically injured parasites may be made recognizable, perhaps by alterations of the surface coat initiating the plasma clotting system. Wilkins & Myers (1966), have indicated that negatively charged colloids tend to be endocytosed at a lower rate than positively charged particles. The coating of damaged or intact trypanosomes either non specifically with fibrin or FDP-like substances, or specifically with antibody could alter surface potential and consequently affect phagocytic discrimination. Vaughan (1965a), suggested that when foreign macromolecules entered the circulation they were exposed to a large and heterogenous population of globulin molecules. It may well be that those which are complentary can bind with available surface groupings, with or without C' fixation. Distortion could occur either revealing hitherto covered groups reacting with phagocytic surface receptors, or initiating local blood clotting with the subsequent deposition of fibrin or fibrinogen on the surface of the foreign cell. Taylor et al (1974), have suggested that adsorbed host components would play a significant role in the normal surface properties of T. brucei and that these would be susceptible to the ionic strength of the surrounding media; and Ketteridge (1971, 1972), has indicated that T. vivax membranes (although markedly different from those of T. brucei) can be coated with what appears to be host sialic acid glycoprotein which may either act as a disguise or affect immune complex interactions. Nevertheless, during the early stages of infection, before the production of opsonizing antibodies, the normal surface charges of both trypanosomes and macrophages may be such as to prevent either agglutination or attachment. This suggested non-specific recognition, therefore, may be relatively short lived and

similar to the process whereby effete red cells or other damaged tissues are removed by the host.

After the initial parasitaemic wave, soluble antigen-antibody complexes are developed. These are capable of activating a large number of interrelated processes including the plasmin, kinin, C' and blood clotting systems, capable of contributing to the observed inflammatory response. In addition, the chemotactic mediation, perhaps involving stable serum components, of migratory monocytes and macrophages would cause cellular accumulation. These or similar complexes are able to specifically sensitize the macrophage surface and may either activate the phagocytic cells permitting them to kill more efficiently and non-specifically, or they may permit specific receptor site interaction with whole trypanosomes.

The trypanosome surface coat itself may be largely anti-phagocytic, perhaps due to the physical masking of the ground matrix by projecting molecules conferring variant specificity. Vickerman (1974), has indicated that cultured parasites lacking this coat may be easily endocytosed in the absence of immune sera. Specific antibodies directed either against selected antigens in the surface coat or against somatic antigens released upon lysis, appear essential for effective attachment and phagocytic uptake.

Seed (1972), has indicated that trypanosome surface antigens consist of at least two separate components, one concerned with protection, the other with agglutination. Consequently, at each antigenic change a minimum of two groups of antigenic determinants must either be modified or replaced (de Raadt 1974). It is possible that antigenic determinants not necessarily connected with variant protection, may similarly exist on or in the glycoprotein surface coat and be specific for either IgM or IgG.

The macrophage-cytophilic and trypanosome-opsonizing antibodies show many similar properties and may well be the same; with reaction differences being due to the method of attachment, whether single, complexed with antigen or with antigen and C' etc. It is suggested that opsonization is independent of the variant antigens and is largely mediated by complexed IgG molecules. During each serotypic increase, opsonization is preferentially resisted, allowing the population to build up. Relapse antibodies are produced and cause structural changes to the susceptible trypanosomes, perhaps mediated through C' fixation. Receptor sites may be altered, removed or the whole surface coat may be effectively stripped off. Those common precipitative antigens present within the trypanosome body and any on the surface coat which are not antiphagocytic permit effective opsonization. Thus specific antibodies with or without related serum components, can mediate phagocytosis. It is possible that phagocytic removal of trypanosomes in an infected host is not so much an active defence mechanism against a rising population, as more a general mopping up and removal of damaged or old individuals which have already been immunologically recognized.

The immunological effects of the various antibodies produced during infection remain controversial. Takayanagi & Enriquez (1973), have indicated that IgM is more effective in agglutinating trypanosomes and in inducing antigenic variation, than is IgG. IgG appears the most effective opsonizing Ig capable of attaching to specific trypanosome surface receptor sites with perhaps its efficiency enhanced due to the powerful agglutinating ability of IgM. Increased effectiveness may also be due to the C'-fixing potentials of both IgM and IgG.

Antigen-antibody complexes can bind to macrophage surface receptor sites and fix C'; these may then be ingested, activate alternate receptor sites, or attach particulate antigen. Alternatively, trypanosome antigen-

antibody complexes could activate C' in the liquid phase, this could dissociate and bind to both trypanosomes and red cells causing surface damage, if not complete lysis. Woodruff et al (1973), have detected C3 on the red cells of humans infected with T. rhodesiense, and high immunoconglutinin titres have been detected in rabbits with T. brucei infections. These antibodies are produced against modified C' components bound to antigen-antibody complexes (Ingram & Soltys 1960) (see Fig. 33).

The variations in parasite attachment recorded during both mouse and rabbit infections and the reported increase in anaemia, could have been modified by the coating of particles with late or early C' components. Consequently as the disease progresses, macrophages are activated; trypanosomes and red cells are coated with C3b complexes and are phagocytosed. Those that escape this early reaction - perhaps by being sequestered in privileged areas, may have surface adherent C3b converted to C3d. This is generally non-phagocytic although specific receptor sites on the macrophage surface may exist, and does not promote IA. Particles so treated may be removed from the circulation at a more controlled rate. This may, therefore, partially reflect an added control mechanism, perhaps facilitating parasite multiplication at the most crucial stages.

Large trypanosome agglutinins produced during infection would also hamper the microcirculation and adsorb to the endothelium of venous drainage channels. These small blood vessels become characteristically 'sticky' during inflammatory processes (Ebert & Grant 1974). Surface phagocytosis would increase and also partly contribute to the observed stasis and thrombosis.

The increased IgM levels would also alter the osmotic balance and in conjunction with the increased amounts of fibrinogen (Boreham & Facer 1974), affect blood viscosity. This may be an important adjunct in

phagocytosis, reducing blood flow and permitting greater cellular contact.

There may be an upper size limit for effective attachment and phagocytosis. One of the disadvantages of the in vitro system is the use of artificial macrophage monolayers. In many instances coalesced peritoneal cells were observed and a number of cytoplasmic bridges were apparent. This may be a result of co-operative phagocytosis and would be important in surface phagocytosis, or it may represent an abnormal cellulor clumping. These may be essential for the effective removal of agglutinins. Furthermore, the presence of a monolayer itself may be necessary to allow adjacent molecular contact.

The antigen-antibody complexes produced during the course of infection are associated with the release of pharmacologically active substances. Kinins, concerned with the chronic aspects of inflammation, are generated and may be largely responsible for the observed oedema and vascular changes, as well as cardiovascular failure and contribute towards haemostasis and tissue anoxia (Goodwin 1974). Large quantities of kinin are produced in experimentally infected cattle just after the second parasitaemic peak, when high levels of circulating common-trypanosome antibodies can be detected (Boreham 1968b). Boreham & Wright (1976), have indicated severe hypotension in experimentally infected rabbits and suggest that this is mediated by kallikrein. Thus trypanosome antigen-antibody complexes activate Hageman Factor (Factor XII) which successively activates kallikrein and causes the release of kinins.

The generation of kinins and other pharmocological mediators by immune complexes would increase vascular permeability and correspondingly widen the chemoattractant field, hence generating a greater influx of activated mononuclear cells. Both FDP's and kallikrein are known to be chemotactic for neutrophils (Wilkinson 1974b), and plasmin can interact with the C' system, splitting C3 to produce the anaphylatoxic and

chemotactic dreivative C3a. Activated mouse macrophages can synthesize, accumulate and release plasminogen activator, and consequently several pathways involving various enzyme cascades may be established for the generation of pharmacologically active peptides (Unkeless et al 1974).

As the initial trypanosome antigen-antibody complexes are subsequently dissociated or engulfed by sensitized macrophages, a negative feedback would be established. This could partly explain the rise and fall in leucocyte activity and numbers seen by Goodwin (1970), during infection. A proportion of these complexes, or those involving heterophile IgM, could similarly adhere to red cells and thus be endocytosed (Assoku 1975; Woo & Kobayashi 1975). Whole trypanosomes may attach by an IA mechanism to host erythrocytes which have previously adsorbed specific complexes (R.A. Nelson 1953, D.S. Nelson 1965) and be removed either immunologically or through a surface phagocytosis effect. In addition to immunologically mediated anaemia a microangiopathic arm could develop due to the increasing number of thromboses in blocked vessels.

Platelet aggregation may also be involved in this context. Davis et al (1974), demonstrated clumping upon the addition of whole T. rhodesiense or parasite-free supernates of disrupted organisms to a variety of infected blood. Investigations rule out the involvement of immunological factors, with the authors suggesting the production of a trypanosome protein enzyme or even a toxin. Although similar results were not observed in humans infected with T. gambiense (Greenwood & Whittle 1976), it indicates that vascular pathology may not necessarily be entirely dependent upon the generation of immune complexes.

Nevertheless, an established immunological method of increased vascular permeability in rabbits is by the complex mediated release of pharmacologically active substances from blood platelets (Henson &

Cochrane 1969). Platelet clumping and degranulation is often associated with an increased local phagocytosis. Luk & Simon (1974) have reported the close association between rabbit and rat macrophages from bone marrow and the activity of platelets. It may be possible that platelets release a chemotactic factor upon degranulation. If this were so, a possible non-immunologic chemotactic mechanism might occur in trypanosome infections. Platelet aggregation could also be the result of fibrin coating. Deposition of fibrin on endothelial linings could cause local thrombi and be important in both macrophage sticking - as an essential prelude to chemotaxis - and surface phagocytosis.

Contact lysis of trypanosomes may be effected by activated macrophages coated either with immune complexes or trypsin-resistant cytophilic antibody (Walker & Demus 1975). Erythrocyte cytolysis may similarly occur (Allison 1974); indeed a possible contributory factor in the aetiology of anaemia in trypanosomiasis could be the production of an erythrotoxic or erythrolytic factor, either via immune complex generation or perhaps directly from the trypanosome itself (Huan et al 1975).

Antigen-antibody complexes additionally assume an importance in chemotactic mediation. Immune complexes are chemotactic for granulocytes due to the formation of heat stable factors from serum containing heat-labile components. This may be a generalized phenomenon, with a variety of cells capable of being stimulated by largely non-specific complexes. (Keller & Sorkin 1965a & b). In the present experiments, complexes involving disrupted trypanosome supernate or heat killed parasites and hyperimmune sera, or, to a lesser extent, normal mouse plasma, generated chemotactic factors capable of affecting the response of mouse peritoneal exudate cells. Despite the fact that incubation was not necessarily effected in fresh serum, but sometimes involved complexes developed in heat-inactivated serum, chemotaxis was still apparent in vitro. A

C'-independent macrophage chemotactic system has recently been described (in Wilkinson 1974b). It is possible that in the systems studied, a number of separate cytotaxins may have been generated including heat labile C' factors and heat stable substances, perhaps including kallikrein. The C' system has, however, been suggested as being of over-riding importance in chemotaxis. Gallin et al (1975), have shown that chemotactic cytotaxins are very often late C' component cleavage products produced by activation of both the alternate and classic pathways. Trypsin digestion of serum or the release of proteases from macrophages and other cells can yield split C' factors such as C5a (Ward et al 1971), able to stimulate leucotactic responses and cause the accumulation of subsequent inflammatory infiltrates (Ward 1975).

Serum lipid and cholesterol levels increase strikingly in T. gambiense infected rabbits (Diehl & Risby 1974), and there is an increase in lipoprotein in rabbits infected with T. brucei (Facer 1974). Turner et al (1975), have indicated that pure lipids may be chemotactic for polymorphs. Membrane damage is extensive in trypanosomiasis, so the possibility of oxidised phospholipids originally present in the membrane capable of generating cytotaxins may exist.

If it is assumed that chemoattractants can direct mobile phagocytes to an area of injury or inflammation and thereby promote phagocytosis and histiolysis, a control mechanism can be visualized. Thus a positive chemotactic gradient would cause directional migration to an area of local activity and phagocyte accumulation would occur. Once there the gradient would no longer exist, due to the high quantities of cytotaxin present, so random migration would predominate. Consequently, cellular activity could be finely restricted to a specific area directly dependent upon the concentration of chemoattractant produced. This would

be reinforced if, as is suggested, both cytotaxins and cytotaxigens can be released from degranulated lysosomes, along with unknown chemotactic inhibitors (Keller et al 1975).

Chemotactically-attracted phagocytes are also of overiding importance in tissue histiolysis; the destruction of basement membranes by cathepsins, elastases, proteases, collagenases etc. appears a common feature in all general inflammations (Wilkinson 1974b), and lysosomal-hydrolase-release by macrophages has been specifically implicated in the destruction of schistosomal tegument (Perez & Terry 1973). Epithelial attachment by macrophages could provide an opportunity for the secretion of hydrolases etc. directly onto the surface, capable of reacting without being rapidly inactivated (Sobelovsky et al 1968). Furthermore, stimulated macrophages are able to release C3 cleavage enzymes, the C3b and C3a so formed can in turn react with the macrophages causing the release of lysosomal enzymes into the medium (Schorlemmer & Allison 1976). Membranes and other tissues are enzymatically disrupted and this, combined with the release of vasoactive substances, increases the vascular permeability and the general inflammatory response. A self-perpetuating positive feedback is thus established, dependent upon the formation of specific immune complexes.

An attempt has been made in Fig. (40), to link the conclusions reached and the speculations advanced, with the present knowledge concerning the general immunopathological events occurring in experimental trypanosomiasis. The contribution of the inter-related processes of chemotaxis and phagocytosis has been emphasized and the way in which these factors are both affected by and affect the various plasma mediated systems has been indicated.

Inflammation is probably the most damaging aspect of trypanosome infections caused by the brucei subgroup and when chronic will have

severe and far-reaching effects on fundamental tissue homoestasis. The inflammatory response itself is a normal host reaction following the initial introduction and subsequent establishment of a viable parasite population. As a result of the presence and liberation of a number of factors, both of host and parasite origin, a complicated and synergistic series of metabolic pathways are activated, a variety of host cells are mobilized, and cellular immune responses are initiated. During this primary stage, the developing inflammation and associated reactions are still aimed at preserving the integrity of living tissue, localising and combatting the existing infection and ridding the infected host of the causative organisms.

Due, however, to the nature and appearance of the trypanosome antigen, the normal inflammatory and immune responses are disrupted. Many of the non-specific component pathways which may themselves lack discriminatory feedbacks are inadvertently activated and assume an abnormal importance, the full effects of which become increasingly apparent as the infection continues.

The repeated insults offered to the host's immune system by the appearance of successive trypanosome populations, and the progressive inability, through a variety of factors, to deal successfully with these challenges, results in continual waves of harmful tissue reactions, each one proportionately more damaging than its predesessor. Type II (cytotoxic) and Type III (complex-mediated) hypersensitivity reactions assume great importance, with repeated allergic sensitization lasting for the duration of the infection.

In some ways the chronically-infected animal is its own worst enemy; for whatever attempts are made to restrict and combat the infection, the complex defensive systems and the well-established effector and control pathways are themselves assuming an ever increasing responsibility for the pathophysiological symptoms and the final demise.

The initial induction of an inflammatory response, the directional migration and accumulation of discriminatory phagocytic defensive cells and the multiple involvement of hormonal, vascular and cytotoxic components - at first so successful in limiting the infection - later assume an effect out of all proportion to their original and intended functions. Instead of healing damaged tissue and assisting in the host curative process, these interdependent allergic responses initially aggravate and finally compound the infection.

Herein lies the paradox. The host's MPS has been activated by the formation and interaction of parasitic complexes, in a far more efficient and selective manner than it would have been by artificial physico-chemical manipulation. But because the MPS is inevitably dealing with an already recognized trypanosome population, immunologically one step ahead of the host response, the repeated physical and metabolic onslaughts mounted against each serotype are merely worsening the condition.

The need, it seems, is not to further enhance either the selectivity or activity of the phagocytic response, but to alter the temporal sequence, such that individual parasites are recognized and phagocytosed before a population build-up has occurred and before circulating immune complexes have been allowed to develop.

Until then, the immediate answer does not appear to lie in attempting the further manipulation of the MPS itself, but in depressing the allergic activities of the pregenerated immune complexes. To this end the use of enzyme inhibitors such as 'trasylol' or EACA, perhaps allied with anti-inflammatory drug treatment, would impair a substantial number of disrupting feedbacks, hence preventing or at least depressing the repeated allergic reactions.

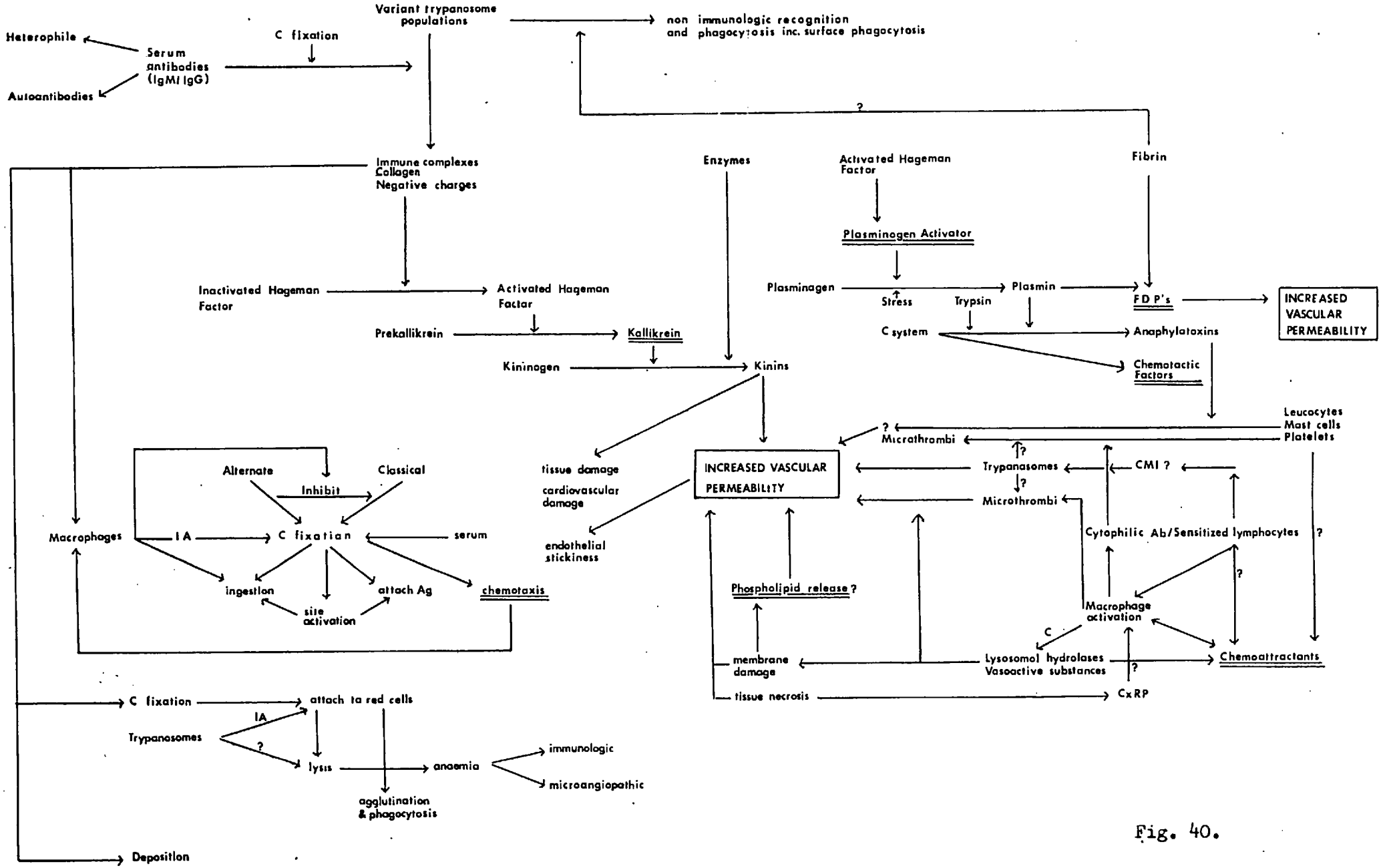

Variant trypanosome populations
non immunologic recognition and phagocytosis inc. surface phagocytosis
Heterophile
Serum antibodies (IgM/IgG)
Autoantibodies
C fixation
?
Immune complexes
Collagen
Negative charges
Enzymes
Activated Hageman Factor
Fibrin
Plasminogen Activator
Inactivated Hageman Factor
Activated Hageman Factor
Plasminogen
Plasmin
Stress
Trypsin
FDP's
INCREASED VASCULAR PERMEABILITY
Prekallikrein
Kallikrein
C system
Anaphylatoxins
Kininogen
Kinins
Chemotactic Factors
Leucocytes
Mast cells
Platelets
Micrathrombi
Alternate
Classical
Inhibit
tissue damage
cardiovascular damage
endothelial stickiness
INCREASED VASCULAR PERMEABILITY
Trypanosomes
CMI ?
Microthrombi
Macrophages
IA
C fixation
serum
Cytophilic Ab/Sensitized lymphocytes
ingestion
attach Ag
chemotaxis
site activation
Phospholipid release?
Macrophage activation
C
membrane damage
Lysosomal hydrolases
Vasoactive substances
Chemoattractants
tissue necrosis
CxRP
C fixation
attach to red cells
IA
Trypanosomes
?
lysis
anaemia
immunologic
microangiopathic
agglutination & phagocytosis
Depositon

Fig. 40.

Fig. 40. Schematic representation of the phagocytic and chemotactic sequences in the pathophysiology of experimental trypanosomiasis.

═══ chemotactic factors.

PART 5. CONCLUSIONS

1. Opsonic and macrophage cytophilic antibody activity is present in the sera of mice and rabbits infected with Trypanosoma brucei. The degree of attachment is dependent upon the presence of specific antibodies, most notably immune IgG; and this activity is capable of being absorbed out of whole and separated serum by live parasites.

2. A number of different and distinct receptor sites are present on the macrophage surface, varying in their susceptibility to a variety of pharmacologically-active chemicals and metabolic inhibitors. 5-hydroxytryptamine either alone or in conjunction with immune sera can enhance the attachment of trypanosomes to mouse macrophages whilst reagents acting with the amino and sulphydryl surface groups depress attachment. Artificially activated macrophages show an enhanced phagocytic response.

3. Antibody-mediated attachment is not dependent upon variant specificity but upon the surface sensitizing effects of immune complexes. Trypanosome antigen-antibody complexes are also chemotactic cytotaxigens for mouse peritoneal exudate cells.

An immune-adherence-type mechanism is of importance, especially in macrophage cytophilic antibody activity in rabbits. The integral C3 component may be generated via either the alternate or classical complement pathways.

4. The trypanosome surface coat may be largely anti-phagocytic. Attachment is dependent both upon the availability and specificity of macrophage receptor sites and the modification of the parasite surface by antibody and complement.

5. Surface phagocytosis of trypanosomes occurs in the absence of immune sera and together with a non-immunological recognition method involving components of the blood clotting and related systems, may contribute to the pathophysiology of the disease.

6. The levels of serum Cx-Reactive Protein increase in rabbits infected with chronic trypanosomiasis and are a reliable indication of pending inflammation. Indomethacin-treatment of infected rabbits results in the depression of both inflammation and Cx-RP levels, but leaves the parasitaemia unaffected.

7. The increased phagocyte response in chronic trypanosome infections is intimately involved in the pathogenesis of the disease and plays a major contribution in local inflammation and anaemia.

APPENDICES

APPENDIX 1.

Buffers and Solutions

(a) Balanced Salt Solution (BSS)

Solution A

10.0g glucose

0.6g KH_2PO_4

3.58g Na_2HPO_4

made up to 1.0l with distilled water

Solution B

1.86g $CaCl_2.2H_2O$

4.0g KCl

80.0g NaCl

2.0g $MgCl_2.6H_2O$

2.0g $MgSO_4.7H_2O$

made up to 1.0l with distilled water

To use: 100ml solution A
100ml solution B
} 800ml distilled water

(b) Barbital Sodium buffer (BS)

42.5g NaCl

2.9g Barbitone

1.9g Sodium barbitone

2.5g $MgCl_2.6H_2O$

0.75g $CaCl_2.2H_2O$

dissolved in 1.0l of distilled water, diluted 4 times before use

---

(c) Barbitone buffer

Barbitone buffer tablets (Oxoid Ltd.) dissolved in 100ml warm distilled water (composition as in (b)).

(d) Gey's Solution

Solution A

35.0g NaCl
1.85g HCl
1.5g $Na_2HPO_4 \cdot 12H_2O$
0.119g KH_2PO_4
5.0g Glucose
50mg Phenol red
distilled water to 1.0l
Autoclaved

Solution B

0.42g $MgCl_2 \cdot 6H_2O$
0.14g $MgSO_4 \cdot 7H_2O$
0.34g $CaCl_2 \cdot 2H_2O$
distilled water to 1.0l
Autoclaved

Solution C

2.25g $NaHCO_3$
distilled water to 100ml
Autoclaved

To use: 20ml soln. A + 5ml soln. B + 5ml soln. C + 70ml distilled water

---

(e) Glycine-buffered Agar

0.1g Glycine
1.0g NaCl
0.5g Sodium azide
dissolved in 100ml distilled water, adjusted to pH 7.0 - 7.2 with 0.1N NaOH.
Stored at 4°C

(f) Michaelis buffer pH 7.4

4.85g Sodium acetate

7.35g Sodium barbitone

8.5g NaCl

dissolved in 980ml distilled water, plus 20.0ml N HCl

(g) Phosphate buffered saline (PBS)

(1) pH 6.0

0.1M NaH_2PO_4

0.1M Na_2HPO_4

Add 1 part of above to 19 parts 0.15M NaCl

(2) pH 7.1

8.5g NaCl

1.07g Na_2HPO_4

0.39g NaH_2PO_4

dissolved in 1.0l distilled water, raised from pH 7.1 by dropwise addition of N NaOH

(h) Phosphate Saline Glucose (PSG) pH 8.0

0.85%	NaCl	300ml
2.5%	Glucose	400ml
0.2M	NaH_2PO_4	15ml
0.2M	Na_2HPO_4	285ml

(i) Physiological saline

8.5g NaCl
0.42g KCl
0.24g $CaCl_2.2H_2O$
1.0g Glucose

made up to 1.0l in distilled water

or:

8.5g NaCl dissolved in 1.0l distilled water (used for general dilutions).

(j) Tris - HCl buffer

0.1M Tris-aminomethane 250.0ml
0.1N HCl 134.0ml

made up to 1.0l in distilled water plus 58.4g NaCl

APPENDIX 2

Collection of antisera

1. CD-1 Mice infected i.p. with c 100 T. brucei 427 in whole mouse blood.

Days post infection	SERUM SERIES A	B	C
	Parasitaemia per ml		
0(1)	0	0	0
1	0	0	0
2	0	0	0
3	2×10^7	0	1×10^5
4	4×10^7	1×10^3	2×10^6
5	4.6×10^8	1.1×10^8	2.9×10^8

1. sera collected 2 h before infection

2. CD-1 mice infected i.p. with c 400 *T. brucei* S42 in whole mouse blood

Days Post Infection	SERUM SERIES Parasitaemia per ml A'	B'
0	0	0
1	0	-
2	0	0
3	0	-
4	0	0
5	1×10^8	-
6	1×10^8	8×10^7
7	1×10^8	-
8	5×10^7	1.1×10^8
9	1.4×10^8	-
10	5×10^7	3×10^7
11	6×10^7	-
12	7×10^7	1.6×10^8
13	2×10^8	-
14	9×10^7	1×10^8
15	1.1×10^8	-
16	9×10^7	8.4×10^8
17	3.3×10^8	-

3. RABBITS

	RABBIT NUMBER			RABBIT NUMBER	
T. brucei 427	S447	S449	*T. brucei* 427	S531	S532
Inoculum in PSG	4.0×10^8 s/c	6.2×10^8 i/v	Inoculum in PSG	1.04×10^9 s/c	1.04×10^9 s/c
Days Post Infection	Parasitaemia		Days Post Infection	Parasitaemia	
0	0	0	5	0	0
3	0	$6_{(1)}$	9	0	1
5	0	0	12	0	0
10	0	47	16	0	0
12	0	1	19	0	1
17	0	$2 \times 10^{5(2)}$	23	0	0
19	0	0	26	0	0
24	1	1×10^5	30	0	0
26	1	10	33	0	0
31	0	17	37	0	-
33	1	6×10^4	40	0	0
38	3	1×10^4	44	0	0
40	0	0	47	1	0
47	0	2×10^3	50	-	1
DIED DAY:-	54	54	DIED DAY:-	64	50

(1) number of parasites per 30 fields wet film x 480

(2) number of parasites per ml blood

3 - Contd.

	RABBIT NUMBER		RABBIT NUMBER		RABBIT NUMBER
T. brucei 427	S556	*T. brucei* 427	S448	*T. brucei* 427	S569
Inoculum in PSG	3.0×10^8 s/c	Inoculum in PSG	2.5×10^8 i/m	Inoculum in PSG	5.0×10^8 s/c
Days Post Infection	Parasitaemia	Days Post Infection	Parasitaemia	Days Post Infection	Parasitaemia
1	0	0	0	0	0
8	2	5	0	7	1
15	0	7	0	14	0
22	1	12	0	21	0
36	0	14	0	28	0
43	0	19	0	35	5
50	0	21	0	36	11
52	0	26	0	37	2
53	2	28	0	38	0
54	0	33	1	39	3
55	3	35	3	40	35
56	5	42	0	41	1×10^7
57	73	47	0	42	8×10^7
		49	0		
DIED DAY:-	58	Aparasitaemic and asymptomatic 12 weeks post infection		DIED DAY:-	43

3. - contd.

	RABBIT NUMBER
T. brucei 427	S468
Inoculum in whole blood	$3.x10^8$ s/c
Days Post Infection	Parasitaemia
2	2
6	0
9	5
13	0
16	0
20	-
23	7
27	6
30	1
34	0
38	0
41	4
44	0
48	0
51	0
56	3×10^4
60	1×10^3
64	1
66	15
73	1×10^4
78	2×10^5
DIED DAY:-	79

	RABBIT NUMBER		
T. brucei 427	S594	S595	S596
Inoculum in PSG	$8.2x10^8$ s/c	$8.2x10^8$ s/c	$8.2 x10^8$ s/c
Days Post Infection	Parasitaemia		
1	0	0	0
8	0	0	0
9	0	-	-
10	0	-	-
11	3	-	-
12	0	-	-
15	0	0	0
16	-	2	-
17	-	1	-
18	-	14	-
19	-	7	-
22	5	0	0
23	-	-	0
24	-	-	0
25	-	-	0
26	-	-	3
29	1	1	0
36	0	1	0
43	1	1	0
DIED DAY:-	57	58	51

3. contd.

	RABBIT NUMBER
T. brucei etat. 4	S576
Inoculum in whole blood	8.3×10^7 s/c
Days Post Infection	Parasitaemia
1	0
5	0
8	0
12	1
15	0
19	5
22	0
26	0
29	0
33	0
DIED DAY:-	40

APPENDIX 3 Results

Agglutinating Antibody Test:

The tests were carried out according to the method of Cunningham & Vickerman (1962), using serial two-fold dilutions of sera in PBS (pH 7.8). The highest dilution of serum giving any agglutination was taken as the agglutination-titre.

a. T. brucei 427 Infected CD-1 mice.

Antigen source: stabilate suspension of trypanosomes in whole mouse blood at -79°C. Obtained by cardiac puncture of 4 + infected mice, 3 days post infection.

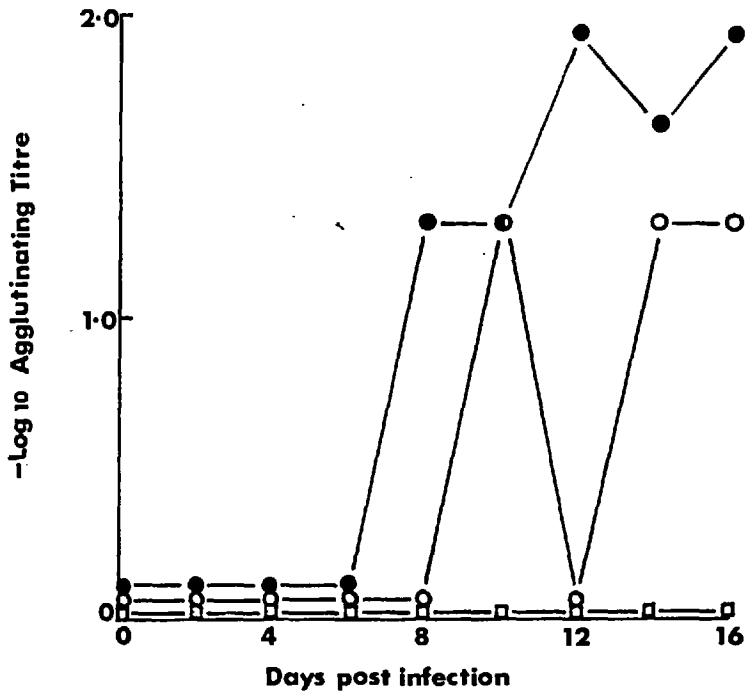
	SERUM SERIES A C
Days Post Infection	Agglutination Titre
0	N/A N/A
1	N/A N/A
2	N/A N/A
3	N/A N/A
4	N/A N/A
5	N/A N/A

N/A: no agglutination

b. T. brucei 427 Mouse hyperimmune 427 sera.

Three separate batches of hyperimmune sera were tested, each representing the pooled sera of six mice.

	1	2	3
TEST	Agglutination Titre		
Hyperimmune 427	1/5,120	1/5,120+	1/5,120+
N.M.S.	N/A	N/A	N/A



(c) Infected CD-1 mice. T. brucei S42.

Results of agglutinating antibody test.

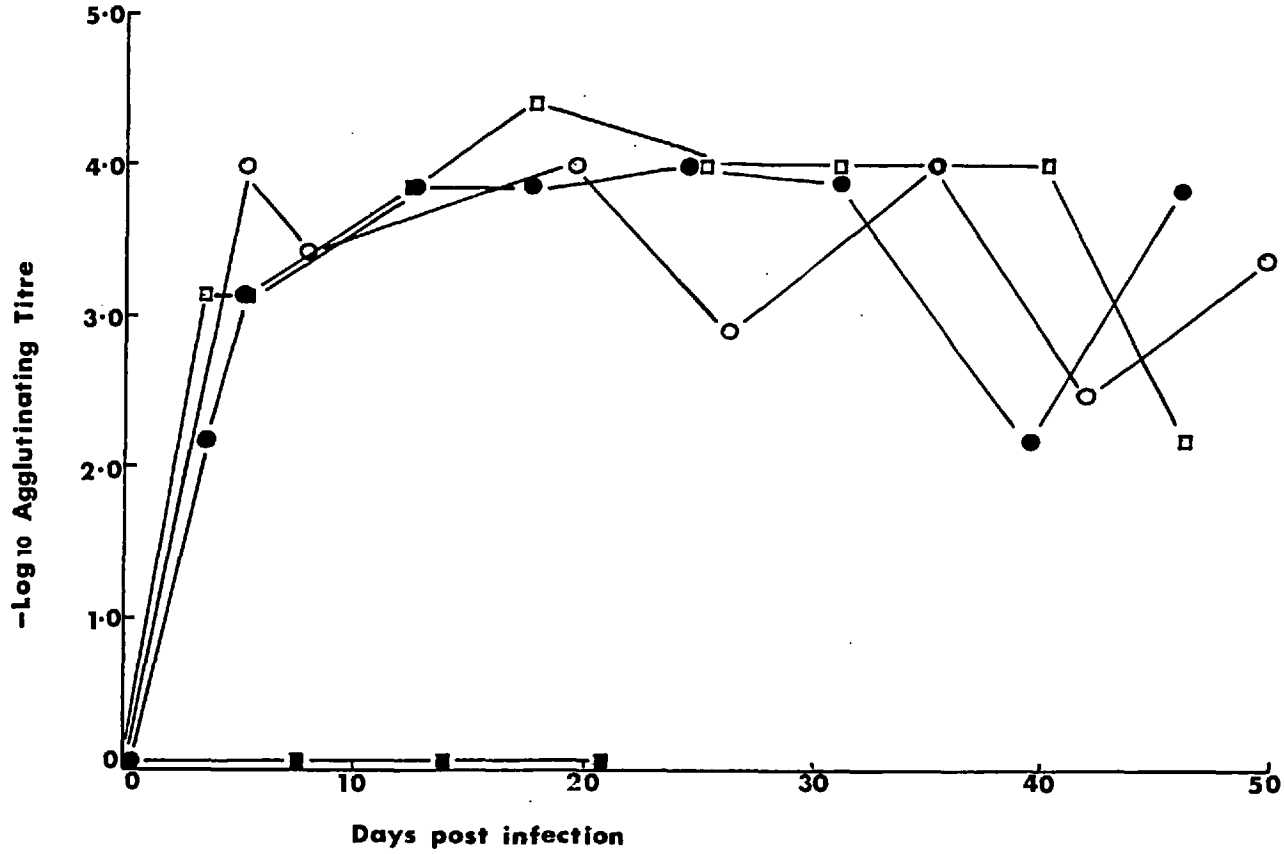
Populations tested against serum series A'

Isolated on days post infection:

●——● day 4

○——○ day 7

□——□ day 18



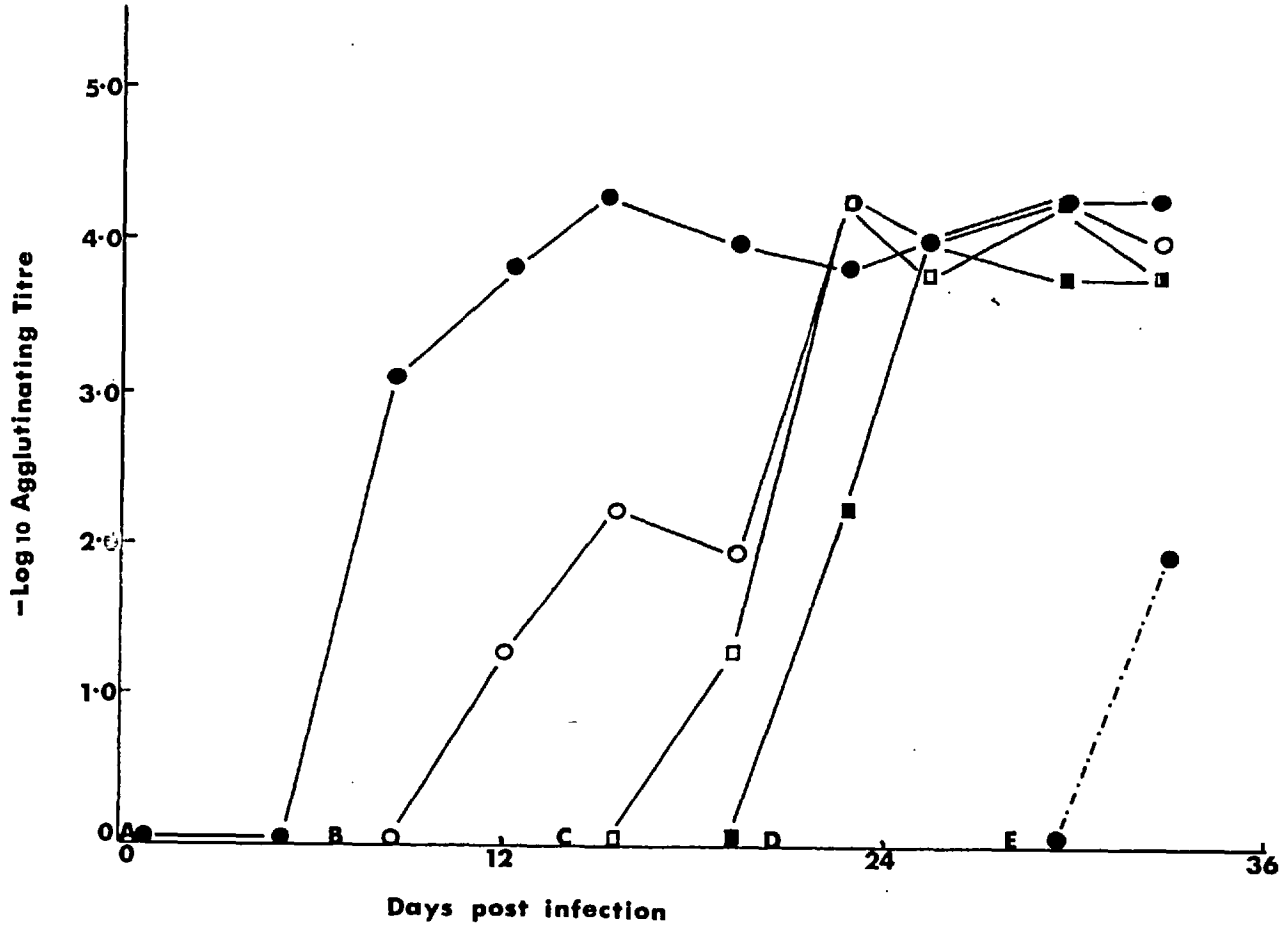
(d) Agglutinating antibody titres. Rabbits infected with T. brucei 427.

●——● Rabbit S447 (s/c)

○——○ Rabbit S448 (i/m)

□——□ Rabbit S449 (i/v)

■——■ Rabbit S507 (uninfected control)



(e) Rabbit S576 infected with T. brucei Etat 4 (s/c)

Results of agglutinating antibody test.

Populations (A - E) isolated at weekly intervals and tested against autologous sera (days 1 - 33 post infection).

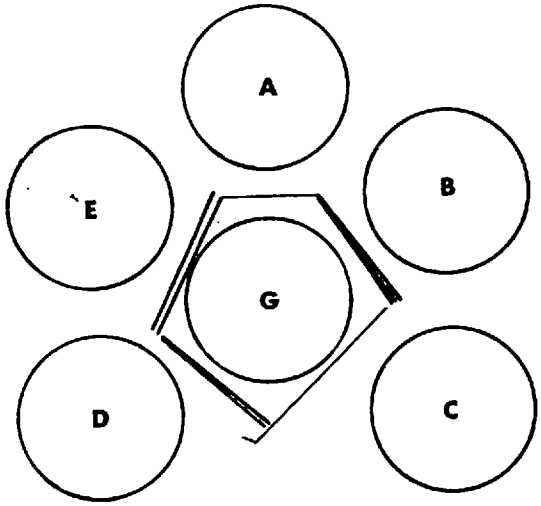
●——● day 0 (basic antigen)

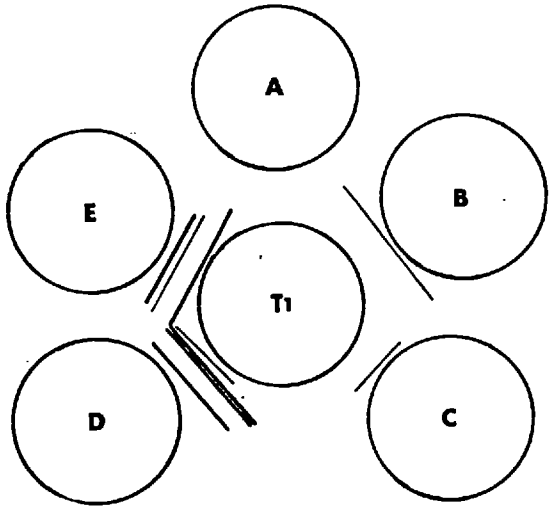
○——○ day 7

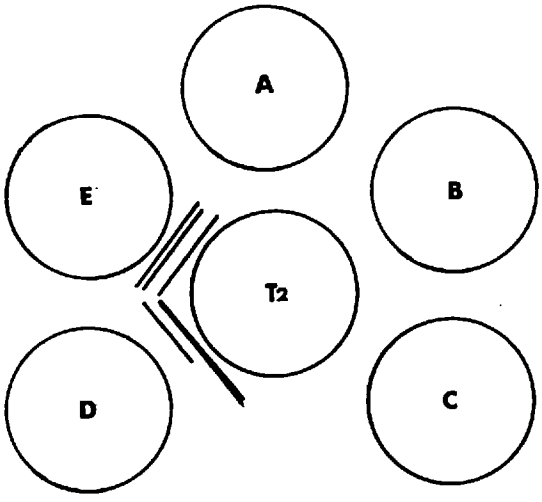
□——□ day 14

■——■ day 21

●-.-.-.-● day 28


A
B
E
G
D
C


A
B
E
T1
D
C


A
B
E
T2
D
C

Ouchterlony plates showing:

a: presence of rabbit γ globulin in normal, immune and hyperimmune rabbit sera.

b: presence of anti-trypanosome antibodies.

A IgG prepared from normal, uninfected rabbit

B IgG prepared from hyperimmune rabbit

C IgM prepared from infected rabbit (S468 - 56 days post infection)

D Whole serum from infected rabbit (S532 - 47 days post infection)

E Whole serum from hyperimmune rabbit

G Goat anti-rabbit γ globulin

T1 T. brucei 427 suspension, disrupted by freeze-thaw and lysed in distilled water

T2 T. brucei 427 suspension, disrupted by freeze-thaw

1. Variant specificity using Homologous Rabbit Sera

RABBIT NUMBER S468

Isolated on: sera from days post infection	DAY 0 Attachment Index[1]	DAY 7 Attachment Index	DAY 14 Attachment Index	DAY 21 Attachment Index	DAY 28 Attachment Index
2	113.0 (21.4)[2]	120.6 (8.5)	138.2 (11.6)	96.2 (14.3)	107.0 (17.2)
9	110.4 (16.9)	91.6 (14.5)	121.8 (8.4)	112.0 (14.0)	105.8 (14.9)
16	273.0 (23.2)	286.8 (9.4)	142.8 (5.4)	234.6 (27.3)	190.6 (13.9)
23	243.0 (13.8)	249.4 (31.7)	486.0 (18.1)	178.8 (13.7)	230.0 (19.5)
34	448.6 (28.9)	459.0 (9.6)	508.6 (10.7)	445.2 (22.4)	413.6 (10.5)
41	397.8 (22.2)	383.2 (25.0)	537.6 (20.4)	487.4 (24.7)	494.0 (14.3)
51	493.6 (15.0)	387.6 (18.4)	433.8 (10.4)	412.2 (18.7)	319.6 (13.8)
60	494.4 (18.2)	419.6 (14.0)	451.0 (7.6)	419.4 (32.5)	314.6 (17.9)
73	464.8 (32.5)	417.0 (13.7)	400.0 (17.8)	463.8 (34.5)	490.0 (23.2)

1. mean of 5 replicates
2. standard deviation in parentheses

RABBIT NUMBER S447

Isolated on: sera from days post infection	DAY 0 Attachment Index	DAY 7 Attachment Index	DAY 14 Attachment Index	DAY 21 Attachment Index	DAY 28 Attachment Index
0	44.2 (6.0)	114.0 (8.9)	180.0 (10.2)	103.2 (20.2)	91.6 (15.0)
3	295.2 (20.1)	87.6 (9.8)	147.8 (14.7)	163.6 (22.5)	115.8 (22.6)
5	444.8 (31.3)	156.4 (16.0)	104.8 (15.2)	164.4 (13.9)	128.4 (10.0)
12	536.8 (35.3)	101.0 (7.7)	162.6 (6.9)	242.0 (29.4)	186.4 (29.5)
17	529.2 (8.8)	438.4 (20.9)	247.8 (7.2)	280.2 (20.2)	265.0 (17.2)
24	531.2 (9.7)	400.2 (21.4)	372.0 (14.1)	373.0 (36.8)	267.8 (21.6)
31	514.0 (11.1)	447.0 (24.8)	421.2 (12.0)	431.2 (37.9)	491.0 (48.8)
33	546.6 (10.3)	440.8 (18.6)	397.4 (12.9)	520.6 (11.5)	440.6 (37.7)
38	517.6 (5.4)	375.8 (20.9)	392.2 (8.3)	503.4 (21.1)	449.0 (23.9)

2. Variant Specificity using Homologous and Autologous Rabbit Sera

RABBIT NUMBER S531

Isolated on: sera from days post infection	DAY 0 Attachment Index	DAY 7 Attachment Index	DAY 14 Attachment Index	DAY 21 Attachment Index	DAY 28 Attachment Index	DAY 35 Attachment Index	DAY 42 Attachment Index	DAY 49 Attachment Index
0	53.2(30.1)	57.0(29.4)	49.2(16.4)	54.2(27.8)	57.2(20.3)	49.4(26.9)	48.4(24.2)	67.4(20.5)
5	119.6(38.5)	203.2(45.5)	53.0(14.6)	88.8(13.8)	55.4(10.4)	55.8(21.3)	77.8(12.6)	73.8(24.0)
12	344.0(59.4)	218.2(40.3)	98.6(15.3)	129.2(38.5)	128.4(31.2)	131.6(39.7)	172.0(67.7)	159.0(28.2)
19	402.6(53.7)	456.6(52.4)	375.6(33.2)	172.0(24.0)	148.0(41.6)	240.6(20.2)	186.6(14.0)	229.8(38.7)
26	463.6(51.6)	462.4(58.1)	487.8(67.3)	392.0(47.9)	432.0(23.7)	395.2(56.2)	242.6(21.9)	388.2(30.5)
33	479 6(32.3)	460.6(33.6)	454.6(51.8)	408.0(61.0)	467.2(35.7)	443.2(41.6)	375.6(33.5)	459.8(51.3)
37	468.2(43.1)	446.8(49.0)	364.8(23.9)	429.6(52.2)	296.2(37.7)	335.2(28.4)	439.2(51.4)	455.4(75.3)
40	521.0(70.8)	465.8(45.2)	466.0(35.1)	344.8(30.4)	378.4(56.3)	411.2(36.4)	446.6(26.5)	445.6(36.7)
47	468.4(51.7)	537.2(56.1)	462.2(31.4)	415.2(64.1)	431.0(43.6)	454.8(50.4)	459.8(25.6)	500.2(36.3)

RABBIT NUMBER S532

Isolated on: sera from days post infection	DAY 0 Attachment Index	DAY 7 Attachment Index	DAY 14 Attachment Index	DAY 21 Attachment Index	DAY 28 Attachment Index	DAY 35 Attachment Index	DAY 42 Attachment Index	DAY 49 Attachment Index
5	81.0(30.6)	78.4(16.2)	62.0(16.0)	53.4(26.2)	77.0(27.1)	56.8(16.6)	65.2(18.9)	51.2(32.7)
12	202.4(46.1)	156.6(30.8)	238.2(24.5)	256.4(20.5)	200.8(41.9)	250.2(36.3)	265.8(21.1)	283.6(35.3)
19	356.8(29.2)	415.2(51.8)	318.6(40.7)	444.2(34.0)	261.4(40.4)	327.8(14.3)	264.2(33.7)	394.0(52.5)
26	383.2(41.7)	471.8(29.2)	217.2(15.1)	347.4(57.7)	348.0(31.2)	236.4(16.3)	446.4(65.7)	434.4(40.8)
33	455.6(30.4)	462.8(29.0)	478.6(52.8)	316.4(48.0)	439.0(30.2)	411.4(56.7)	433.0(28.6)	475.2(23.7)
40	461.0(33.7)	492.2(22.1)	405.2(69.8)	363.2(33.8)	421.0(37.8)	423.4(31.4)	319.6(51.6)	487.8(61.4)
47	498.2(38.5)	493.4(46.3)	475.6(53.4)	439.0(35.2)	469.0(23.8)	372.4(32.8)	415.6(33.6)	367.6(25.9)

3. Variant Specificity - using Autologous Rabbit Sera

RABBIT NUMBER S576(2)

Isolated on: sera from days post infection	DAY 0 Attachment Index(1)	DAY 7 Attachment Index	DAY 14 Attachment Index	DAY 21 Attachment Index	DAY 28 Attachment Index
1	85.0(17.0)	131.0(93.3)	66.0(43.8)	109.0(87.7)	57.5(41.7)
5	108.0(36.8)	210.5(95.4)	207.0(90.5)	161.0(25.4)	80.0(58.0)
8	235.5(54.4)	157.5(51.6)	144.0(77.8)	234.5(51.6)	367.0(76.4)
12	140.5 (2.1)	402.5 (6.4)	354.5(81.3)	402.5 (6.4)	386.5(62.9)
15	501.0(39.6)	493.5(115.2)	266.5(177.5)	437.0(22.6)	313.5(64.3)
19	479.0(67.9)	474.5(61.5)	352.0(91.9)	492.5(34.6)	502.5 (6.4)
22	338.5(78.5)	543.0(43.8)	403.5(129.4)	366.5(34.6)	521.0(10.0)
26	491.5(55.9)	414.5(98.3)	554.0(66.5)	591.5 (0.7)	407.0(121.6)
29	415.0(96.2)	411.5(21.9)	495.0(90.5)	400.5(10.6)	468.5(14.8)
33	448.5(128.0)	501.0(21.4)	467.5 (7.8)	426.5(119.5)	470.5(75.7)

1. mean of 2 replicates

2. Infected with cloned strain T. brucei Etat 4

CHEMOTAXIS

Results of Individual Experiments

(1)

Compartment A	Compartment B	C.I.[1]
Peritoneal Cells	Gey's Solution[3]	0
Peritoneal Cells	T.C.M.	3.6
Peritoneal Cells	Stored casein in Gey's	27.0
Peritoneal Cells	Casein in Gey's[2]	100

1. per 5 fields counted (x 480)
2. 5 mg/ml
3. without penicillin or streptomycin

(2)

Compartment A	Compartment B	C.I.[1]
Peritoneal Cells	Gey's Solution	0
Peritoneal Cells	Casein in Gey's	100
Peritoneal Cells	Histamine in Gey's[2]	9.4
Peritoneal Cells	A.T.P. in Gey's[3]	14.7
Peritoneal Cells + A.T.P.[3]	A.T.P. in Gey's	1.1

1. per 10 field counted (x 480)
2. 0.2 mg/ml
3. 0.33 mg/ml

(3)

Compartment A	Compartment B	C.I.[1]
Peritoneal Cells	Gey's Solution	0
Peritoneal Cells	Casein in Gey's	100
Peritoneal Cells	N.M.P.[2]	16.4
Peritoneal Cells	Trypsin[3] + N.M.P.	143.8
Peritoneal Cells	5-HT[3] + N.M.P.	12.3

1. per 10 fields counted (x 480)
2. 10% normal mouse plasma in Gey's
3. 0.2% w/v in Gey's + 10% N.M.P.

(4)

Compartment A	Compartment B	C.I.[1]
Peritoneal Cells	Gey's Solution	0
Peritoneal Cells	Casein in Gey's	100
Peritoneal Cells	N.M.S.[2]	34.9
Peritoneal Cells	Inactivated N.M.S.[3]	- 4.4
Peritoneal Cells	Inac. N.M.S. + N.G.P.S.	-15.5[5]
Peritoneal Cells	N.G.P.S.[4]	1.1

1. per 10 fields counted (x 480)
2. 10% normal mouse serum in Gey's
3. heated to 56°C for 30 min
4. 10% normal guinea pig serum in Gey's
5. very few cells 30 per field on Compt. A side of membrane

(5)

Compartment A	Compartment B	C.I.[1]
Peritoneal Cells	Gey's Solution	0
Peritoneal Cells	Casein in Gey's	100
Peritoneal Cells	N.M.S.	16.0
Peritoneal Cells	N.M.S. + casein	125.3
Peritoneal Cells	Inac. N.M.S. + casein	8.7
Peritoneal Cells	N.G.P.S. + casein	132.4

1. per 10 fields counted (x 480)

(6)

Compartment A	Compartment B	C.I.[1]
Peritoneal Cells	Gey's Solution	0
Peritoneal Cells	Casein in Gey's	100
Peritoneal Cells	Excess casein in Gey's	42.6
Peritoneal Cells	Inac. Calf Serum	20.49
Peritoneal Cells + Gey's	Gey's Solution	24.59
Peritoneal Cells + Casein	Casein in Gey's	9.83

1. per 5 fields counted

(7)

Compartment A	Compartment B	C.I.[1]
Peritoneal Cells	Gey's Solution	0
Peritoneal Cells	Casein in Gey's	100
Peritoneal Cells	Histamine[2] in Gey's	5.99
Peritoneal Cells	A.T.P.[2] in Gey's	35.27
Peritoneal Cells	N.M.S.	10.62
Peritoneal Cells	N.M.S. + casein	54.45

(1) per 10 fields counted (x480)
(2) 0.42 mg/ml

(8)

Compartment A	Compartment B	C.I.[1]
Peritoneal Cells	Gey's Solution	0
Peritoneal Cells	Casein in Gey's	100
Peritoneal Cells	Autolysed muscle	2.4
Peritoneal Cells	Autolysed liver	6.5
Peritoneal Cells	Trypsin[2] + muscle	7.5
Peritoneal Cells	Trypsin[2] + liver	-13.1
Peritoneal Cells	N.R.S.[3]	9.6
Peritoneal Cells	Inactivated N.R.S.[3]	-4.9

(1) per 10 fields counted (x480)
(2) 0.2% w/v in TCM at 37°C for 24 h
(3) normal rabbit serum

(9)

Compartment A	Compartment B	C.I.[1]
Peritoneal Cells	Gey's Solution	0
Peritoneal Cells	Casein in Gey's	100
Peritoneal Cells	N.M.P.	-2.6
Peritoneal Cells	T. brucei supernatant + N.M.P.	28.9
Peritoneal Cells	T. brucei supernatant + hyp. 427	17.3
Peritoneal Cells	T. brucei sediment + N.M.P.	-5.8
Peritoneal Cells	T. brucei sediment + hyp. 427	-3,3
Peritoneal Cells	T. brucei supernatant	5.6
Peritoneal Cells + supernatant	T. brucei supernatant	-5.8
Peritoneal Cells	Disrupted trypanosomes + N.M.P.	-3.3

(1) per 10 fields counted (x480)

Compartment A	Compartment B	C.I.[1]
Peritoneal Cells	Gey's Solution	0
Peritoneal Cells	Casein in Gey's	100
Peritoneal Cells	N.M.P.	16.4
Peritoneal Cells	Live T. brucei + N.M.P.	29.6
Peritoneal Cells	Live T. brucei + inac. N.M.P.	23.0
Peritoneal Cells	Live T. brucei	1.1
Peritoneal Cells	Live T. brucei + N.G.P.S.	13.4
Peritoneal Cells	Live T. brucei + inac. N.G.P.S.	29.2
Peritoneal Cells	Live T. brucei + hyp. 427	4.1
Peritoneal Cells	Live T. brucei + inac. hyp.	4.3
Peritoneal Cells	Supernatant + hyp. 427	64.6
Peritoneal Cells	Supernatant + inac. hyp. 427	57.2
Peritoneal Cells	Heat killed + N.M.P.	34.1
Peritoneal Cells	Heat killed + hyp. 427	66.2
Peritoneal Cells	Hyperimmune 427	7.7
Peritoneal Cells	Inac. hyp. 427	4.5

(1) per 10 fields counted (x480)

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