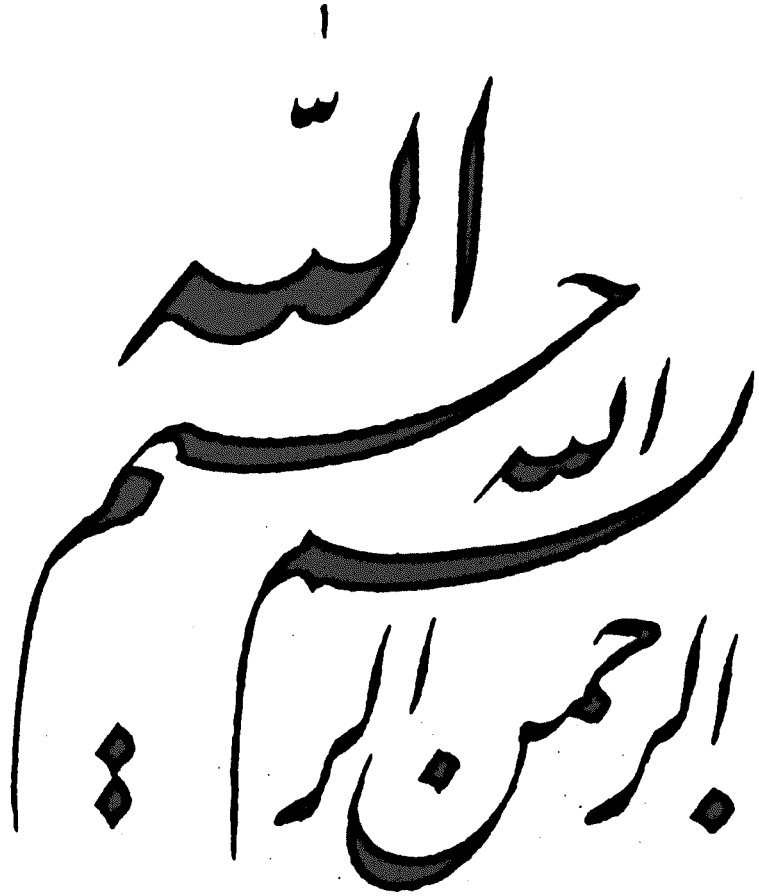

In the Name of God

the Merciful, the Compassionate

BIOCHEMICAL AND IMMUNOLOGICAL CHARACTERIZATION OF THE LIPOGENESIS-INDUCING MALARIAL TOXIN

THESIS SUBMITTED IN ACCORDANCE WITH THE REQUIREMENTS OF THE UNIVERSITY OF LIVERPOOL FOR THE DEGREE OF DOCTOR IN PHILOSOPHY

BY

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ABSTRACT

Biochemical and immunological characterization of the lipogenesis-inducing malarial toxin. **Sedighed Zakeri**

Hypoglycaemia is an important manifestation of Falciparum malaria, particularly in children and pregnant women, but its pathophysiology remains unclear. Previous studies have shown that toxic malaria antigens (TMA) released by *Plasmodium yoelii* induce hypoglycaemia in mice and act synergistically with insulin in stimulating lipogenesis in rat adipocytes *in vitro*. It is therefore of some importance

a) to identify whether these phenomena are also a feature of human malaria parasite, *Plasmodium falciparum*,
b) to understand the mode of action and
c) to characterize the active components.

These aims were designed to increase our knowledge and understanding of the complications of malaria, particularly hypoglycaemia and for the development of anti-disease vaccines and therapy.

Rat adipocytes were used to measure the incorporation of ^{14}C- labelled glucose into lipids following incubation with insulin, TMA, or a combination of both. This assay was used during the thesis to evaluate the "toxicity" of molecules. We confirmed that *P. falciparum* TMA (which are released into the culture supernatant when schizonts rupture) act in synergy with insulin to increase lipogenesis by more than 250% ($p < 0.001$). Control preparations prepared from non-parasitized cells grown under similar conditions, had no effect ($p < 0.001$).

A novel finding was that the short term exposure (less than 4 h) of adipose cells to TMA did not stimulate the lipogenesis, while 10 nM insulin increased stimulation of lipogenesis 3- to-6 fold after a 60 min exposure. In contrast, long term exposure of adipose cells to TMA caused a marked stimulation of lipogenesis. A maximal increase was observed after 12-24 h of exposure, at a level comparable to that observed with insulin. The difference in time-course suggested different mechanisms are involved by TMA and insulin. However, the blockage of lipogenesis by cytochalasin B (a strong inhibitor of glucose transporter) indicated that both insulin and TMA acted on the glucose transport system.

The presence of TMA in the serum of squirrel monkeys infected with different species of malaria parasites (*P. vivax, P. falciparum* and *P. brasillianum*) and different strains of *P. falciparum* confirmed that such molecules may have role to play *in vivo*.

Monoclonal antibodies (McAbs) were produced against TMA of *P. falciparum* IRBC which blocked the lipogenesis-inducing activity of the TMA in the rat adipocytes. We also found antibodies against the lipogenesis-inducing molecule(s) in a malaria endemic community. This finding is important because it opens up the possibility of looking at anti-toxic immunity in malaria patients, if a more quantitative assay can be developed.

Partial characterization of TMA showed that TMA is unaffected by protease digestion, but that its functional effects are abolished by lipase treatment. The Folch extraction (chloroform/methanol 2:1 v/v) showed that the biological activity is found in the polar lipids fraction of solvent extraction of malaria culture supernatants. A variety of techniques were used for more detailed characterization of the TMA. All the different fractions collected from either HPLC or silica gel column chromatography, showed lipogenesis-inducing activity in the rat adipocyte assay. The results suggest that the active principle could reside in a common structure such as a fatty acid moiety which is present in all the lipid fractions. Such a molecule(s) may perhaps be responsible for TMA activity after its release from the parent lipids. Enzymatic treatment of the lipid fraction which co-migrated with PC standard by phospholipase C (PLC) showed that the activity of the fraction was associated with to the 1,2-diacylglycerol (1,2-DAG) moieties released from the polar lipid. When this exogenous 1,2-DAG was added to the adipocytes cultures (short and long term cultures) it induced stimulation of lipogenesis in rat adipocytes. The activity of the 1,2-DAG sample was inhibited by cytochalasin B which revealed that the action was on the glucose transport system was similar to that obtained with boiled supernatant of *P. falciparum* culture. No lipogenesis activity was obtained from polar lipids and more specifically the 1,2-DAG isolated from NRBC.

Gas chromatography (GC), Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) techniques were applied to identify the structures of fatty acids and 1,2-DAG species of *P. falciparum* IRBC and NRBC, respectively. GC results showed that the compositions of the fatty acid mixtures derived from IRBC and NRBC samples were the same although it is possible that some minor fatty acid components which were present at the limits of detection could have varied between the two samples. Likewise GC-MS and LC-MS showed the apparent similarity in the compositions in the major 1,2-DAG species recovered from NRBC and IRBC. Further work need to elucidate the structure of the 1,2-DAG from *P. falciparum* IRBC which showed high activity in the rat adipocytes.

Regarding to recent reports on contamination of *P. falciparum* culture with mycoplasma, we re-examined all our results and we confirmed that TMA inducing-lipogenesis activity was specifically obtained from *P. falciparum* IRBC, but not from mycoplasma contamination.

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and

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To Azadeh and Mohammad-Reza for making me laugh during this time

LIST OF ABBREVIATIONS

BSA	bovine serum albumin
bp	base pairs
1,2-DAG	1,2-diacylglycerol
°C	degrees centigrade
DAG	diacylglycerol
dH_2O	distilled water
ddH_2O	double distilled water
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphate
CPM	counts per minutes (for radioactive isotopes)
EDTA	ethylenediamine tetra-acetic-acid
ELISA	enzyme linked immunosorbent assay
FAME	fatty acid methyl ester
FCS	foetal calf serum
g+g	L-glutamin+gentamycin
GC-MS	gass chromatography-mass spectrometry
HAT	hypoxanthine-aminopterin-thymidine (medium)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HPLC	high performance liquid chromatography
HT	hypoxanthine-thymidine (medium)
IFAT	indirect fluorescent antibody test
Ig	immunoglobulin
IFN-γ	interferon gamma
IRBC	infected red blood cell
I.V	intravenous
KRB	krebs-ringer bicarbonate buffer
LC-MS	liquid chromatography
LPS	lipopolysaccharide
MRA	mycoplasma removal agent
M	molar
McAb	monoclonal antibody
mg	milligram
ml	millilitre
NCTC	NCTC-135 basal medium
OD	optical density
OPI	oxaloacetate-pyruvate-insulin
PA	phosphatidic acid
PBS	physiological buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PCV	packed cell volume
PE	phosphatidylethanolamine
PEG	polyethylene glycol
PG	phosphatidylglycerol

Pg	picogram
PI	phosphatidylinositol
PS	phosphatidylserine
S.C	subcutaneous
SEM	standard error of mean
RBC	red blood cell
TNF	tumour necrosis factor
TAG	triacylglycerol
v/v	volume per volume
w/v	weight per volume
U.V	ultraviolet
μg	microgram
μl	microlitre

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Malaria as an important Tropical disease

1.1.1 Historical aspect

Malaria, one of the earliest recorded diseases having been chronicled in the ancient medicinal literature of India, Egypt and China, still poses one of the most serious health concerns in the world today. The disease remains one of the leading causes of morbidity and mortality in the tropical and sub-tropical areas, and there are an estimated 300-500 million cases of malaria each year, resulting in over 1 million deaths, mainly of children under five years old in Africa (WHO, 1997). Malaria has been estimated to represent 2.3% of the overall global disease burden and 9% in Africa, ranking third among major infectious disease threats, after pneumococcal acute respiratory infections (3.5%) and tuberculosis (2.8%). The disease is often linked to the movement of refugees or populations seeking work and to environmental change, including forestry, mining and water development projects (WHO, 1996a). The geographical distribution of malaria is shown in Fig. 1.1 (WHO, 1996b).

In 1717, Lancisi noted the presence of some black pigment in human spleens and brains, but did not associate these findings with malaria (Wernsdorfer & McGregor, 1988). On November 1880, a young French army physician named

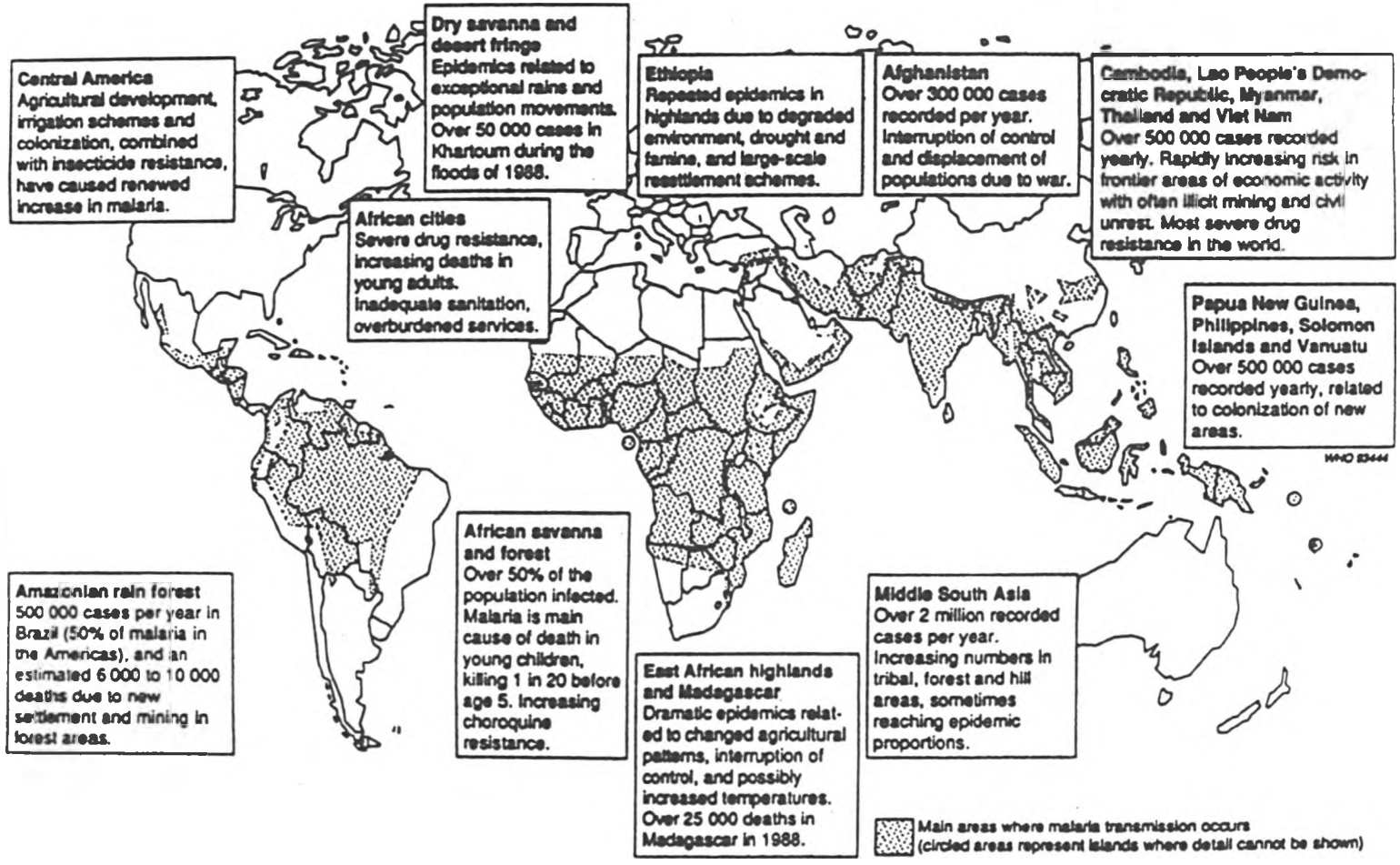


Fig. 1.1 The geographical distribution of malaria. (WHO/TDR/1996).

Alphonse Laveran first noticed pigmented bodies, during examination a drop of blood from a young soldier under the microscope, who had been admitted for severe intermitted fever (Wernsdorfer & McGregor, 1988).

However, the route of natural transmission of malaria remained a mystery. The elucidation of the mystery of the life-cycle of the parasite in an Anopheles mosquito and its transmission by bite did not come until the 20th of August 1897 and was due to a British surgeon Ronald Ross who had identified the *Anopheles* mosquito as the malaria vector (Hagan & Chauhan, 1997).

The tissue stages of primate malaria parasites remained obscure until Garnham discovered the exoerythrocytic stages of *Hepatocystis kochi* developing in the liver parenchyma cells of infected monkeys. The investigations were quickly followed by the identification of the full life cycle of *P. cynomolgi*, *P. vivax* and *P. falciparum* in monkey and man (Shortt & Garnham, 1948; Garnham 1966).

So, today it is accepted that the disease of malaria is a result of infection with a protozoa of the genus *Plasmodium*, which is inoculated into the blood of the host by the bite of an infected blood feeding female anopheline mosquito.

1.2 The malaria parasite and its life cycle

Malaria is a parasitic disease caused by protozoa of the genus *Plasmodium* that parasitize reptiles, birds, or mammals. *Plasmodium* is the only Genus in the

Plasmodiidae Family of the Sub-Order *Haemosporina* which belong to the Phylum *Apicomplexa* (Hommel & Gilles, 1998).

Four *Plasmodium* species are recognized to be specific of humans: *P. malariae, P. ovale, P. falciparum* and *P. vivax*. Under natural conditions, these four species need to infect human hosts to complete their life cycle. A characteristic of all *Plasmodium* species is *Anopheles* spp. as arthropod host and man as the only natural vertebrate host, with the exception of *P. malariae* where higher non-human primates may be involved occasionally (Wernsdorfer & McGregor, 1988). The real drama and tragedy of the disease is caused by the combination of the *Plasmodium falciparum* transmitted by the extremely persistent and efficient *Anopheles gambiae* complex of mosquito vectors, which is responsible for the deaths of some one million children in Africa each year (WHO, 1995)

1.2.1 The liver stage

The malaria parasite is an intracellular protozoan which has been characterised as having both sexual and asexual stages in its life cycle (Garnham, 1966). Although the life cycles of the four species of human malaria differ slightly, they all tend to follow a similar cycle to the one described below (Fig. 1.2).

An infected female mosquito will inoculate the host with sporozoites, while taking its blood meal, which are the infective stage of the parasite. The sporozoites travel quickly from the site of the bite to the liver via the bloodstream, less than 60

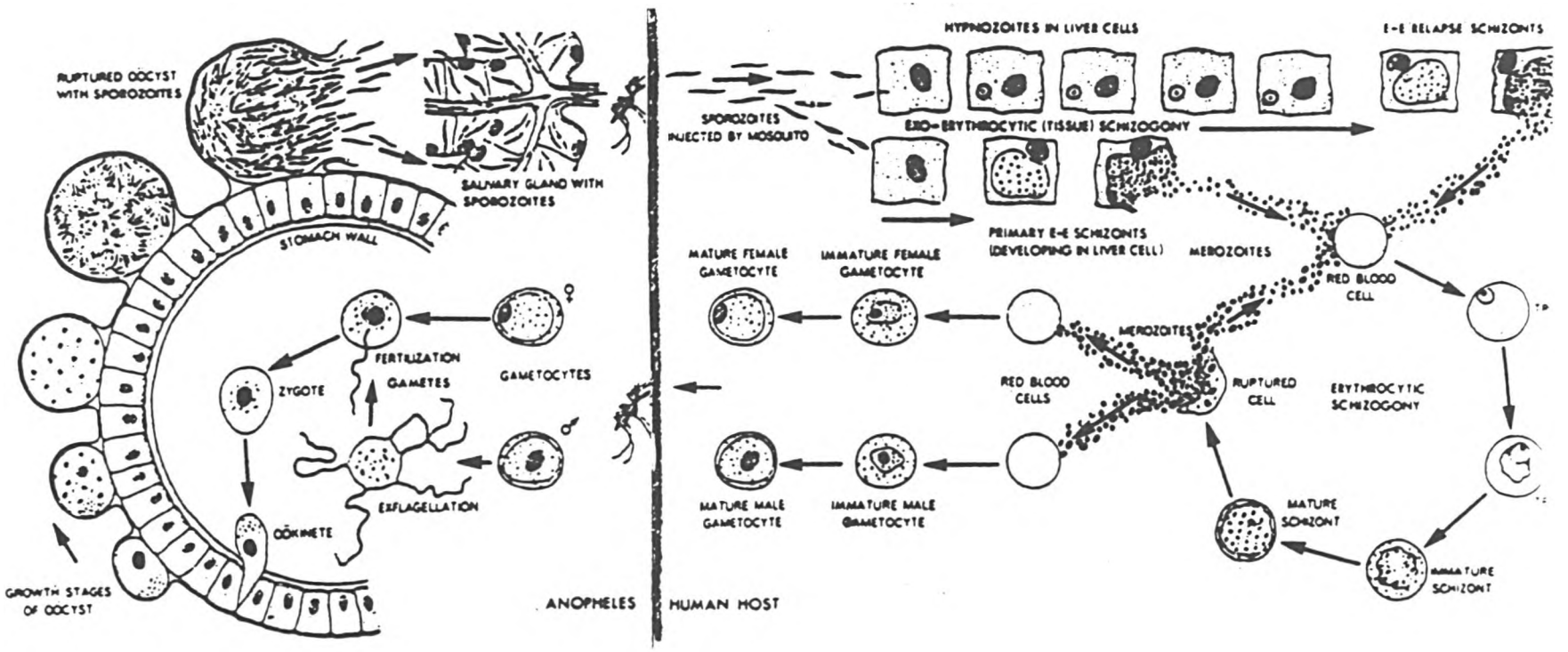


Fig. 1.2 The life cycle of the malaria parasite. (after Gilles & Warrell, 1993).

minutes in *P. vivax* or *P. falciparum* infections. The sporozoite is specifically designed to recognize and invade hepatocytes but it may, in order to reach the hepatocyte, need to cross the Kupffer cells. The rate at which sporozoites are capable of finding a host cell is both parasite-and host-species dependent; for example, *P. vivax* and *P. falciparum* are regarded as highly infective and can induce an infection with 10 sporozoites or less.

Exoerythrocytic (EE) schizogony is characteristic of each species with a minimum maturation time of 5.5 days in *P. falciparum* and 15 days in *P. malariae*, but at the end of the process, a large number of merozoites are produced (about 10,000 in *P. vivax* and 30,000 in *P. falciparum*). No pigment is present in this stage, nor in the sporozoite or microgamete stage. The schizont then ruptures and the merozoites escape into the sinusoids of the organ and invade erythrocytes. Merozoites have a very short extracellular viability (e.g. probably less than 30 minutes) and can only invade erythrocytes.

1.2.2 Erythrocytic stage

The cycle in the blood is initiated by the discharge of merozoites from the exoerythrocytic (EE) schizonts in the liver into the circulation. The minimum time from infection by mosquito bite until the first appearance of merozoites in erythrocytes is termed the "prepatent period". The period is constant and characteristic for each species of parasite. The prepatent period lasts for 5.5 days in *P. falciparum*, 9 days in *P. ovale*, 15 days in *P. malariae*, and 8 days in *P. vivax*

infections. The "incubation period" is different in that signs and symptoms only arise when the parasitaemia reaches a sufficient density and is usually at least two days longer than the "prepatent period". Immunity starts to develop probably when parasites first appear in the blood, and certainly when the products of the first cycle of schizogony are thrown into the blood stream.

After invasion of the erythrocyte, the parasite loses its specific invasion organelles and differentiates into a round "ring form" located within a parasitophorous vacuole in the red cell cytoplasm. Growth follows and the cytoplasm exhibits varying degrees of amoeboidicity according to the species of *P. falciparum*. After about 12-24 hours, movement slows down, the vacuole appears and grains of pigment (haematin) appear in the cytoplasm. The pigment is the residue of the digestion of the haemoglobin of the red blood cell.

Nuclear division continues until the parasite reaches maturity and schizogony occurs with the formation of a specific number of merozoites. The process of schizogony in the blood occurs at regular tertian (every 48 hours) (Fig. 1.3) or quartan (every 72 hours) intervals; the merozoites escape into the plasma and enter new erythrocytes. The rupture of the schizonts is accompanied by the typical malaria paroxysm.

1.2.3 Gametocytes and sporogony in the mosquito

Some of the merozoites will, after invading an erythrocyte, develop into sexual

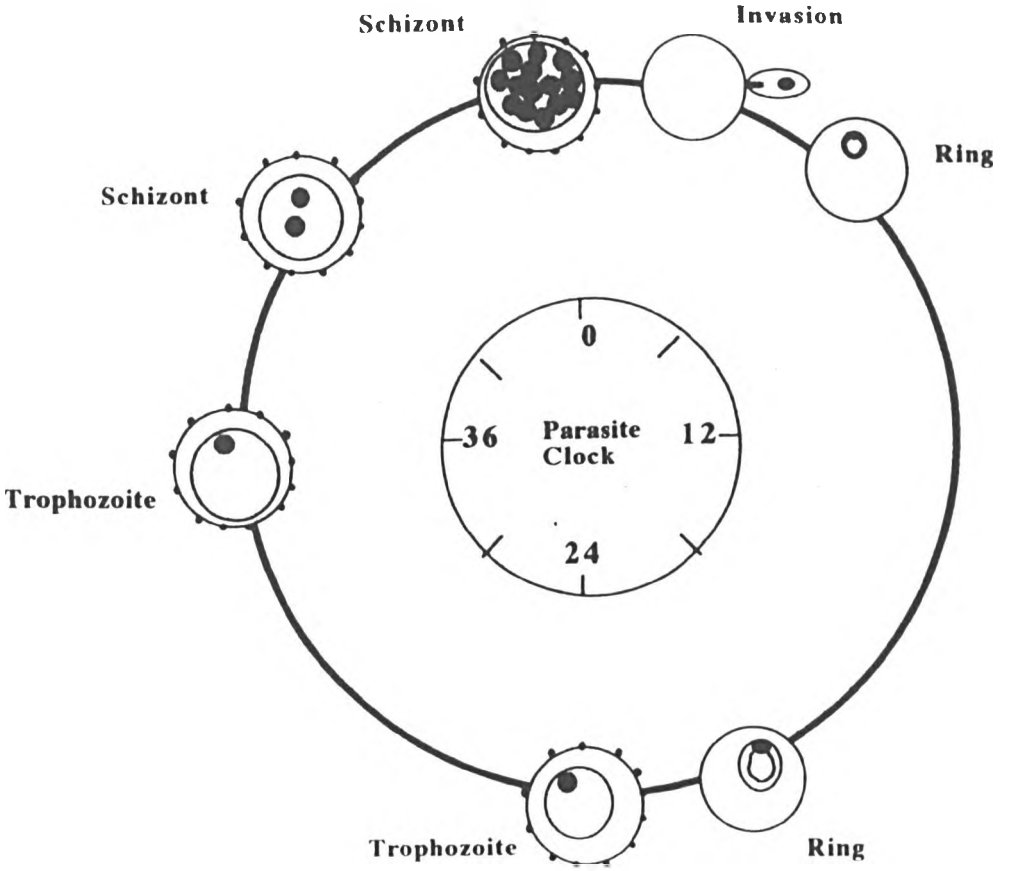


Fig. 1.3 Timing of the intra-erythrocytic cycle of *Plasmodium falciparum*. (after Hommel & Gilles, 1998).

forms-the macrogametocytes (female) and microgametocytes (male). The precise nature of the stimulus which leads to gametocytogenesis remains unknown. The work of Hawking and co-workers showed that in certain species of *Plasmodium* (*P. knowlesi*), the life of the gametocyte was confined to a single night, in other words, this stage was only infectious to mosquitos during the hours of darkness (Hawking *et al.* 1968). However, the " Hawking phenomenon" is limited to parasitaemia of a synchronous nature; if, as is usually the case in human malaria, no strict synchronicity exists, then viable gametocytes may present in the blood at any hour of the day or night. The duration of gametocytogenesis is variable from one species to another, requiring generally between one and two times longer than the duration of the asexual erythrocytic development (e.g 3-4 days in *P. vivax*); the notable exception to this generalization is *P. falciparum*, where gametocytes require 8-10 days to reach maturity.

The gametocytes are taken up by the suitable mosquito vector when it blood feeds on a malaria-infected host. The blood is taken into the midgut of the insect, where the macrogametocyte rapidly escapes from the erythrocyte to form a macrogamete. Microgametogenesis proceeds after about ten minutes, the nucleus divides three times and into eight portions, each of eight nuclei formed combines with cytoplasm to form eight thread-like microgametes ("exflagellation"). Microgametes are very motile, highly differentiated stages, which move actively towards the macrogametes. The invasion of the macrogamete by the microgamete starts the process of fertilization and sexual stage of the parasite's life cycle (Fig. 1.2). The two nuclei fuse and the zygote is formed. Within 18 hours or less of the mosquito taking

the blood meal, the zygote has elongated into the slowly motile ookinete about 20 μm in length and this traverses to reach the single cell layer of the midgut epithelium and finally near to the external surface, it rounds up into the oocyst, 24-72 hours after the blood meal has been taken.

Depending on environmental temperature, the oocyst grows to reach a diameter of 40 to 60 μm between 4 and 21 days from the time of the infective bite. Each infected mosquito may carry a few hundred oocysts, but normally the number of oocysts per mosquito is much smaller.

At the end of the maturation period, sporozoites are formed, the number of which varies from species to species (e.g. approximately 10-20,000 in *P. falciparum*). They are about 10-15 μm long, curved with narrowed ends and are highly motile. The immature sporozoites, which emerge through the oocyst wall into the haemolymph. To complete their maturation, sporozoites must migrate to the salivary glands. Their infectivity to man increases from a low degree in the mature oocyst, until they become highly infective after about a day's residence in the glands; the infectivity then declines with age. When the mosquito bites, the sporozoites pass with the saliva into the skin of the vertebrate host.

So, the completion of the sporogony takes between 7 days to 8 weeks, depending on the parasite species, the nature of the vector and, more importantly, the environmental conditions (particularly temperature and relative humidity).

1.2.4 Relapse and recrudescence phenomenon

One notable difference in the life cycles of the four species of human malaria is that a proportion of the sporozoites produced by the tertian malarias, namely *P. vivax* and *P. ovale*, which invade the liver cells, are able to transform into dormant stages called hypnozoites (Fig. 1.2). These parasites produce a relapse of the disease, months to years after the initial infection. However, the mechanism behind development of these parasites, or the subsequent initiation of relapse of disease are so far poorly understood (Wernsdorfer & McGregor, 1988).

Parasites which do not form hypnozoites (e.g. *P. malariae* or *P. falciparum*) may, nevertheless, produce secondary waves of parasitaemia which are called a "recrudescence" (Fig. 1.2). Such secondary waves of parasitaemia may be explained either by a long-term survival of erythrocytic stages or the continuation, at a low or undetectable level, of erythrocytic schizogony in the peripheral bloodstream, resulting from time to time in detectable parasitaemia and clinical attacks of malaria; the basis for this latent survival of parasites has not, so far, been documented. The main practical difference between relapse and recrudescence is the fact that, while latent erythrocytic stages are susceptible to standard chemotherapy, hypnozoites require a specific treatment (e.g. with primaquine) (Hommel & Gilles, 1998).

1.3 Clinical features of malaria

Malaria causes an acute febrile illness which may be characterized by periodic

febrile paroxysms occurring every 48 or 72 hours with afebrile asymptomatic intervals and a tendency to recrudesce or relapse over periods of months to many years. The severity and course of an attack of malaria depends on the species and strain of infecting parasite and hence on the geographical origin of the infection; on the age, genetic constitution, state of immunity, general health and nutritional status of the patient and on any chemoprophylaxis or chemotherapy which has been used. Synchronicity is important as many of the symptoms of the disease are related to synchronous rupture of meronts with the release of cellular debris and a variety of parasite proteins, glycoproteins, glycolipids and phospholipids into the circulation. This may explain why, in non-immune subjects, the symptoms of *P. vivax* malaria (highly synchronous) are often worse than those of the less synchronous *P. falciparum* early in uncomplicated infections.

The best known symptom of malaria is the febrile paroxysm and usually comprises three successive phases. The **cold stage** starts with a sudden inappropriate feeling of cold. Mild shivering quickly turns into violent teeth chattering and shaking of the whole body. Although the core temperature is high and rising quickly, there is intense peripheral vasoconstriction; the skin is cold, dry, pale and cyanosed. The patient may vomit and febrile convulsions may develop at this stage in young children. These stage last for 15-60 minutes after which the shivering ceases, the patient feels some waves of warmth and the **hot stage** (flush phase) ensues. Patients quickly become unbearably hot. Temperature reaches its peak of 40-41°C or more. During this phase the patient may become confused or delirious. The hot stage lasts from 2 to 6 hours. In the **sweating stage**, the fever declines over the next 2 to 4

hours, symptoms diminish and the patient usually gets a deep sleep and on waking, feels weak. The total duration of the typical attack is 8 to 12 hours. Most paroxysms start between midnight and midday or, at the latest, in the early afternoon (Gilles & Warrell, 1993).

1.3.1 Complications of severe Falciparum malaria

Falciparum malaria is the most serious form of malaria. Attacks may be uncomplicated or associated with serious and often fatal complications. Many of the underlying processes leading to severe malaria are probably common to all patient groups, but there are important differences between non-immune adults and African children. In endemic areas, people have been frequently infected so they can tolerate *P. falciparum* parasitaemia with no symptoms. However, in non-immune people, such as travellers in malarious regions, falciparum infection nearly always causes symptoms and must be regarded as a potentially fatal disease.

The incubation period of Falciparum malaria is between 9-14 days but can be as short as 7 days (Hommel & Gilles, 1998). The classical febrile malarial paroxysm followed by an afebrile asymptomatic interval is not a feature of falciparum infection. The symptoms are non-specific with headache, pains in the back and limbs, simulating influenza; anorexia, vomiting or mild diarrhoea, a feeling of chill rather than a distinct cold phase (as in vivax malaria). The fever is continuous or remittent as in enteric fevers but not tertian. In non-immune individuals the disease can progress very rapidly to severe life-threatening malaria unless appropriate treatment

is started (Gilles & Warrell, 1993).

Parasitaemia greater than 10% is not uncommon in *P. falciparum* infections and levels of 30-60% have been observed (**hyperparasitaemia**). At these high levels, the clinical state of patients tends to deteriorate rapidly. Cerebral malaria, acute renal failure, massive haemolysis, haemoglobinuria, profound anaemia and signs of hepatic and pulmonary involvement may develop within hours (Looareesuwan *et al.* 1983). Parasite counts made before and during treatment have considerable prognostic value. Schizonts are frequently detectable in peripheral blood in patients with hyperparasitaemia.

Cerebral malaria is the most common presentation of severe malaria in adults. They have usually been ill for 4-5 days with fever, slowly lapsing into coma, with or without convulsions. The term cerebral malaria should be confined to patients with demonstrable asexual forms of *P. falciparum* who are in coma. They are unrousable, making only non-purposive, non-localising movements in response to painful stimuli or even more deeply comatose (Plum & Posner, 1980). The syndrome commonly develops over a period of days but may, however, appear suddenly with rapid onset of coma. In South-East Asia, where malaria is seasonal, cerebral malaria is frequently found in adults and socio-economic factors influence its incidence. In Africa, cerebral malaria is unusual in adults. Because of intense transmission, immunity is gained in childhood through repeated infection (Wernsdorfer & McGregor, 1988). So, in Africa, the combination of coma and low parasitaemia, in adults or older children, is therefore less likely to indicate a diagnosis of cerebral

malaria than it is in Asia. However, in young children (between six months and five years) presenting in coma, the finding of *P. falciparum* in the blood should indicate cerebral malaria until proved otherwise. More than 95% of adults and more than 85% of children who recover from cerebral malaria show no persistent neurological sequelae which suggests that much of the pathology must be transient and reversible (Gilles & Warrell, 1993).

In parts of Africa malarial **anaemia** kills as many children as cerebral malaria, its peak incidence being at the younger age of less than 2 years. It is also common in pregnant women and in adults with severe malaria in whom it correlates with parasitaemia (Hommel & Gilles, 1998).

Pulmonary oedema is the most dreaded complication of malaria, may develop at any stage of the disease. It is sometimes precipitated by excessive parenteral fluid therapy and it may present late when the patient appears to be recovering.

Acidosis frequently accompanies pulmonary oedema and occasionally follows hyperparasitaemia, shock, renal failure and hypoglycaemia. High plasma lactate levels and decreased pH in arterial blood are features indicative of acidosis (White *et al.* 1985a). The condition is usually fatal.

Hypoglycaemia, commonly accompanied by lactic acidosis, is now recognized as an important manifestation of falciparum malaria (Molyneux *et al.* 1989; Taylor *et al.* 1988). Although hypoglycaemia may occur in any severe infection, and is an

important problem in malnourished children (Bennish *et al.* 1990), it appears to be particularly common in severe falciparum malaria, where it is associated with increased mortality (White & Ho, 1990).

A proportion of patients with severe malaria infections become hypoglycaemic before receiving any treatment (Migasena, 1983; White *et al.* 1987), others become hypoglycaemic during treatment with quinine, an effect which has been attributed to quinine-induced hyperinsulinemia (White *et al.* 1983; Okitolonda *et al.* 1987). Most reports of quinine-related hypoglycaemia have come from studies of adults with severe malaria and pregnant women (Davis *et al.* 1990). In Thailand chloroquine-resistant strains of *P. falciparum* are prevalent. Therefore, quinine remains an important antimalarial drug which is an important cause of hypoglycaemia by stimulation of pancreatic insulin release (White *et al.* 1983). White and co-workers have performed on large series patients with cerebral malaria in eastern Thailand. They found two different clinical situations in which hypoglycaemia complicating malaria occurs (White *et al.* 1983). In one series, patients developing hypoglycaemia within the first 24 h of treatment had high plasma lactate and alanine concentrations, and relatively low or unmeasurable plasma quinine and insulin concentrations. In a second group, hypoglycaemia occurring after the first 24 h in quinine-treated patients was usually associated with hperinsulinaemia (White *et al.* 1983).

The results in group one suggested that such early hypoglycaemia could be related to insulin resistance and so, was likely to be due to insulin-independent impaired hepatic glucose production, with accumulation of gluconeogenic substrates

and associated lactic acidosis. Glucose administration in these patients should reverse hypoglycaemia (White, 1986). However, in group two, hypoglycaemia was due to treatment with quinine. These patients are often receiving infusions of dextrose-containing solutions and there is evidence that glucose and quinine act synergistically on the pancreatic beta-cell (Henquin, 1982; Davis *et al.* 1990). As the patient with malaria improves and the parasitaemia clears, insulin sensitivity and plasma quinine concentrations (White *et al.* 1985a) will increase and there is a risk of profound hyperinsulinaemic hypoglycaemia. In a patient unable to take sufficient carbohydrate by mouth, inhibition of insulin secretion would seem the best therapeutic. Long-acting somatostatin analogues can reduce quinine-stimulated insulin secretion (Phillips *et al.* 1986) but have their own side-effects (such as hypotension) and are expensive (Davis *et al.* 1990).

In West Africa *P. falciparum* is still largely sensitive to chloroquine, and despite increasing evidence of resistance in East Africa, this drug remains the main anti-malarial treatment for most of the continent (White *et al.* 1987). In African children, hypoglycaemia is an important and treatable manifestation of severe malaria and is unrelated to antimalarial treatment (White *et al.* 1987). Taylor and co-workers also reported that in Malawian children with falciparum malaria, hypoglycaemia was not a complication of treatment with slowly infused intravenous quinine (Taylor *et al.* 1988). In addition study by Kawo and co-workers in children with severe falciparum malaria in Tanzania showed that hypoglycaemia occurred in children who have never received quinine and they had low serum concentrations of insulin (Kawo *et al.* 1990). They also reported that occurrence of hypoglycaemia in these children correlated with the time since the last meal (some had a starvation period of up to 90

h), so, depletion of liver glycogen is the most likely explanation for the hypoglycaemia in patients. Glucose requirements are two to four times greater in infants than in adults, probably because of the large size of the brain in relation to body mass. Glycogen reserves are sufficient for about twelve hours without food, and then the child is dependent on gluconeogenesis for maintenance of normoglycaemia (Pagliara *et al.* 1973; Dahlquist *et al.* 1979). Dekker and co-workers reported that prolonged (not short term) fasting is a factor that contributes to hypoglycaemia (Dekker *et al.* 1997). They also suggested that glucose production in children with uncomplicated falciparum malaria is largely dependent on gluconeogenesis, however, gluconeogenesis is potentially limited by insufficient precursor supply (Dekker *et al.* 1997).

The relative incidence of the many complications of malaria vary, perhaps depending on endemicity and age. In Africa, compared with South East Asia for example, renal failure, pulmonary oedema, circulatory collapse and disseminated intravascular coagulation are rare. In holoendemic (continuous sustained exposure) areas anaemia tends to dominate, whereas in hyperendemic (seasonal exposure) areas there is a relative higher prevalence of cerebral cases (Pasvol *et al.* 1995).

1.4 The pathophysiology of malaria

Of the four species of malaria parasites known to infect man, *Plasmodium vivax, P. ovale* and *P. malariae* undoubtedly cause major morbidity, but result in little mortality. *P. falciparum*, by contrast, is responsible for over one million deaths in Africa each year.

Infection by malaria parasites may lead to a variety of clinical syndromes (see above), depending on a combination of different elements, including the " virulence" of the parasite isolate and a variety of host-related factors such as the status of host immunity and its genetic make-up. Pathological processes in malaria are the result of the erythrocytic cycle and, for most malaria species, it is the rupture of the schizont which will trigger-off the major events leading to the characteristic symptoms of the malaria paroxysm. In the case of *P. falciparum* this process results in the following changes to the infected erythrocyte: altered membrane transport mechanisms, decreased deformability and other mechanical changes, development of knobs beneath the surface membrane, expression of variant surface antigens (strain specific), development of cytoadherent and rosetting properties (Fig. 1.4). The secondary effects of these changes are related to the host's immunological response to parasite antigens and altered red cell surface membranes, changes in regional blood flow and vascular endothelium and systemic complications of altered biochemistry, anaemia, tissue and organ hypoxia and cytokine production.

The pathogenesis of malaria could be due to direct or indirect effects of the parasite on the host. Direct effects of parasite is when mature schizonts rupture and release merozoites, malaria antigens, pigment and malarial toxins. Production of cytokines is an important indirect effect of the parasite on the host, particularly tumour necrosis factor (TNF-α) and IL-1 (Clark *et al.* 1989; Grau & de Kossodo, 1994), which are induced by the release of parasite products during schizont rupture

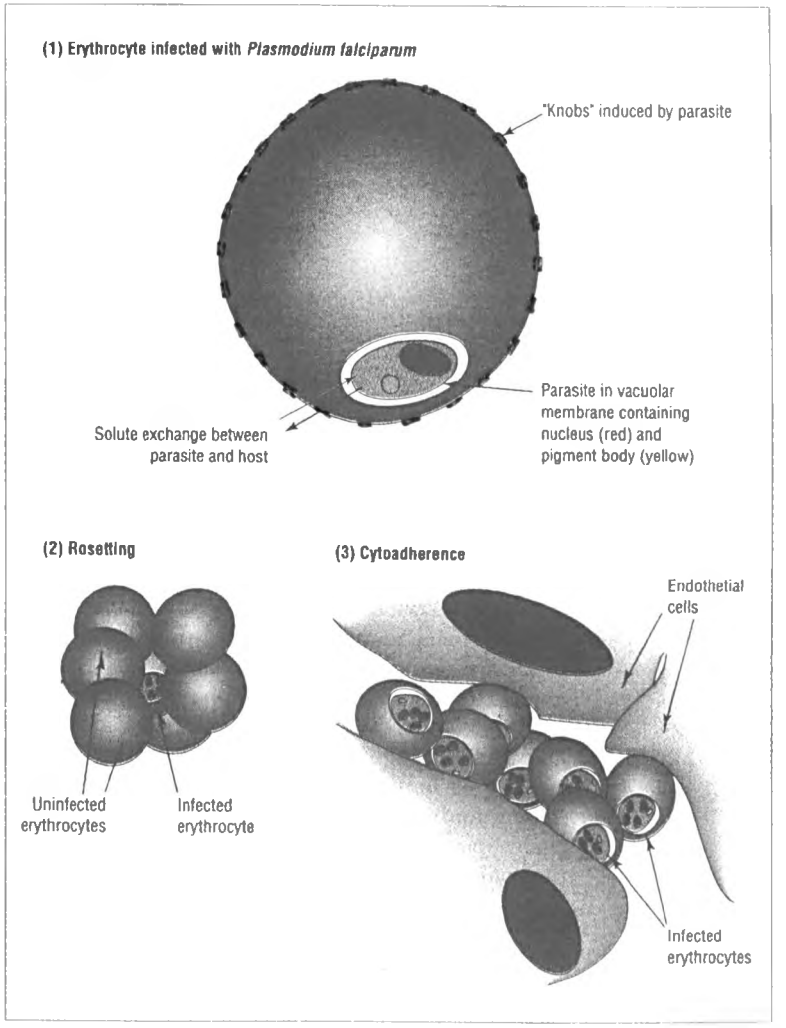


Fig. 1.4 Important processes in host-parasite interaction. (after Krishna, 1997).

1) The infected erythrocyte undergoes complex alterations which enable the parasite to regulate soluble exchange with the host. Parasites also express proteins on the surface of the red cell that are important in pathophysiology some of these proteins aggregate in accretions termed "Knobs".

2) Rosetting in the adherence of uninfected red cells to an infected red cell, forming aggregates that may impede microcirculatory flow

3) Cytoadherence is the adhension of infected red cells containing relatively mature parasites to endothelial cells lining capillaries and postcapillarly venules. Cytoadherence results in microvascular obstruction and impaired delivery of oxygen and possibly other metabolic precursors to vial organs.

and appear to play a central role in the pathophysiology of most of the symptoms of malaria.

1.5 Metabolism of malaria parasites

The nutrients required by *Plasmodia* are obtained from the cytoplasm of the host erythrocyte and from the nutrients available in the plasma. The presence of the parasite in the red cell increases its permeability through the membrane for the passage of nutrients, but the biochemical exchanges are very complex. Alterations in erythrocyte membrane proteins, carbohydrates and lipids might be responsible for the increased osmotic fragility and greater permeability which are characteristic of infected red blood cells.

1.5.1 Lipid metabolism

Infection of a red blood cell (RBC) by a malaria parasite causes changes both in the fluidity and the composition of the host cell membrane. The growth of the parasite within the host erythrocyte and the subsequent formation of 6-20 merozoites requires a large increase in total membranes. The ovoid merozoite (1-2 μm across) is surrounded by a complex of three membranes (pellicle), and contains a nucleus, mitochondria, endoplasmic reticulum, a cytostome, and apical organelles called rhoptries and micronemes. The latter organelles probably contain membrane-active substances that are released during the invasion process and act on the host cell membrane to induce endocytosis. This process gives rise to the parasitophorous

vacuolar membrane (PVM) that surrounds the intracellular parasite (Aikawa, 1988).

It has been clearly established that the lipid content of the erythrocyte increases considerably after *Plasmodium* infection and that there are many differences between the lipid composition of malarial parasites and that of the original host erythrocyte. More specifically, total phospholipids (PL), as well as in neutral lipids, diacylglycerols (DAG), and triacylglycerols (TAG) also increased (Vial *et al.* 1990). Changes in the lipid composition and degree of saturation of the constituent fatty acids of membrane lipids are generally accompanied by alterations in the physicochemical properties of the membrane.

Plasmodia appear to lack the capacity to synthesize fatty acids *de novo* from acetate. Thus, the host fatty acids required for the synthesis of plasmodial membranes must pass across the host cell membrane. The mechanism by which these fatty acids and other membrane lipids are exchanged between the plasma and inserted in the various membrane is not known (Holz, 1977; Cenedella *et al.* 1969; Rock, 1971; Vial *et al.* 1982). Parasites exhibit an intense biosynthesis of neutral lipids and a variety of phospholipids biosyntheses. They are also limited in their capacity for saturation and desaturation as well as chain lengthening and chain shortening reactions. Therefore, the erythrocytic stages of *Plasmodium* must satisfy their lipid requirements by relying on dynamic exchanges with the blood plasma, an activity similar to that of the red cell in which the parasite grows and reproduces (Holz, 1977; Sherman, 1984, 1985).

Red cells infected with malaria parasites show an increase in total phospholipids, and a decrease in the cholesterol/phospholipid ratio (Holz, 1977; Sherman, 1979, 1984). PL levels rise by around 50% from the ring stages to 500-700% just before *P. knowlesi* merozoite release (Angus *et al.* 1971b; Vial *et al.* 1984; Beaumelle & Vial, 1986; Van Der Schaft *et al.* 1987) and the proportion of two main PLs phosphatidylcholine (PC) and phosphatidylethanolamine (PE) is increased. Phosphatidylinositol (PI) is also greatly increased, but sphingomyelin (SM) and phosphatidylserine (PS) decrease sharply (Van der Schaft *et al.* 1987). Maguire and Sherman also reported that membranes of human red blood cells infected with the malaria parasite *P. falciparum* display a decrease in sphingomyelin content (Maguire & Sherman, 1990). This change would result in a more permeable red cell membrane, both of which have been observed in the malaria-infected cell.

The overall fatty acid (FA) composition of the erythrocyte is changed after infection. The amount of saturated FA, especially palmitic acid (16:0) is increased. In terms of polyunsaturated acids, there is an increase in both oleic and linoleic acid, whereas arachidonic acid decreases sharply (Angus *et al.* 1971a; Beaumelle & Vial, 1986).

Malaria parasites use a variety of complex metabolic pathways for phospholipid biosynthesis, and to ensure its development, the parasite depends on the host for supplies of the PL polar-head groups and FA molecules that it cannot synthesize. Hence, if one could interfere in this vital parasite metabolism, it would probably provoke the death of the parasite. This could form the basis for a new chemotherapy.

1.6 Malaria, Toxin(s), cytokines and disease

Ninety-three years ago, in a study mainly concerned with yellow fever, Rosenau reported that serum taken from a malaria patient at crisis caused fever in a normal recipient (Jakobsen *et al.* 1995). 87 years ago Sir Ronald Ross wrote that "....It is now almost certain that (malaria) fever is connected with the discharge of some toxic substance from each mature sporoid at the moment when its spores are scattered in the serum " (Kwiatkowski, 1995). Later, Collier suggested that the symptoms of malaria were due to parasite-derived molecules which, while not classical toxins, induced the body to over-produce toxin factors (Jakobsen *et al.* 1995). This pyrogenic toxin receives frequent mention in the early malaria literature but the topic fell into neglect until Clark pointed out a number of clinical similarities between bacterial endotoxaemia and severe malaria (Clark, 1978). He suggested that the two pathologies might be mediated by soluble factors produced by host macrophages in response to the infection (Clark *et al.* 1981). Although the concept of malaria as a toxic disease is not a new concept, recent findings regarding cytokines have made it possible to find rational explanation for much of malaria.

There is considerable interest in the parasite component(s) that are responsible for stimulating the production of TNF (a polypeptide also known as cachectin, which is secreted by monocytes and macrophages) and other endogenous pyrogens, not only because these are the putative stimuli of malaria fever but also because of the part they may play in severe malarial pathology (Fig. 1.5). It is recognized that not only fever, chills and rigors, but many of the other clinical and pathological consequences

of this parasitic disease, such as anaemia, diarrhoea, hypertriglyceridaemia, renal tubular necrosis, neutrophil accumulation in the pulmonary vasculature, hypotension and abortion, which commonly occur in human malaria can be induced by administration of recombinant tumour necrosis factor (TNF) (Clark, 1987). It was also shown that TNF may increase sequestration by upregulating endothelial adhesion molecules that bind parasites, such as ICAM-1 (Berendt *et al.* 1989). Clark and colleagues have proposed a novel way in which sequestered parasites could cause coma (excessive local concentrations of TNF-α may stimulate endothelium to release sufficient nitric oxide to disturb neurotransmission in the surrounding brain) (Clark *et al.* 1991). Therefore, it is important to identify the postulated toxin(s) and to understand the mode of action.

It would be wrong to focus exclusively on the pathological aspects of TNF because it has a number of anti-parasitic properties (Clark *et al.* 1992b). In biological terms, the protective effects of TNF may be much more important than its pathological consequences, which cause fatality in only a minority of infections. Fever is itself inhibitory to parasite growth (Kwiatkowski, 1989). The classical malaria literature provides evidence that parasites grow exponentially during the first few days of erythrocytic infection, but growth starts to level off shortly after the onset of fever, and may then remain at a roughly constant level for several weeks (Kitchen, 1949). This suggests that maybe TNF and fever are density-dependent regulators of parasite growth, acting to protect the host from high parasitaemia irrespective of

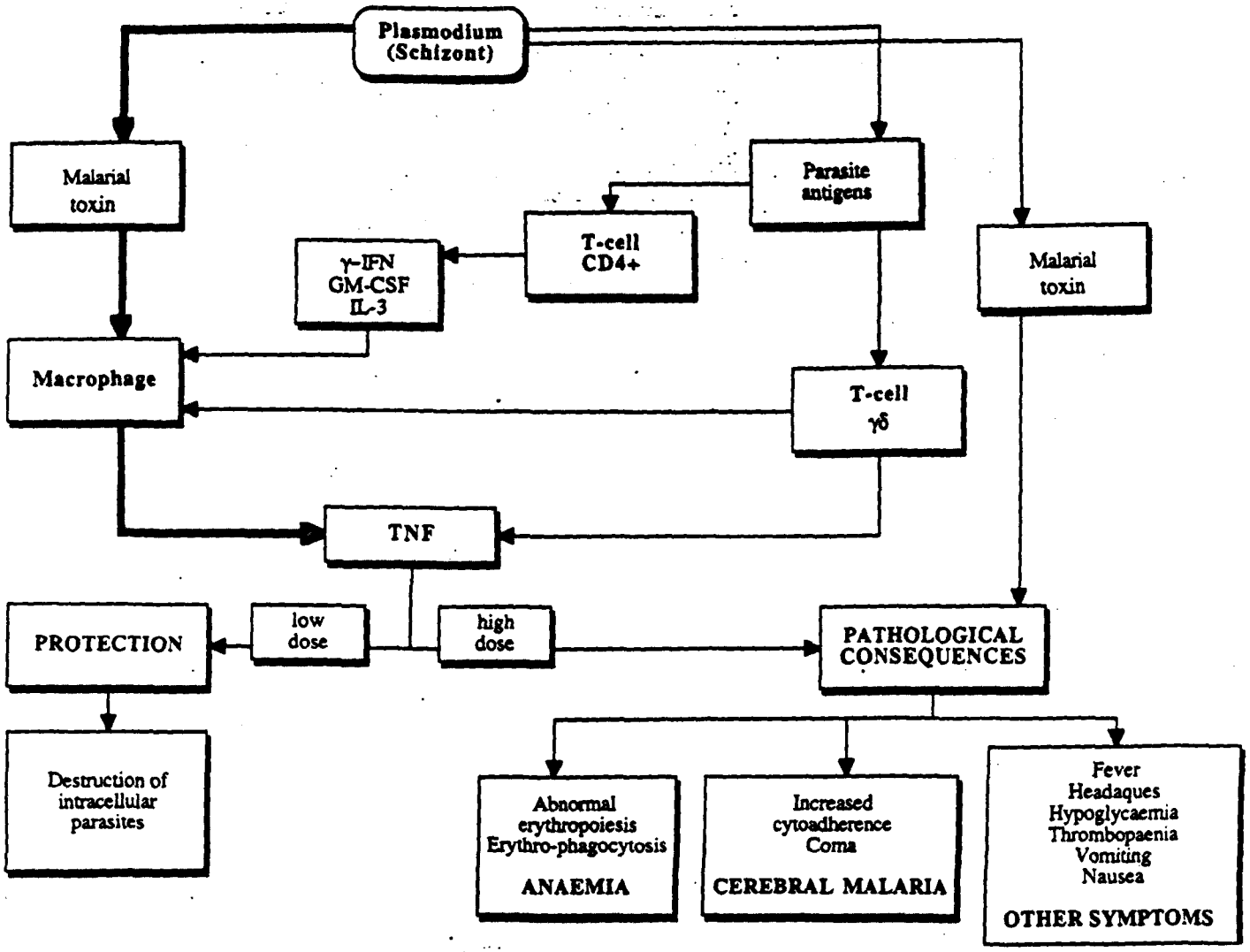


Fig. 1.5 Role of malaria toxin and TNF in the pathophysiology of malaria. (after Hommel & Gilles, 1998).

immune status (Kwiatkowski, 1991). If the parasitaemia falls to a low level, then TNF production and fever are switched off and the parasite is able to recover.

Research carried out by Playfair and co-workers has identified a class of parasite-derived components that have been termed Toxic Malaria Antigen (TMA) and are present in the supernatant of suspensions of cells infected with the rodent parasites incubated overnight (Bate *et al.* 1988) and in the medium of *P. falciparum* cultures (Taverne *et al.* 1990). Such supernatants can stimulate macrophages to secrete TNF (Bate *et al.* 1988) and also human monocytes to secrete Il-1 (Jakobsen *et al.* 1991) and IL-6 (Jakobsen *et al.* 1993a). IL-1 synergises strongly with TNF, both in nitric oxide production and the induction of hypoglycaemia in mice infected with *P. vinckei* (Rockett *et al.* 1994). In another study Rockett and co-workers demonstrated that partially purified *P. falciparum* toxin (malarial extracts) stimulate macrophages to make TNF and nitric oxide (NO) (Rockett *et al.* 1996). It is now evident that nitric oxide, as well as being involved in pathology (Rockett *et al.* 1994) is part of the host's defence against the malaria parasite, with reports of all parasite stages being targeted (Nussler *et al.* 1991; Taylor-Robinson *et al.* 1993).

Attempts to characterize the malaria toxin were made in the first half of the century. One of the earliest of these aimed to show that a major component of the toxin was malarial pigment. Crystalline haematin from uninfected erythrocytes was solubilized and injected into rabbits, who developed fever about an hour later (Kwiatkowski, 1995). This finding was challenged by subsequent investigators but even if correct, it is of doubtful relevance as it is now known that malaria pigment

is a unique polymer of haem groups linked by an iron-carboxylate bond, known as haemozoin, which would have been absent from Brown's erythrocyte extracts (Slater *et al.* 1991). However, study by Pichyangkul and colleagues showed that particles of true malaria pigment, extracted from erythrocytes infected with *P. falciparum*, stimulate a strong TNF response when they are ingested by human monocytes (Pichyangkul *et al.* 1994). They also found that most of the TNF-inducing activity was removed from native pigment particles after protease digestion. So, it is unclear how much of this TNF-inducing activity is due to haemozoin itself, and how much is due to proteins and lipids that are associated with the haemozoin. These findings suggest that pigment can induce TNF due to some other active moiety, linked to polypeptide, that binds to the haemozoin.

Another early idea was that the malaria toxin might resemble bacterial lipopolysaccharide (LPS) (endotoxin). A number of investigations used the Limulus amoebocyte lysate (LAL) assay to detect circulating endotoxin-like substances in malaria patients but most did not (Glew & Levin, 1975; Greenwood *et al.* 1975). Parasite lysates were also found to be LAL-negative (Felton *et al.* 1980), and it was concluded that an endotoxin-like structure was unlikely to be responsible for malaria fever. In addition, LPS and malaria toxin can be distinguished in many ways. The activity of LPS, but not of the toxin, is blocked by polymyxin B (Bate *et al.* 1988, 1989), and phosphatidylinositol, inositol monophosphate and partial structures derived from the toxin or from normal erythrocytes all inhibit the activity of the toxin but not that of LPS (Bate *et al.* 1992a; Bate & Kwiatkowski, 1994a). Similarly malaria hyperimmune antiserum (Bate *et al.* 1988, 1990) and antisera made against PI-

containing liposomes or inositol monophosphate (Bate *et al.* 1992d) do not affect TNF induction by LPS but abolished TMA activity.

TNF-inducing activity of ruptured schizont residues appears to be within a single molecule or a closely related family of molecules. The evidence for this stems from studies of TNF-induction by lysates of the rodent parasite *P. yoelii* (Bate *et al.* 1988). These investigators found that TNF release appeared to depend on a phospholipid structure, in that the activity of crude parasite lysates was greatly reduced after treatment with either phospholipase C, or mild alkali (a deacylating procedure), or hydrofluoric acid (which causes dephosphorylation). A phosphatidylinositol (PI)-like moiety was suggested by the observation that the activity was strongly inhibited in the presence of either PI derivatives (Bate *et al.* 1992b) or anti-PI antibodies (Bate *et al.* 1992d). Subsequent work showed that nanomolar concentrations of monoclonal antibodies (McAbs) which recognize PI can deplete the TNF-inducing activity of crude lysates of *P. falciparum* by over 95% (Bate & Kwiatkowski, 1994c). The same McAb can also inhibit TNF-induction by different strains of *P. falciparum*. They also reported that antisera raised against lysates of *P. yoelii* can inhibit the TNF-inducing activity of *P. falciparum* and *P. vivax* (Bate *et al.* 1992c). Taken together, these results point to a major TNF-inducing toxin whose biological activity depends on a PI-like moiety which is relatively conserved among different plasmodial strains and species.

Glycosylphosphatidylinositol (GPI)-anchor components of the merozoite surface antigens MSP-1 and MSP-2 which are distinct from similar structures in

Leishmania (Schofield *et al.* 1996) have also been claimed to induce the production of modest amounts of TNF and IL-1 (Schofield & Hackett, 1993) and nitric oxide (Tachado *et al.* 1996). GPI exerts these effects by initiating an endogenous signalling pathway that involves non-receptor protein tyrosine kinases (PTK) and protein kinase C (PKC) (Tachado *et al.* 1996). It has also been demonstrated that malarial GPI regulates glucose metabolism in adipocytes (Schofield & Hackett, 1993). Late stages of *P. falciparum* parasite have been shown to produce several glycolipids and two putative GPI anchor precursors (Gerold *et al.* 1994). The primary function of GPI anchors is to provide a means of protein attachment to the plasma membrane, but in the higher eukaryotes there is also evidence that they can participate in signal transduction (Ferguson, 1994). Evidence for the GPI hypothesis came from a study where myristate and palmitate labelled forms of MSP-1 and MSP-2 from *P.falciparum* were affinity purified, and shown to possess TNF-inducing activity (Schofield & Hackett, 1993). However, the well known GPI anchor of the trypanosome variant-specific glycoprotein does not induce TNF (Bate *et al.* 1992b), indicating that the structure of these molecules may be crucial for cytokine induction.

The malaria toxin could be a modified host structure, because it has been shown that TNF-inducing factors with broadly similar properties are present, at much lower level of activity, in sonicated erythrocytes, saponin-lysed and Triton X-114 extracts of uninfected erythrocyte membranes (Bate & Kwiatkowski, 1994a). However, they revealed that a *P. falciparum*-infected erythrocyte contains the capacity to stimulate approximately 200 times as much TNF as a non-infected erythrocyte, even after extensive sonication. This indicates that the TNF-inducing activity of

malaria-infected erythrocytes cannot be simply explained by erythrocyte lysis and is more likely owing to the synthesis of new TNF-inducing factors or to biochemical modification of erythrocyte components. GPI anchors are associated with several erythrocyte membrane proteins (Roberts *et al.* 1988) and they are also actively synthesized by malaria parasites. However, Bate and co-workers reported that TNF secretion is not inhibited by glucosamine or mannose, which have been noted to inhibit cell-signalling events attributed to GPI-anchors in other systems (Machicao *et al.* 1990). They also have tested a number of purified GPI-anchored antigens from other sources and none was found to stimulate TNF secretion (Bate & Kwiatkowski, 1994a). They concluded that if a GPI moiety is responsible for the TNF induction by lysed erythrocytes or malaria parasites, it probably has an unconventional structure.

However, with regards to the recent reports on *Mycoplasma spp.* ("mycoplasma") contamination of *P. falciparum* culture (Turrini *et al.* 1997; Rowe *et al.* 1997), it is essential that the validity of all published work on TNF-inducing activity of TMA be re-examined by further investigations (see Chapter seven).

1.6.1 The role of TMA and TNF on induction of hypoglycaemia

Studies in South-East Asia (White *et al.* 1983; Looareesuwan *et al.* 1985), India (Narula *et al.* 1985), Africa (White *et al.* 1987; Okitolonda *et al.* 1987; Taylor *et al.* 1988),and Papua New Guinea (Currie & Kevau 1988) have shown that hypoglycaemia is a common and important complication of *Plasmodium falciparum* malaria. It is a common and important complication of falciparum malaria in both

adults (White *et al.* 1983; Okitolonda *et al.* 1987; Looareesuwan *et al.* 1985) and children (White *et al.* 1987; Taylor *et al.* 1988). Because hypoglycaemia can result in permanent neurological sequelae or death, prompt detection and treatment are important (Marks & Rose, 1981).

Although hypoglycaemia is caused by an imbalance between the production and disposal of glucose, the pathophysiology of hypoglycaemia in malaria is incompletely understood. Therefore, a better understanding of pathogenetic mechanisms might lead to new therapeutic approaches for complicated falciparum malaria. Several hypotheses have been proposed, for example increased glucose consumption by both host and parasites (Sherman, 1979; White *et al.* 1983), impaired and abnormal gluconeogensis (White *et al.* 1983) and overproduction of cytokines such as TNF and IL-6 (Clark *et al.* 1992b). In some studies, hypoglycaemia has been attributed to the metabolic side-effects of antimalarial drugs which are used for treatment. Mefloquine is one of anti-malaria which appears to have significant effects on pancreatic beta cell function at very low plasma free concentrations (Davis, 1997), However, quinine is the most potent of drugs which have ability to stimulate insulin release *in vitro* (Henquin *et al.* 1975) and *in vivo* (White *et al.* 1983). A study by Looareesuwan and co-workers showed that some patients with severe falciparum malaria had profound hypoglycaemia and hyperinsulinaemia before quinine was started (Looareesuwan *et al.* 1985). In that study they suggested that insulin release may be triggered by products of the malaria parasite such as endotoxin-like substances. The parasite products which are released after schizont rupture, have both indirect (via TNF-induction) (Bate *et al.* 1989) and direct effects (via lipogenesis

inducing) (Taylor *et al.* 1992a,b) on host cells.

TNF-inducing extracts from parasitised erythrocytes with *P. yoelii* injected into mice cause a rapid drop in blood glucose and this could be prevented by prior immunization with supernatant or with phosphatidylinositol (Taylor *et al.* 1992a). This activity also appears to be due to a phospholipid-containing component, in that it was unaffected by digestion with pronase, was abolished by lipase digestion and was inhibited by inositol monophosphate. The toxin was reported to stimulate the release of insulin *in vivo* (Elased & Playfair, 1994) and appears to synergize with low levels of insulin to increase lipogenesis rather acting on their own (Taylor *et al.* 1992b). Mice infected with *P. chabaudi* develop profound self-limiting hypoglycaemia at the time of crisis (60% parasitaemia) which correlates with hyperinsulinemia (Elased & Playfair 1994) and may be related to the ability of the parasite products to enhance lipogenesis into adipocytes (Taylor *et al.* 1992b). Hypoglycaemia also occurs in the terminal phase of the *P. yoelii* infection which is associated with hyperinsulinaemia (Elased & Playfair, 1996). Therefore, hypoglycaemia in rodent malaria could be secondary to a hyperinsulinaemia that is probably stimulated directly by products of the parasite. If hyperinsulinemia is a feature, therapy might be directed at this. Treatment along these lines might be more logical than injection of glucose, which is likely to further stimulate insulin secretion. Hypoglycaemia with normal plasma insulin is more likely to be due to depletion of liver glycogen or glucose starvation, and here, of course, glucose therapy would be indicated.

Disease severity and death are correlated with high levels of circulating TNF

(Grau *et al.* 1989; Kwiatkowski *et al.* 1990) and a small dose of TNF given to mice with a subclinical malaria infection induced hypoglycaemia (Clark *et al.* 1987). It was proposed that TNF might be responsible for the occurrence of hypoglycaemia in malaria (Clark *et al.* 1992b). However, the hypoglycaemia in both lethal *P. yoelii* and non-lethal *P. chabaudi* infection was not prevented by pretreatment with a monoclonal antibody that neutralizes TNF, nor by pentoxifylline (Elased *et al.* 1996). In addition, Taverne and co-workers reported a lack of correlation between the TNF-inducing capacity of extracts of parasitized red blood cells (toxin) and their ability to cause hypoglycaemia (Taverne *et al.* 1995). Elased and co-workers also reported that injection of recombinant human TNF into normal mice caused an immediate increase in the concentration of blood glucose rather than a decrease, they also found that highly purified TNF did not cause hypoglycaemia, except in the presence of traces of LPS (Elased *et al.* 1996). It is possible that the hypoglycaemia reported by Clark and co-workers (Clark *et al.* 1992a) following administration of recombinant TNF to infected mice was due to contamination with LPS or to parasite molecules acting like LPS or both (Elased *et al.* 1996). However, work on the role of TNF in diabetes, obesity and glucose homeostasis indicates that its over-expression decreases levels of circulating insulin (Evans *et al.* 1989), blocks the action of insulin, by suppressing insulin-induced phosphorylation of the insulin receptor (Feinstein *et al.* 1993; Hotamisligil *et al.* 1994), and induces insulin resistance.

These findings provide further support to the view that the different symptoms of malaria are not caused by a single TNF-inducing entity and suggest that TNF is not the principal mediator for hypoglycaemia and the cause of hypoglycaemia is likely

to be multifactorial. It is, therefore, possible that toxic malaria antigens might induce hypoglycaemia by stimulating the secretion of insulin or act directly to alter glucose utilization by fat tissue without the mediation of any cytokine (Elased *et al.* 1996).

1.7 Immunity to malaria

Malaria is an important cause of morbidity and intense effort has gone into the development of a human malaria vaccine, but little is known about the disease process and the human immune responses to the parasite. There is little known about the mechanisms by which immunity is acquired and maintained, and how it acts to limit rather than to eliminate parasitaemia. What we do know is that resistance to malaria develops slowly and that in young children there is high parasitaemia, morbidity and mortality (Snow *et al.* 1994) and it is not until later in childhood that clinical symptoms decline together with parasite levels (Marsh, 1992). Older children, nevertheless, often carry a substantial parasite load yet are asymptomatic with no accompanying fever or other adverse symptoms, a state known as "clinical tolerance" or "anti-toxic immunity". Adults rarely have a high parasitaemia and appear to have acquired a degree of non-sterile immunity (Facer & Tanner, 1997).

In stable endemic areas, children born to immune mothers appear themselves to be relatively immune to malaria for a period. It is not rare to find low numbers of parasites in cord blood, but these do not give rise to overt infections. This contrasts with the situation in less stable areas where clinically severe congenital malaria does occur. The child remains protected for a period of between 3 and 6 months following

birth, after that children become increasingly susceptible to the more severe clinical manifestations of malaria. In malaria-endemic areas children pass through a period when they commonly have high parastaemia without serious disease, i.e. a concept of antimalarial immunity which is not directly antiparasitic but is "antitoxic", either by neutralizing toxic parasite products or by inducing a tolerance of the hosts against acute infection.

Fig. 1.6 shows potential immune mechanisms against different stages of the malaria parasite. Protective immunity to the asexual blood stages of malaria parasites involves both cellular and antibody mediated mechanisms (Taylor-Robinson, 1995). Antibodies inhibit erythrocyte invasion, promote opsonization of merozoites and infected erythrocytes by monocytes, and detach sequestered infected erythrocytes from the endothelial cells of brain capillaries. Cellular immunity to *P. falciparum* is mediated through macrophages and neutrophils, with and without specific antibody, and by cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), and reactive intermediates of oxygen and nitrogen (Taylor-Robinson, 1995).

1.7.1 Antibodies

In man, serum concentration of IgM, IgG and IgA all increase following infection. The rise in IgM and IgA levels is only transient, whereas all subclasses of IgG remain high (Facer, 1980).

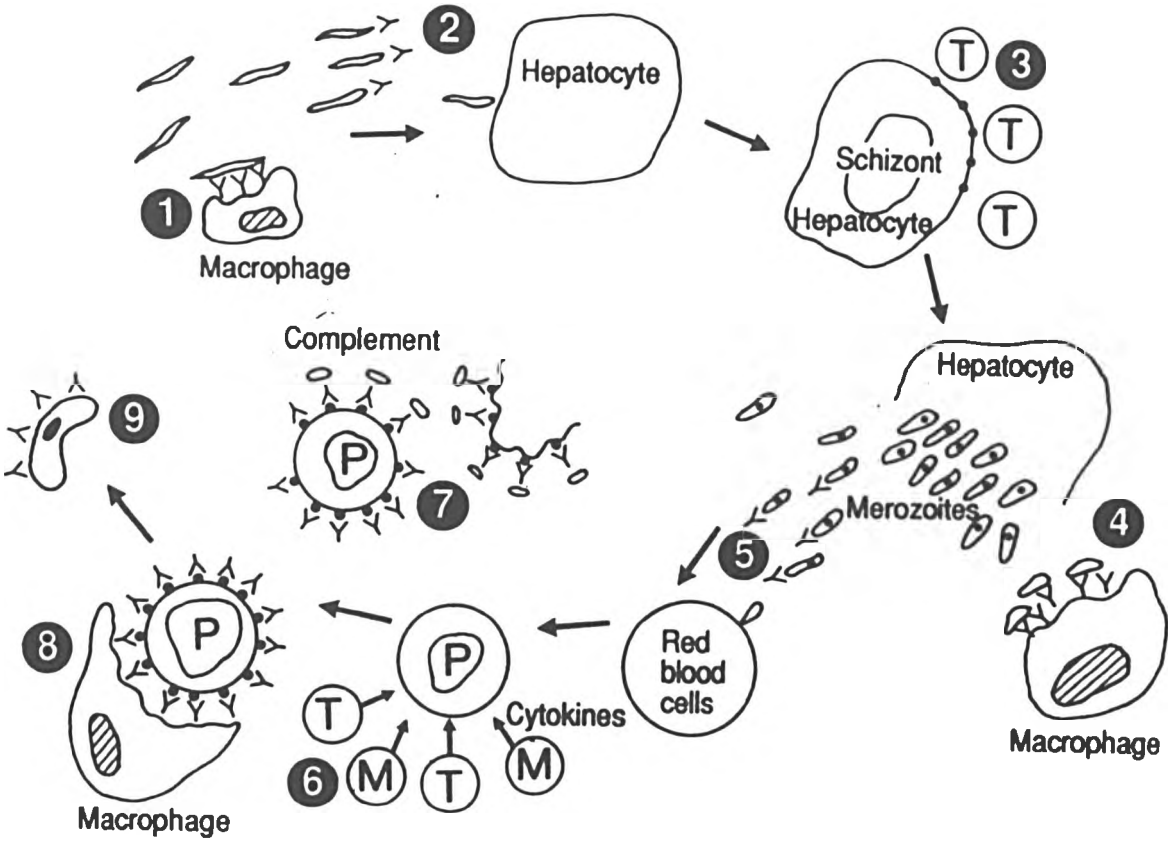


Fig. 1.6 Potential immune mechanisms against the malaria parasite.
1. Antibody-mediated opsonization of sporozoites.
2. Blocking of hepatocyte invasion.
3. Cell-mediated responses to processed antigens at the surface of the infected hepatocyte.
4. Opsonization of merozoites.
5. Blocking of merozoite invasion and red cells.
6. Intracellular killing of parasites by soluble mediators.
7. Complement activation by antibody binding to neoantigens on the surface of infected red cells, leading to lysis.
8. Opsonization of infected red cells.
9. Antibody to the surface of gametocytes (carried into the mosquito where it blocks normal process of fertilization and development). This list is not exhaustive, but indicates the complexity of antimalarial immunity. T, T cell; M, macrophage; P, parasite. (after Gilles & Warrell, 1993).

Anti-parasite immunity is the ability of immune mechanisms to inhibit parasite growth. Based on experimental studies in animal models, both antibodies (Bouharoun-Tayoun *et al.* 1990; Newbold *et al.* 1992) and effector T cells (Quaki *et al.* 1994) play important roles. Antibodies recognize opsonic determinants on the surface of the infected red cell leading to phagocytosis of cells by macrophages or neutrophils or recognize merozoites and inhibit red cell invasion. Antibodies of the former specificity have been shown to correlate with the acquisition of clinical immunity in humans. There are reports that antibodies from immune adults could passively transfer protection to non-immune recipients and these antibodies mostly are of the IgG class (Cohen *et al.* 1961; Bouharoun-Tayoun *et al.* 1990) and that these antibodies were of the cytophillic classes (Bouharoun-Tayoun & Druihle 1992).

In analysis of the isotype content of sera from individuals with defined clinical states of resistance (hyperimmune from African adults living in a hyperendemic region) or susceptibility to malaria (children and primary attack), Bouharoun-Tayoun and Druihle have found profound differences in the distribution of each Ig subclass and particularly in the ratio of cytophilic versus noncytophilic antibodies (Bouharoun-Tayoun & Druihle 1992). In non-protected subjects, IgM predominated, while in a primary attack, IgG2 was predominant; in both, there were low levels of antimalarial antibodies. In protected subjects, two cytophilic isotypes (IgG1 and IgG3) were found to predominate. The latter antibodies cooperate with blood monocytes,whilst the former antibodies did not and can actually inhibit the *in vitro* effect of the latter antibodies. From these findings Bouharoun-Tayoun & Druilhe concluded that cooperation between monocytes and antibodies would appear to be an essential

effector mechanism in the acquired state of protective immunity, though others can be involved (Bouharoun-Tayoun & Druihle 1992). In parallel to above findings, it has been shown that IgG3 antibodies generated against merozoite surface antigen 2 (MSP2) following natural exposure are potentially highly effective mediators of protection because of their cytophilic nature and their ability to fix complement; in addition they may block the invasion of merozoites into new erythrocytes, thereby interrupting the erythrocytic life cycle. However, while IgG3 antibodies to MSP2 may be very effective in controlling parasitaemia, it is noted that their effectiveness will be dependent on the maintenance of this response through repeated exposure to parasites as the IgG3 antibody response is not normally long lasting. Failure of the anti-MSP2 response to switch downstream to an IgG1 response can be expected to give the parasite a survival advantage (Ferrante & Rzepczyk, 1997).

1.7.2 T cells and macrophages

It is well documented that T cells are crucial for malaria immunity (Ho & Webster, 1989). T cell function is not only necessary for antibody production but also provides memory for a secondary response upon rechallenge. During the early days of infection, the immune response is essentially of a TH-1 type, which is not sufficient to eliminate parasites, but later, the immune response switches to an essentially TH-2 mode, with antibody mediated mechanisms playing a major role in the eventual elimination of parasites (Hommel & Gilles, 1998).

In humans, T cell stimulation by parasites might be equally effective in

triggering TNF-α secretion and consequent disease (Currier *et al.* 1992). T cells from non-exposed donors can respond to parasites at a density of 1 parasitized red cell per μl of blood or less. These T cells typically secrete IFNγ and either themselves secrete or direct the secretion of TNFα. Good found in over 90% of non-exposed adults and 50% of children may contribute significantly to disease pathogenesis following activation of T cells *in vivo* by malaria parasites (Good, 1995). So, T cells can play a significant role in parasite elimination, but a consequence of this elimination is the activation of T cells and liberation of cytokines that may result contribute to the pathogenesis of the disease.

T lymphocytes bearing heterodimeric T-cell receptors composed of $\gamma\delta$ chains ($\gamma\delta$ T cells) have been proposed to represent a primitive immune surveillance system. $\gamma\delta$ T cell expansion has been reported in *P. falciparum* malaria infection (Roussilhon *et al.* 1994). The response of $\gamma\delta$T cells may be beneficial to the host, as they have been shown to exert direct anti-parasitic activity (Elloso *et al.* 1994). TNF and IFN-γ produced by $\gamma\delta$ T cells in response to parasite-infected red cells (Behr *et al.* 1994; Goodier *et al.* 1995) may promote macrophage activation that could also result in parasite killing. The antigen specificity of $\gamma\delta$ T cells in malaria has not been defined. However, peripheral blood $\gamma\delta$ T cells from non-exposed individuals respond to antigens of the *P. falciparum in vitro*. This response, largely due to stimulation of T cells by components of parasite expressed on the schizont stage and released at schizont rupture (Jones *et al.* 1996).

Results from a number of different model systems suggest that while T cells

can control parasitaemia, antibody may be required to completely eliminate parasites (Roberts & Weidanz, 1979; Taylor-Robinson & Phillips 1994), but the process is under T cell control.

1.7.3 Anti-disease immunity

It is common to find 5-10 years old African children who are asymptomatic yet have levels of parasitaemia that would be associated with fever and disease symptoms in a non-exposed malaria-native individual. It is obvious that these children must have anti-disease immunity, which results in decreased clinical manifestations of disease.

The mechanisms leading to anti-disease immunity is not completely understood. However, it is proposed that at schizogony, large amounts of parasitized red blood cell (PRBC)-derived antigens (lipids, glycolipids, glycoproteins) are released into the circulation over a short period of time (including malaria "toxin") which act on host cells to induce secretion of a variety of factors including cytokines responsible for clinical symptoms (Playfair *et al.* 1990). The lipidic nature of the immunogens involved is suggestive of T cell-independent mechanisms, which may explain why anti-disease (or "anti-toxic") immunity is acquired only slowly and requires frequent boosting in order to be sustained.

Anti-disease immunity would reduce the risk of severe disease but not effectively protect against superinfection. These features may explain asymptomatic

P. falciparum infections in the presence of high parasitaemias in African children. The short-lived nature of anti-toxic immunity might also explain the rapid loss of acquired protection against the clinical malaria seen in African malaria immune adults who are away from endemic regions for periods greater than 6 months.

1.8 Malaria control

100 years after Ronald Ross discovered the role of the mosquito in the life cycle of the malaria parasite, efforts to control the disease stand at what could prove to be a historic watershed.

Malaria is already estimated to kill between 1.5 and 2.7 million people every year. Another 300 to 500 million people have the disease, and one-third of all humanity lives in zones where they risk catching it. Malaria kills one person, often a child under five, every 12 seconds. Nine out of ten cases occur in sub-saharan Africa, and two-thirds of the rest are concentrated in just six countries. These are, in decreasing order of malaria prevalence, India, Brazil, Sri Lanka, Vietnam, Colombia and the Solomon Islands (Butler, 1997).

By the year 2000, the current WHO Global Malaria Control Strategy aims to reduce malaria mortality by at least 20%, compared with 1995, in at least 75% of the affected countries (WHO, 1993). This strategy recognizes that multiple varied transmission and operational drug-resistance patterns worldwide lead to different epidemiological patterns. Because malaria represents a moving target, control

programmes need to be identified and monitor epidemiological patterns (WHO/ World Bank, 1996). However, pregnant women and children under five years old continue to constitute two of the most important risk groups.

To control malaria, better ways must be found to apply existing control methods, improved tools must be developed (Engers & Godal, 1998). In sub-Saharan Africa, where transmission rates are so high, new approaches are needed to control malaria and its mosquito vectors. New drugs must be found to prevent and treat the disease. Outside Africa, much can be done using existing control techniques given adequate commitment. Mosquitoes have grown resistant to pesticides, and the harm that these cause to the environment has restricted their use further. New drugs, and elusive vaccine, are also needed by all malarious countries, not to mention the tourists and other travellers who visit them (Butler, 1997).

The development of an effective malaria vaccine represents one of the most important strategies for providing a cost-effective addition to currently available malaria control interventions. A universally effective malaria vaccine is not available, despite significant advances in understanding mechanisms of protection, identifying targets of protective immune responses and designing vaccine delivery systems that induce the required immune responses against the desired target.

The aim of a vaccine is to reduce morbidity/mortality due to malaria and this may be achieved in one of two different ways. One is by interrupting the infection at one or other of the stages of the parasite life-cycle, or second, by means of an anti-

disease vaccine, which would reduce the pathophysiological effects triggered by the release of malarial toxin(s) (Playfair *et al.* 1990).

It has been recognized for many years that children living within malaria endemic regions develop what is known as "anti-toxic" immunity, manifested as a progressive reduction in disease severity, following repeated malarial attacks and occurring several years parasitaemias begin to fall. The concept of malarial toxin becomes important as there is re-emerging evidence of an age-old belief that malaria is a metabolic disorder, perturbing many aspects of the immune system including the cytokines and their receptors. The toxin theory of malaria pathology leads to certain novel ideas for therapy and prevention. It is better, if the toxin could be made both safe and immunogenic, it may make an effective "anti-disease" vaccine (Playfair *et al.* 1990).

In order to interrupt the infection a multistage, multivalent, multi-immune response vaccine would be ideal, but design of such a vaccine is not easy. It requires to find the unique antigenicities of the different stages of the parasite, but most antigens are not expressed at all stages of the parasite's life cycle, and some may be expressed only during a single stage.

Finally, even if a perfect vaccine can be found, it is unlikely to solve malaria on its own, but will need to be integrated into an appropriate control strategy, adapted to each endemic situation, such as early diagnosis and prompt treatment, selective controls and preventive measures against the parasite as well as vector, regular assessment of a country's malaria situation, in particular the ecological, social and

economic determinants of the disease and involvement of primary health care in endemic area (WHO, 1993).

1.9 Insulin and its action on adipose tissue

Insulin plays an a central role in metabolism, not only by controlling blood glucose levels but also in the regulation of amino acid transport and control of lipid breakdown (Cheatham & Kahn, 1995). It is thus an important anabolic hormone which acts on a variety of tissues including liver, fat and muscle. Insulin regulation of glucose homeostasis has two major components: stimulation of peripheral glucose uptake and suppression of hepatic glucose output.

Adipose tissue, which consists of adipocytes or fat cells, is amorphous and widely distributed in the body under the skin, around the deep blood vessels and in the abdominal cavity (Lehninger, 1982). This tissue is not an inactive store house and like other cell types in the body, fat cells are active.

It was first demonstrated by Krahl that the uptake of glucose by adipose tissue incubated *in vitro* is stimulated by addition of insulin (Krahl, 1951). It has been shown that insulin (in the presence of glucose) decreases the rate of release of free fatty acid (FFA) (Vaughan, 1960; Raben & Hollenberg, 1960). The available evidence also suggested that in adipocytes, regulation of glucose transport is considered to depend on the action of insulin in mobilizing the glucose transporters (Suzuki & Kono, 1980; Cushman & Wardzala, 1980).

Polymyxin B is a antibiotic and has been recognized as a new agent inhibiting the action of insulin on glucose transport (Amir *et al.* 1987) and also inhibits insulin-induced glucose incorporation into lipids in a dose-dependent manner (Cormont *et al.* 1992) in isolated rat adipocytes and in isolated rodent muscles (Henriksen *et al.* 1989; Gremeaux *et al.* 1987) *in vitro*. It also blocks *in vivo* the hypoglycaemic effect of insulin (Amir & Shechter, 1985). Although the exact mechanism for its effect is not completely known, however this anti-insulin effect results from an inhibition of insulin-induced glucose transport both in skeletal muscle and in adipose tissue (Amir *et al.* 1987; Gremeaux *et al.* 1987). Cormont and co-workers reported that polymyxin B was interfering with the exocytotic process of the GLUT4 vesicles, perhaps at the fusion step between vesicles and plasma membranes (Cormont *et al.* 1992).

The ability of insulin to stimulate glucose metabolism is markedly diminished in large adipocytes from older rats (Richardson & Czech, 1978; Salans & Cushman, 1978) and rats fed with high-fat diets (Susini & Lavau, 1978) compared to small adipocytes from younger animals. In both cases, the decreased insulin effect cannot be attributed to major alterations of insulin binding or to the ability of insulin to stimulate glucose transport, and therefore must be due to a limited capacity to metabolize glucose (Susini & Lavau, 1978; Richadson & Czech, 1978; Olefsky, 1977; Salans & Cushman, 1978). Marked reduction in the activities of the key fatty acid synthesis enzymes seem to be responsible for the decreased fatty acid synthesis by these cells (Fried *et al.* 1981).

It has long been established that the plasma membranes of virtually all

mammalian cells possess a transport system for glucose of the facilitative diffusion type; these transporters allow the movement of glucose across the plasma membrane down its chemical gradient either into or out of cells. However, glucose transport into certain tissues of mammals is under both acute and chronic control by circulating hormones. In fact, research efforts in the transporter field over the past 5-10 years have shown that, rather than being mediated by a single transporter expressed in all tissues, glucose transport is mediated by a family of six facilitated glucose transporters, known as GLUTS which are products of distinct genes and are expressed in a highly controlled tissue-specific fashion (Bell *et al.* 1990).

The transporter family are included:

GLUT1: The erythrocyte-type glucose transporter

GLUT2: The liver-type glucose transporter

GLUT3: The brain-type glucose transporter

GLUT4: The insulin-responsive glucose transporter

GLUT5: The small-intestine sugar transporter

GLUT6: A pseudogene-like sequence

GLUT7 : The hepatic microsomal glucose transporter

GLUT1 and GLUT4 are largely responsible for removing glucose from the blood stream. GLUT1 and GLUT4 are present in many tissues and cells including fat tissues. GLUT1 is present in many tissues and cells (Flier *et al.* 1987), but it is expressed at highest levels in the brain but is also enriched in the cells of the blood-tissue barriers such as the blood-brain/nerve barrier, the placenta, the retina, etc. In

addition, GLUT1 has been identified in muscle and fat, tissues which exhibit acute insulin-stimulated glucose transport, but only at very low levels in the liver, the other major tissue involved in whole-body glucose homeostasis (Flier *et al.* 1987). GLUT4 is constitutively expressed in adipose and muscle. The most important property of GLUT4, which distinguishes it from other isoforms, is its propensity to remain localized in intracellular vesicles in the absence of insulin (Gould & Holman, 1993), However, in the absence of insulin GLUT1 is found predominantly on the cell surface. Insulin stimulates the translocation of GLUT4 to the plasma membrane, which facilitates glucose uptake (Lienhard *et al.* 1992). In rat adipose cells, insulin produces an approximate 15 fold increase cell-surface GLUT4 and 4 fold increase in cell surface GLUT1 (Holman *et al.* 1990). Insulin can then specifically recruit this transporter to the surface under metabolically appropriate conditions (Fig. 1.7). In the absence of insulin (the basal state), glucose transport is rate limiting for metabolism, but in the presence of insulin plasma membrane abundance of GLUT4 transporter will increase (Gileman & Rees 1983).

In 1980, several laboratories reported that insulin enhances the rate of glucose uptake into isolated rat adipocyte primarily through a rapid, reversible and energy-dependent translocation of glucose transporters from a large intracellular pool to the plasma membrane (Suzuki & Kono, 1980). With the formulation of this translocation hypothesis, subsequent adipocyte studies revealed that insulin resistance was associated with a depleted number of intracellular glucose transporters in multiple animal models, including streptozotocin-induced diabetes (Karnieli *et al.* 1981) and aged obese rats and high fat-feeding (Hissin *et al.* 1982).

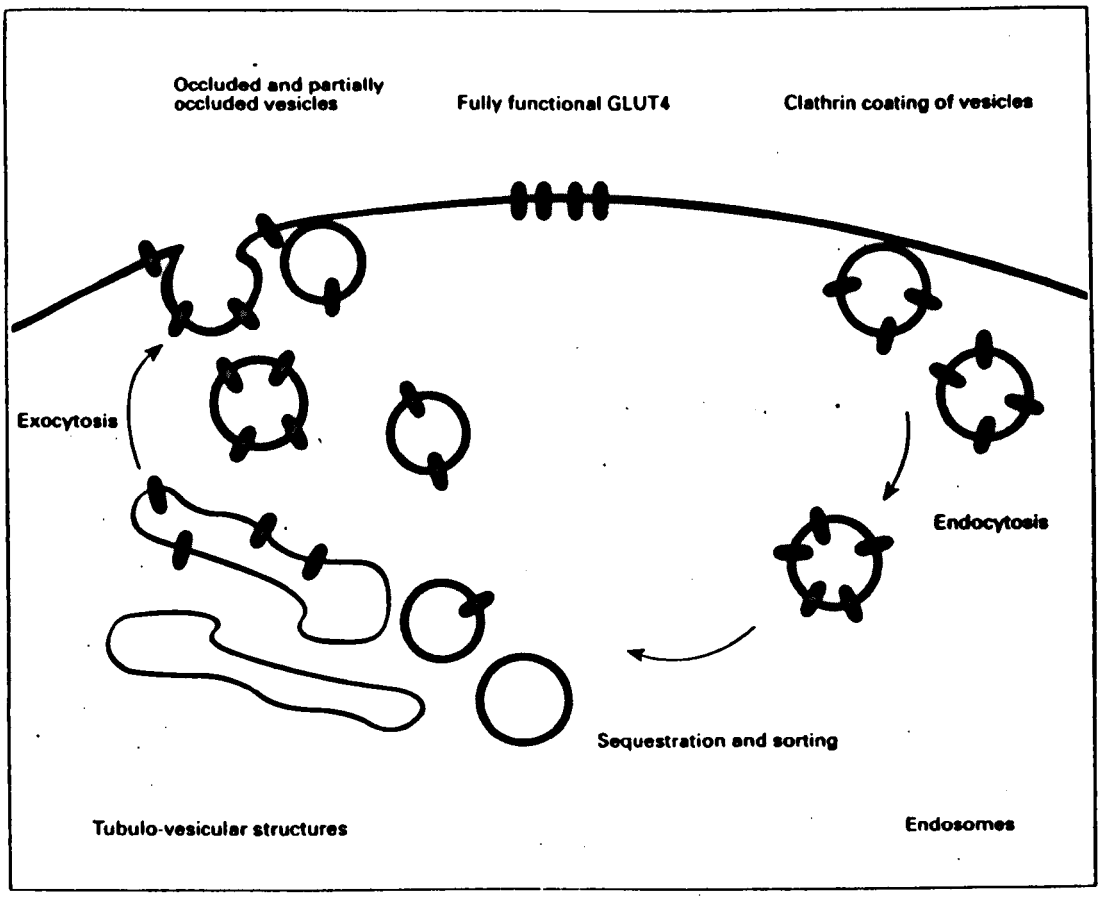


Fig. 1.7 Insulin regulation of GLUT 4 translocation. GLUT 4 is predominantly present in intracellular tubulo-vesicular structures in the absence of insulin. Upon insulin stimulation, exocytosis is increased and GLUT 4 vesicles dock and fuse with the plasma membrane.
(after Gould & Holman, 1993).

Thus, at least one case of insulin resistance can be attributed to cellular depletion of glucose transporter proteins.

Insulin action is mediated through the insulin receptor, a transmembrane glycoprotein with intrinsic protein tyrosine kinase activity. The insulin receptor is present in virtually all vertebrate tissues, although the concentration varies from as few as 40 receptors on circulating erythrocytes to more than 200/000 receptors on adipocytes and hepatocytes (White & Ronald Khan, 1994). The insulin receptor is a tetrameric structure with bilateral symmetry comprised of two α-subunits that are each linked to a β-subunit and to each other by disulfide bonds (Olefsky, 1990). The α-subunits are located entirely outside of the cell and contain the insulin binding site(s), whereas the intracellular portion of the β-subunit contains the insulin-regulated tyrosine protein kinase. 1 or 2 insulin molecules can bind to each receptor and the affinity of insulin for the plasma membrane receptor at physiological pH (7.2) is relatively high (Hammond *et al.* 1997). The unoccupied α-subunit of the insulin receptor inhibits the tyrosine kinase activity of the β-subunit. Removal of α-subunits by proteolytic cleavage relieves this inhibition. On binding of insulin to the extracellular α-subunits, the β-subunits become phosphorylated on tyrosine residues (Ballotti *et al.* 1987) , which activate the intrinsic tyrosine kinase activity present in the β-subunit (Khan *et al.* 1989). This autophosphorylation and activation of the intrinsic kinase sets in motion the various sets of signalling cascades that ultimately result in modulation of metabolic process. Autophosphorylation of receptors enables the phosphorylation of various substrates. One of the cellular substrates for the insulin receptor tyrosine kinase is insulin receptor substrate-1 (IRS-1) (White *et al.* 1985b;

Sun *et al.* 1991). IRS-1 undergoes tyrosine phosphorylation in response to insulin and acts as a multisite docking protein to interact with Src homology 2 (SH2) domains of various signal-transducing molecules to propagate the insulin signal downstream (Sun *et al.* 1991). Phosphatidylinositol 3-kinase (PI-3-kinase) is one such SH2 domain-containing protein whose activity is stimulated by insulin (Ruderman *et al.* 1990; Kelly *et al.* 1992) via binding of phosphorylated IRS-1 (Fig. 1.8). This kinase activity is requisite for the many biological effects of insulin, including the critically important activation of glucose transport (Morgan & Roth, 1987).

1.10 Insulin second messengers

Some of insulin's short-term metabolic effects could, to some extent, be replicated in fractionated cell systems by unidentified factors that were isolated from insulin treated cells (Jarett *et al.* 1985; Seals, 1985). The factor(s) was variously suggested to be of a peptide nature, but it was never isolated and its structure not identified. When it was demonstrated that a low molecular mass water-soluble and insulin-mimicking oligosaccharide could be released from cell membranes by a phosphatidylinositol (Glycan)-specific phospholipase C, as well as by insulin, (Saltiel & Cuatrecases, 1986), the concept of a second messenger acting in insulin signalling gained new interest.

Insulin or a phospholipase can release a carbohydrate-containing compound that in cell-free assays mimics several of insulin's short-term effects, which is a property required of a second messenger of the hormone. This compound also

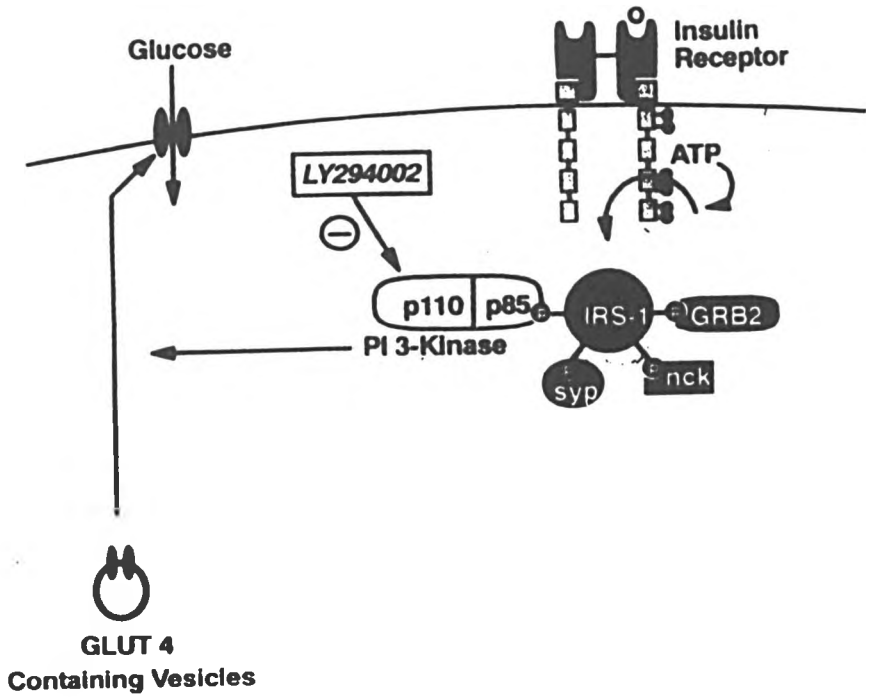


Fig. 1.8 The model shows insulin-regulated pathways of GLUT4 and some of the components involved. When insulin binding to and activating its cell surface receptor, tyrosine residues and rapid phosphorylation of an immediate downstream substrate molecules, insulin receptor substrate 1 (IRS-1). In its phosphorylated form, the IRS-1 has been shown to act as a docking protein that forms a signalling complex with phosphatidylinositol 3-kinase. PI 3-kinase activity is required for glucose transport.(after Cheatham *et al.* 1994).

reproduces effects of insulin when added to intact cells. Some of the effects of insulin such as amino acid uptake also appear to be mimicked.

Indirect analyses indicate that a bacterial phosphatidylinositol (glycan)-specific phospholipase C (PIG-PLC)-released an insulin-mimicking substance which is a phosphoinositol-glucosamine-containing oligosaccharide. This is a structural motif that is also found in phosphatidylinositol-glycan anchors of many plasma membrane proteins. Because neither the PIG-PLC-released substance nor the parent phosphatidylinositol-glycan has been purified, its structure has not been elucidated and remains unsettled. However, based on susceptibility of the substance to specific enzymatic or chemical modifications the basic structure is a phosphatidylinositol in glycosidic linkage with a hexosamine-containing glycan. Based on incorporation of radiolabelled glucosamine it has been shown that the hexosamine is a glucosamine (Suzuki *et al.* 1991; Mato *et al.* 1987). The free insulin-mimicking oligosaccharide has usually been referred to as phospho-oligosaccharide (POS) or inositolphosphoglycan (IPG).

When cells are treated with insulin, IPG is generated by a phospholipase C activity within a couple of minutes and accumulates inside the cell (Stralfors, 1997). Hydrolysis of the parent glycolipid in response to insulin implies that a phosphatidylinositol-glycan-specific phospholipase C is present and can be activated by insulin receptors.

A prominent effect of insulin that does not appear to be controlled by IPG is

activation of glucose transport (Kelly *et al.* 1987), but diacylglycerol, which is generated in parallel with IPG, can stimulate glucose transport and may be a second messenger counterpart for control of this membrane transport function. Insulin activation of PIG-PLC activity has been demonstrated as the release of water-soluble IPG and the release of radiolabelled diacylglycerol (Suzuki *et al.* 1991; Saltiel *et al.* 1987) which is lipophilic and remains in the cell membranes. In addition, diacylglycerol has been shown to be produced from phospholipase C hydrolysis of phosphatidylcholine (Farese *et al.* 1992).

Addition of 1,2-diacylglycerol has been shown to stimulate glucose uptake in isolated adipocytes (Stralfors, 1988; Farese *et al.* 1993). 1,3-diacylglycerol was a poor activator of glucose transport (Stralfors, 1988). Long-chain diacylglycerols such as dimyristoyl, dipalmitoleoyl and dioleoylglycerol (exogenous) were most effective, while short-chain diacylglycerol such as dihexanoyl and dioctanoylglycerol (exogenous) were ineffective (Stralfors, 1988). In rat adipocytes, however, addition of short-chain diacylglycerols had no effect on glucose transport, while the addition of exogenous long-chain diacylglycerols activated glucose transport (Stralfors, 1988).

Insulin stimulation of glucose transport is known to involve the translocation of a specific insulin-regulated glucose transporter isoform (GLUT) from intracellular membrane depots to the plasma membrane. Stimulation of glucose transport in adipocytes by 1,2-dimyristoylglycerol appears to result from translocation of GLUT4 to the plasma membrane (Gustavsson *et al.* 1995). The mechanism of action of the 1,2-diacylglycerol is, however, not completely clear.

Together, the molecular pathways for insulin's signal transduction from its cell surface receptor to the cell's interior metabolic machinery remain in many ways unknown. Two molecules have been proposed as second messengers transducing the insulin signal into the target cell. One is a phosphooligosaccharide/ inositolphosphoglycan and the other is diacylglycerol, both deriving from the same plasma membrane glycolipid, which is hydrolysed in response to insulin treatment. To establishing these compounds as second messengers without any doubt, IPG needs to be purified and its detailed structure solved and the mechanisms of action of IPG and of diacylglycerol need to be identified (Stralfors, 1997).

1.11 Cytokines and glucose homeostasis

During the course of certain inflammatory processes and infectious diseases, particularly during the acute phase, the host reacts not only by mounting an immune response against the causal infective agent but also with endocrinologically mediated metabolic responses (Beisel, 1977; Miller *et al.* 1980; Powanda *et al.* 1980; Sidey *et al.* 1987). Microorganisms, their products or substances released on tissue aggression, could directly induce metabolic derangements in the host. Alternatively, factors released during the immune response could also mediate such alterations (Besedovsky *et al.* 1985). Because monocytes-macrophages are cells critically involved in early host defence, cytokines are likely candidates to elicit endocrine and metabolic responses.

Interleukin 1 (IL-1), an immune-derived cytokine released during these processes, is proposed to function as a mediator of such alterations, since

administration of low subpyrogenic doses of human rIL-1 to mice and rats produced hypoglycaemia. In mice, this effect was paralleled by increased insulin blood levels. When IL-1 was repeatedly injected, mice remained hypoglycaemic for at least 14 h after the last injection. Furthermore, these animals responded normally to a challenge with glucose, thus suggesting the proper function of the pancreas was preserved (Del Rey & Besedovsky 1987). These authors concluded that administration of IL-1 to mice leads to alterations of glucose homeostasis. They also reported that following injection of human recombinant tumor necrosis factor (TNF) to mice, neither hypoglycaemia nor changes in insulin levels were observed. In addition Satomi and co-workers reported that highly purified TNF does not induce hypoglycaemia (Satomi *et al.* 1985). However, in view of TNF's capacity to induce IL-1 production (Dinarello, 1985), it is not excluded that comparable endocrine and metabolic alterations could be observed at a later time after TNF administration.

TNF-α is an endotoxin-induced secretory protein, synthesized principally in the phagocytic cells of the immune system which plays an important role in both the immune and inflammatory responses (Vilcek & Lee, 1991) and also has many catabolic actions. In adipocytes, these catabolic actions include a decrease in the activity of lipoprotein lipase (LPL) (Fried & Zechner, 1989), a decrease in the expression of the glucose transporter GLUT4 and an increase in lipolysis, suggesting an increase in the activity of hormone sensitive lipase (Patton *et al.* 1986). TNF-α is expressed in adipose tissue, and is expressed at higher levels in the adipose tissue of obese rodents and humans (Hotamisligil *et al.* 1995; Kern *et al.* 1995). Therefore, the production of TNF-α by adipose tissue could be a local regulator of fat cell size,

and the overproduction of TNF in the adipocytes of obese mammals could limit adipocyte size through some combination of catabolic action (Saghizadeh *et al.* 1996). TNF is also expressed by both rat and human muscle, and is also expressed by muscle cells in culture.

Besides TNF-α, other cytokines, such as TNF-β, IL-1, IL-6 and interferon, also have profound effects on lipid metabolism (Grunfeld & Feingold, 1991).

CHAPTER TWO

MATERIALS AND METHODS

2.1 *In vitro* culture of *Plasmodium falciparum*

2.1.1 Introduction

Of the four species of malaria parasites that infect man, only *P. falciparum* can be maintained in long term continuous culture *in vitro*; while the intraerythrocytic stages of this parasite can readily be cultured. By continuous passage, all the other stages can only be maintained intermittently *in vitro* (e.g. sporozoites from mosquitoes can infect hepatocytes and can mature to the merozoite stage, gametocytes can readily transform to ookinetes, but have only occasionally been cultured to mature oocyts). *In vitro* cultivation provides a wonderful opportunity for investigations into the biochemistry, immunology and molecular biology of the erythrocyte stages of *P. falciparum*.

Despite many years of attempts to establish a continuous culture of malaria parasites, this was not achieved until 1976. In 1971, Trager developed a "flow vial" in an attempt to mimic more closely the natural environment of parasites *in vivo*. Some success was achieved, though parasites did not survive for more than two generations (Trager, 1971).

The first continuous culture of *P. falciparum* was reported by Trager and Jensen (1976). Their technique employed a modified flow-vial with RPMI 1640 medium supplemented with HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid) buffer and 10% human serum.

In 1977 the technique was simplified and adapted for use with a candle-jar. The culture being maintained in plastic Petri-dishes and the medium has to be changed at least once a day (Jensen and Trager 1977). These techniques often with minor modifications, are now universally employed for the maintenance of *P. falciparum in vitro*.

2.1.2 Culture system

All malaria culture work was carried out using standard aseptic techniques in a laminar flow cabinet. All containers such as culture flasks, centrifuge tubes, universal bottles and other plastic wares were presterilized by the manufacturers. Glassware and Eppendorf tubes and tips were sterilized by conventional autoclaving (120°C, 15 atmospheres, 30 min). Before use, media and reagents were sterilized by filtration through a 0.2 μm acrylic filter (Nalge Company, U.S.A.). Hands were rinsed regularly by 70% ethanol when working in the laminar flow safety cabinet to prevent contamination. In situations where mycoplasma-free culture were required, specific precautions were taken including the wearing of a mask and gloves.

2.1.3 Culture media

RPMI 1640 stock solution was prepared in a 1 litre volume by adding 10.43 g of RPMI 1640 powdered medium (Gibco, U.K.), and 2.0 g of sodium hydrogen carbonate (Sigma, U.K.) to double distilled water (ddH_2O). The solution was stirred continuously for 1-2 h on a magnetic stirrer. This stock medium was sterilised by filtering through a 0.2 μm filter (Nalge company U.S.A). The medium stock solution was stored in 500 ml aliquots at 4°C in tightly capped bottles for up to two weeks.

2.1.4 Culture serum

Human AB serum was kindly supplied by the North West Regional Blood Transfusion Centre, Liverpool. 100-250 ml bags of serum were supplied and, to reduce batch variation effects, 2-4 bags of serum were thawed at 37°C in a water bath, pooled and stored in 20 ml aliquots at -20°C until use.

2.1.5 Uninfected erythrocytes

Human O^+ whole blood was kindly supplied by the North West Regional Blood Transfusion Centre, Liverpool. This blood was supplied in citrate phosphate dextrose adenine and had been tested for anti-HIV and anti-hepatitis B antibodies. When received, the blood was aliquoted under aseptic conditions to sterile 250 ml culture flasks and stored at 4°C for up to three weeks.

The red blood cells were prepared for culture by centrifugation of whole blood (800 X g, 10 min). The serum and buffy coat were then removed using a presterilised unplugged Pasteur pipette and the remaining packed erythrocytes were washed three times by resuspending them in sterile RPMI stock solution. After each wash, the erythrocytes were sedimented by centrifugation (800 X g, 10 min) and the supernatant discarded. After washing was completed, erythrocytes were stored as packed cells at 4°C for up to three days.

2.1.6 Parasite cultivation procedure

Complete culture medium was prepared by adding to 100 ml of the stock solution of RPMI 1640, 2.5 ml of a 1 M HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesultfonic acid) (final concentration 25 mM) buffer solution (Sigma, U.K.) 10% human serum and 0.3 ml of a 50 mg/ml gentamycin sulphate solution (Sigma, U.K.). Complete medium was stored at 4°C for not more than one week. Cultures were maintained in presterilised tissue culture flasks (Costar, U.S.A). Infected erythrocytes at a parasitaemia of 0.1-5% were mixed with 5 ml complete medium in a 25 cm^2 tissue culture flask at a 10% haematocrit (i.e. 0.5 ml PCV for 5 ml).

Cultures were initiated by seeding a red cell/complete medium suspension with parasitised red cells from either another culture flask or from cyopreserved stocks to give the required haematocrit. Cultures were usually initiated at 0.1% parasitaemia.

The medium was changed every 24 h. Spent medium was removed aseptically

from above the static cell layer with a sterile unplugged Pasteur pipette linked to a vacuum pump. Pre-warmed fresh complete culture medium was then added in volumes of 5 ml to flasks of 50 ml and 15 ml to flasks of 200 ml capacities. New cultures were flashed for 30 second with a gas mixture consisting of 3% O_2, 6% CO_2, 91% N_2 (British oxygen company, U.K.). All cultures were regassed daily after changing the medium and incubated at 37°C.

Flasks were subcultured when the target parasitaemia had been reached ($\geq$10%). The subculturing procedure was as follows: fresh red cell/medium suspension at the required haematocrit was added to a new flask labelled with the name of isolate. Most of the medium was removed from the donor flask and the cell layer was resuspended in the remaining medium. A small sample of this suspension was used to seed the new culture flask at the required starting parasitaemia. The remainder of the original culture was either discarded, cryopreserved as described in section 2.1.8 or used for experiments.

2.1.7 Routine monitoring of parasitaemia:

Thin blood films were made from each culture flask by spreading a drop of packed erythrocytes on a new clean glass microscope slide. Films were air dried and then fixed in methanol. Slides were stained in a 10% solution of Giemsa stain (BDH, Ltd) in buffer pH 7.2 for 20 min. Blood films were then washed under running tap water, dried and examined under oil immersion at 100 X magnification on a light microscope.

The parasitaemia was calculated from the number of infected red blood cells per 1000 red blood cells.

2.1.8 Cryopreservation and retrieval of parasite

The cryopreservation and retrieval techniques used throughout these studies were based on the method of Diggs *et al.* (1975). This procedure for cryopreservation is as follows:

Cultures of high parasitaemia ($\geq$ 5%), predominantly at ring stage, were transferred aseptically to a sterile centrifuge tube and centrifuged (800 X g, 10 min). The supernatant was removed and fresh RPMI 1640 was added. Ring-stage infected blood was washed twice with RPMI to remove traces of serum and the first volume (i.e. 0.4 X PCV) of cryoprotective solution (Appendix 1) was added directly to the packed cells at a rate of 0.75 ml/min with continuous gentle shaking. The cells were left to stand for 5 min. After 5 min, 1.2 X PCV of cryo-solution was added at the same rate and the cells were left for a further 5 min. 2.4 X PCV of cryo-solution was then added, the cell suspension was shaken and 1 ml aliquot was pipetted into 1.8 ml sterile round-bottomed cryotubes (Nunclon, U.K.). These cryotubes were left at -70°C for 24 h in a polystyrene box (to allow slow freezing), then transferred to liquid nitrogen for long term storage.

Cryopreserved cultures were retrieved as follows:

Cryotubes were removed from the liquid nitrogen and thawed at 37°C in a water bath. The contents of the tube were then transferred aseptically to sterile 50 ml centrifuged

tubes and two volumes of the infected cells of 27% sterile sorbitol solution in buffer (Appendix 1) were added. The first volume was added over 8 min and the second volume over 5 min with continuous gentle shaking. The tubes were left to stand for 5 min and then two volumes of the infected cells of 5% sorbitol solution were added over 10 min with continuous gentle shaking. After leaving to stand for 5 min, the cells were centrifuged (400 X g, 10 min). The supernatant was removed and two volumes of 5% sorbitol solution were added over 8 min and the cells were left to stand 5 min before being centrifuged as before. The supernatant was removed and the pellet resuspended in 2 ml RPMI and recentrifuged. The supernatant was discarded and the pellet resuspended in 5 ml of complete culture medium made up to a 10% haematocrit with washed uninfected erythrocytes. The flask was gassed and placed in an incubator at 37°C. After 24 h, the culture was checked for parasites and the medium changed daily and the culture was checked for growth of parasites a few days later.

2.1.9 Synchronization of parasite cultures

Highly synchronous cultures were used throughout the work which was described in this project. Parasites were synchronised regularly by the method of Lambros and Vanderberg (1979). This technique selectively lyses the late stage parasites that are more permeable to sorbitol, causing them to swell and eventually lyse.

The culture with a high proportion of ring stage parasites was transferred

aseptically to sterile centrifuge tubes and centrifuged (800 X g, 10 min) and the supernatant was discarded. The pellets were resuspended in nine volumes of 5% sterile D-sorbitol solution in ddH_2O, incubating at 37°C for 10 min after shaking vigorously and centrifuged to deposit intact cells. These cells were washed with sterile RPMI 1640 and recentrifuged. The pellets were resuspended in complete medium and the suspension placed back into culture for 48 h. After 48 h, a thin blood film was prepared from the synchronized culture as described above. If the culture was still asynchronous, synchronization of parasite cultures were repeated to obtain highly synchronous cultures.

2.1.10 Parasite isolates

Four strains of *P. falciparum* were employed throughout this study. These isolates included two cloned laboratory lines and two wild isolates. Details of the strains can be found in table 2.1.

Table 2.1- The strains of *P. falciparum* which were employed during this study

Isolates	Source	Laboratory / Wild
3D7*	Dutch airport worker	cloned laboratory
K1*	Thai patient	cloned laboratory
CY16	Thai patient	wild isolate
CY27	Thai patient	wild isolate

In most experiments CY27 has been used for further characterisation of TMA
(* Strains 3D7 and K1 were kindly provided by Prof. D. Walliker, University of Edinburgh)

2.2 Preparation of Toxic Malaria Antigen (TMA)

2.2.1 Boiled culture supernatant

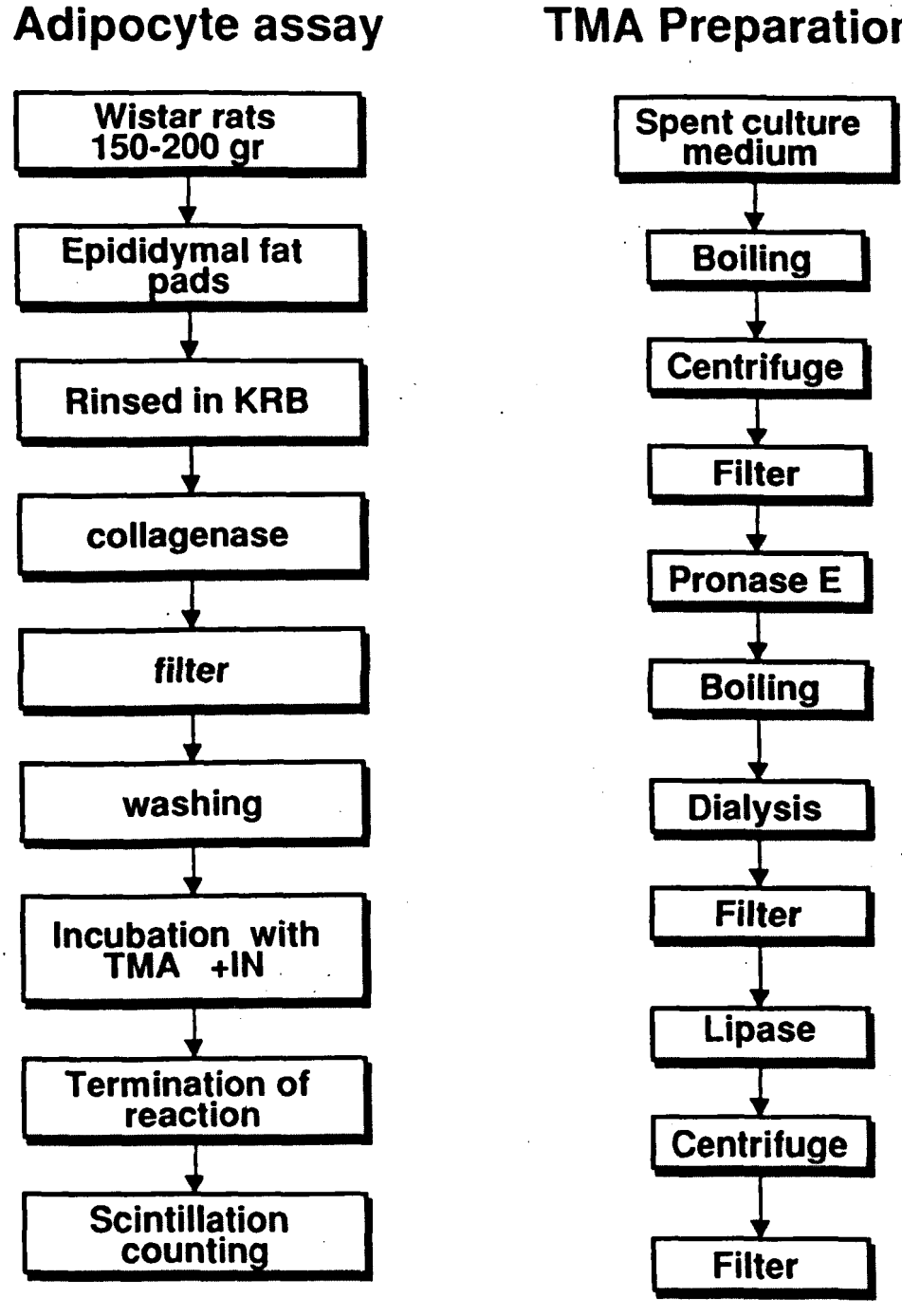
Highly synchronous parasite cultures were achieved as described in section 2.1.9. At ≥10% parasitaemia, the late trophozoite stage, parasites were incubated in complete medium at 37°C for 12-16 h. Before collection of spent culture medium, a blood film was prepared from the parasite culture to make sure all schizont stages of the parasite had ruptured and reinvasion had occurred. In this situation spent culture medium from the *P. falciparum* culture was collected. Whole culture was aseptically transferred to 50 ml sterile centrifuge tube and centrifuged (800 x g, 10 min). The supernatant was transferred to another sterile 50 ml tube and boiled for 10 min, this was recentrifuged at 20000 g for 10 min to remove denaturated proteins. The supernatant was then passed through a 0.2 μm filter and stored at 4°C for further use (Fig. 2.1).

A control culture was maintained in parallel with an infected culture, using uninfected red blood cell from the same batch. Spent culture medium from the normal erythrocyte culture was prepared as described above for the *P. falciparum* culture.

2.2.2 Boiled culture supernatant (pigment free)

Pigment was isolated from *P. falciparum* culture supernatant with a slight modification from that previously described by Pichgangkul and co-workers (Pichgangkul *et al.* 1994). Parasite cultures were synchronized by two treatments with

Fig. 2.1


Adipocyte assay
Wistar rats 150-200 gr
Epididymal fat pads
Rinsed in KRB
collagenase
filter
washing
Incubation with TMA +IN
Termination of reaction
Scintillation counting
TMA Preparation
Spent culture medium
Boiling
Centrifuge
Filter
Pronase E
Boiling
Dialysis
Filter
Lipase
Centrifuge
Filter

5% sorbitol (section 2.1.9). Late trophozoite stage parasites (≥10% parasitaemia) were cultured for 12-16 h at 37°C to allow parasite rupture. The parasite cultures were then centrifuged at 800 x g at room temperature for 10 min to remove erythrocytes. The supernatant was collected and centrifuged again at 20,000 X g for 45 min at 4°C to remove all pigment from supernatant. The supernatants were collected, boiled for 5 min and passed through 0.22-μm filter unites. Supernatant prepared in this way was called boiled supernatant free of pigment.

2.3 Enzymatic digestion

2.3.1 Digestion with pronase E

Boiled supernatant was incubated for 24 h at 37°C in 10 μg/ml of pronase E (Sigma, U.K.) under sterile conditions with continuous gentle shaking. After 24 h, the reaction was terminated by 5 min boiling of whole supernatant and centrifuged (20,000 X g, 10 min). The supernatant was dialysed using Visking Dialysis Tubes size 2 (Medicall International Ltd) against phosphate buffered saline (PBS pH 7.4) for three days (changing of PBS every day). No protein was then detectable by the Bio-Rad assay (limit of detection is <1 μg/ml). The pronase digested samples were sterilized by filtering through 0.22 μm filters and stored at 4°C (Fig. 2.1).

2.3.2 Digestion with lipase

Pronase digested supernatants were incubated overnight at 37°C with 5 U/ml

of lipase (wheat germ lipase bound to agarose beads, Sigma, U.K.) with continuous gentle shaking. The enzyme reaction was terminated by centrifugation at 800 X g, 10 min and the supernatant was filtered through a 0.22 μm filter and stored at 4°C for further use (Fig. 2.1).

2.4 Preparation of malaria antigens from the serum of infected monkeys

Splenectomized squirrel monkeys (*Saimiri sciureus*) were infected with *P. falciparum* , *P. brazilianum*, and *P. vivax*. Serum was collected at different levels of parasitaemia from 0.1%- 46% and was stored at -20°C between 1980-1995. All serum samples were provided by Professor M. Hommel. Serum samples were diluted 1:3 with dH_2O and boiled for 5 min, then centrifuged at 20000 X g for 10 min to remove denatured proteins. The samples were passed through a 0.22 μm filter and stored at 4°C for further analysis.

2.5 Rat adipocyte preparation

Male Wistar rats, weighing 170-250 gr were used in this study. They were fed on standard or CRM diet (Appendix 3). The animals were kept at constant light and temperature. The rats were killed by dislocation of the neck. The epididymal fat pads were immediately remov2ed under sterile conditions with as little handling as possible. They were rinsed in sterile warmed Krebs-Ringer bicarbonate (KRB) buffer

(37°C) containing 1% BSA (Appendix 1), then cut into a few small pieces.

Fat cells were isolated by a modification of the method originally described by Rodbell (1964). The minced tissues were transferred to a sterile 50 ml centrifuge tube containing, sterile collagenase (1.5 mg/ml), 1% Bovine Serum Albumin (BSA, Sigma, U.K.), 0.1 ml glucose (0.5 M glucose at 5 mM final concentration) and KRB buffer (Appendix 1). This mixture was gassed (3% O2, 6% CO2, 91% N2) for 5 min and then shaken for 30 min at 37°C. Isolated fat cells were separated from undigested tissue by sterile filtering through a nylon mesh (10XX) (Fig. 2.1).

The suspension of cells was centrifuged in sterile 50 ml centrifuge tubes for 5 min at 60 g. The fat cells floated to the surface and the stromal-vascular cells (capillary, endothelial, mast, macrophage and epithelial cells) were sedimented. The stromal-vascular cells were removed by aspiration and the fat cells were washed by suspending them in 10 ml of pre-warmed glucose free KRB buffer containing 1% BSA (Appendix 1). This procedure was repeated four times. For a set of experiments, fat cells were usually obtained from the pooled adipose tissue of 2-3 rats. After a final wash, cells were suspended in sterile pre-warmed washing buffer.

2.5.1 Primary culture of isolated adipocytes

The primary culture of isolated adipocytes was carried out using standard aseptic technique in a laminar flow cabinet. All containers, medium, washing buffer and incubation buffer were sterilized.

Adipocytes were isolated as described above under sterile conditions. Freshly prepared adipocytes were added to 1.5 ml of sterile KRB containing 1% BSA and 25 mM HEPES (final concentration), 5 mM glucose and the indicated concentration of bovine insulin with or without a 100 μl aliquot of Toxic Malaria Antigen (TMA) preparations at a final concentration of 2-3 X 10^5 cells/ml in 25 cm^2 culture flask. When total lipid, polar lipid or diacylglycerol (DAG) was used, the lipid preparation was resuspended in PBS-chloroform. Final concentration of chloroform (0.5% v/v) did not influence cell viability or morphology.

Cells were incubated at 37°C for various times between 60 min to 24 h in the absence (control) or in the presence of TMA or insulin, or a combination of both (Fig. 2.1). All flasks were gassed for 60 sec (3% O_2, 6% CO_2, 91% N_2) and then transferred to a 37°C incubator and shaken gently (50-100 cycles/ per min) for 60 min, unless otherwise stated. Between 2-3 replicate flasks were incubated in each experiment.

2.6 Lipogenesis assay

Lipogenesis in isolated rat adipocytes was measured by the incorporation of [U-^{14}C]-glucose into labelled lipid. Freshly prepared cell suspensions were transferred to a sterile polystyrene 25 cm^2 flask suspended in 1.5 ml vols of KRB, 1% BSA, 5 mM [U-^{14}C]-glucose pH 7.4 (Appendix 1), for 1-24 h at 37°C. The assay was terminated by the addition of 5 ml vols of propan-2-ol/hexane/0.5 M H_2SO_4 (Dole, 1956) (Appendix 1).

2.6.1 Routine extraction of total lipid of adipose cells

The total lipid was extracted into hexane by adding 3 ml hexane and 1.5 ml water and this was vortexed for 60 sec. The samples were then allowed to stand for at least 10-15 min to permit two sharp layers to develop.

The top layer, which contained the lipids in hexane, was transferred to a wash tube containing 7.5 ml dH_2O and then well mixed. After separation of the two phases, 2.5 ml of the top separation was transferred to 5 ml "cocktail T" scintillation fluid (BDH, U.K.), then mixed and counted, using a LKB Rackbeta 1211 scintillation counter (Fig. 2.1).

2.6.2 Blanks, controls and counting standards

Blanks were prepared by adding cells to the medium containing the [U-^{14}C]-glucose and stopping the incubation immediately. Controls consisted of cells incubated in complete medium without test agents and insulin (basal) along with the flasks containing cells in complete medium and test agent.

The counts recorded were corrected for the scintillator background and control. The blanks and controls have been treated in the same way as samples.

2.7 Lipid extractions

2.7.1 Lipid extraction from *P. falciparum* culture supernatant

The boiled supernatants of *P. falciparum* culture were prepared as described in section 2.2.1. Total lipids were extracted from boiled supernatant of *P. falciparum* cultures and normal erythrocyte cultures by using a chloroform/methanol (2:1 v/v) extraction (Goad *et al.* 1985). The lipid was extracted by stirring the above mixture 2-3 h. The organic phase was washed with saturated NaCl in ddH_2O. The chloroform layer was removed, rotary evaporated and the total lipid extract was redissolved in a known volume of cyclohexane (200-500 μl) and kept at -20°C. The aqueous phase was used for a second extraction (1 h) with chloroform/methanol (2:1 v/v) as mentioned above. The remaining aqueous phase was collected and kept at -20°C for further analysis.

2.7.2 Extraction of lipid from human erythrocytes

Extracts of total lipids were prepared with a slight modification from that previously described by Folch *et al.* (1957). Fresh whole blood was washed three times with RPMI to remove white cells and serum and then 5 ml of packed cells were extracted with 150 ml of chloroform/methanol (2:1 v/v). The appropriate volume of methanol was first added to cells and the chloroform was then added separately. This procedure reduced aggregation of individual red cells into a large mass. The extraction was carried out overnight. Any precipitated protein and aggregated cells

were removed by filtration through Whatman paper No.1. The recovered cell debris was mixed with chloroform/methanol (2:1 v/v) for a second extraction (2 h). The chloroform solutions from the first and second extractions were pooled and any remaining precipitated protein was removed by filtration through a sintered glass funnel. The chloroform was removed by rotary evaporation and the residue redissolved in a known amount of chloroform/methanol (1:1 v/v) and kept at -20°C for further analysis.

2.8 Isolation of individual lipids by Thin Layer Chromatography (TLC)

Thin layer chromatography is one of the most effective techniques for separation of intact complex lipids (phospholipids, glycolipids, etc.).

Although the more modern technique of high performance liquid chromatography (HPLC) has many applications in lipid analysis, several features still make TLC particularly useful for this purpose. For example, good results on a quantitative basis can be obtained relatively simply and rapidly and many samples can be analyzed simultaneously alongside standards. The separated classes can be visualized and samples can be recovered for further analysis.

2.8.1 General TLC procedure

Basically, a lipid mixture is applied to an absorbent coated in a thin layer on

an inert support (glass or aluminium). The point of sample application is termed the origin. The mixture is then resolved into its components by different migration, as a stream of solvent passes through the layer of adsorbent by capillary action. In a given solvent system each lipid component has a characteristic mobility that can be described as its Rf value. The Rf value is defined as the distance travelled by the component divided by the distance travelled by the solvent front, both distances being measured from the origin. Since many lipids are colourless, the separated lipid components have to be rendered visible by chemical reagents.

2.8.2 Preparation of TLC plates

For the self preparation of chromatographic layers, adsorbents were purchased in the form of powders. Silica gel G contains $CaSO_4$ binder (Keiselgel 60G). TLC plates were prepared as followed:

1) Clamp clean glass plates (20 cm X 20 cm X 0.5 mm) into the spreading rack and wipe with acetone.
2) Place the adsorbent applicator on top of the plates at one end of the rack, with the gap corresponding to the thickness of the final layer (0.5 mm in this study) next to the glass.
3) Shake 40 g silica gel G vigorously with 80 ml distilled water in a stoppered flask to form a slurry, and pour quickly into the reservoir of the applicator.
4) Spread the slurry smoothly across the top of 5 plates to the other edge of the rack.
5) Allow plates to sit in rack for 2-5 min until gel has started to form.

6) Carefully remove the coated plates from the spreading rack and leave for 15 min at room temperature, then place in oven at 120°C for 1 h to activate.

7) Remove plates and store in airtight box until required.

8) If stored for a few days the plates can be reactivated by heating at 120°C for 30-60 min before using.

Pre-coated silica gel on aluminium sheets (20 cm x 20 cm x 250 μm) (Merk) were also used during this study for analytical and preparative work.

2.8.3 Development tanks

For the development of 20 X 20 plates, rectangular glass tanks of approximate dimensions 10 X 21 X 21 cm are commonly used. The chromatography tank has a heavy glass lid which acts to seal the tank and therefore maintain an atmosphere saturated with solvent vapour within the tank.

2.8.4 Pre-washing

For best analytical results, the TLC plate should be pre-washed before use to remove impurities that could a) darken the background in the visualization process, b) cause a dark line along the solvent front, c) contaminate compounds eluted from the plates in preparative TLC procedures. This is done by developing the plate to the top in diethyl ether and then allowing it to dry in the fume hood before use.

2.8.5 Sample application

Care must be taken not to damage the adsorbent layer during this procedure, and TLC plates should only be handled by the edges, to avoid contamination with lipids from the fingers. For analysis by standard 20 X 20 cm TLC the sample should ideally be applied at a distance of approximately 2 cm from the lower edge of the plate. A spot of diameter not more than 2 mm, or a narrow streak of 5 to 10 mm lengths, both give good resolution. Samples can be identified by lightly writing with a pencil at the top of plate above where the solvent front will be.

The sample is applied in as non-polar a solvent (e.i. cychlohexane) as possible, however, for some lipid samples their solubility requires the use of chloroform/methanol (2:1 v/v). Methanol alone should not be used for sample application as it is a polar solvent and tends to produce large spots and wide streaks.

2.8.6 Standards

Lipid classes can be identified by comparison of Rf values of samples to standards. The application of lipid standards, alongside the lipid being analyzed, greatly aids in the identification of the components present in the lipid sample. Within any laboratory, the Rf values of lipid classes in a given solvent system are not always constant due to day-to-day variations in temperature, humidity and perhaps even the batch of plates used.

2.8.7 Development of plates

After the application of samples and standards, the plate was transferred to a development tank containing sufficient solvent to cover the adsorbent up to a level of about 5 mm from the bottom edge. The solvent system to be used should be added to the tank about 30 min before the plate is introduced. The component solvent should be thoroughly mixed.

When solvent systems containing large proportions of polar solvents such as methanol are employed, the tanks can be lined with filter paper to help saturate the atmosphere. This will prevent the so-called edge effects occasionally observed in TLC, which arise from non-uniform saturation of the tank atmosphere. However, with non-polar solvents, the lining of tanks is not always necessary.

2.8.8 Neutral lipid separation

The total lipid extract was fractionated into lipid classes using thin layer chromatography. Thin layer plates were prepared as described in section 2.8.3. The total lipid extract was applied to the plate and the plates were developed in petroleum/diethyl ether/acetic acid (40:10:1 v/v/v) (Rolph *et al.* 1989). Phospholipid, sterol, free fatty acid, triacylglycerol and steryl ester bands were identified using co-chromatography with authentic samples, at Rf values of 0, 0.15, 0.25, 0.40, 0.60 and 0.90, respectively. The plates were visualised by spraying with 0.005% berberine in ethanol and looking under U.V. light at 366 nm. The appropriate bands were scraped

from the plates and the phospholipids were eluted from the silica gel using chloroform/methanol (1:1) under vacuum; sterols, triacylglycerols and steryl esters were eluted in diethyl ether.

2.8.9 Separation of phospholipids into classes

Most of the TLC systems described for the separation of polar lipid are based on the use of chloroform and methanol as major components of the developing system. In this study for separation of the phospholipids a variety of solvent systems (detailed below) were employed to obtain the best separation of the phospholipids into the major phospholipid classes using a standard phospholipid mixture containing PC, PE, PG, PI, PS as phospholipid markers. The plates were allowed to develop and phospholipid bands were identified using co-chromatography with the individual markers of the different phospholipid classes. Also a variety of visualising sprays were used to identify the phospholipid classes.

2.8.10 Solvent systems

The following solvents were used for separation of the phospholipids and glycolipids classes by one-dimensional TLC:

a) Chloroform/methanol/water (65:25:4 v/v/v) (Lepage 1964)

The above solvent (100 ml) was added to the tank about 30 min before the plate is introduced, to allow the atmosphere in the tank to become saturated. The Rf values obtained were as follows PC, 0.40; PE, 0.7; PG, 0.6; PI, 0.35; PS, 0.30;

PA+DPG run together at a Rf near to 1.0.

b) Chloroform/methanol/water (137:20:6 v/v/v)

The solvent (100 ml) was placed into a covered TLC tank and allowed to equilibrate for 30 minutes to allow the atmosphere in the tank to become saturated. The Rf values obtained were as follows: PC, 0.30; PE, 0.63; PG, 0.48; PI, 0.11; PS, 0.22; PA, 0.63; DPG, 0.8.

c) Chloroform/methanol/water (65:35:5 v/v/v), Chloroform/methanol/water (25:75:5 v/v/v) were also used. The best separation was obtained with chloroform/methanol/water (65:25:4 v/v/v) as solvent system.

2.9 Visualising sprays (detection systems)

After development, the plates must be treated or stained by some method to reveal the position of lipids. In general, this staining can be divided into two main categories; general stains that will enable virtually all lipids to be visualised nonspecifically and specific stains that will only stain certain types or classes of lipids.

Some of the stains contain corrosive materials, such as strong acids so specially spraying, must be done in a suitable fume cupboard.

2.9.1 General stains

a) Sulphuric acid (20% v/v)

Plates can be sprayed with simple solutions of strong oxidizing acids such as sulphuric acid (20% v/v). This method can detect as low as 1μg of lipid, but the method is destructive, i.e. the samples will be destroyed during the visualization process.

Sulphuric acid (20% v/v in water) was the most often used during this study. Plates were sprayed with sulphuric acid solution, then heated in an oven at 180°C for times ranging from 15-30 min and lipids appeared as brown/black.

b) Berberine hydrochloride (Sigma, U.K.)

A 0.005% solution in ethanol (w/v) yielded a spray that is nondestructive and therefore suitable for preparative work. Lipids appeared as fluorescent green spots when viewed under ultraviolet light at 366 nm.

2.9.2 Specific stains

These spray reagents (Henderson & Tocher, 1992), generally involve a chemical or chemicals in the reagent reacting with specific group in the lipids that results in the lipid being stained or made visible in some way.

a) **Phospholipids**

Molybdenum blue (1.3% molybdenum oxide in 4.2 M sulphuric acid) was used to detect phospholipids. Plates were sprayed with molybdenum blue, dried and heated at 110°C-120°C. Phospholipids (1-2 μg) appeared as blue-green spots.

b) **Glycolipids**

Orcinol-sulphuric acid reagent (200 mg orcinol (Sigma, U.K.) in 100 ml 75% sulphuric acid) was used for the visualization of glycolipids. Glycolipids appeared as blue/purple spots on a white background after spraying and heating the plate at 100°C for 10-15 min. The reagent is stable for one week if kept in the dark and refrigerated.

2.10 High-Performance Liquid Chromatography (HPLC)

HPLC is a major technique used for lipid analysis. In this study, HPLC was employed for the separation of phospholipids into the individual phospholipid classes.

TLC has advantages and disadvantages compared to HPLC: TLC is a very simple and cheap technique, but it is difficult to be quantitative. In addition, during TLC, the lipids are exposed to the air on the dry support surface and may undergo oxidation. However, using HPLC the sample is always in solution and thus not exposed to the atmosphere in dry form so reducing the risk of oxidative degradation.

2.10.1 Equipment

The basic equipment for HPLC consists of a high pressure pump-solvent delivery system with microprocessor control and a stainless steel column and injector system, a suitable detector, a fraction collector and a recorder plus integrator.

2.10.1.1 Mobile phase, Columns, Detectors

The actual choice of the mobile phase will be decided by the nature of the sample and the type of chromatography being employed, but for lipid analysis in general organic, rather than aqueous, solvents are usually used.

In this study HPLC was done on kontron T-414 LC pump fitted with a 5 μm silica column (250 X 4.6 mm Altech HPLC Column Eco-nosphere C18 5 micron). A LKB 2238 unicord SII ultra-violet detector set at 205 nm was employed.

2.10.1.2 Solvent system

This was adapted from Patton *et al.* (1982) to separate all major phospholipid classes. The solvent mixture was propan-2-ol/hexane/ethanol/potassium phosphate buffer/acetic acid, and was mixed as described below.

The hexane, propane-2-ol, ethanol and acetic acid were of HPLC grade; the potassium phosphate buffer (pH 7.0) was made using double deionised redistilled

water (ddH_2O). Propanol-2-ol (490 ml) was mixed with the hexane (367 ml). 25 mM potassium phosphate buffer (pH 7.0)(62 ml) was added to 100 ml absolute ethanol and this was mixed with the propan-2-ol/hexane mixture. This solvent mixture was degassed under vacuum through a 0.5 μm filter using a millipore filtration apparatus. Glacial acetic acid (0.6 ml) was added to solvent mixture after filtration. The final solvent composition is hexane-2-propanol- 25 mM phosphate buffer-ethanol-acetic acid (367:490:62:100:0.6 v/v/v/v/v).

2.10.2 Lipid application and phospholipid separation

Lipid was extracted from *P. falciparum* culture supernatant and NRBC culture supernatant as described in section 2.7.1. The extract was dried under nitrogen (N_2) and redissolved in hexane-2-propanol-water (40:54:6 v/v/v) for HPLC of phospholipids (50 mg total lipid/500 μl solvent). Total lipid (10 mg/100 μl) was injected into a 100 μl Rheodyne loop. The individual phospholipid classes were collected and stored at -20°C until required for analysis. The order of elution of the phospholipid classes was authenticated by injecting PC (l-α-phosphatidylcholine dilinoleoyl 18: 2,[*cis*]9-12 Synthetic Sigma, U.K.) PI (l-α-Phosphatidylinositol from bovine liver Sigma, U.K.), both at 20 μg/100μl of solvent, on a mixture of both PC and PI (10 μg each/100 μl). The flow rate was 0.5 ml/min at the start and then changed to 1.0 ml/min after 60 min. The PI standard was eluted with flow rate 0.5 ml/min after 30 min and PC standard was eluted with flow rate 1.0 ml/min after 90-100 min.

2.11 Column chromatography

Chromatography of lipids on a column of suitable material is a useful procedure for fractionation of lipid mixtures on a preparative scale.

2.11.1 General fractionation procedure on a silica gel column chromatography

In adsorption chromatography, compounds are bound to the solid adsorbent by polar and ionic forces and to a smaller extent by non-polar or Van der Wals forces. Separation of lipid mixtures thus takes place according to the relative polarities of the individual lipids.

Several types of adsorbents have been used in column chromatography of lipids. Of these, the most effective adsorbent is silica gel.

Materials:

A suitable commercial grade of silicic gel (Kieselgel 60 Merk 70-230 mesh), and reagent-grade chloroform-methanol and distilled water have been used. A glass column, 3.0 cm (inside diameter) by 50 cm (length) was used with a small plug of glass wool at the bottom of the tube to retain the adsorbent and there was a Teflon stopcock in the end of column.

2.11.2 Column preparation

A slurry of 20 gr silica gel was prepared in a 100 ml beaker in about 30-50 ml of chloroform. The slurry was poured into the 3 X 50 cm chromatography column, and the stopcock opened and the tube tapped gently to dislodge air bubbles and aid in settling of the silicic acid in the column. The solvent level was allowed to drop to the top of the silica gel, and the bed was then washed with 3 column volumes (36 ml) of chloroform. The height of the column was about 12-14 cm. The column was left for two hours before use in order to allow for settling of silica gel in the column.

2.11.3 Application of samples and elution of column

With the solvent level at the top of the silica gel a solution of about 200 mg of total lipids in 5 ml chloroform was added (Pasteur pipette) down the sides of the column. The solvent was allowed to run into the column and the chloroform washing (1-2 ml) of the container was also applied to the column to ensure quantitative transfer of lipid mixture to the column.

The column was eluted at a flow rate of about (3 ml/min) with the following solvents:

Solvents	Volume	Fractions
1) chloroform	150 ml	1
2) chloroform	150 ml	2
3) 90% chloroform + 10% methanol	200 ml	3
4) 80% chloroform + 20% methanol	200 ml	4
5) 65% chloroform + 35% methanol	200 ml	5
6) 50% chloroform + 50% methanol	200 ml	6
7) 30% chloroform + 70% methanol	200 ml	7
8) 30% chloroform + 70% methanol	200 ml	8
9) 100% methanol	200 ml	9
10) 90% methanol + 10% ddH_2O	200 ml	10

The solvents were removed by rotary evaporation and lipid fractions were redissolved in a known volume of chloroform/methanol (1:1 v/v) and kept at -20°C.

2.11.4 Composition of column fractions

a) Chloroform eluate: for the lipids of most animals, plants and microbial tissues this fraction contains the neutral lipids: i.e., sterols, steryl esters, fatty acids, cholesterol.

b) Methanol eluate: with lipids from all sources, this fraction contains mostly phosphatides with only traces of glycolipids. In this study, we used a mixture of chloroform/methanol to elute different polar lipids according to their polarity. With an increased methanol as elution solvent, more polar lipids were eluted. In fraction 10 the eluting solvent was 90% methanol/10% dH_2O (9:1 v/v), which was to elute more polar lipid than 100% methanol. Each of the above fractions was further fractionated by analytical TLC. Lipid spots were detected with general and specific stains as described in section 2.11.1 and 2.11.2 with sulphuric acid, Molybdenum

blue and orcinol-sulphuric acid, respectively.

2.12 Analysis of phospholipids

The phospholipids were further analyzed by hydrolysis and transmethylation of the fatty acids located at the *sn-1* and *sn-2* positions of the molecules. The fatty acid methyl esters (FAMES) were analyzed by gas chromatography (GC) (section 2.14.1 and 2.14.2).

2.12.1 Sulphuric acid in methanol

A 2.5% solution of sulphuric acid in methanol was freshly prepared for each experiment. This was added (2.5 ml) to the phospholipid extracts blown to dryness in a screw cap (with Teflon lining) methylating tube and the reaction was incubated at 70°C for two hours. The solution was then cooled and neutralised by addition of a solution of 5% sodium chloride saturated with hydrogen carbonate (5 ml). The fatty acid methyl ester (FAMES) were partitioned into hexane (3 X 2 ml). The hexane extract was reduced to dryness under nitrogen and the FAMES were redissolved in 50μl hexane and analyzed by Gas Chromatography (GC).

2.12.2 Gas Chromatography

The GC analysis of FAMES acids was carried out on a Hewlett Packard (5800 series II 5890) gas chromatography coupled to a Spectra Physics Integrator (SP 4270).

The FAMES were analyzed on a Hewlett Packard carbowax column (0.33 mm film thickness X 0.33 μm internal diameter X 25 m length), by injecting on to the column at 50°C followed by a slow heating rate of 10°C/min to a final temperature of 220°C which was held for 20 min. The FAMES of interest emerged between 10-15 min.

2.13 Mass spectrometry (MS)

Mass spectrometry is used for the structure elucidation of lipids, and their detection and quantification at trace levels. A mass spectrometer (MS) is a device for producing and mass measuring ions. Mass spectrometry is unique in its ability to yield often complete structure assignments without needing to use other physico-chemical techniques. The complexity of the mass spectrometric instrumentation has meant that analyses have traditionally been conducted by highly-trained operators. In the conventional mode of mass spectrometry (MS), the compound in the gas phase is ionized by bombardment with electrons in the ionization chamber. The resulting mass spectrum consists of a characteristic pattern of peaks representing molecular fragments with different mass to charge (*m/z*) ratios, produced by electron bombardment and/or by "cracking" during volatilization of the compound. Some of these peaks or patterns of peaks are structurally diagnostic.

Liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) analyses were kindly performed by Mr Mark Prescott in the School of Biological Sciences, Liverpool University.

For the LC-MS work the HPLC reverse phase column employed was ODS2, 5μ (4.62cm x 250mm) supplied by Waters Ltd. The column was eluted with propionitrile/hexane (9:1 v/v) at a flow rate of 0.8ml/min. The LC-MS instrument comprised a Waters 600MS solvent delivery system coupled to a VG Quattro mass spectrometer. The ionisation was by Atmospheric Pressure Chemical Ionisation (APCI) in the positive ion mode. The source temperature was 120°C; vaporiser 200°C; discharge voltage 3.4kV; cone voltage 90V; nebuliser gas: nitrogen at 100p.s.i. (7bar); mass range *m/z* 200 - 700; scan time 3 secs.

For the GC-MS analytical work the column used was a 25m BP-1 capillary column supplied by SGE. The equipment was a Hewlett-Packard gas chromatography, Model 5890 coupled to a Hewlett-Packard quadrupole mass analyser, Model 5970 with a Model 9144/9153C data handling computer system. The samples were applied to the column by on column injection at 50°C and the GC oven was then programmed at 25°C/min to 150°C and the at 6°C/min to a final oven temperature of 290°C; the carrier gas was helium at 12p.s.i. Mass spectra were recorded in the EI positive ion mode over the range *m/z* 50 - 750. The 1,2-DAG samples for analysis by GC-MS were converted to their trimethylsilyl (TMS) ethers derivative by dissolving in 50μl pyridine and adding 50μl BSTFA [N,O-bis (trimethylsilyl) trifluoroacetamide] followed by warming at about 60°C for 20-30 minutes before injection of 1-3μl onto the GC column.

2.14 Treatment of fractions 3,4 and 7 eluted from silica gel column chromatography with phospholipase C (PLC)

Fraction 7 (eluted from a silica gel column chromatography) contained polar lipids that co-migrated with PI-PC standards. TLC and molybdenum spray showed that this fraction contained phospholipids. Because of the high activity of these fractions with the adipocyte assay and the large amount of material compared with other fractions, this fraction was chosen for further characterization with PLC enzyme. Fraction 3 and 4 which mostly contain glycolipid (possibly monosugar) were also hydrolysed with PLC enzyme.

2.14.1 Procedure

Reagents were added in the following order and to give a final volume of 5 ml: 2 ml of a 0.25 M Tris-HCl pH 7.3 was added to

0.5 ml of 63 mM calcium chloride,

0.1 ml serum albumin (1.3 mg/ml, fatty acid free, Sigma, U.K.),

0.5 ml of 0.7 M ammonium sulphate,

1.4 ml ddH_2O as the incubation buffer,

and 4 mg of fraction 7 was redissolved in 0.5 ml absolute ethanol then added to the incubation buffer. The final concentration was 100 mM Tris-HCl, 6.3 mM calcium chloride, 0.026 mg/ml bovine serum albumin, 70 mM ammonium sulphate. To the 5 ml final volume 40 units (10 μl) of PLC was added with shaking 100 cycle/min at 37°C overnight. The enzyme reaction was stopped by 5 min boiling

followed by extraction of the lipids with chloroform/methanol (2:1 v/v) as described in 2.7.1. The aqueous phase was reduced to dryness under nitrogen and samples redissolved in a known volume of chloroform (200-500 μl) and kept at -20°C.

2.14.2 Isolation of individual lipids

The lipid extract from above was fractionated into lipid classes using thin layer chromatography. Thin layer plates were purchased from Merk.

The total lipid extract was applied to the plate and the plate was developed in chloroform/absolute ethanol (98.4:1.6 v/v) for 30 min. The standards were SE, 1,3 DAG, 1,2 DAG, FS, PC and PI.

This solvent system separated diacylglycerol from sterols. The lower and origin bands were eluted first with chloroform/methanol (1:1 v/v) then with diethyl ether (both samples were pooled) while the top bands, including diacylglycerol were eluted in diethyl ether. All fractions were redissolved in a known volume of chloroform and kept at -20°C for bioassay and analysis.

2.15 Preparation of lipid fractions for bioassay

During this study different lipid fractions from TLC plates, HPLC and column chromatography have been tested in the adipocyte assay (described in section 2.5 and 2.6).

Neutral lipid was redissolved in cychlohexane and polar lipid in chloroform/methanol (1:1) and diacylglycerol in chloroform. The 1,2-DAG (50 μg in 50 μl chloroform) which was under investigation was mixed with 450 μl sterile PBS (pH 7.4) or 2 mM of sodium taurodeoxycholate (NaTDC) and vortxed for 1-2 min or sonicated for 1-2 min. An aliquot (100 μl) of this emulsified mixture was added to the adipocyte assay (final volume 2.0 ml) to give a final concentration of 1,2-DAG of 5 μg/ml. The total lipid extract and polar lipid samples were prepared for bioassay the same way as mentioned for 1,2-DAG.

2.16 Inhibition of glucose transporter by cytochalasin B

Cytochalasin B was purchased from Sigma (U.K.) and dissolved in dimethyl sulphoxide (DMSO). Consequently all control incubation mixtures contained 1% (v/v) DMSO, the same concentration as in the incubation mixtures with cytochalasin B. This amount of DMSO did not effect glucose metabolism.

Isolated fat cells suspensions were incubated with 3 x 10^{-9} M insulin, 50 μl/ml of TMA preparation or combination of both with 2 μg/ml cytochalasin B in zero time and cells were incubated at 37°C for 2 h (synergy with insulin) or 24 h (stimulation of lipogenesis). In control culture flask, the fat cells incubated with only 2 μg/ml of cytochalasin B.

2.17 Monoclonal antibody production

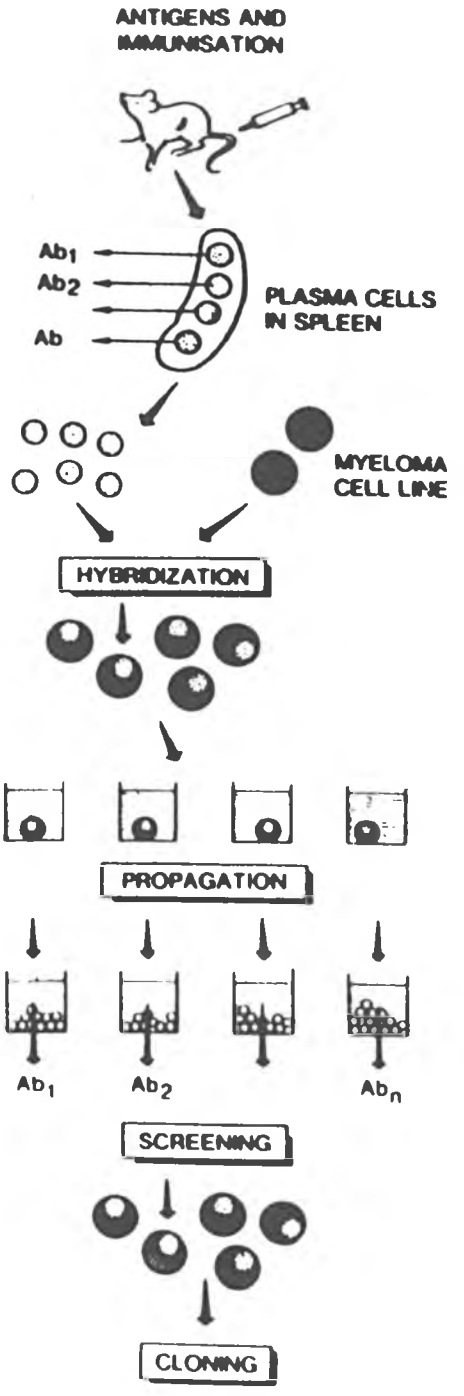
2.17.1 Introduction

Serum contains many different types of antibodies that are specific for many different antigens. The use of these mixed populations of antibodies (polyclonal) creates a variety of different problems in immunochemical techniques. Kohler and Milstein (1975) developed a technique for producing a homogeneous population of antibodies of known antigen specificity. They did this by fusing B lymphocytes from an immunized mouse to mouse myeloma cells to produce hybrid cells that both proliferate indefinitely and secrete a monoclonal antibody specific for the antigen used to immunize the donor mouse (Fig. 2.2). Spleen cells are generally useful as a source of lymphocytes. After fusion, the hybridomas producing antibody of the desired specificity are identified and cloned by growing the cultures from single cells. Since each hybridoma is a clone derived from a single B cell, all the antibody molecules produced are identical in structure, including their antigen binding site. Such antibodies are therefore called monoclonal antibodies. Monoclonal antibodies are now used in most serological assay, as diagnostic probes, and as therapeutic agents.

2.17.2 HAT selection

Cell fusion for the immortalisation of spleen cells requires a means of selection for mixed hybrids (Myeloma-spleen cell hybrids). The favoured selection procedure depends on the capacity of cells to use the "Salvage" pathway for

Fig. 2.2 Monoclonal antibodies production



guanosine production when the main *de novo* biosynthetic pathway is blocked (usually by the antibiotic aminopterin). This salvage pathway relies on the presence of the enzyme hypoxanthine guanine phosophoribosyl transferase (HGPRT). Cells lacking the enzyme will die when grown in media containing aminopterin thymidine because both pathways necessary for the formation of the purine precursors of DNA are then blocked. However, a cell which contains a non-functional HGPRT will grow in the selection medium if fused with a cell containing a functional HGPRT. Myelomas which are $HGPRT^-$ are needed for successful selection of myeloma ($HGPRT^-$) and spleen cell ($HGPRT^+$) hybrids in the hypoxanthine-aminopterine-thymidine (HAT) medium. Selection of $HGPRT^-$ cells is carried out in the presence of the toxic base analogous, 8-azaguanine which are incorporated into DNA via HGPRT.

2.17.3 Preparation of antigen and mouse immunization

The classes of molecules and structures most commonly used as immunogens cover an enormous range of natural and synthetic materials including proteins, nucleic acids, carbohydrates, lipids, bacteria, viruses etc. The amount of antigen necessary to induce a good immune response will depend on the individual antigen and host animal.

In this study, inbred Balb/c strain mice (male, 6-12 week old) have been used. Two different routes of injection were used;

a) Subcutaneous injection (SC):

P. falciparum boiled supernatant was extracted as described in section 2.7.1 and polar lipid was separated by TLC with the solvent system petroleum/ether/diethyl ether/acetic acid (40:10:1) as described in section 2.9.

The polar lipid extract was used as antigens for production of monoclonal antibodies. The polar lipid (200-400 μg) was dissolved in 50 μl chloroform/methanol (1:1) then mixed with sterile PBS (pH 7.4). Antigens in PBS were mixed with an equal volume of the FREUND'S complete adjuvant, vortexed vigorously (it is better to prepare 2-4 ml of antigen solution then sonicate) until a thick emulsion was formed. The emulsion should be very thick and not disperse when a drop of it is placed on the surface of a saline solution. 0.2 ml of this emulsion was administered into the loose skin on each side of the back.

For subsequent injections Freund's incomplete adjuvant was used. 3-4 boosters being given subcutaneously at intervals of two weeks, with the final boost 1-2 weeks prior to fusion.

Because lipids are not good antigens, we used adjuvants to induce a strong antibody response to antigens. Freund's complete adjuvant (FCA) consists of a water-in-oil emulsion containing killed and dried *Mycobacterium. tuberculosis* bacteria and is generally used in primary immunization. FCA contains no bacteria (IFCA) and is commonly used in booster immunizations to prevent possible hypersensitivity

reactions occurring to the bacteria in the adjuvant.

b) Intravenous injection (IV):

In this process, boiled supernatant of *P. falciparum* culture was used as antigen. Antigen in aqueous form is delivered directly into the bloodstream. A maximum of 0.2 ml could be injected in this procedure. This process was an intense course of twice weekly intravenous injections, for three weeks. A final boost being given three to four days prior to fusion.

In the U.K., under the Animal (scientific procedures) Act 1989, any procedures to be carried out on an animal which may cause pain must be performed by a licensed worker. All such procedures performed in this project were carried out by licensed staff of the Animal Unit in LSTM.

2.17.4 Preparation of myeloma cells

The myeloma cell line used in this study was NS.1 (HAT sensitive), a variant of the BALB/c myeloma line. It is maintained in Iscove's (ISC) + 10% FCS medium (Appendix 2). They were incubated in an atmosphere of 8% CO_2 at 37°C in a humidified incubator. NS-1 were subcultured each time to 3 X 10^4 cells/ml. The day before fusion, NS-1 were counted using Trypan blue exclusion (section 2.17.5) and two flasks of 20 ml were set up at a cell density of 5 X 10^5 in Iscove's + 10% FCS + 8-azaguanine (Appendix 2) to select for cells deficient for the enzyme

hypoxanthine guanine phosphoribosyl transferase (HGPRT).

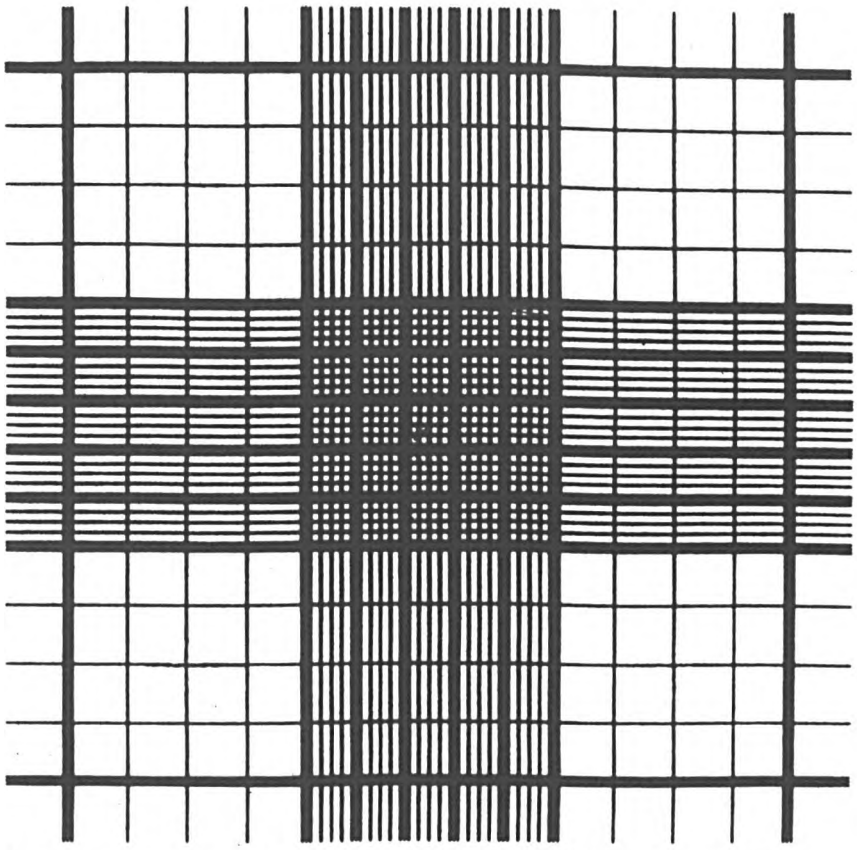
2.17.5 Estimation of cells using the trypan blue exclusion method and a haemocytometer

During this study a Neubauer haematocytometer was regularly used to count the myeloma NS-1 cells and spleen cells. The counting of cells with a haematocytometer was made in conjunction with the determination of cell viability using trypan blue exclusion dye (Sigma, U.K.) Trypan blue is taken up by dead cells.

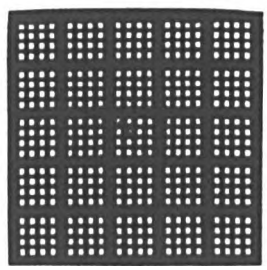
The cells were resuspended by gently tapping the base of the culture flask. 0.1 ml of the cell suspension was mixed with an equal volume of trypan blue stain (Sigma, U.K.) by pipetting up and down in an Eppendorf tube. After 5 min (not longer because viable cells start to take up the stain), a drop of the suspension was applied to the edge of the cover slip over the counting chamber and the chamber allowed to fill by capillary action.

A Neubauer chamber was used for counting cells. This consists of a glass slide containing a counting chamber engraved with a grid forming nine 1 X 1 mm squares when a cover slip is placed over the counting chamber, a volume of 0.1 mm^3 (10^{-4} cm^3) is created by each of these squares (Fig. 2.3). If 1 cm^3 is equivalent to 1 ml, then a count of the cells in one square multiplied by 10^4 gives the number of cells per ml. The cell count per ml determined using the following equation:

Fig. 2.3 Diagram of haemocytometer's chambers



Large Square



Small Square

Cells per ml = Average number of cells per 1 large square X 10^4 X dilution factor

or;

Cells per ml = Average number of cells per 5 small square X 5 X 10^4 X dilution factor

2.17.6 Media required for fusion

Several media were used to carry out the fusion. All of these media have been given in Appendix 2.

2.17.7 Fusion protocol

The protocol described here is based on techniques described by Pontecorvo, 1976 and adapted by Atkinson, 1991.

1) The immunized mouse was given an i.v. boost with the antigen preparation 4-5 day before fusion and in the case of subcutaneous injection 1-2 weeks before fusion.

2) On the day of fusion, mice were killed by CO_2 inhalation. A sample of blood was collected by cardiac puncture to use as a reference serum. The collected blood was left at 4°C overnight to clot, and then the serum removed and centrifuged at high speed for 1 min and kept at -20°C for further use.

3) The mouse was pinned out, and the abdomen washed with 70% ethanol. With

sterile dissecting instruments, the abdomen was incised and the spleen removed aseptically (take care not to rupture the spleen). It was placed in a sterile Petri dish containing 8 ml pre-warmed Iscove's washing medium (Appendix 2). All subsequent manipulations were performed inside the Laminar flow hood.

4) Excess fat and tissue were cut off and separated from the spleen which was passed through three further washes of Iscove's washing medium in sterile Petri dishes. The spleen was gently teased out by using a sterile needle, bent to an angle of 90° and forceps.

5) The cell suspension was transferred into a 15 ml conical tube and left to stand to allow the larger fragments to settle. The supernatant was transferred to a second 15 ml centrifuge tube, leaving the large fragments at the bottom of the tube and spun at 800 X g for 10 min.

6) The supernatant was removed and the pellet suspended in pre-warmed sterile 2.5 ml 0.85% NH_4Cl (ammonium chloride) to lyse the red blood cells. The cells were pipetted up and down few times and left for 2-3 min in a 37°C water bath, then 10 ml Iscove's + 10% FCS (Appendix 2) was added and the suspension was centrifuged at 800 X g for 10 min. The supernatant was discarded and the spleen cell pellet was suspended in 10 ml Iscove's + 10% FCS and counted (section 2.17.5). There should be approximately 10^8 spleen cells in 10 ml.

7) NS-1 cells were counted (section 2.17.5) using trypan blue and haemocytometer. NS-1 cells and spleen cells were combined at a ratio of 1 NS-1 cell : 10 spleen cells (1:10) in a 50 ml conical centrifuge tube or number of spleen cells counted per ml divided to number of NS-1 cells counted /ml = number of mls from original NS-1 culture to add to 10 ml spleen cells.

8) The suspension was spun 800 X g for 10 min. The supernatant was discarded and the cell resuspended in 10 ml of Iscove's washing medium and spun again to remove any remaining serum.

9) The supernatant was removed, the pellet resuspended by tapping the base of the conical tube. 0.3 ml of warmed polyethylene glycol mixture (PEG) (Appendix 2) was added over period of 60 seconds under constant agitation.

10) Immediately afterwards 15 ml of Iscove's washing medium were added gradually over a period of 90 seconds. The cells were left to stand at room temperature for 10 min. The cells spun at 800 X g for 10 min.

11) The supernatant was removed and the cells resuspended in 30 ml OPI-HAT medium (Appendix 2). The suspension was distributed in 96-well flat bottomed plates, approximately 100 μl or one drop from a 10 ml pipette per well. The plates were placed in a 37°C humidified incubator in an atmosphere of 8% CO_2.

12) After 24 h in a 37°C incubator, the wells were fed with one drop of Iscove's + OPI + HAT.

13) After 6 days, wells were examined for the presence of hybridoma colonies using an inverted microscope. The wells were fed by removing half of the supernatant in each well and feeding with a drop of Iscove's + OPI + HAT.

14) On day ten, the wells were again checked, scored and when the hybridoma reached a suitable colony size they were assayed using ELISA (100 μl supernatant was taken from colonies when the size of the hybridoma was about quarter of the well or larger). The wells were fed again.

2.17.8 Monoclonal antibody-screening test

The selection of useful hybrid cells must be based upon a specific antibody-screening test. The first antibody screen after fusion will be required when, by observation, the density of viable hybrid cells reaches approximately one third of the confluent level. This will usually occur between 10-14 days post-fusion, although longer periods may be required if the initial seeding density was low.

The ELISA method for detecting hybridomas producing antibodies of the desired specificity was used throughout this study. All buffers required for ELISA have been given in Appendix 1.

1) The microtitre plate (Immulon 1) was coated with 100 μl /well of *P. falciparum* boiled supernatant in [dilution (1:5) in coating buffer (Appendix 1)]. The plate was kept at 4°C overnight.

2) The plate was washed to remove unbound antigens three times with washing buffer (Appendix 1).

3) The test samples of culture supernatant (100 μl per well) were added and incubated 90 min at 37°C. Negative wells were incubated with normal mouse serum (1:100 dilution in washing buffer) and positive wells with 1:100 dilution of reference mouse serum used for same fusion.

4) Washing step repeated as step 2.

5) 100 μl of the 2-antibody (Alkaline-Phosphate labelled Goat anti-mouse immunoglobulin antibody (1:750) were added per well and the plate incubated for 90 min at 37°C.

6) Unbound antibodies were removed by washing

7) The reaction was visualised by adding a substrate P-Nitrophenol phosphate (Sigma fast PNPP) for the detection of alkaline phosphate actively. One PNPP tablet and one Tris-buffer table, dissolved in 20 ml of deionized water, provides 20 ml of ready-to use substrate. The OD reading for PNPP substrate was 405 nm.

2.17.9 Expansion of positive hybridomas

After a positive well was identified, the cells were transferred to three wells of a new 96 well micro culture plate each containing 200 μl of Iscove's + OPI-HT

(Appendix 2). After successful growth in the transfer wells, a doubling dilution of cells to the next 3 wells was made by transferring all the cell suspension to the first new well, half from this to the next well and so on. The plates were incubated in the conditions described previously and examined daily. When confluent growth was obtained, the supernatant was assayed again by ELISA (section 2.17.8) and any positive hybridomas were now transferred to a 25 cm^2-flask containing 1 ml OPI-HT. The flasks were incubated at a 45° angle for the 24 h. If the cells were showing successful growth rate fresh medium was increased to 5 ml gradually and the flasks were incubated lying flat. When sufficient growth was observed the cells were transferred to an 80 cm^2 tissue culture flask containing 10 ml pre-warmed fresh medium and gradually increased to 20 ml over a few days. For use in the adipocyte assay, positive hybridomas were transferred to pre-warmed Iscove's+ 10% FCS instead of OPI-HT (to remove insulin from medium).

The supernatant was checked one more time by ELISA and positive hybridomas were frozen in liquid nitrogen, the culture supernatant was kept frozen at -20°C for subsequent assays.

Each expanded positive colony was coded according to the fusion in which they were produced (SZ 1-6) and the plate (I, II, III,....) and well number (A_2, B_6,....) of the hybrid e.g SZ1 IIA_2.

2.17.10 CLONING

The original positive well will often contain more than one clone of hybridoma cells, and many hybrid cells have an unstable assortment of chromosomes. In order to ensure that cells that produce the antibody of interest are truly monoclonal and that the secretion of this antibody can be stably maintained, cloning was done by limiting dilution. This is the simplest and most popular method of hybridoma cloning.

2.17.10.1 Cloning by limiting dilution

The positive hybridoma cells were transferred to a 50 ml sterile conical tube and centrifuged at 800 x g for 10 min. The supernatant was discarded and the pellet was resuspended in 5 ml of OPI-HT. Cells were counted using trypan blue and a haemocytometer (section 2.17.5) and the volume of the suspension was adjusted to give approximate counts of 0.5, 1 and 5 cells per well with each well receiving 100 μl of cell suspension. A drop of OPI-HT medium was added to each well and plates were incubated at 37°C in an 8% CO_2 incubator and checked for clones after 5-10 days. When sufficient growth was obtained the wells were assayed by ELISA (section 2.17.8) and the positive cells expanded as described in section 2.17.19.

2.17.11 Isotyping of monoclonal antibodies

The Ouchterlony double-diffusion assay is the most common method for determining class and subclass of a monoclonal antibody. Isotype kits are

commercially available (Fig. 2.4). Mouse monoclonal typing kits (The Binding Site) containing an immunodiffusion plate and six antisera including IgM, IgA, IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 were used. The diffusion gel has a series of ten rosettes, each consisting of a large central well surrounded by six smaller wells. The antisera should be reconstituted with the volume of distilled water indicated and gently mixed until completely dissolved. Plates were taken out of the pouches in a dust free environment and left open until all condensation was removed. 75 μl of the test monoclonal antibodies were placed in the central well and 10 μl of the antisera distributed to the surrounding six wells. Plates were tightly closed and incubated at room temperature 24-48 hours until a precipitation arcs develop between them. The line of precipitation between the antisera and test well indicates its isotype.

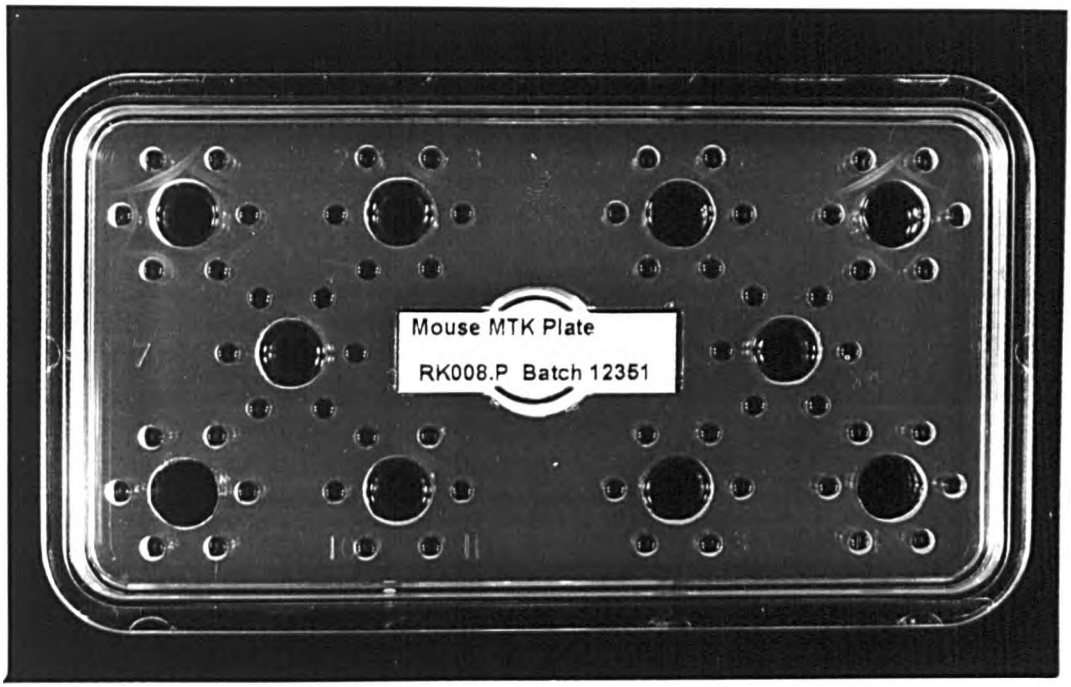
2.17.12 Freezing of NS-1 cells and hybridomas

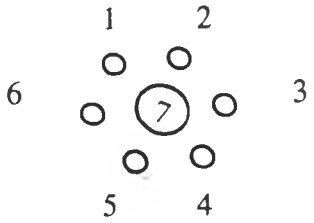
Hybridomas and NS-1 cell lines can be stored in liquid nitrogen for long term storage. For freezing of cells only cells that are healthy and rapidly dividing should be used.

The cells were counted with trypan blue and a haematocytometer (section 2.17.4) and frozen at a cell density of 5 x 10^6 to 5 x 10^7 cells per ml. The cell suspension was transferred to a sterile 50 ml conical tube and centrifuged at 800 X g for 10 min. The supernatant was collected and transferred to another tube and frozen down at -20°C as an antibody source. The pellet was resuspended in 1.5 ml Iscove's + 20% FCS and 1.5 ml Iscove's + 20% FCS + 20% dimethylsulfoxide (DMSO) as cryoprotectant. 1 ml of the cell suspension has about 5 x 10^6 cells per ml

Fig. 2.4 Isotyping monoclonal antibodies

+ Control +Control





1- IgM 2-IgG1 3- IgG2a 4- IgG2b 5- IgG3b 6- IgA

7- test supernatant

Wells 1-6 are immunoglobulin wells (10 μl Ig/well) and Well No. 7 is test well (75μl test supernatant/well).

and was transferred to a cryotube. The vials were kept at -70°C overnight in polystyrene box, in order to slow freezing. Cells can be stored temporarily at -70°C and will remain viable for several weeks or months. Long-term storage and high efficiency recovery require storage at -185°C (Liquid nitrogen).

Approximately 1 week after each batch has been transferred to the liquid nitrogen freezer, one vial was removed, thawed and retrieved (section 2.17.13). After 24 h viability of the cells were checked. This will determine the survival rate of the cells.

2.17.13 Retrieval of the frozen NS-1 cells and hybridomas

The vials of cells were removed from liquid nitrogen and thawed in a 37°C water bath. The vials were sprayed with 70% ethanol, and cell suspension was transferred to a 15 ml sterile centrifuge tube containing 20 ml of Iscove's + 20% FCS medium. Cells were centrifuged at 800 X g for 10 min and the pellet resuspended in 5 ml of OPI-HT medium. In case of NS-1 cells, they resuspended to 5 ml Iscove's + 10% FCS.

A dilution of the cells (1:10) were also set up at this time, to give the cell line good opportunity to re-establish. Cells were incubated at 37°C and 8% CO_2. The cultures were examined daily and when sufficient growth was achieved, the cells were transferred to a flask containing 20 ml of fresh OPI-HT medium. NS-1 cells were transferred to Iscove's + 10% FCS medium.

2.18 Lipid ELISA assay

ELISA was performed utilizing as antigen polar lipid extracted from *P. falciparum* boiled supernatant antigens which were prepared as described in section 2.7.1. The ELISA was a slight modification of that previously described by Facer & Agiostratidou (1994). All of buffers which were used for this ELISA have been given in Appendix 1).

20 μg of total polar lipid or different fractions of polar lipid were redissolved in chloroform/methanol (1:4). Polystyrene 96 well flat-bottomed microtitre plates for ELISA (Sigma, U.K.) were coated with 20 μg /well of solution of polar lipids in 1:4 chloroform/methanol. Evaporation of the solvent solution occurs during 3 h incubation of lipid at 37°C. The plates were incubated (without prior washing) with 100 μl of neat monoclonal antibodies or 1:100 dilution of patient serum (plasma samples were diluted 1:100 with PBS/10% FCS). 1:100 dilution of normal human serum, normal mouse serum and neat NS-1 supernatant were used as negative control. The plates were left for 2 h at 37°C. After washing three times with PBS, 100 μl of alkaline phosphate conjugated goat anti-human Ig (1:1000 in PBS/10% FCS) or anti-mouse Ig (1:750 in PBS/10% FCS) were added to the wells. Following incubation for 2 h at 37°C, plates were washed. Enzyme activity was measured by addition of 100 μl of the substrate p-nitrophenyl phosphate (Sigma fast PNPP). Absorbance was read in an ELISA reader at 405 nm after 30 min incubation at room temperature.

2.19 Assay for macrophage stimulation

2.19.1 Collection and cultivation of mouse peritoneal macrophages

CD1 female mice at least 6 weeks old were used. Mice were inoculated intraperitoneally with 1 ml of 4% thioglycollate medium and the cells harvested by peritoneal lavage 4-5 days in sterile cold RPMI containing 1 unit/ml of heparin and kept on ice. For each experiment 6-9 mice were treated and macrophages from each individual mouse collected separately and after examination under the microscope for contamination, cells were pooled. Cell suspensions were centrifuged at 800 X g for 10 min at 4°C, the supernatant was discarded and the pellet resuspended in 1 ml RPMI and counted with trypan blue using a haemocytometer (section 2.17.5). The cells were resuspended in endotoxin free RPMI 1640 + 5% FCS + 1 ml streptomycin and penicillin.

Cells were then dispensed into 96 well microtitre plates (Costar, U.K.) in 100 μl volumes to a concentration of 5 X 10^5 - 1 X 10^6 / well. Plates were incubated 2 h at 37°C in 5% CO_2 in air to allow macrophages to adhere. Non-adherent cells were removed by washing with sterile RPMI 1640 (Endotoxin free). The cells were then incubated overnight with test and control agents in triplicate in RPMI 1640 + 5% endotoxin-free FCS. The culture supernatants were harvested for determination of TNF levels. Macrophages incubated in LPS or medium alone were always included as positive and negative controls respectively. The dilution of a TMA preparation was compared with the concentration of LPS that induced the same amount of TNF-α

release.

2.19.2 TNF-α capture ELISA

TNF-α levels were determined by capture of ELISA (Genzyme, Cambridge MA). All buffers required for this experiment are given in Appendix 1. The Immuol 4 plates were incubated with 100 μl of monoclonal Hamster anti-mouse TNF-α at concentration of 6 μg/ml in carbonate buffer. The plates were incubated overnight at 4°C.

The following day, plates were washed with washing buffer (PBS + 0.05% Tween 20) for 3 min 3 times. The wells were blocked with 200 μl of 1% BSA in PBS for 2 h at 37°C. Then without washing, plates were blot dried and then incubated with 50 μl samples and 50 μl ELISA washing buffer for 60 min at 37°C. They were washed again and incubated with 100 μl/well of a horseradish-peroxidase (HRP)-conjugated goat anti-mouse TNF-α as second antibody (3 μg/ml in PBS/Tween 20 containing 1% BSA). They were washed as before,then incubated for 10-30 min at room temperature with 100 μl/well tetramethylbenzidine (TMA) as substrate. The enzyme reaction was stopped with 50 μl/well of 1 M solution of H_2SO_4. Absorbance was read in an ELISA reader at 450 nm.

Note: The standard curve should be prepared in cell culture media when measuring samples obtained from cell culture supernatants and in serum when measuring serum samples. Prepare standard curve covering an assay range from 31 to 2000 pg/ml.

2.20 Nitric oxide assay (NO assay)

The Grieses reaction (Ding, *et al.* 1988) was used to measure the amounts of nitrit as an indicator of NO production/accumulation during culture.

Macrophages were obtained from female CD-1 mice as described in section 2.19.1. Macrophages were incubated with test and control agents overnight. 50 μl of samples (supernatants) in duplicate were added to 50 μl of 1:1 mixture of 1% sulphanilamide in 2.5% H_3PO_4 and 0.1% naphtylenediamide-dihydrochloride in 2.5% H_3PO_4. After 10 min of reaction the absorbance was read at 570 nm. Nitrite was determined with reference to a standard curve using sodium nitrite ($NaNO_2$) at concentrations ranging from 0.5 to 100 μm in culture medium.

2.21 Detection of mycoplasma contamination in *P. falciparum* cultures by the polymerase chain reaction (PCR)

2.21.1 Mycoplasma PCR kit

The use of PCR to type clinical isolates is becoming common place because of the sensitivity and speed of this method. This technique has also been applied to mycoplasma species by targeting species-specific DNA sequences. The mycoplasma PCR Kit used in this study was purchased from Stratagene (Cambridge, U.K.). The primer set can detect most mycoplasma infections from 1 μl of cell culture

supernatant. The mycoplasma PCR Kit included PCR primers, an internal control template (that can be used to confirm polymerase-mediated amplification in all PCR samples), positive control template DNA (non-infective genomic DNA from *Mycoplasma orale*), StrataClean resin, and Rehydration buffer.

2.21.2 Template preparation of *P. falciparum* culture supernatant

Two cloned laboratory lines, K1, 3D7 and two wild isolates CY16, CY27 were thawed and retrieved (section 2.1.8) for this experiment. *P. falciparum* boiled supernatant from all four strains were prepared as described in section 2.2. 10 μl of resin were added to the boiled supernatant. Mix them by gently flicking the tube. Briefly centrifuge (5-10 sec) to pellet the resin (2 times). The supernatant is the template. Remove an aliquot of the treated supernatant to a fresh tube and dilute 1:10 and 1:100 with UV-irradiated water.

2.21.3 Preparation of the Reaction Mixture for Use in PCR

The recommended optimal reaction conditions for mycoplasma detection include the following:

10 mM of Tris-HCl (pH 8.3-8.8)

50 mM KCl

1.5-2.5 mM $MgCl_2$

200 μM of each dNTP

1 U of Taq DNA polymerase/reaction

The recipe below is for one reaction (50 μl) which should be adjusted for the number of samples to be tested

1) Add 5 μl of 10X buffer to 30.2 μl of H_2O (UV-irradiated)

2) UV irradiate at 12000 $\mu J/cm^2$ for 10 min

3) add the following:

   0.4 μl of dNTPs (25 mM stock, 200 μM final concentration in PCR)

   0.4 μl of Taq polymerase (2.5 U/μl stock, 1 U/reaction final concentration in PCR)

   2 μl of primer mix

4) Add either 2 μl of internal control template or, if the template is not used, added 2 μl of H_2O instead. The total of the reaction mixture for PCR should be 40 μl

5) Aliquot 40 μl of the reaction mixture into each PCR tube

6) Add 10 μl of the diluted template

7) Add 10 μl of the negative control (UV-irradiated H_2O or an extract from a cell line Known to be negative for Mycoplasma) to the appropriate tube

8) Add 10 μl of the positive control to the appropriate tube

9) Layer each tube with 60 μl of light mineral oil.

2.21.4 PCR program

The following PCR program has been designed to detect all strains (*M. orale, M. hyorhinis, M. fermentans, Acholeplasma. laidlawii, M. arginini*) of mycoplasmas.

Stage one : One cycle of

94°C	5 min and
55°C	1 min, 45 sec

Stage two: three cycles of

72°C	3 min
94°C	45 sec
55°C	1 min, 45 sec

Stage three: 40 cycles of

72°C	3 min
94°C	45 sec
55°C	45 sec

Stage four: one cycle of 10 min at 72°C

2.21.5 PCR product analysis on agarose gel

The PCR products are analyzed using standard agarose gel electrophoresis. 10 μl of PCR product has been mixed with 2 μl of dye blue and run on 2% agarose gel (This ensures good differentiation between PCR products associated with the internal control template and those due to mycoplasma) prepared with TCA buffer. The results are recorded by Baby Imager (Appligene, France) .

2.22 Treatment of infected *P. falciparum* culture with Mycoplasma Removal Agent (MRA)

Mycoplasmas are small free-living prokaryotes lacking a cell wall, and are common contaminants of eukaryotic cell lines (Rottem, 1993). Mycoplasmas can infect and survive in long term *in vitro* cultures of *P. falciparum* (Turrini *et al.* 1997).

Mycoplasma Removal Agent (MRA) has been developed by Dainippon Pharmaceutical Co., Ltd (in Japan) in cooperation with ICN Biomedicals. for cell culture. This agent has been shown to be effective in the elimination of various types of mycoplasma from contaminated cultures. The product contains 50 μg of 4-oxo-quinoline-3-carboxylic acid derivative in 1 ml of water. This drug inhibits mycoplasma DNA gyrase. It does not seem to be toxic to *P. falciparum* at the concentration used to kill mycoplasma.

0.5 μg/ml of MRA was added to standard culture medium. *P. falciparum* culture medium containing MRA was changed daily for seven days. The cell cultures were transferred to the fresh medium several times without MRA to confirm that regrowth of the contaminating mycoplasma has not occurred. Mycoplasma contamination was detected by PCR (section 2.21).

Statistics

Lipogenesis data were expressed as arithmetic mean $\pm$ S.E.M. Statistical significance was assessed using analysis of variance (ANOVA). Values of $P < 0.001$ were considered significant.

CHAPTER THREE

SYNERGISTIC EFFECT BETWEEN INSULIN AND TMA ON LIPOGENESIS IN RAT ADIPOCYTES

3.1 Introduction

Each year, approximately 5% of the worlds population is infected with the malaria parasite, *Plasmodium falciparum*. About 1% of cases are fatal, often as a result of the complex pathological syndrome known as cerebral malaria. Although the human disease caused by infection with *P. falciparum* is well documented clinically, much of its pathogenesis remains obscure. The changes seen in its severe forms are complex, and not directly attributable to the presence of protozoa inside erythrocytes.

Hypoglycaemia is a complication of severe malaria in both human and rodent models; however, the mechanism(s) leading to hypoglycaemia have not been elucidated.

Apart from cases proved to be due to hyperinsulinaemia following quinine therapy, there is no agreed mechanism to explain the hypoglycaemia. Reduced levels of liver glycogen and gluconeogensis, increased glucose consumption by both host and parasite, and even simple fasting, have all been proposed (Taylor, 1992a). Research carried out by Playfair and co-workers have identified a class of parasite derived components that have been termed Toxic Malaria Antigens (TMA) and which

are released by asexual blood stage parasites under *in vitro* conditions. The active components of these preparations are not fully characterised, although they appear to be conserved amongst murine and human malaria parasites (Taverne *et al.* 1990).

Previous laboratory studies have shown that TMA released by blood stage *Plasmodium yoelii* induce hypoglycaemia in mice (Taylor *et al.* 1992a). More remarkably they also synergize with insulin both *in vitro* (to enhance lipogenesis) (Taylor *et al.* 1992b) and *in vivo* (to induce hypoglycaemia in mice). It is therefore possible that they may play a part in the pathogenesis of malaria.

The main aim of the present chapter was to evaluate the ability of TMA released by the human malaria parasite *P. falciparum in vitro* culture to stimulate lipogenesis and to synergize with insulin in the lipogenic pathway of rat adipocytes.

The second aim was to determine whether the molecules released during *in vitro* culture were also present *in vivo*, therefore, we tested serum samples from squirrel monkeys infected with different species of malaria parasites at different levels of parasitaemia.

3.2 Results

3.2.1 Adipocyte assay optimization:

Rat adipocytes were used to measure the incorporation of ^{14}C-labelled glucose into lipids following incubation with insulin, TMA or a combination of both.

TMA were prepared in the three following ways:

1) Overnight incubation of 10^8/ml parasites at the late trophozoite stages in RPMI.
2) Overnight incubation of 10^8/ml parasitized erythrocytes at the late trophozoite stage in PBS (pH 7.4).
3) Collection of spent culture medium of *P. falciparum*.

All three different TMA preparations have been tested in the rat adipocyte assay. Results showed no significant difference between them and all three preparations showed synergistic activity with insulin. In subsequent experiments we used the third method of preparation. Fig. 3.1 (A) demonstrates that, when using a synchronized culture where most of parasites had reached the trophozoite stage, all the schizonts ruptured when parasites were incubated for up to 12-16 h. Fig. 3.1 (B) revealed that reinvasion occurs after the schizonts were ruptured and merozoites were released. Spent culture medium was collected and boiled for 5 minutes, and it was called "TMA".

TMA from cultures with different levels of parasitaemia have also been tested. Preparations with parasitaemia $\geq 10\%$ had higher level of activity (293%) compared

to preparations from cultures with low (2-6%) parasitaemia (140%-200%). All subsequent preparations were from cultures of *P. falciparum* with ≥10% parasitaemia.

Different TMA concentrations have been examined in order to find synergy between TMA and insulin (Fig. 3.2). The results revealed that 50 μl/ml of TMA preparation had the highest synergistic effect with insulin. With this concentration, there was no synergy between insulin and the control preparations. This volume was used in all subsequent studies.

A dose-dependent synergy effect between TMA and insulin has been shown in Fig. 3.3. At maximally effective concentrations, insulin alone increased lipogenesis by 2-2.5 fold, but when combined with the TMA the response to the same concentrations ($3x10^{-9}$-10^{-8} M) of insulin was increased 5-6 fold. $3x10^{-9}$ M insulin concentration was used in all experiment during this study.

In comparison with insulin, TMA alone stimulates lipogenesis by 65% (mean of 22 experiments); the same preparation acted in synergy with insulin to increase the response to $3x10^{-9}$ M insulin by 293%.

3.2.2 TMA preparation from different strains of *P. falciparum*

During this study we used two cloned laboratory lines and two wild isolates of *P. falciparum* (section 2.1.10). All of them showed a synergistic effect with insulin

in the rat adipocytes *in vitro*. Fig. 3.4 and 3.5 demonstrated that, there was no significant difference between cloned laboratory lines and wild isolates in their activity to induce lipogenesis and act in synergy with insulin *in vitro*.

Control preparations consisted of boiled supernatant from NRBC culture (grown under similar conditions as *P. falciparum* parasite) and culture medium alone (section 2.2.1). Neither control preparations from uninfected erythrocytes, nor the culture medium showed any synergy with insulin.

3.2.3 Stage-specific synergistic effect

P. falciparum culture with $\geq 10\%$ parasitaemia at ring stages was synchronized (section 2.1.9). Boiled supernatant from highly synchronized cultures of ring (Fig. 3.6 A) and schizont stages (Fig. 3.6 B) of *P. falciparum* were collected. Both samples were tested in the rat adipocytes *in vitro*. The effect was stage-specific and TMA from highly synchronized cultures of ring stages of *P. falciparum* (at $\geq 10\%$ parasitaemia) showed no effect on adipocytes in synergy with insulin, while supernatant preparations released during schizogony showed a synergy (Fig. 3.7).

3.2.4 Enzyme digestion

In order to characterise TMA, the boiled supernatants of *P. falciparum* and NRBC cultures were incubated with pronase E and lipase (section 2.3.1). Pronase treatment of *P. falciparum* culture supernatants did not affect their ability to act in

synergy with insulin on rat adipocytes *in vitro*; in fact the activity of TMA was slightly increased after digestion with protease. In contrast, lipase treatment of TMA abolished the effect (Fig. 3.8).

3.2.5 TMA-like activity in serum of monkeys infected with malaria parasites

3.2.5.1 Synergistic effect

Splenectomized squirrel monkeys were infected with *P. falciparum, P. vivax. P. brasillianum.* Serum was prepared as described in section 2.4. In order to determine whether the molecules released during *in vitro* culture were also present *in vivo*, we tested infected and non-infected serum blind from squirrel monkeys infected with different species of malaria parasites and at different levels of parasitaemia. The results showed that similar activity was present in infected serum but not in non-infected monkey serum samples, and that TMA from serum acted in synergy with insulin *in vitro* (Fig. 3.9). TMA preparations from monkeys infected with *P. falciparum*, *P. vivax* and *P. brasillianum* were similarly active in their synergistic effect with insulin.

3.2.5.2 Dose-dependent synergy between insulin and TMA from the serum of monkeys infected with *P. falciparum*

TMA-like activity has been found in serum of monkeys infected with *P.*

falciparum. T1-T9 were individual infected monkey serum with different parasitaemias. They showed different degrees of synergism. No activity was found in different normal monkey sera (Fig. 3.10).

In comparison with uninfected monkey serum samples, serial dilutions of infected serum from an animal with 46% parasitaemia showed synergy with insulin in a dose-dependent manner. The assay could pick up the equivalent of a 0.3% parasitaemia (1:128 dilution) (Fig. 3.11).

3.3 Discussion

The classical symptom of malaria is periodic fever. A century ago, Golgi observed that these fever paroxysms occur when schizonts rupture from host erythrocytes to release a new generation of merozoites. He described the cause of the fever to pyrogenic toxins that are released by the parasite during this act of replication (Kwiatkowski, 1995). Taylor and co-workers showed, using the murine malaria parasite *P. yoelii*, that a supernatant of blood stage parasites incubated overnight induced hypoglycaemia when injected into normal mice (Taylor *et al.* 1992a). They also found, by measuring lipogenesis and lipolysis in adipocytes *in vitro*, that these toxic antigens appeared to synergize with insulin (Taylor *et al.* 1992b). However, the nature of these toxic agents remains unknown.

The aim of this chapter was to test whether TMA (the active components which are released when schizonts rupture *in vitro*) from *P. falciparum* could, by

analogy with *P. yoelii,* stimulate lipogenesis and synergize with insulin in inducing lipogenesis in adipocytes.

During optimization of the rat adipocyte assay in this study, some strains of Wistar rats have shown resistance to insulin and in consequence, there was not any stimulation of lipogenesis (1-1.2 fold increase compared to effect of insulin alone) in rat adipocyte cells by TMA alone or in synergy with insulin. Sometimes, there was stimulation by insulin, but there was no synergy between TMA and insulin. In order to find out if the implementation of the test itself or the TMA preparation was the cause of failure, several variables were considered.

TMA were prepared by three different methods using a biological approach. Although there was no significant difference between them, the third preparation method was chosen and used for further experiments. Overnight incubation of parasites in PBS (pH 7.4) and RPMI showed that, while most of the schizonts ruptured reinvasion did not occur at all. We preferred to collect samples according to the third method which was considered similar to actual physiological conditions.

In order to understand why there was no synergy between TMA and insulin, TMA preparations were concentrated by a Minicon B15 Concentrator 10 X (Lexington, Mass, USA). Samples have also been concentrated by dialysing against polyethylene glycol (20,000 Mol.Wt), then dialysed against PBS (pH 7.4). TMA preparations were not incubated with polymyxin B because, it is believe that polymyxin B inhibits insulin action in isolated adipocytes (Cormont *et al.* 1992).

However, Results showed no synergy even after concentration of TMA preparations and/or eliminated of the incubation with polymyxin B. Epididymal fat pads were immediately removed after death and with as little handling as possible, followed by a rinse in buffer. Different batches of collagenase have been used, but there was still no synergy between TMA and insulin. Finally, we decided to used Wistar rats which had been bred on different diets. Wistar rats with CRM diet showed a high response to insulin and the combination of insulin and TMA. The ability of insulin to stimulate glucose metabolism is markedly diminished in adipocytes from rats fed high-fat diets (Lavau *et al.* 1979; Susini & Lavau, 1978). The effect of diet on lipogenesis was not clear. The comparison between CRM with the standard diet revealed that levels of fat in the CRM diet were a little lower than with the standard diet (Appendix 3). The reason for this sensitivity is not clear, but it could be because of the CRM diet. The rats fed on the standard diet showed synergy (1-1.2 fold), but those rats on the CRM diet were always sensitive to insulin and insulin + TMA (increase in lipogenesis 2-4 fold compared with insulin). In subsequent experiments we used rats with CRM diet and we never had any problem.

The most significant observation which arose during this study was that, Toxic Malaria Antigens (TMA) prepared from human malaria, *P. falciparum* increase lipogenesis and act synergistically with insulin *in vitro* to enhance lipogenesis. Adipocytes were chosen here as a convenient test system. Glucose uptake in adipose and muscle tissue is controlled by insulin and the GLUT 1 and GLUT 4 proteins have been identified in muscle and fat tissues which exhibit acute insulin-stimulated glucose transport. Other tissues (such as skeletal muscle and /or liver) are also likely to be

involved in this synergistic interplay between insulin and the antigens *in vivo*.

P. falciparum TMA were like rodent malaria TMA, heat-stable and only released during schizogony. The idea that malaria parasites secrete a toxin that directly causes the illness and pathology seen in infected individuals is not a new concept (Kitchen, 1949). This concept was modified by Clark and co-workers (Clark *et al.* 1981) suggesting that the role of a malarial " toxin" was simply to induce release of inflammatory cytokines. This toxin also had the ability to enhance glucose uptake into adipocytes (Taylor *et al.* 1992b) and induce the secretion of insulin from islet cells (Elased & Palyfair, 1994).

Boiled supernatants from ring stages of *P. falciparum* cultures did not affect lipogenesis in adipose cells. However, boiled supernatants from schizont stages (from the same population of parasites and parasitaemia) can act in synergy with insulin to increase lipogenesis in the rat adipocytes. The results revealed that TMA were stage-specific and that they were released only from schizont stages. This result may also explain why the classical symptom of malaria is periodic.

Similarly to the preparations from rodent malaria parasites, the ability of *P. falciparum* TMA resists protease digestion and was increased by digestion with protease. Supernatants from uninfected human erythrocytes digested with protease did not stimulate lipogenesis or synergize with insulin in the lipogenic pathway. The increase in activity of TMA after digestion with protease E suggested that the triggering molecules were not proteins. However, the activity may be associated with

proteins.

Lipase treatment of pronase digested samples abolished the functional effects of TMA on lipogenesis. This results suggests that the triggering activity of TMA was either lipid or, associated with a lipid component.

A comparison between cloned laboratory lines with fresh isolates suggested that there was no measurable difference between isolates in their activity to induce lipogenesis. This result revealed that, these molecules are conserved among different strains of *P. falciparum* parasite.

Two different control preparations have been used;

a) culture medium of malaria parasite

b) control preparations from non-parasitized cells grown under similar conditions as *P. falciparum* parasite.

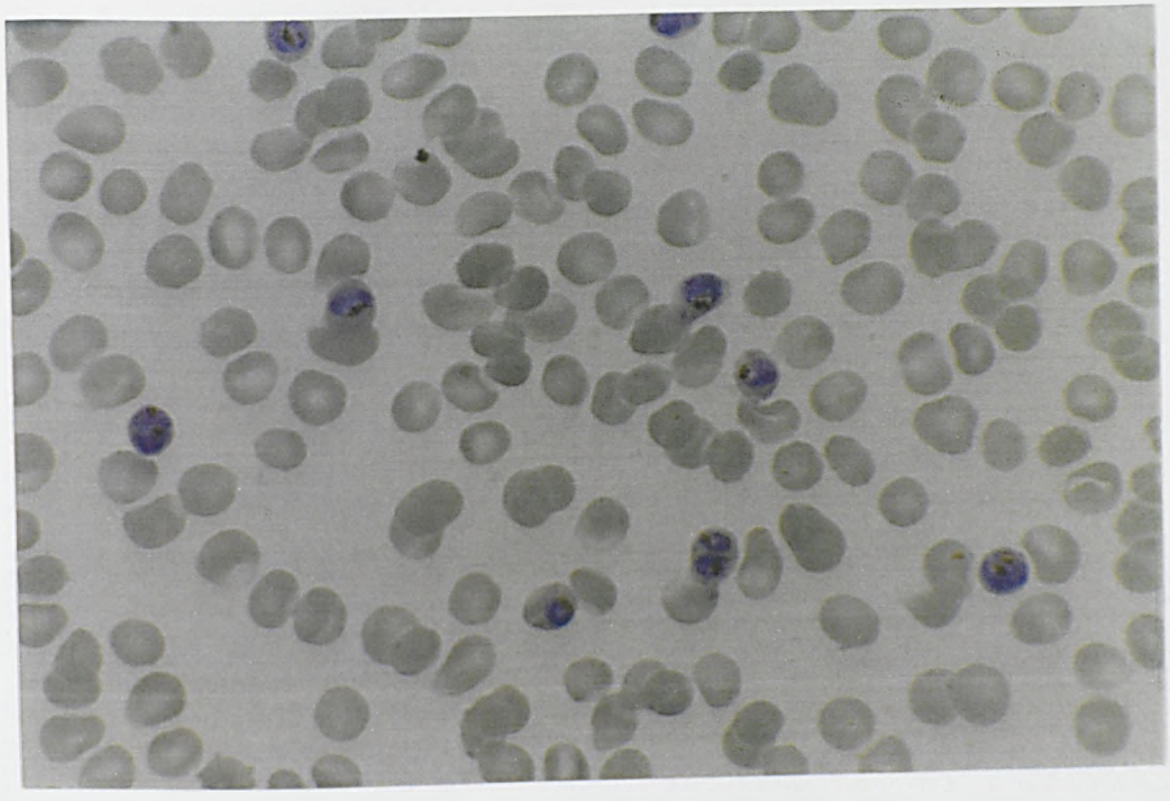
Using the first control proved that culture medium containing 10% serum did not stimulate lipogenesis. The second control was used because *P. falciparum* culture contained approximately 90% normal red blood cells (NRBC). In order to prove that the active component was not released during culturing of NRBC, we cultured non-parasitized red blood cells in parallel with infected red blood cells. No activity was found in these control samples.

There is no published work on the activity of *P. falciparum* TMA *in vivo*. We found that TMA-like activity was present in the serum of infected monkeys while no

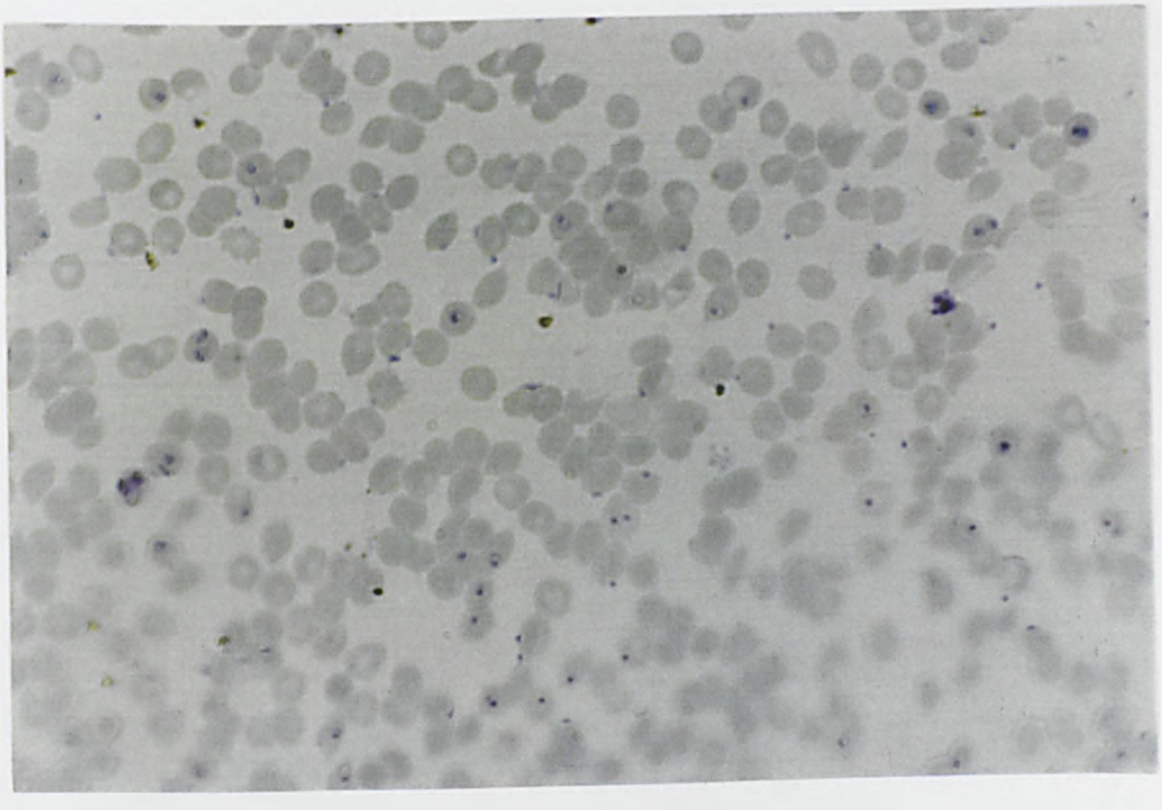
active molecules were present in normal monkey serum. These molecules seem to be heat-stable and unaffected by protease digestion. The active components, presented in the serum of all monkeys infected with three different malaria species. TMA-like molecules in monkey serum synergize with insulin in a dose-dependent manner in the rat adipocyte assay. The assay could pick up 0.3% parasitaemia or more, and below this level no synergy effect with insulin was detected.

To summarise, results of this Chapter suggest a similarity between *P. falciparum* TMA and the molecules previously described in *P. yoelii*. TMA preparations were resistant to protease and their activity abolished by lipase. Therefore, the active components should be lipid. This study also demonstrated the presence of TMA-like activity *in vivo* (monkey serum). Characterization of the triggering moiety was the ultimate objective.

Fig. 3.1 A) Synchronous *P. falciparum* culture at the trophozoite stage

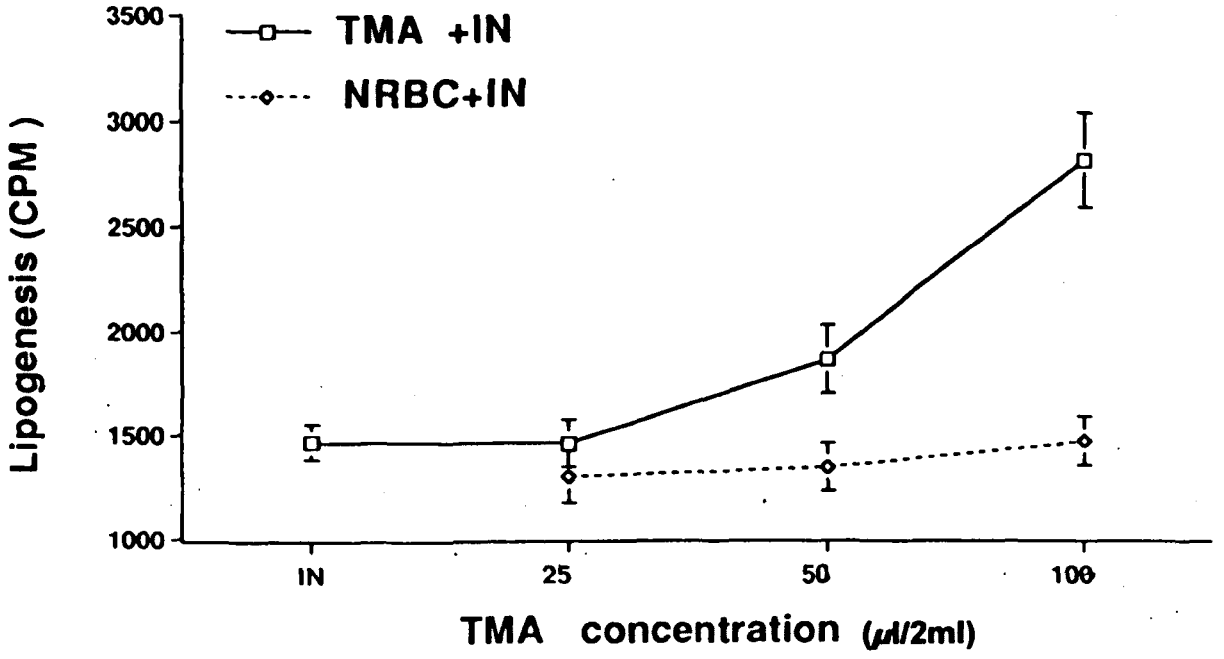


B) Synchronous *P. falciparum* culture after rupturing of schizonts



Highly synchronous of parasites culture was achieved as described in section 2.1.9. The late trophozoite stage, parasites were incubated in complete medium at 37°C for 12-16 h. A) presents a parasite culture before incubation at the late trophozoite stage (X1000). B) demonstrates the same parasite culture after most of schizonts had ruptured, and after merozoites had been released and reinvasion has occurred (X1000).

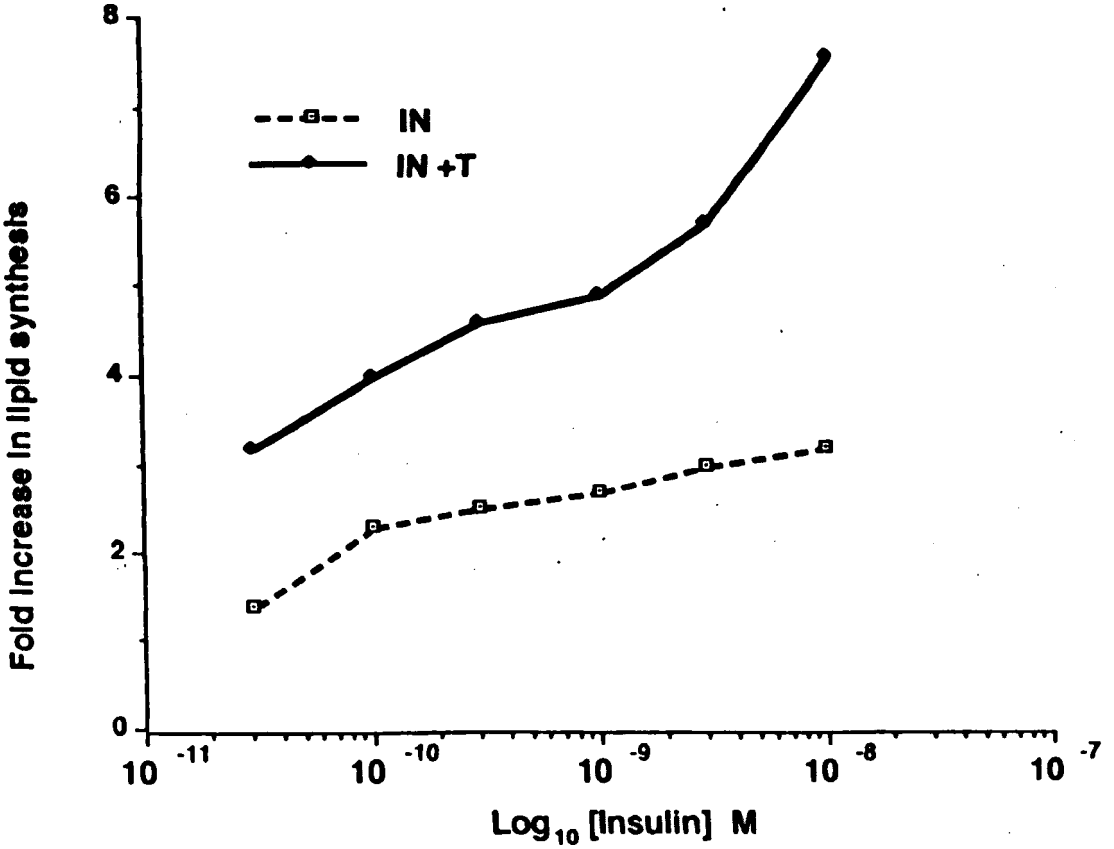
Fig. 3.2 EFFECT OF TMA CONCENTRATION IN SYNERGY WITH INSULIN



Boiled supernatant of *P. falciparum* culture (TMA) were prepared as described in the Materials and Methods. Briefly, adipocytes were isolated from the epididymal fat pads of male Wistar rats according to the method of Rodbell (1964). 3 x 10^5 cells were incubated in Krebs-Ringer buffer pH 7.4 containing 1% de-fatted bovine serum albumin, 5 mM [U-^{14}C] glucose (final concentration), 3 x 10^{-9} M insulin and different volumes of TMA as indicated, at 37°C with gentle shaking for 60 minutes. Reactions were terminated by adding Doles mixture as described in the Materials and Methods. The solid lines show a synergistic effect between TMA and insulin. The hatched line is the NRBC control. 50 µl/ml (or 100 µl/2ml final volume) of TMA showed the most significant difference in synergy with insulin compared with the control preparation. Results represent the mean (± SEM) of samples in triplicate.

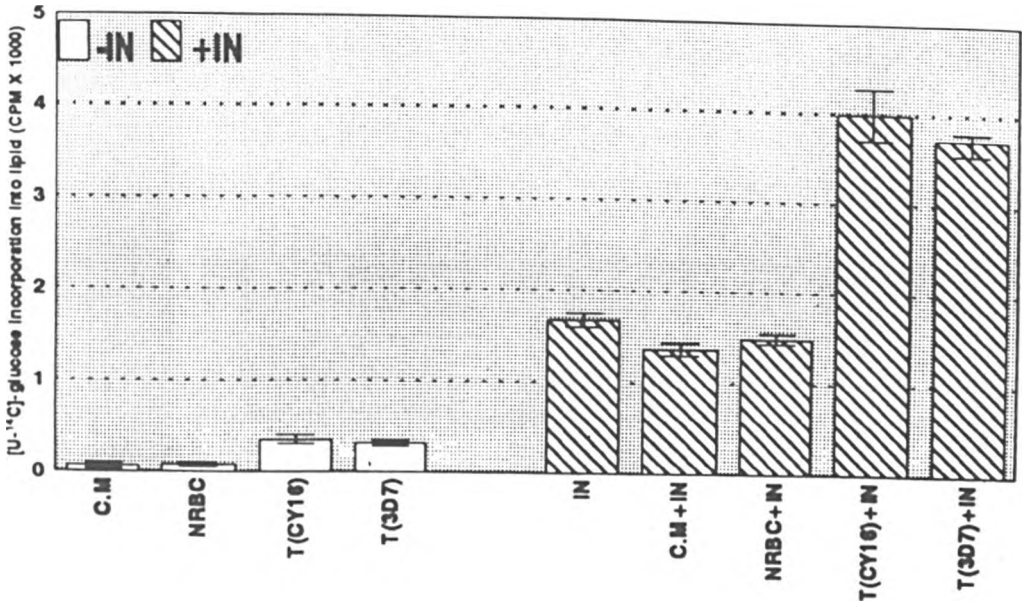
Basal value=450 CPM
TMA alone=468 CPM

Fig. 3.3 DOSE-DEPENDENT STIMULATION OF LIPOGENESIS WITH INSULIN AND INSULIN +TMA



The hatched line shows the effect of insulin concentrations alone on lipogenesis. The solid line shows synergistic effect of TMA with insulin. Adipocytes were isolated according the Rodbell method (1964). The adipose cells incubated with samples for 60 minutes in the presence of 5mM [U-^{14}C]glucose. Incubation of cells with $3x10^{-9}$ M insulin concentration showed a 4-5 fold increase in lipogenesis (compared with same concentration of insulin alone). This concentration was used in subsequent experiments. Values are means of four experiments in triplicates.

Fig. 3.4 SYNERGISTIC EFFECT OF TMA WITH INSULIN ON LIPOGENESIS IN RAT ADIPOCYTES

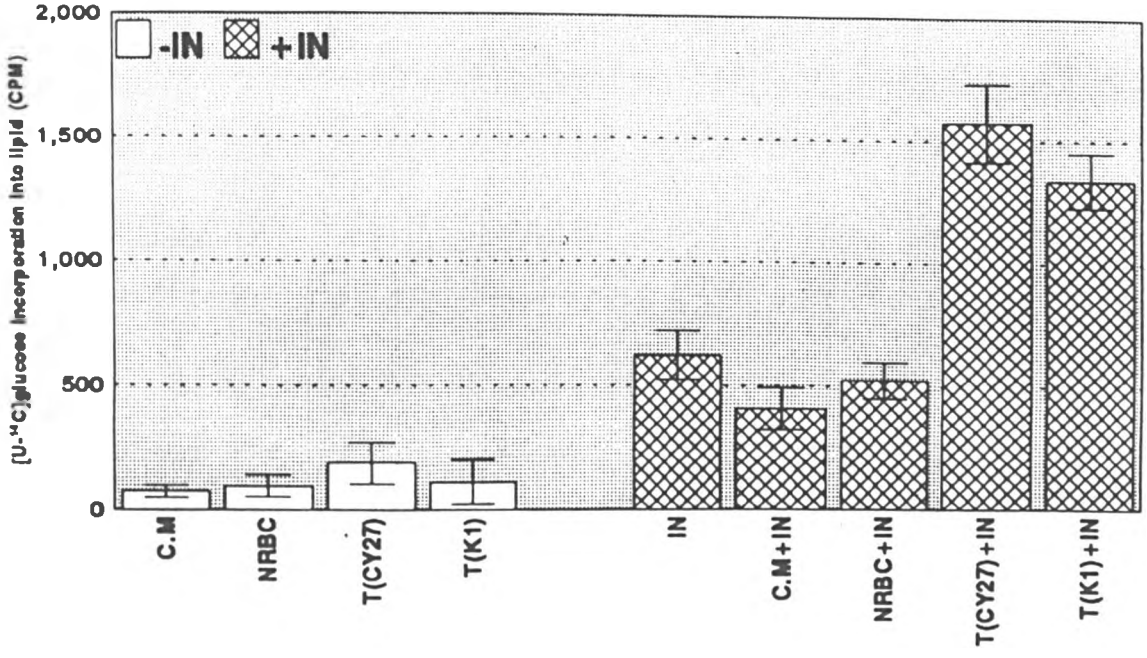


The left panel shows the effects of TMA (50 μl/ml) added *in vitro* on incorporation of [U-^{14}C]glucose carbon into lipid by rat adipocytes. The right panel shows the synergistic effect of TMA (50 μl/ml) with insulin (3 x10^{-9}) on lipogenesis *in vitro*. Cells were incubated at 37°C for 60 minutes in Krebs-Ringer bicarbonate buffer containing 5mM [U-^{14}C]glucose with indicated TMA, insulin and combinations of both. Values are means of four independent experiments (± SEM), each performed in triplicate (0-time control value was subtracted from treatment groups values). ANOVA shows a significant effect of boiled supernatant of *P. falciparum* culture compared with controls values ($P < 0.001$). No significant difference between cloned laboratory line (**3D7**) and wild isolate (**CY16**) of *P. falciparum* strains (P=0.723).

(**3D7**) and wild isolate (**CY16**) of *P. falciparum* strains (P=0.723).

C.M = Boiled culture medium
NRBC = Boiled supernatant of normal red blood cells
T(CY16) = Boiled supernatant of *P. falciparum*
T(3D7) = Boiled supernatant of *P. falciparum*
IN = Insulin

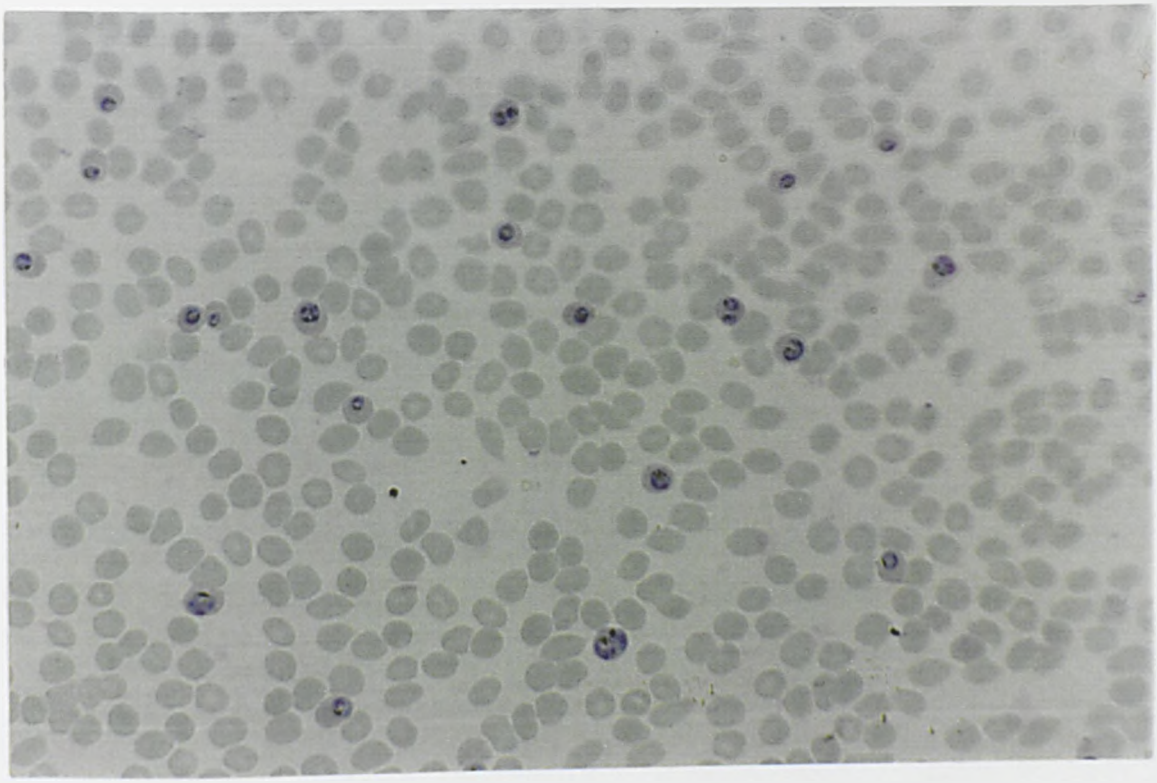
Fig. 3.5 TMA AND INSULIN EFFECT ON LIPOGENESIS IN RAT ADIPOCYTES



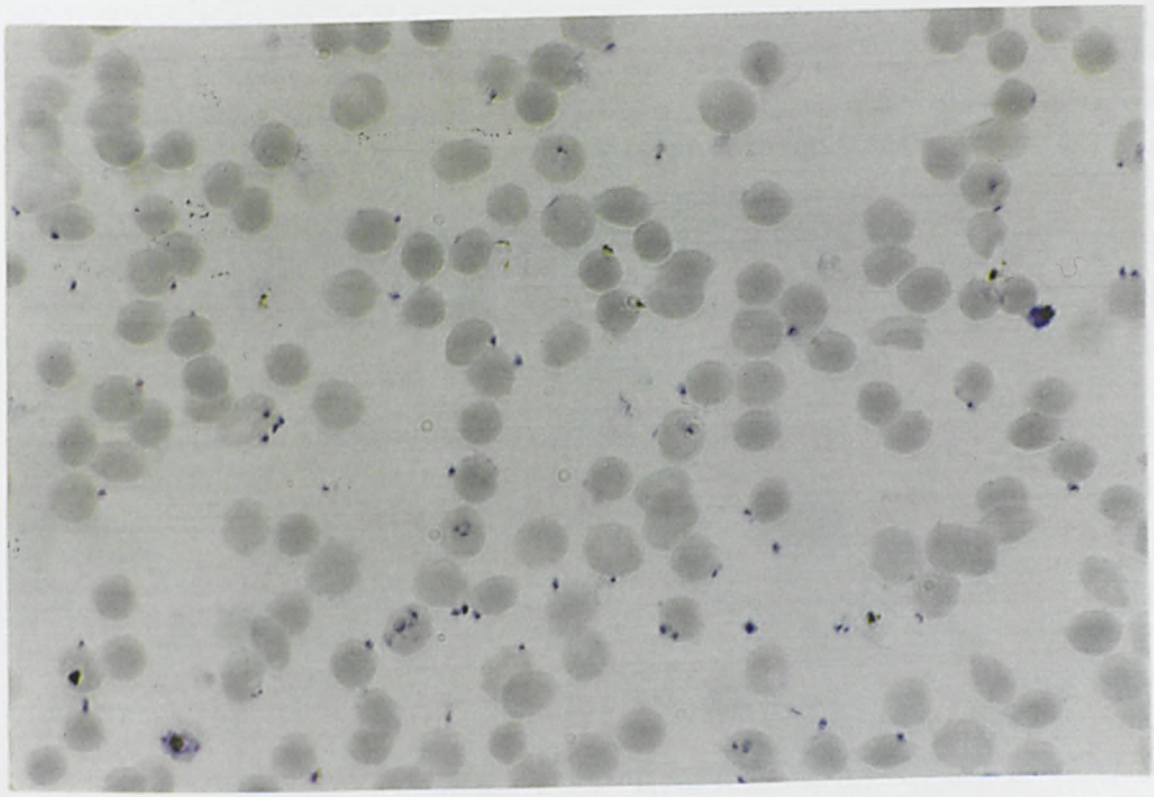
The procedure employed was the same as that described for experiment shown in Fig. 3.4. In this assay two other *P. falciparum* strains K1 and CY27 were used. Values are means of four independent experiments
(± SEM), each performed in triplicate (0-time control value which determined nonspecific activation of lipogenesis was subtracted from treatment groups values). ANOVA shows a significant effect of boiled supernatant of *P. falciparum* culture compared with controls values
($P < 0.001$). No significant difference between cloned laboratory line (**K1**) and wild isolate (**CY27**) of *P. falciparum* strains (P=0.723).

C.M=Boiled culture medium
NRBC=Boiled supernatant of normal red blood cells
T(CY16)=Boiled supernatant of *P. falciparum*
T(3D7)=Boiled supernatant of *P. falciparum*
IN=Insulin

Fig. 3.6 A)

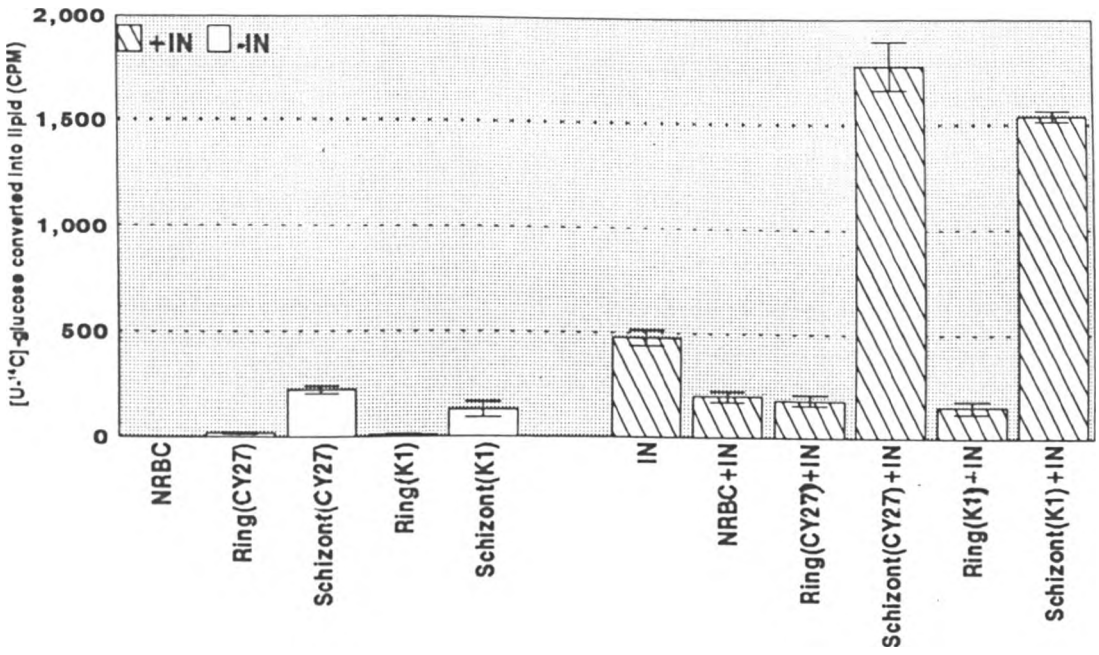


B)



Highly synchronous parasite culture was achieved as described in section 2.1.9. Parasites at the ring stage were incubated in complete medium at 37°C for approximately 12 h (Fig. 3.6 A) (X 400). Spent culture medium was collected and boiled for 5 minutes. The same parasites at trophozoite stages were incubated in fresh warmed medium until schizont ruptured (Fig. 3.6 B) (X 1000).

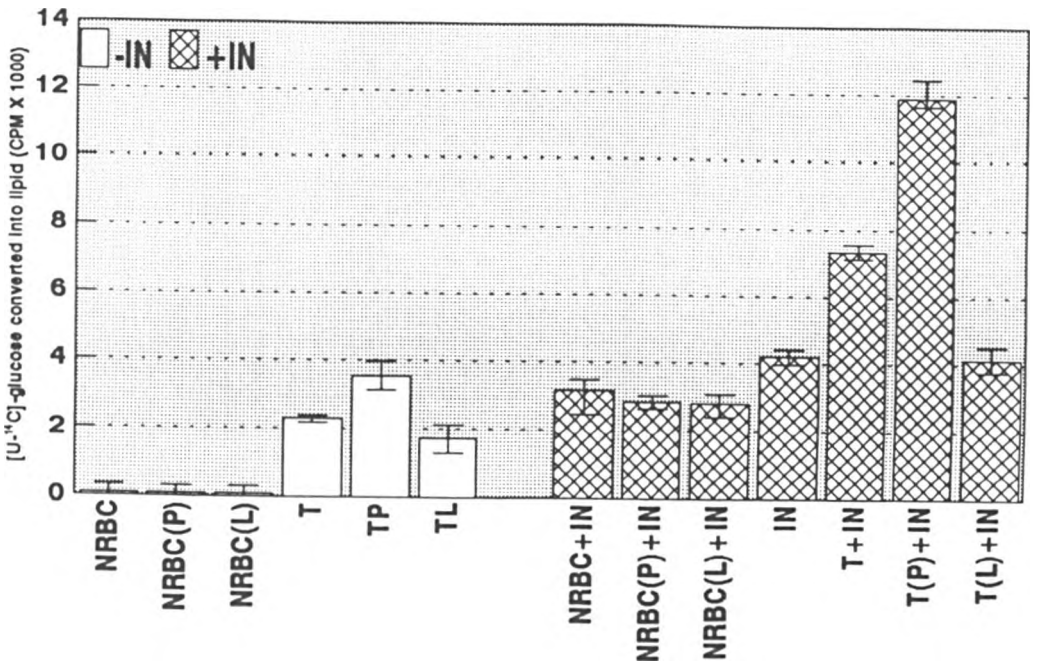
Fig. 3.7 EFFECT OF THE TMA FROM SYNCHRONISED CULTURES OF DIFFERENT STAGES OF *P. falciparum* ON LIPOGENESIS



Adipocytes (3 x 10^5 cells/ml) were incubated for 60 minutes at 37°C with 3 x10^{-9} M insulin, 50 μl/ml TMA (prepared from ring and schizont stages of *P. falciparum* culture supernatants) and combination of both.
The open bars represent the effect of TMA (alone) added *in vitro* on incorporation of [U-^{14}C]glucose carbon (5mM final concentration) into lipid by rat adipocytes. The shaded bars represent the synergy effect of insulin + TMA from different stages of *P. falciparum* cultures on lipogenesis. Values are means of four independent experiments (± SEM), each performed in triplicate (0-time control value which represented nonspecific activation on lipogenesis was subtracted from treatment groups values). ANOVA shows a significant effect of boiled supernatant of *P. falciparum* culture (schizont stages) compared with controls values (ring stages) ($P < 0.001$).

NRBC = Boiled supernatant of normal red blood cells
IN = Insulin

Fig. 3.8 TREATMENT OF TMA WITH PROTASE AND LIPASE

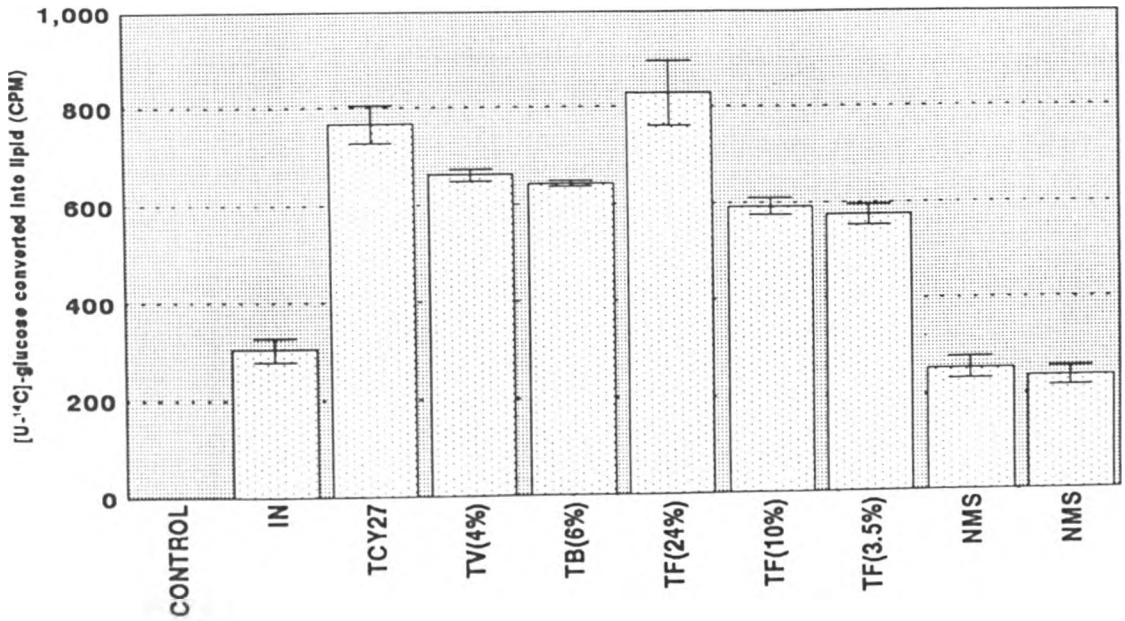


The activity of boiled supernatants of *P. falciparum* culture were compared before and after digesting with 10 μg/ml pronase E (**P**) and 5 μg/ml of lipase (**L**). The open bars show effect of 50 μl/ml TMA (**T**), digested TMA with pronase E (**TP**) and pronase digested TMA with lipase (**TL**) in the absent (-) of insulin on lipogenesis in rat adiposytes. The shaded bars show effect of 100 μl T, TP and TL in present (+) of insulin (3×10^{9}) on lipogenesis. TP samples showed synergy with insulin, however, TL samples showed no synergy. ANOVA shows a significant difference between two treatments ($P < 0.001$).

NRBC = Boiled supernatant of normal red blood cells

IN = Insulin

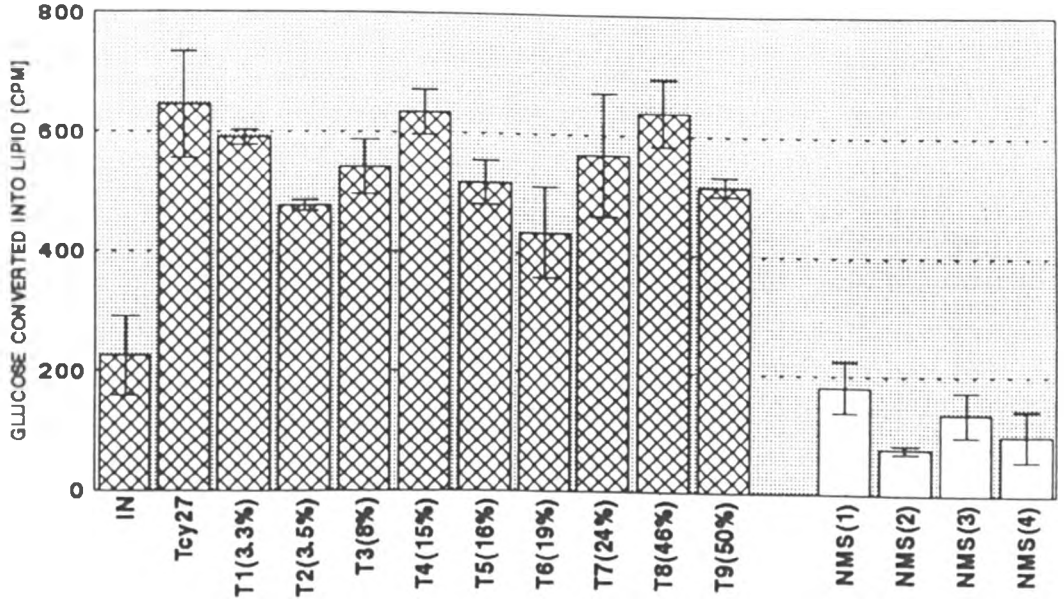
Fig. 3.9 INSULIN SYNERGY OF TMA FROM THE SERUM OF MONKEYS INFECTED WITH DIFFERENT SPECIES OF MALARIA PARASITE



3 x10^5 isolated fat cells were incubated for 60 minutes at 37°C in 1.5 ml KRB buffer (pH 7.4), containing 5mM [U-^{14}C]-glucose (final concentration), 3 x10^{-9} M insulin and 50 μl/ml test agents. Serum of monkeys infected with *P. vivax, P. falciparum* and *P. brasillianum* showed similar activity as TMA of *P. falciparum in vitro*. The results demonstrated that active component are conserve between different species of malaria parasite. Values are means(± SEM) of two experiments in duplicate (0-time and basal control values were subtracted from treated group values).

IN=Insulin
TCY27=TMA of boiled supernatant of *P. falciparum* culture *in vitro*
TV=Infected monkey serum with *P. vivax*
TF=Infected monkey serum with *P. falciparum*
TB=Infected monkey serum with *P. brasilianum*
NMS=Normal monkey serum

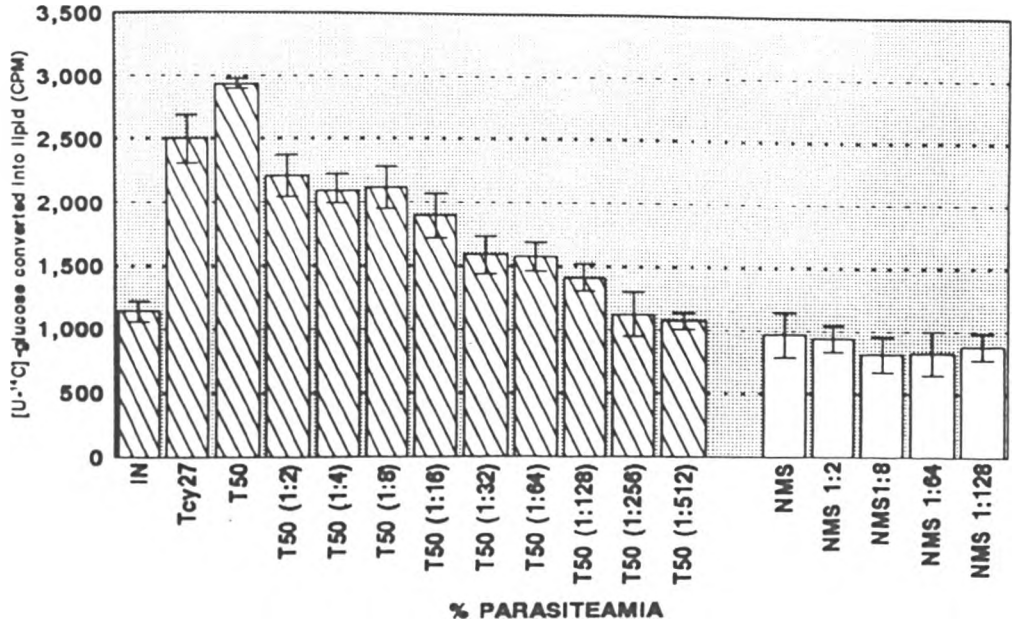
Fig. 3.10 INSULIN SYNERGY OF TMA FROM THE SERUM OF MONKEYS WITH *P. FALCIPARUM*



The effect of Normal Monkey Serums (NMS) and TMA-like activity from monkey serum infected with *P. falciparum* (T) (NMS and T were prepared as described in the Materials and Methods) on the conversion of [U-^{14}C]glucose by rat adipocytes in the presence of insulin. 3 x10^5 isolated fat cells were incubated for 60 minutes at 37°C in 1.5 ml KRB buffer (pH 7.4), containing 5mM ^{14}C-glucose (final concentration), 3 x10^{-9} M insulin and 50 µl/ml test agents. The results demonstrated TMA-like activity in serum of monkeys infected with *P. falciparum* with different levels of parasiteamia. Values are means (± SEM) of two experiments in duplicate (0-time and basal controls values were subtracted from treated groups values).

T = individual infected monkey serum (parasitaemia in bracket) with *P. falciparum*,
NMS = Normal Monkey Serum
T(CY27) = TMA of boiled supernatant of *P.falciparum* culture *in vitro*; which is used as a control.
IN = Insulin alone

Fig. 3.11 DOSE-DEPENDENT SYNERGY BETWEEN INSULIN AND TMA



The procedure employed was the same as that described for experiment shown in Fig. 3.9.

Serum of monkey infected with 46% parasitaemia and normal monkey serum were diluted in KRB buffer (log 2). Results revealed synergy effect of TMA-like activity in monkey serum with insulin in dose-dependent manner in rat adipocytes. Values are means (± SEM) of three experiments in duplicate

T50= Monkey serum with 46% parasitaemia

NMS= Normal Monkey Serum

IN=Insulin alone

CHAPTER FOUR

TIME COURSE OF ^{14}C-GLUCOSE INCORPORATION INTO LIPIDS *IN VITRO*

4.1 Introduction

One of the most important physiological effects of insulin is its ability to increase glucose transport in cardiac and skeletal muscles and adipose tissue (Levine *et al.* 1955). In rat adipose cells, insulin can produces an approximate 20-30 fold increase in glucose transport (Holman *et al.* 1990; May & Mikulecky, 1982).

In 1982 Marshall described for the first time a method for maintaining isolated adipocytes in primary culture (Marshall, 1982). Before that most study of insulin had been done with short term cultures of fat cells. Viability of such cultured cells was established by multiple criteria. After two days in culture, adipocyte cell numbers and specific insulin-binding capacity remained unchanged, the cells remained impermeable to L-[^{3}H]glucose and trypan blue indicating integrity of the plasma membrane, and adipocytes were able to synthesize both protein and functional insulin receptors (Marshall, 1983).

Glucose uptake in adipose and muscle tissues is controlled by insulin. The hormone induces translocation of glucose transporters from an intracellular location to the plasma membrane (Cushman & Wardzala, 1980; Kono *et al.* 1981). The

intracellular signal pathway via the insulin receptor leading to translocation has not completely been elucidated.

Insulin-stimulated increase in glucose transport in fat and muscle cells is associated with a redistribution of glucose transporter proteins from an intracellular pool to the plasma membrane (Oka & Czech, 1984; Wardzala & Jeanrenaud, 1981). A number of studies have demonstrated that several glucose transporter protein isoforms are present in mammalian cells. GLUT1 and GLUT4 are two glucose transporter proteins in insulin-sensitive rat adipocytes and rat skeletal muscle. Both GLUT4 and GLUT1 are redistributed from low density microsomes to the plasma membrane in rat adipocytes exposed to insulin (Zorzano *et al.* 1989).

Cytochalasin B, a metabolite of the mould *Helminthosporum dermatioideum* inhibits cell division by preventing cytoplasmic cleavage (Carter, 1967). Cytochalasin B has been shown to be a powerful inhibitor of glucose uptake in several cell types (Czech *et al.* 1973; Mizel & Wilson, 1972). It has been postulated that cytochalasin B acts by modifying the activity of the contractile microfilamentous system of the cell. Microtubules and microfilaments have been identified in adipose tissue (Soifer *et al.* 1971), it seems possible that they may have a role to play in the metabolism of adipocytes.

Detailed studies of [^{3}H]cytochalasin B-binding to plasma membranes and the inhibition of glucose transport by cytochalasin B in purified isolated rat adipose cell plasma membranes have now permitted the demonstration that insulin stimulates glucose transport in the rat adipose cell primarily by increasing the number of

functional glucose transport systems in this cell's plasma membrane (Wardzala *et al.* 1978). This same assay has been further used to identify a large intracellular pool of glucose transport systems associated with the microsomal membranes of the isolated rat adipose cell and to demonstrate that insulin increases the number of glucose transport systems in this cell's plasma membrane through the translocation of glucose transport systems from this pool (Cushman & Wardzala, 1980).

Cytochalasin B inhibits glucose metabolism to both CO_2 and lipids in fat-cells. This drug specifically inhibits the glucose-transport system of isolated fat-cells (Loten & Jeanrenaud, 1974). Prolonged incubation with this agent causes no further inhibition.

It has been demonstrated in Chapter three that the boiled supernatant from *P. falciparum* culture (or TMA) had a synergistic effect with insulin on isolated fat cells *in vitro*. The incubation times were between 0.5-2 h, but, TMA were never shown to increase lipogenesis by themselves. TMA from *P. yoelii* were also incubated up to 4 h in the presence of [U-^{14}C]glucose and in the absence of insulin with isolated fat cells, but, there was no increase in lipogenesis similar to that observed with insulin (Taylor, personal communication).

The aim of this present chapter was to evaluate the effect of TMA on primary cultures of isolated adipocytes for up to 24 h compared to insulin. We also tried to understand the mechanism of action of TMA by using Cytochalasin B as an inhibitor of the glucose-transport system.

4.2 Results

4.2.1 Effect of TMA from *P. falciparum* on lipogenesis in long term cultures of isolated rat adipocytes

Isolated rat adipocytes were prepared as described in Materials and Methods. The isolated cells (Fig. 4.1) have been cultured as in section 2.5.1.

The addition of 3 X 10^{-9} M or 10 nM insulin to isolated adipose cells after 60 minutes incubation in the presence of [U-^{14}C]glucose, elicited a rapid 3-6 fold increase in glucose uptake over that observed in control cells. Exposure of cells to 50 μl/ml TMA in long term culture also increased glucose uptake. However, in contrast to the action of insulin, the stimulatory effects of TMA developed more slowly. Fig. 4.2 shows the size differences in fat cells after 4 h incubation with NRBC supernatant, TMA preparation, insulin and a combination of insulin + TMA in primary culture of isolated rat fat cells. With increased incubation time, the size of cells was changed. Figs. 4.3 and 4.4 also show the increase in size of fat cells after 12 and 24 h incubation with TMA and insulin. The increased size of isolated adipose cells during incubation with TMA and insulin (compared to controls) could be related to increased glucose uptake and conversion to lipid.

The maximal effect of TMA was observed between 12-24 h. Fig. 4.5 shows that cells exposed to 10 nM insulin or 50 μl/ml TMA for 0.5 and 24 h, increase their glucose uptake. Between 0.5-3 h the effect of TMA on lipogenesis were

approximately similar to controls. However, after 12-24 h, TMA showed an activity similar to insulin. In addition, exposure of cells to TMA and insulin for 0.5-3 h continued to show a synergistic effect, while prolonged incubation of cells with both agents, revealed additive effects.

Incubation of cells in the absence of insulin and TMA (basal control) or with boiled supernatant of NRBC cultures (control) did not stimulate lipogenesis in rat adipocytes (0.5-24 h).

4.2.2 Inhibition of the glucose transport system by cytochalasin B

In order to understand the underlying mechanism of the effect of TMA on fat cells, cytochalasin B was used as an inhibitor of glucose-transport. Cytochalasin B was added to long term and short term isolated rat adipose cultures as in the Materials and Methods. This experiment showed that in short term incubation of cells with TMA, insulin and cytochalasin B, the synergistic effect was completely inhibited (Fig. 4.6). In long term incubation of cells (24 h) with TMA or insulin with cytochalasin B, lipogenesis was inhibited by 100% by this drug (Fig. 4.7).

4.3 Discussion

The results presented in this chapter demonstrate a marked stimulatory effect of 50 μl/ml of TMA on lipogenesis in primary cultures of isolated rat adipocytes. The extent of the maximal increase in conversion of glucose to lipid in fat cells exposed to TMA was 6-10 fold (after 24 h) above controls, equivalent to that observed in cells exposed to 10 nM insulin. However, the onset of the increase in lipogenesis elicited by TMA was much slower than that elicited by insulin. While the insulin effect is essentially increased significantly above base line after 30 min of incubation, the stimulation of lipogenesis in cells exposed to TMA reached the levels observed for insulin only after exposures of 12 to 24 h. Rats with the CRM and the standard diet were sensitive to insulin and TMA alone after 24 h incubations, but only rats which received the CRM diet showed a synergistic effect with insulin in short term culture.

The results of the experiment described in this chapter demonstrate that, TMA have an effect on lipogenesis alone, but that, compared to insulin, this effect is slower to develop. However prolonged treatment (12-24) of cells with TMA may involve an increase in the activity of glucose transporters by TMA.

To understand the mechanism by which TMA exert its effect, cytochalasin B was used as a glucose transport inhibitor. It was previously reported that insulin stimulates glucose transport in intact isolated rat adipose cells through an apparent translocation of glucose transport systems from a specific intracellular membrane pool to the plasma membrane (Suzuki & Kono, 1980).

The ability of isolated adipocytes to metabolise glucose to both CO_2 and lipid is inhibited by cytochalasin B in a dose-dependent manner (Loten & Jeanrenaud, 1974). Insulin-stimulated glucose metabolism was inhibited by cytochalasin B to the same extent as basal glucose metabolism. Inhibition of TMA-stimulated lipogenesis proved that the TMA stimulated lipogenesis in rat adipocytes occurred by means of the glucose transporter. 2 μg of cytochalasin B completely inhibited the effect of TMA and insulin on lipogenesis. This concentration of cytochalasin B was without effect on several other metabolic functions of fat-cells (such as basal ATP, conversion of pyruvate, lactate or arginine into CO_2 and lipids and also the anti-lipolytic effect of insulin) (Loten & Jeanrenaud, 1974).

The inhibition of the synergistic effect between TMA and insulin in short term culture and the inhibition of the effect of TMA alone in long term cultures by cytochalasin B proved that TMA stimulated lipogenesis via the glucose transport system in rat adipose cells.

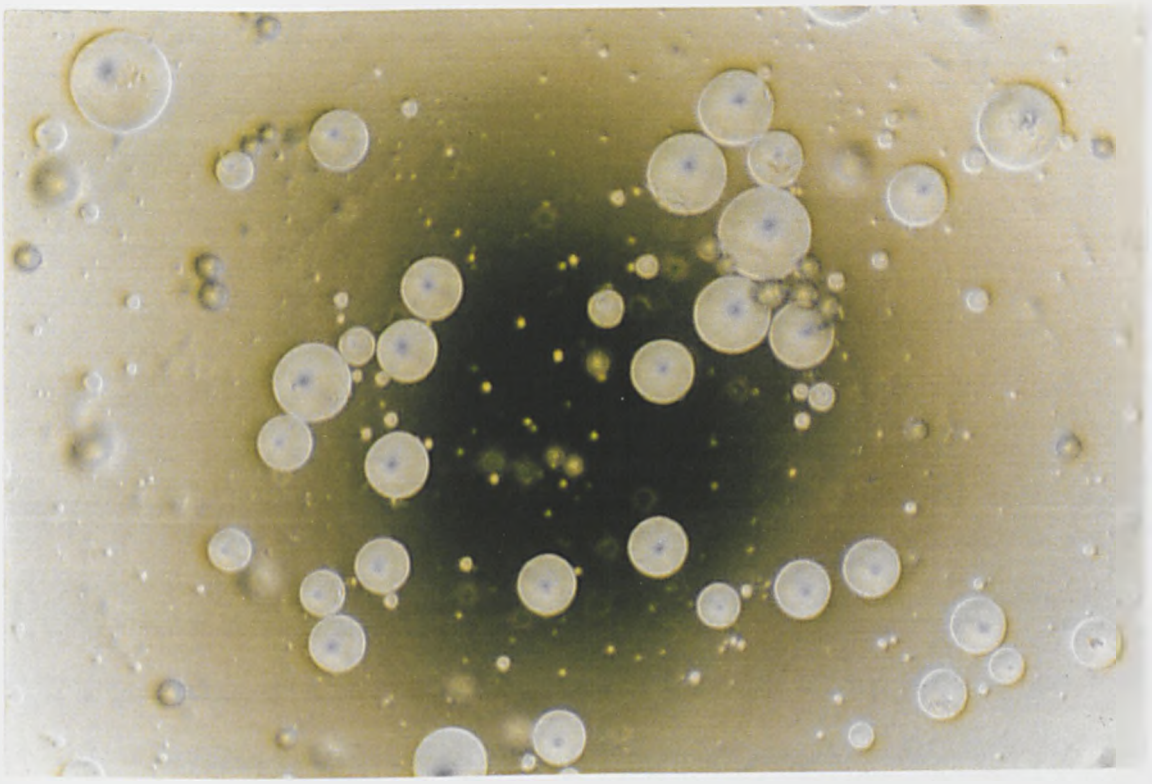
Previous studies with *P. yoelii* showed that supernatants of blood stage parasites incubated overnight, appeared to synergize with low levels of insulin rather than acting on their own. The way in which these molecules induce hypoglycaemia *in vivo* is not established (Taylor *et al.* 1992a). This study, demonstrated that TMA from *P. falciparum* not only synergised with insulin but also to act on their own.

Hypoglycaemia is a serious complication of *P. falciparum* malaria (WHO 1990) and administration of glucose as an emergency therapy can be

unsatisfactory because glucose stimulates insulin release and initiates the need for increasing amounts of hypertonic glucose to maintain normoglycaemia. Work on murine malaria suggested that, hypoglycaemia may be largely secondary to increased insulin secretion (Elased & Playfair, 1994) and malaria toxin can also stimulate pancreatic β-cells directly to secrete insulin (Elased, unpublished work). Hence, malaria toxin can directly stimulate insulin secretion and this toxin synergise with elevated insulin to increase lipogenesis on peripheral tissues such as adipose tissue. In addition, prolonging the presence of such molecules alone also stimulated glucose uptake, result to reducing more blood glucose level.

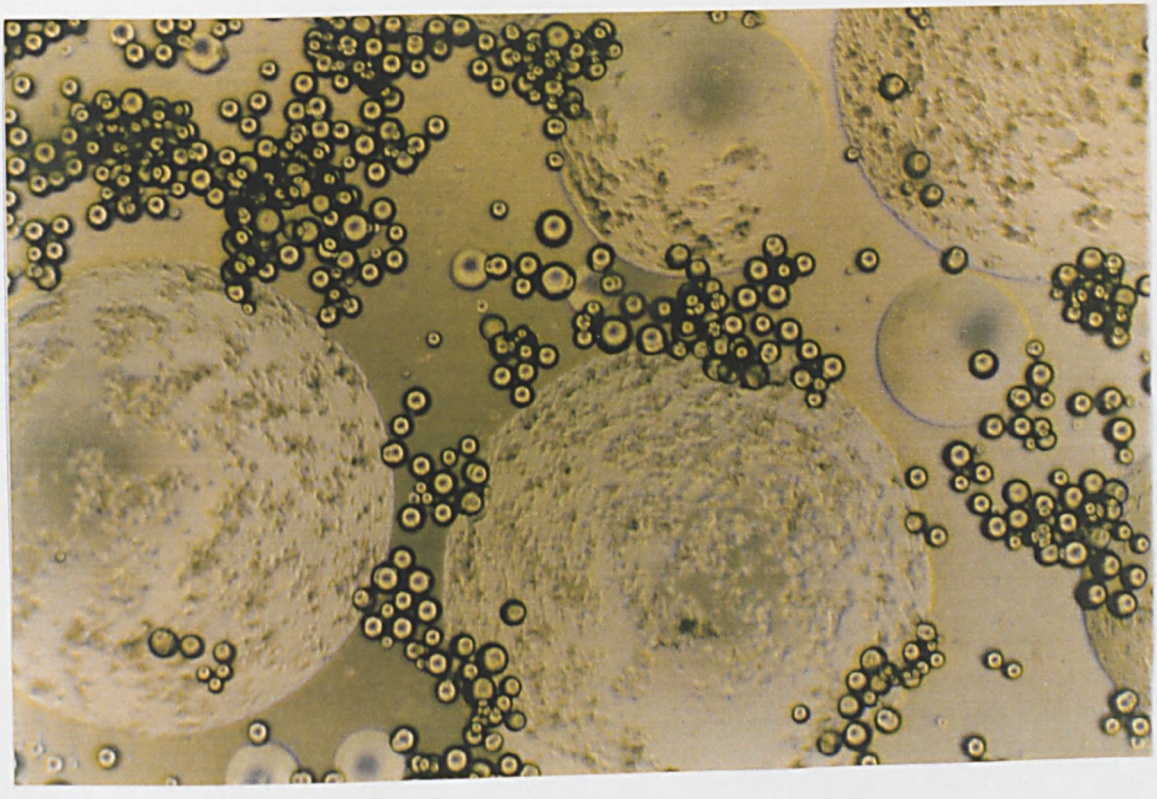
Therefore, the increased level of insulin and the action of TMA may explain in parts one mechanism leading to hypoglycaemia in malaria patients. The results described in this chapter are based on *in vitro* experiments, but this effect of *P. falciparum* TMA may also exert *in vivo*.

Fig. 4.1 Free fat cells at zero time before incubation with different agents



Male Wistar rats weighing 170-190 g were used. Isolated adipocytes were obtained using a method modified from Rodbell (1964). The epididymal fat pads were removed and incubated in KRB buffer containing 1% defatted BSA and collagenase at 37°C for 30 minutes. Photograph shows free fat cells before incubation with any agents (X40).

Fig. 4.2 A) Incubation of isolated fat cells with insulin (4 h)



B) Incubation of isolated fat cells with TMA (4 h)

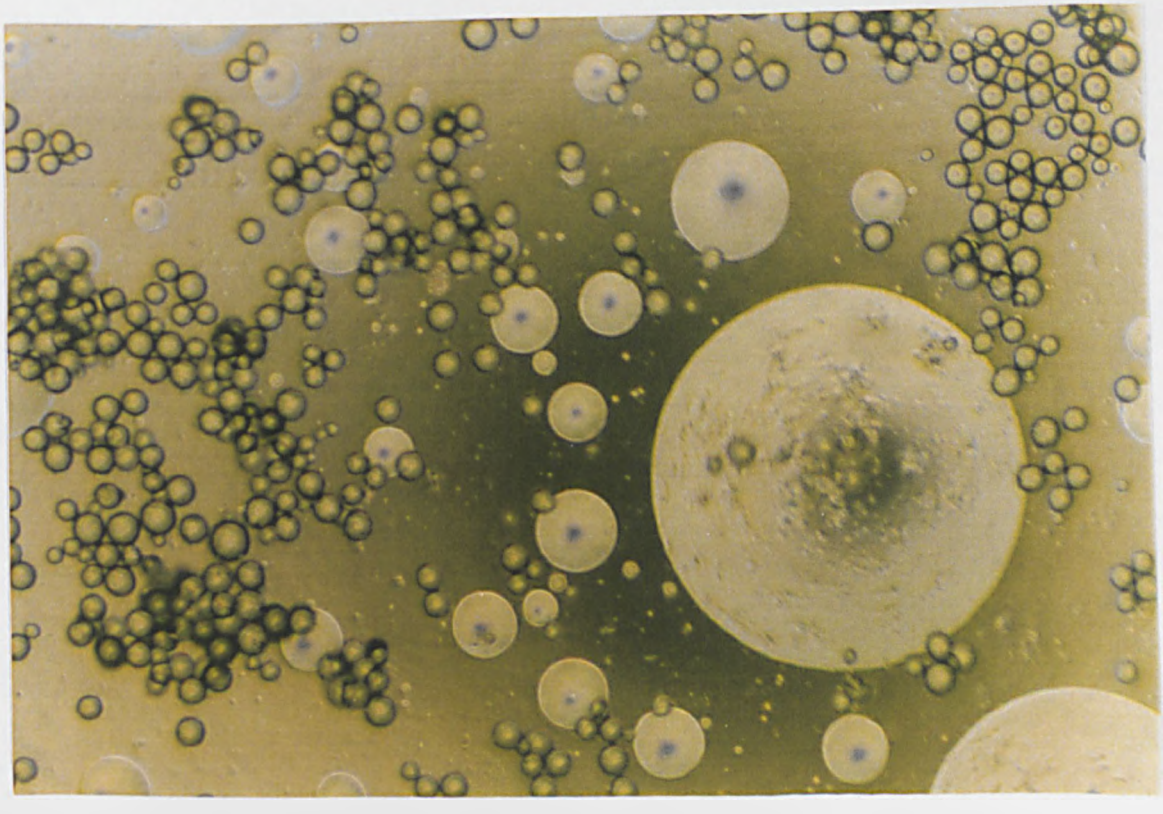
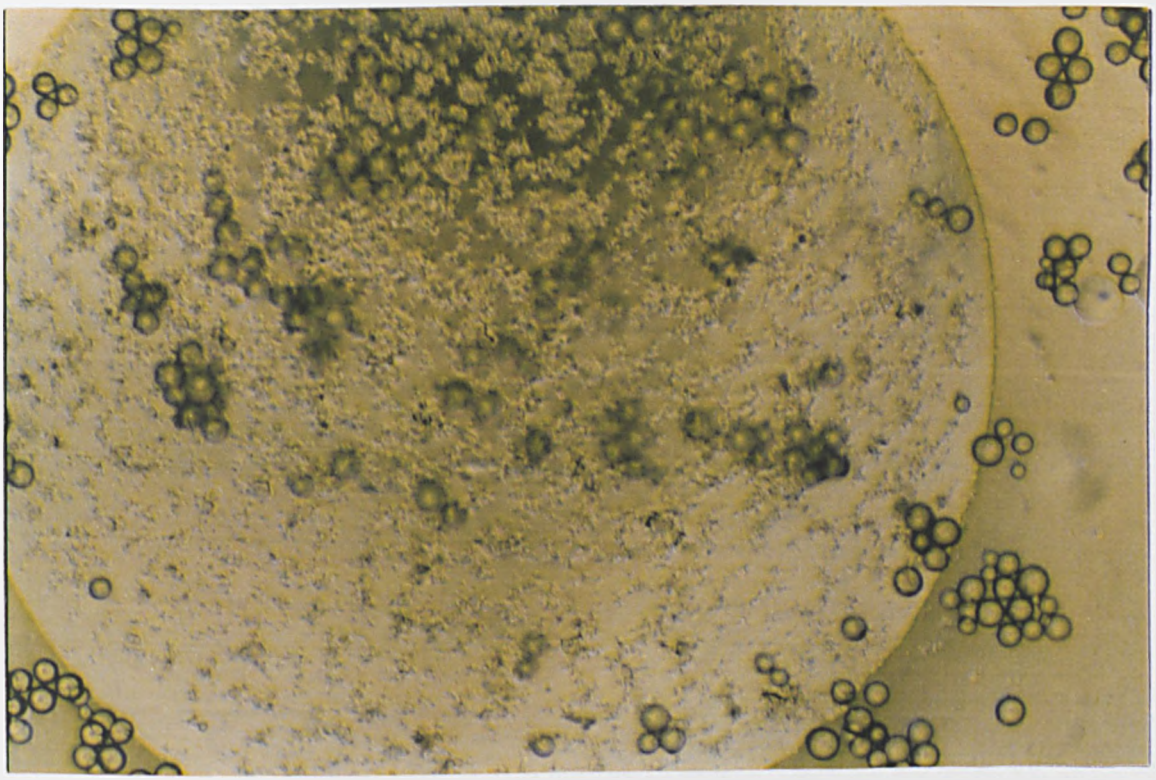


Fig. 4.2 Continued

C) Incubation of isolated fat cells with TMA + IN after (4 h)



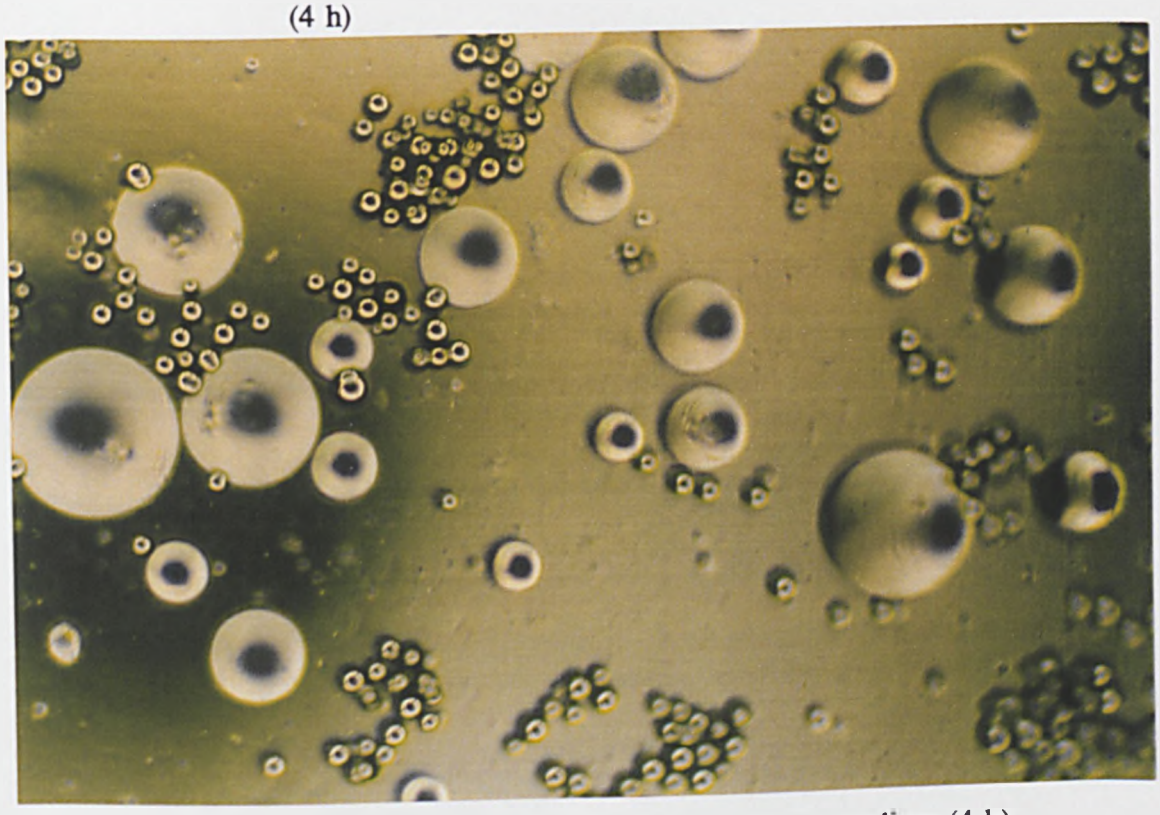
Male Wistar rats weighing 170-190 g were used for this experiment under standard or CRM diet. Isolated adipocytes were obtained using the method modified from Rodbell (1964) under aseptic conditions. Isolated adipocytes (5 x 10^5 cells/ml) were incubated at 37°C in sterile conditions in KRB buffer containing a final concentration of 25 mM Hepes buffer in airtight 25 cm^2 sterile culture flasks. Cells were exposed to 50 μl/ml TMA and 10 nM insulin or a combination of both at 37°C for the times indicated with gentle shaking. The reaction was stopped and ^{14}C-lipid was extracted as described in the "Materials and Methods". Photographs shows the size differences in fat cells after 4 h incubation with (X40):

A) Insulin B) TMA C) IN+TMA D) NRBC supernatant (control)
E) Cells+incubating buffer (control)

There was difference in size of fat cells due to stimulation of lipogenesis by the above agents. There was marked increase in the size of the fat cells when they were incubated with TMA + IN (synergy effect) compared to controls.

Fig. 4.2 Continued

D) Incubation of isolated fat cells with NRBC boiled supernatant (4 h)



E) Basal incubation of cells with incubation medium (4 h)

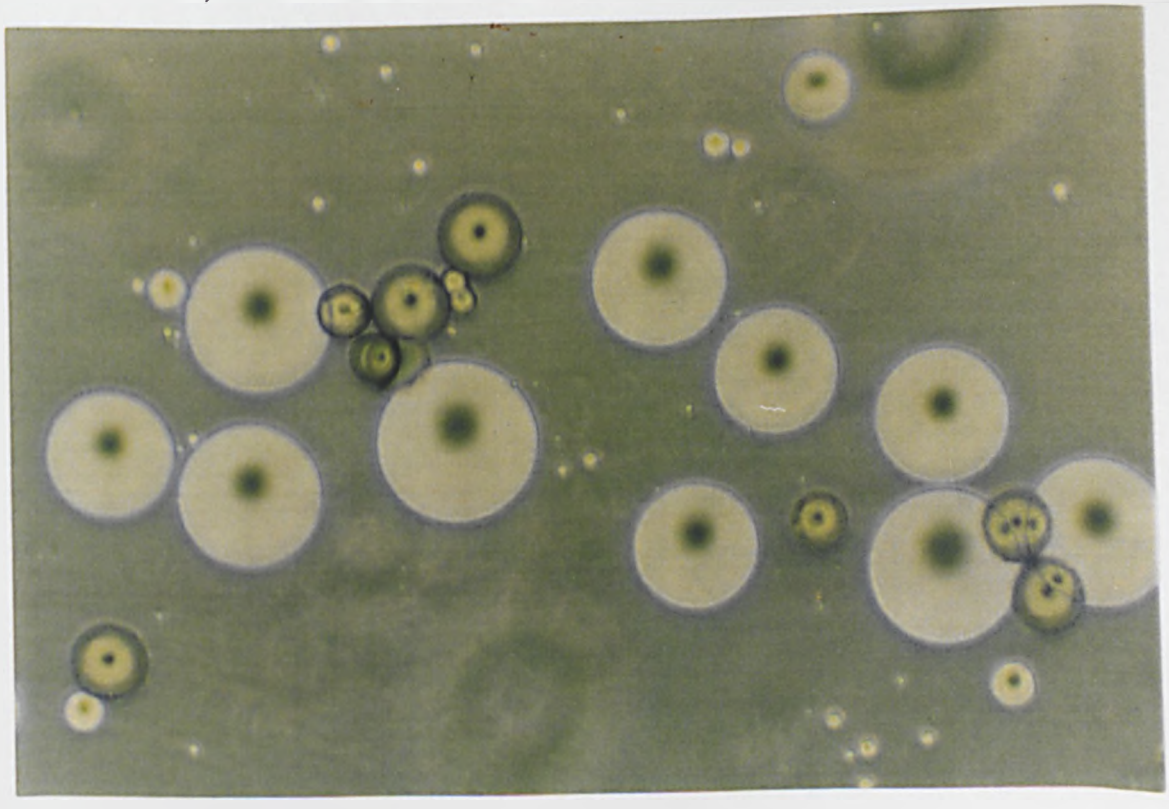
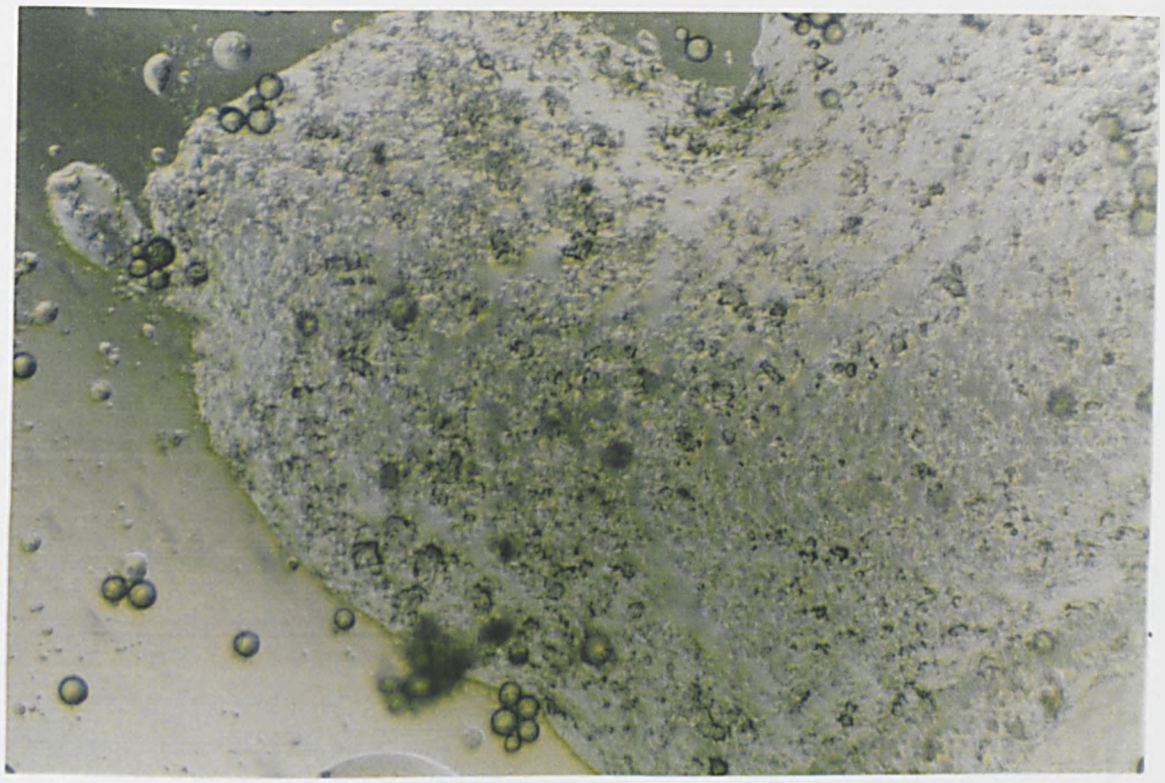


Fig. 4.3 A) Incubation of isolated fat cells with insulin (12 h)



B) Incubation of isolated fat cells with TMA (12 h)

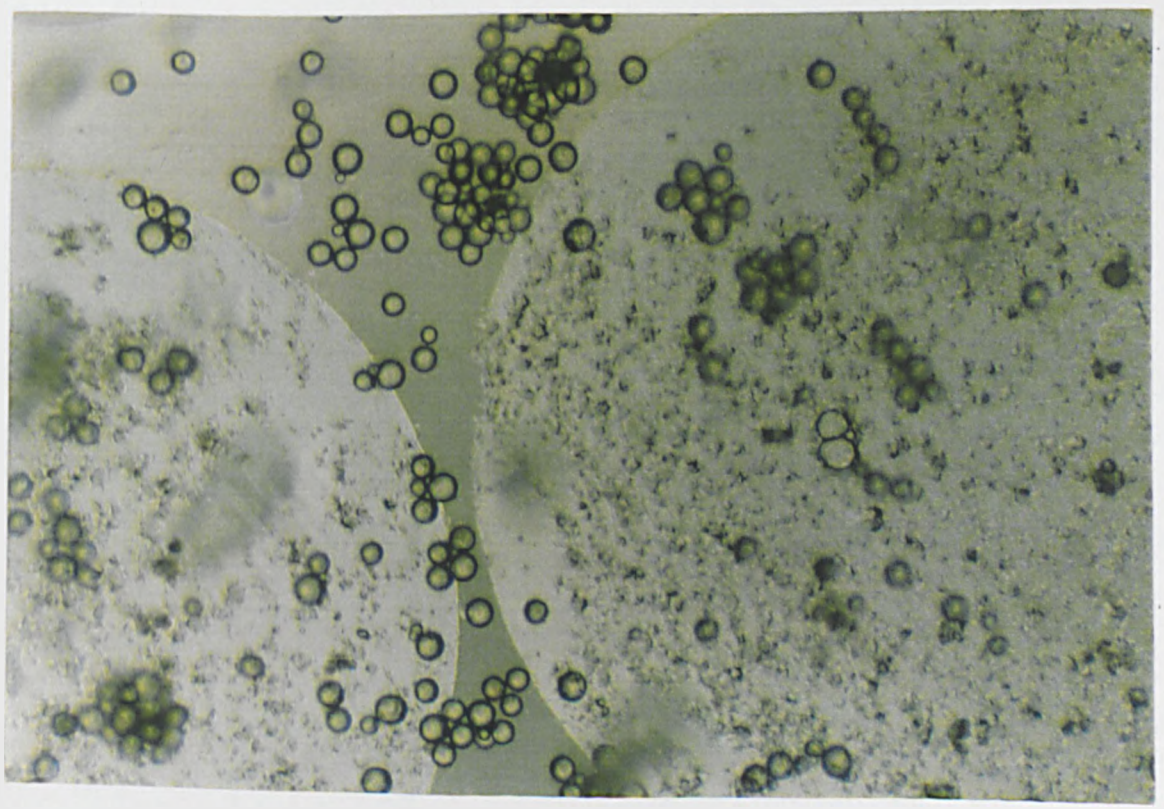
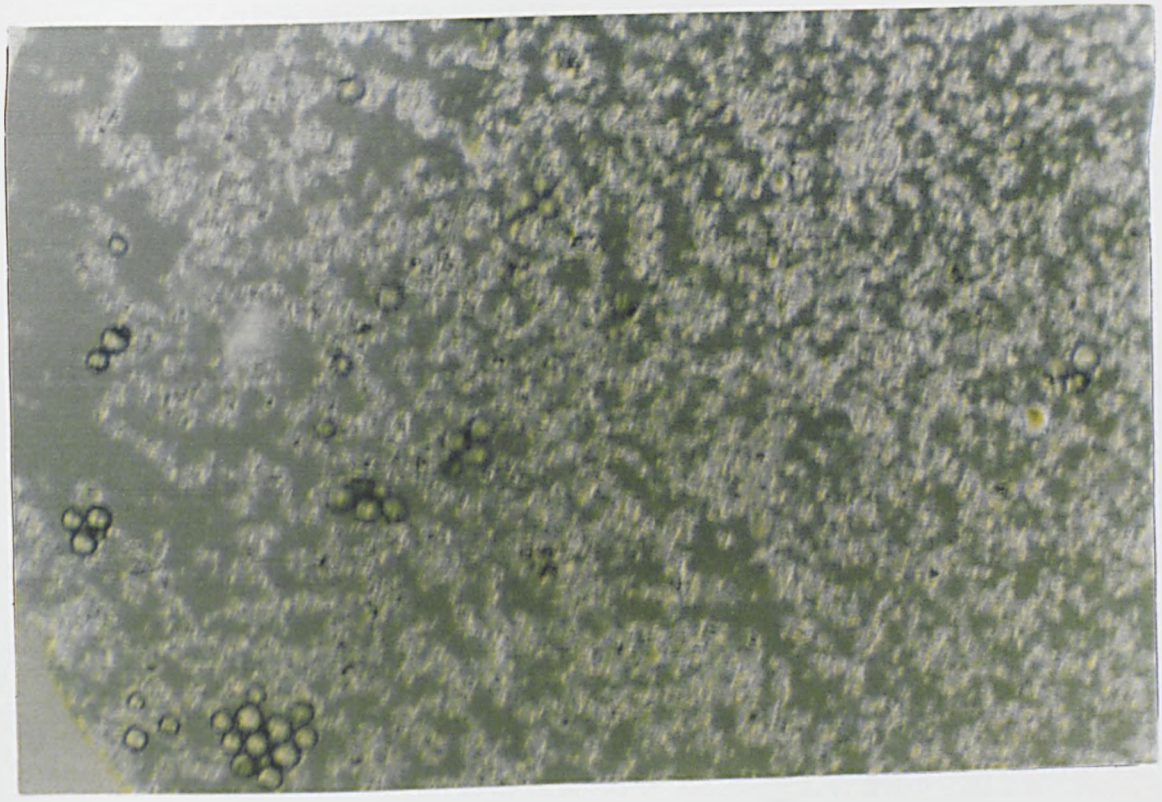


Fig. 4.3 Continued

C) Incubation of cells with T + IN (12 h)

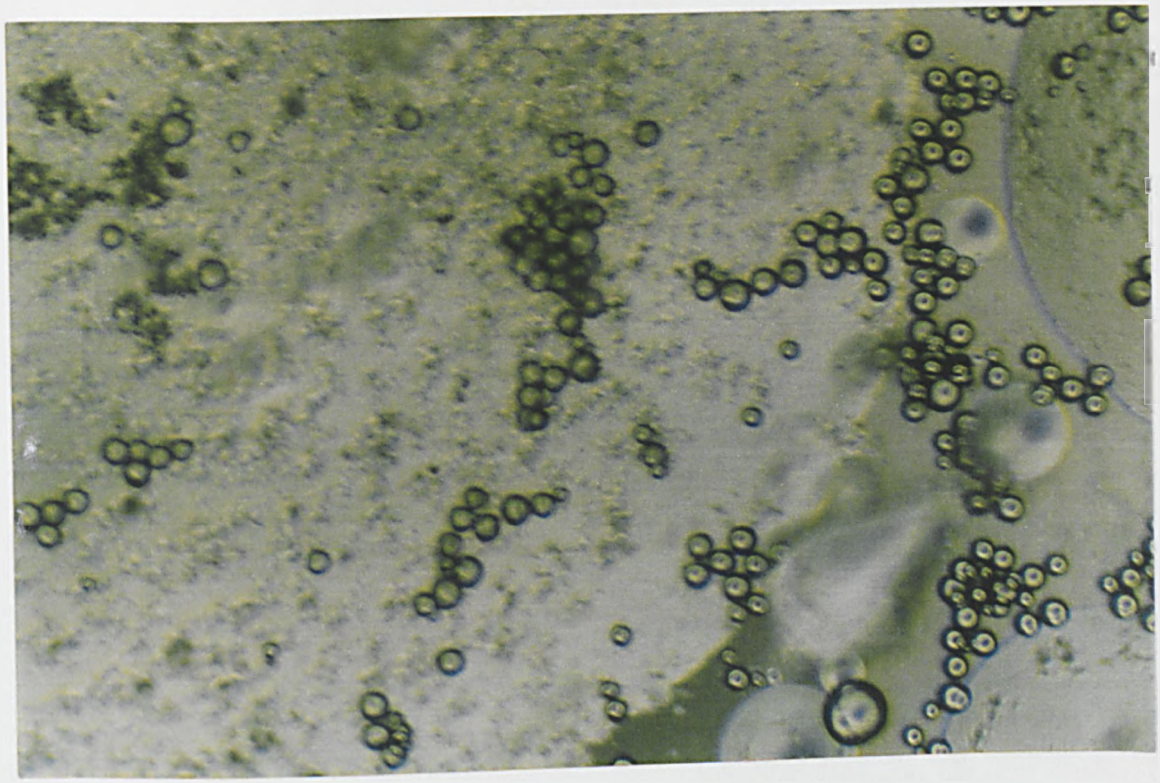


Male Wistar rats weighing 170-190 g were used for this experiment under standard or CRM diet. Isolated adipocytes were obtained using the method modified from Rodbell (1964) under aseptical situation. Isolated adipocytes (5 x 10^5 cells/ml) were incubated at 37°C in sterile condition in KRB buffer containing a final concentration of 25 mM Hepes buffer in airtight 25 cm^2 sterile culture flasks. Cells were exposed to 50 μl/ml TMA and 10 nM insulin at 37°C for the times indicated with gentle shaking. The reaction was stopped and ^{14}C-lipid was extracted as described in "Materials and Methods". Fig shows that the size differences in isolated rat fat cells after 12 h incubation with (X40):

A) Insulin B) TMA C) IN+TMA

There was a increase in size of all fat cells incubated with above agents after 12 h.

Fig. 4.4 A) Incubation of isolated fat cells with insulin (24 h)



B) Incubation of isolated fat cells with TMA (24 h)

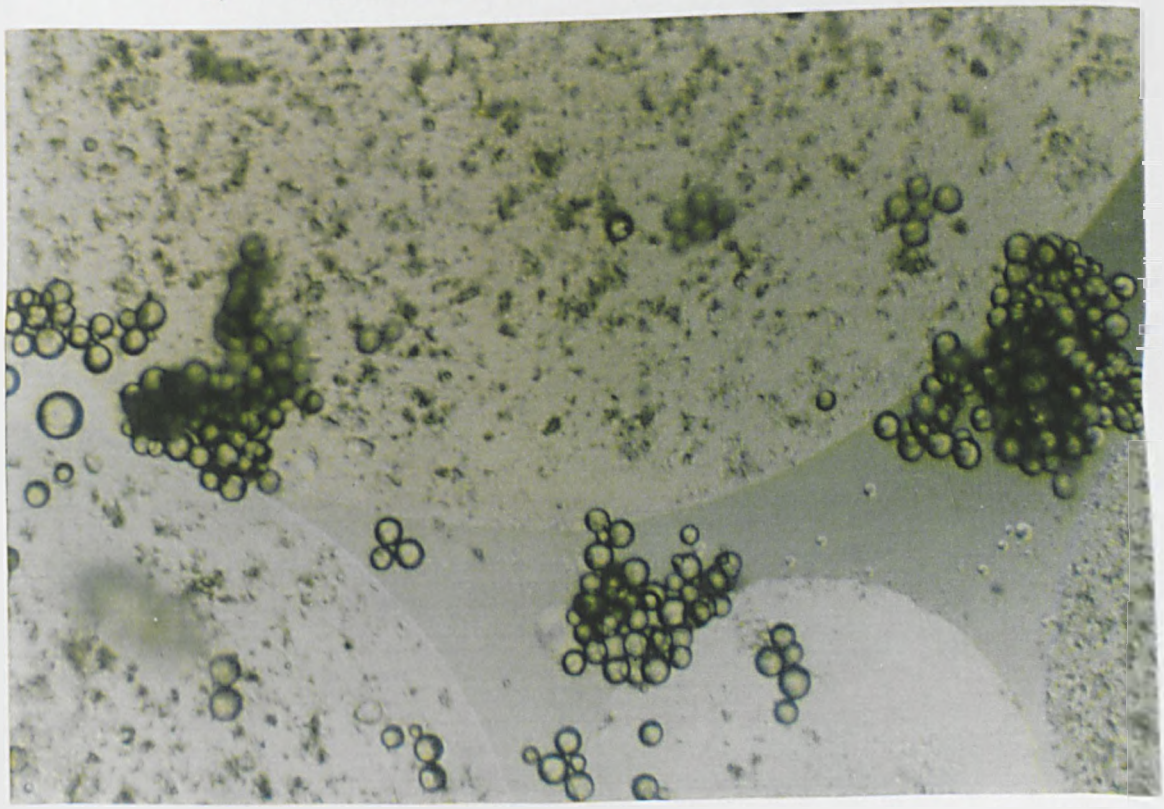
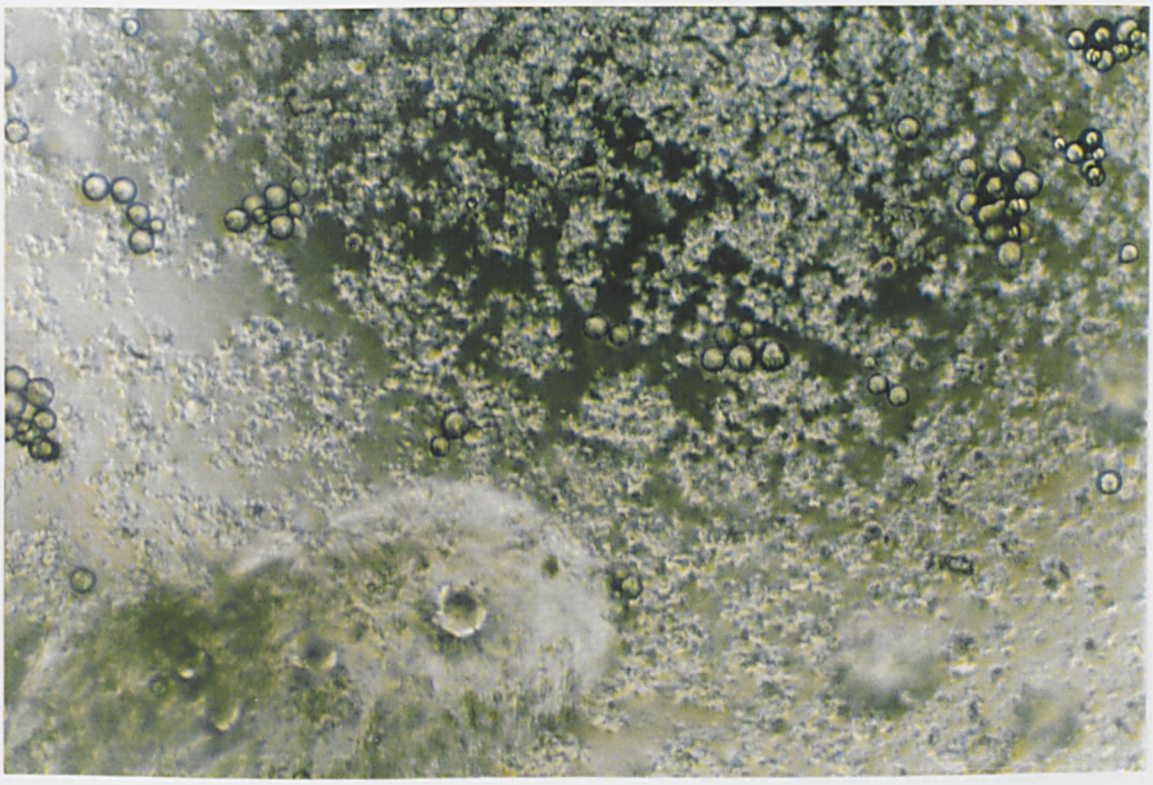


Fig. 4.4 Continued

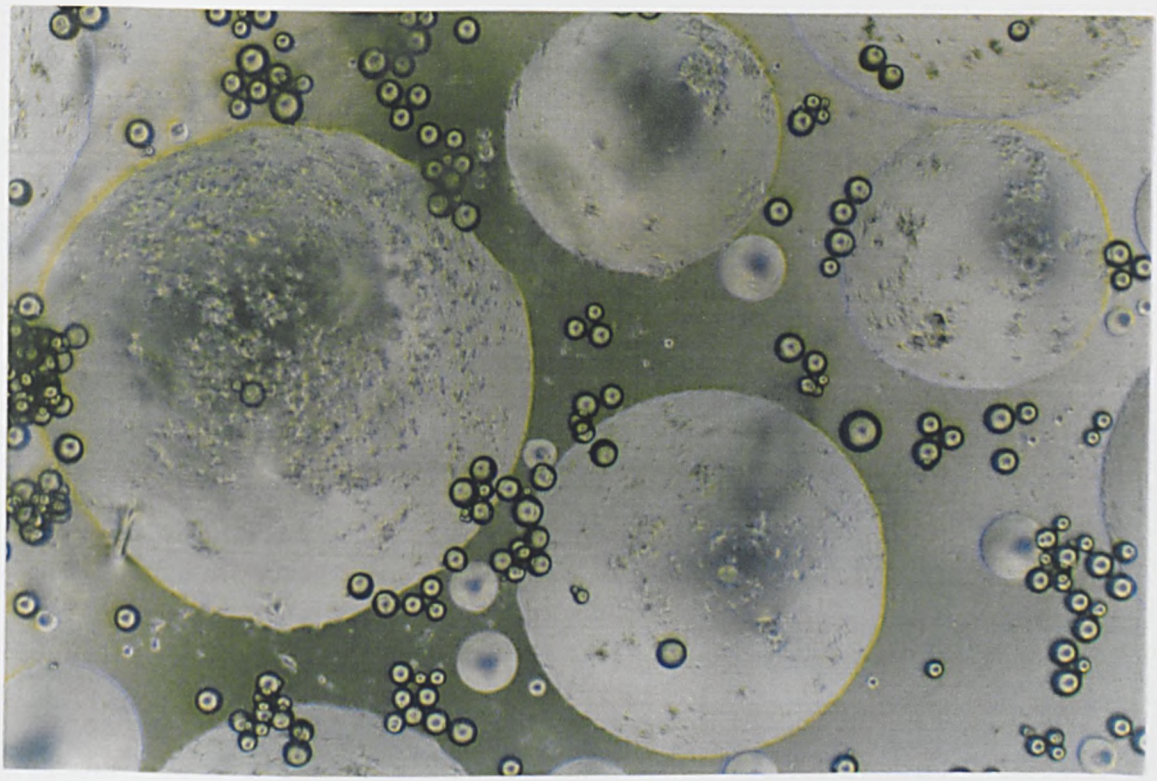
C) Incubation of cells with TMA + IN (24 h)



Isolated adipocytes were obtained using a method modified from Rodbell (1964) under aseptic conditions. The procedure employed was the same as that described in Fig. 4.2, except that the incubation time was 24 h. The size of cells increased at the same level even after 24 h cells incubation with TMA and insulin (X40). Stimulation of lipogenesis by TMA and insulin was similar after prolonged incubation. The size of controls increased in comparison to basal level at zero time but not the same as after incubation with TMA and insulin.

Fig. 4.4 Continued

D) Incubation of isolated fat cells with NRBC supernatant (24 h)



E) Incubation of cells with incubation medium (basal) (24 h)

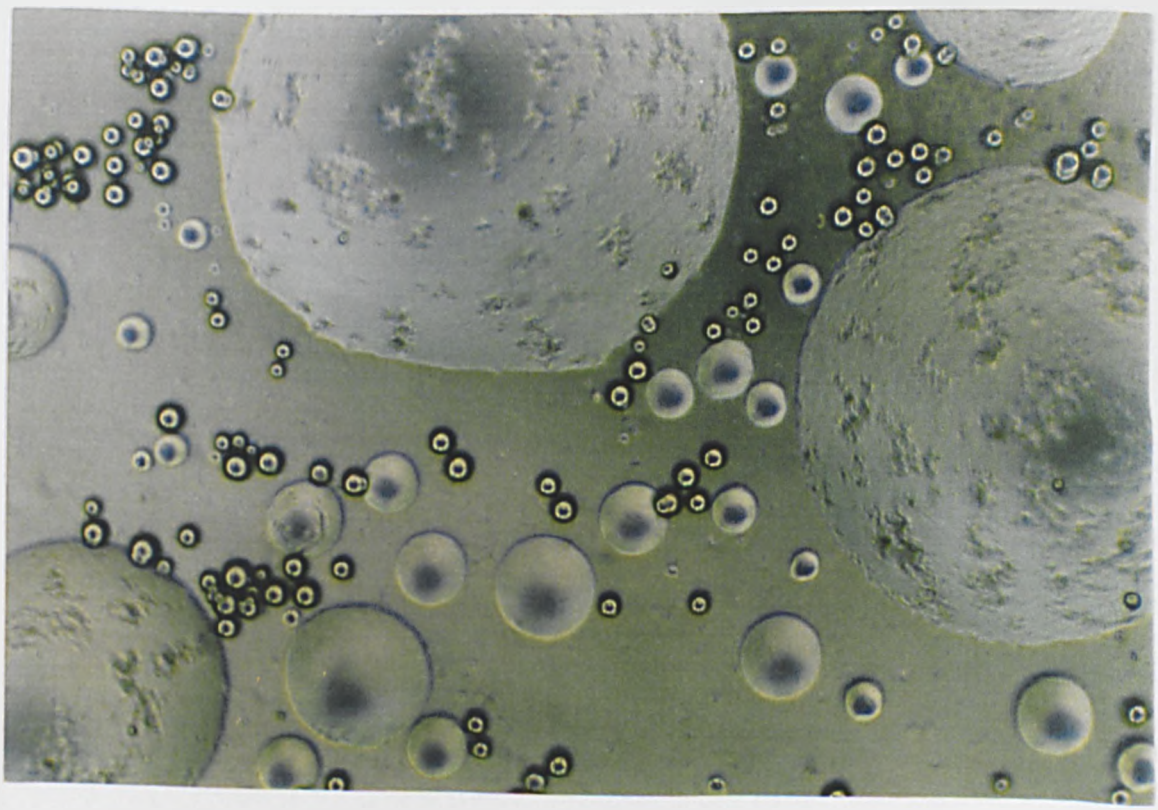
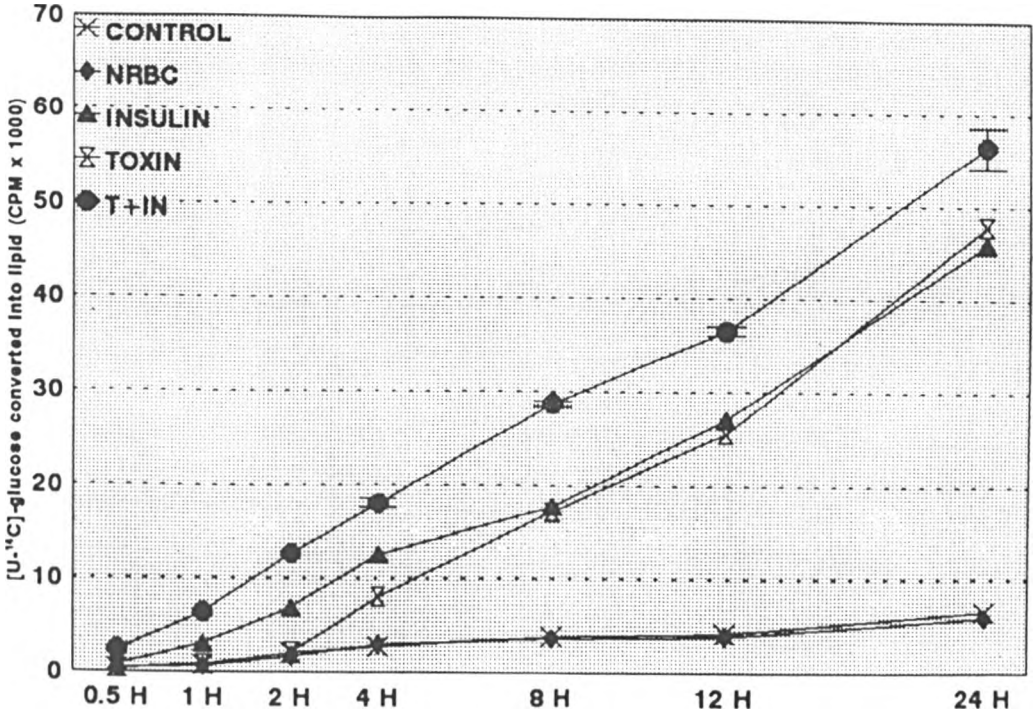
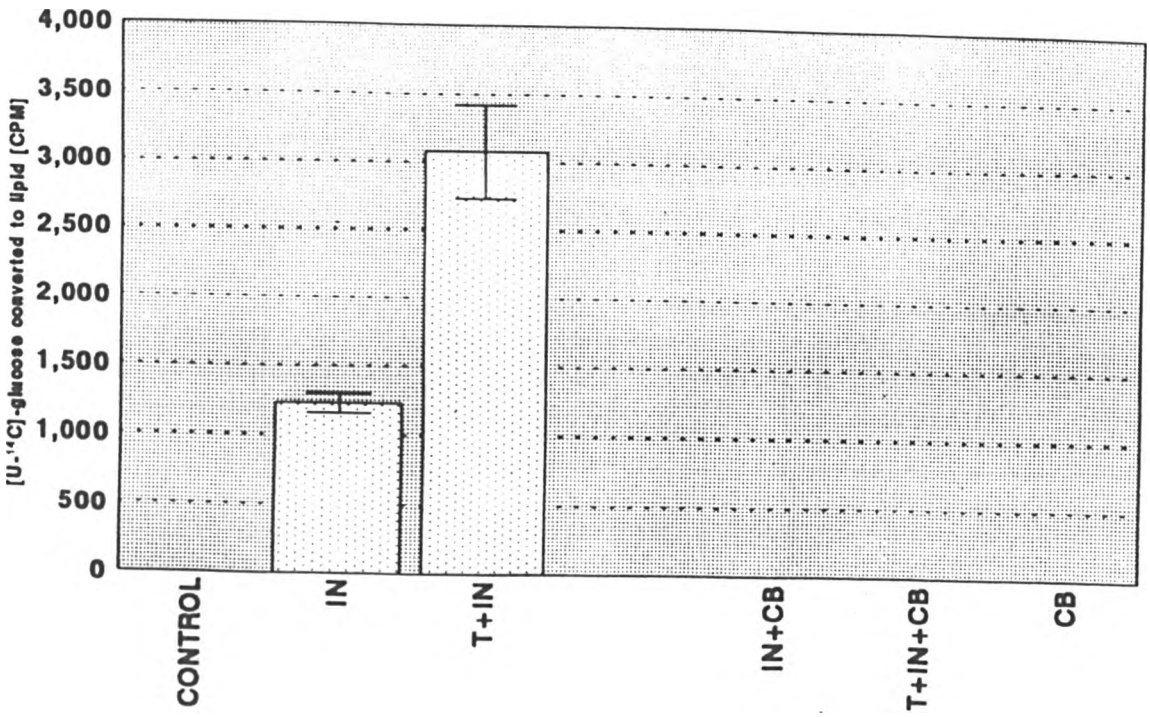


Fig. 4.5 TIME COURSE EFFECTS OF INSULIN AND TMA ON LIPOGENESIS IN RAT ADIPOCYTES



Male Wistar rats weighing 170-190 g were used for this experiment under standard or CRM diet. Isolated adipocytes were obtained using the method modified from Rodbell (1964) under aseptical situation. Isolated adipocytes (5 x 10^5 cells/ml) were incubated at 37°C in sterile conditions in KRB buffer containing a final concentration of 25 mM Hepes buffer in airtight 25 cm^2 sterile culture flasks. Cells were exposed to 50 μl/ml TMA and 10 nM insulin at 37°C for the times indicated with gentle shaking. The reaction was stopped and ^{14}C-lipid was extracted as described in "Materials and Methods". Result shows that during culturing of cells with 50 μl/ml TMA and 10 nM insulin or a combination of both, TMA acting by their own after 12-24 h incubation up to insulin level. However, in short term incubation TMA can only act in synergy with insulin. The cells were also incubated in absence of TMA and insulin as basal control and NRBC supernatant 50 μl/ml as second controls. The results presented are the means ($\pm$ SEM) of duplicate determinations from three different experiments.

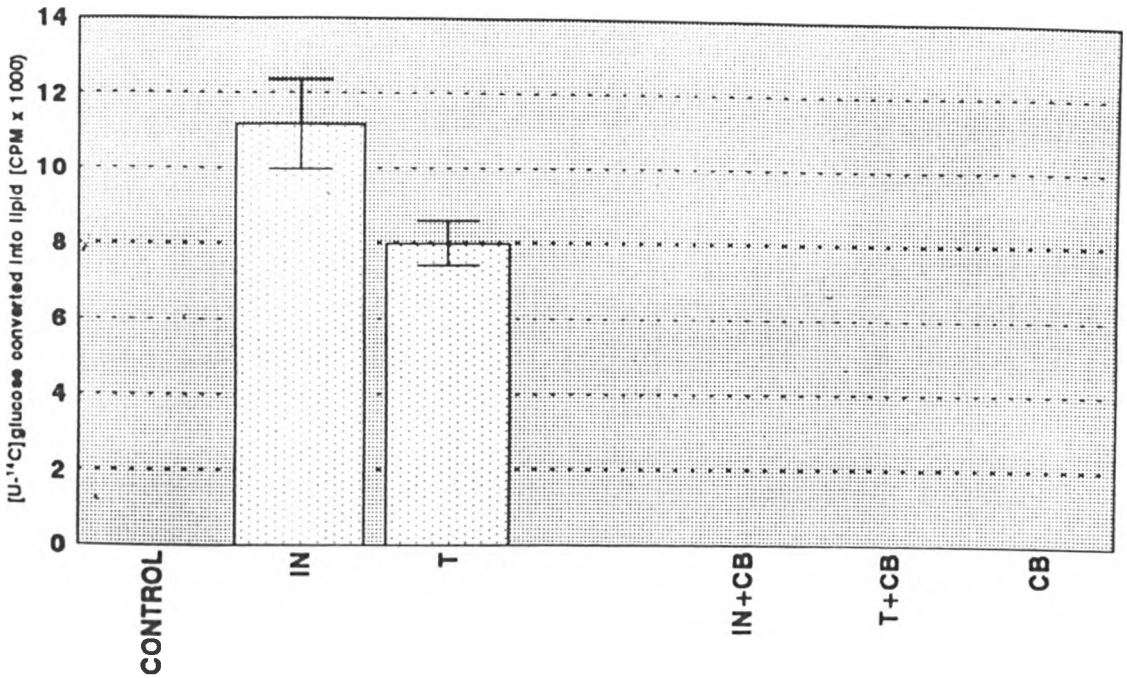
Fig. 4.6 INHIBITORY EFFECT OF CYTOCHALASIN B ON SYNERGY BETWEEN TMA AND INSULIN



Isolated adipocytes were obtained using a method modified from Rodbell (1964). Isolated adipocytes (5 x 10^5 cells/ml) were incubated at 37°C in KRB buffer containing 1% defatted BSA, [U-^{14}C]glucose and 1% (v/v) dimethyl sulphoxide. Cells were exposed to 50 μl/ml TMA and 10 nm insulin and combination of both. At zero time of incubation, 2 μg/ml cytochalasin B was added to culture flasks containing TMA or insulin or combination of both. The cells were also incubated with 2 μg/ml of cytochalasin B as control. After 60 minutes incubation, conversion of [U-^{14}C]glucose into lipid was measured as described in the Materials and Methods section. Results demonstrated that cytochalasin B at that concentration inhibited the synergy effect of TMA on lipogenesis by 100%. The results presented are the means ($\pm$ SEM) of duplicate determinations.

T=*P. falciparum* toxin malaria antigen(s)
IN=Insulin
CB=Cytochalasin

Fig. 4.7 INHIBITORY EFFECT OF TMA AND INSULIN ON LIPOGENESIS BY CYTOCHALASIN B



Isolated adipocytes were obtained using a method modified from Rodbell (1964) under aseptical situation. Isolated adipocytes (5 x 10^5 cells/ml) were incubated sterile at 37°C in KRB buffer containing a final concentration of 25 mM Hepes buffer and 1% (v/v) dimethyl sulphoxide in airtight 25 cm^2 sterile culture flasks. Cells were exposed to 50 μl/ml TMA and 10 nm insulin. At zero time of incubation 2 μg/ml cytochalasin B was added to the culture flasks containing TMA or insulin. The cells also incubated with 2 μg/ml of cytochalasin B as control. After 24 h incubation, conversion of [U-^{14}C]glucose into lipid was measured as described in the Materials and Methods section. The results demonstrated that cytochalasin B at that concentration inhibited the effect of TMA on lipogenesis by 100%, similar to that obtained by incubation with insulin. The results presented are the means (± SEM) of duplicate determinations.

T = *P. falciparum* toxin malaria antigen(s)
IN = Insulin **CB** = Cytochalasin B

CHAPTER FIVE

CHARACTERIZATION OF TOXIC MALARIA ANTIGEN OF *P.falciparum* INFECTED RED BLOOD CELLS

5.1 Introduction

Cerebral malaria (CM) is a severe complication of *Plasmodium falciparum* infection, whose physiopathology is still poorly understood (Wyler, 1983). It is caused by replication of malaria parasites, obstruction of blood flow by sequestrated infected erythrocytes, and excessive production of cytokines (Clark *et al.* 1989; Kwiatkowski, 1991). A major challenge has been to explain how the parasites, while restricted to red cells, can cause tissue injury and biochemical alterations in a range of uninvited tissues and organs. For the past decade the concept has been developing that these changes are caused by the effect of some active component, or "toxin", released from the parasite. This active component stimulates murine macrophages to secrete TNF (Bate *et al.* 1989), human monocytes to secrete IL-1 (Jakobsen *et al.* 1991) and IL-6 (Jakobsen *et al.* 1993a). It has been reported that TNF-inducing molecules from cells infected with rodent malaria parasites or with *P. falciparum* and *P. vivax* are antigenically related (Bate *et al.* 1992c). Damaged uninfected erythrocytes also induce TNF-α release, although this activity is less than those in infected red blood cells (Bate & Kwiatkowski, 1994a). The toxic activity resists digestion by proteases and depends upon a phospholipid structure (Bate *et al.* 1992b). Characterisation of the molecular structure of *P. falciparum* TNF-inducing TMA,

with a range of monoclonal antibodies raised against *P. falciparum* TMA or phosphatidylinositol, has confirmed the phosphatidylinositol (PI)-like structure of *P. falciparum* TMA (Bate & Kwiatkowski, 1994c).

In addition to the phospholipid-based toxins described above, protein associated with haemozoin, the breakdown product of haemoglobin, also induced the production of IL-1β and TNF-α (Pichyangkul *et al.* 1994). These molecules showed no resistance to pronase digestion. Glycosylphosphatidylinositol (GPI)-anchor components of the merozoite surface antigens MSP-1 and MSP-2 have also been shown to produce TNF and IL-1 (Schofield & Hackett, 1993). Late stages of the parasite have been shown to produce several glycolipids and two putative GPI anchor precursors (Gerold *et al.* 1994). It is unknown whether the induction of cytokine secretion during malaria is caused by release of PI-containing moieties into the circulation during the merozoite reinvasion and rupture of erythrocytes or by a unique IMP-containing GPI-like malaria parasite toxin.

TNF-inducing extracts from parasitized erythrocytes of *P. yoelii* injected into mice cause a rapid drop in blood glucose (Taylor *et al.* 1992a). This activity also appears to be due to a phospholipid-containing component, in that it was unaffected by digestion with pronase, was abolished by lipase digestion and inhibited by inositol monophosphate. The active constituent(s) of these preparations are not fully characterised, although they appear to be conserved amongst murine and human malaria parasites (Appear *et al.* 1990).

This study was started to assess the ability of TMA prepared from *P. falciparum* to synergise with insulin *in vitro* or induce lipogenesis alone. In Chapter three, we have proved that TMA of *P. falciparum* can enhance insulin signalling in the lipogenic pathway in rat adipocytes. Chapter four also demonstrated that TMA alone can stimulate lipogenesis. The lipid nature of TMA was suggested by lipase treatment which destroyed the biological activity. Therefore, the preliminary studies have shown a similarity between the TMA of *P. falciparum* and the molecules which had previously been described from *P. yoelii*. To investigate whether lipids of RBCs infected with *P. falciparum* are responsible for specific biological activities, detailed structural analysis was necessary. In order to further characterize the toxin(s) we have extracted lipid from *P. falciparum* IRBC culture supernatant and analysed the lipids by different biochemical techniques. Such studies, and more detailed analysis of the effects of active component on host cells, are needed in order to understand the role of this active molecule(s) in the pathophysiology of malaria.

5.2 Results

5.2.1 Separation of pigment from spent culture medium of *P. falciparum*

A synchronous *P. falciparum* culture at trophozoite stages (>10% parasitaemia) was incubated for 12-16 hours at 37°C. The spent culture medium was collected after rupturing of infected red blood cells and the merozoite reinvasion had occurred. Malaria pigment was separated from the spent culture supernatant as described in section 2.2.1. The supernatant with and without pigment was subjected to the bioassay. Fig. 5.1 demonstrates that there is no significant difference between incubation of the rat adipocytes with boiled supernatant of *P. falciparum* with (Tb) and without pigment, in long term culture of fat cells for 24 h at 37°C. The results revealed enhanced activity in the absence of pigment in our preparations. We also tested pronase treatment (Tp pronase digested *P. falciparum* culture supernatant was prepared from the same batch of Tb) of both preparations and still there was no difference in their ability to stimulate lipogenesis in the rat adipocytes. Hence, malaria pigment is not responsible for biological activity of TMA in *P. falciparum* culture supernatant.

5.2.2 Separation of lipid classes by thin layer chromatography (TLC)

Boiled supernatant of *P. falciparum* CY27 strain was extracted as described in section 2.7.1. The total lipid extract was chromatographed by TLC (section 2.8). Fig. 5.2 shows the analysis of a total lipid extract of *P. falciparum* infected red blood

cells (IRBC). The results showed separation of neutral lipid classes into steryl esters (SE), triacylglycerol (TAG), free fatty acid (FFA), free sterol (FS) and polar lipid (PL) by TLC. All lipid fractions were identified using co-chromatography with markers. The NRBC supernatant (as control, comparing to IRBC with *P. falciparum*, the same batch of erythrocytes were used) was extracted in the same way and total lipid classes were separated by TLC. The results revealed that the majer classes of lipid in both samples (IRBC and NRBC) and control were the same with this technique.

5.2.3 Bioassay of different total lipid classes from *in vivo* and *in vitro* cultures of *P. falciparum*

Boiled supernatants of *P. falciparum* strains CY27 and K1 were extracted as described in section 2.7.1. Separation of the total lipid extract (TLT) was achieved by TLC (section 2.8.8). Each fraction was separated as described in Materials and Methods. After each separation the fractions were assayed for lipogenesis-inducing activity in the rat adipocyte assay. The NRBC supernatant was treated in the same way and each fraction was then tested by bioassay in the same experiment as the above samples. Fig. 5.3 demonstrates the synergistic effect between lipid fractions and insulin *in vitro*. During the extraction of boiled supernatant of *P. falciparum* IRBC and NRBC supernatant from both the aqueous and organic phases were collected and tested in the bioassay. The organic lipid phase was separated by TLC and all the different fractions were presented to the fat cells by making an emulsion in PBS. The results showed no synergy between the aqueous phase samples with

insulin, therefore no active molecules were present in the aqueous phase. The total lipid extract of showed a synergy effect with insulin compared with a positive control (a boiled supernatant from *P. falciparum* culture). In order to investigate that activity related to a particular lipid class, the total lipid extract was separated into the lipid classes by TLC, with each fraction eluted separately and then tested by bioassay. The results showed no synergy between insulin and steryl esters (SE), triacylglycerol (TAG), free fatty acid (FFA) and free sterol (FS) of samples from *P. falciparum* IRBC or NRBC. However, the polar lipid (PL) fraction of IRBC showed the synergistic effect compared to the basal value. The PL of the NRBC preparation did not show any synergy with insulin in the promotion of the lipogenic pathway. The fractions from both strains of *P. falciparum* showed similar activity in synergy with insulin in the rat adipocyte assay. Taken together, these data prove that the TMA activity of *P. falciparum* spent culture was associated with the lipid fraction and the total activity of the lipid was related to the polar lipid but not to the neutral lipid fractions. No activity was found in the aqueous phase following lipid extraction and no synergy was detected with the fractions separated from the NRBC total lipid. The comparison of data suggest that the active component (polar lipids) was conserved between *P. falciparum* strains.

Chapter three describes how a similar activity of *P. falciparum* TMA was detected in serum from monkeys infected with *P. falciparum* (*in vivo*). In order to characterize the active components, infected monkey serum (36% parasitaemia) and normal monkey serum (NMS) were extracted as described in Materials and Methods. Total lipid was separated as above and different fractions were eluted from the TLC

plate. Analysis of the total lipid extract of monkey serum showed a similar pattern to that described in Fig. 5.2 for total lipid extract of *P. falciparum* IRBC culture. All the different fractions were added in PBS to the rat isolated fat cells. The aqueous phase, and all the different fractions (neutral and polar lipids), were tested in the rat adipocytes assay (Fig. 5.4). The results show a synergistic effect between lipid of monkey serum and insulin *in vitro*. The results revealed the synergistic effect only with the PL fraction of infected monkey serum. No synergy was identified between the PL fraction of NMS and insulin. The results again confirmed the similarity between TMA of *P. falciparum* culture (*in vitro*) and an active component in an infected monkey serum (*in vivo*).

5.2.4 Analysis of the PL fraction of *P. falciparum* culture supernatant by TLC

For further identification of the PL fraction of IRBC, we analyzed the PL by TLC which was developed in different solvent systems as described in Materials and Methods. Fig. 5.5 shows the result of IRBC and NRBC PL analysis by TLC. It was found that by using the solvent system (chloroform/methanol/water, 65:25:4 v/v/v) considerably wider separations of fractions which co-migrated with phosphatidylinositol (PI) and phosphatidylcholine (PC) standards were achieved. Two major and visible fractions were identified which co-migrated with PI and PC markers. There was no difference in the patterns of the IRBC and NRBC PL separations.

5.2.5 Bioassay of different PL classes

The polar lipid fractions of *P. falciparum* IRBC and NRBC were separated into different PL classes by TLC as described in Materials and Methods. As mentioned above, only two major fractions were identified in both samples. Because we do not know anything about the active molecules and it is possible that the active molecules were only present in minute amounts, it was wise to examine the whole area of the TLC plate from where samples were applied at the origin up to the solvent front. Thus the plate was divided into zones of the same size to provide different fractions (12 fractions). In order to find which part contained the active molecules, all fractions were subjected to assay for lipogenesis-inducing activity in the rat adipocyte assay. All samples were prepared in a small amount of chloroform/methanol (1:1 v/v) and diluted with PBS for presentation to the assay (5 μl/ml). This amount of solvent showed no effect on lipogenesis (Fig. 5.3).

The results of the bioassay showed no synergy between insulin and PL fractions of NRBC. However, there was synergy to varying extends in all 12 fractions of the PL of the IRBC even where these was no visible lipid spot on the TLC. The most activity was from the fraction which was co-migrated with the PI standard (Fig. 5.6). In some experiment the higher synergistic activity was related to fractions three (F3) and four (F4) which were co-chromatographing with PI and PC as standards, respectively, but always all 12 fractions showed some synergistic activity compared to the basal and control values (NRBC P classes).

5.2.6 Analysis of PL by HPLC

The 12 fractions of polar lipids (PL) of the IRBC, which were separated by TLC, all showed activity in the bioassay. Since characterization of all the fractions would be very difficult, it was decided to see if HPLC purification of an active principle could be applied instead of TLC. Fig. 5.7 shows a chromatogram of the total lipid extract (TLT) of *P. falciparum* IRBC. Chromatography was regularly completed in about 2 h by changing the flow rate as indicated in section 2.12.

Analysis of the total lipid extract by HPLC showed similar peak patterns for the IRBC and NRBC samples except in the PC region. The PC of NRBC appeared as a larger and wider peak in comparison to the PC peak which was obtained from IRBC.

The collected fractions were identified by comparing retention times of authentic PI and PC (Sigma U. K.) as markers. The eluate between peaks was also collected. All collected fractions were bioassayed in long term cultures (24 h) of the rat adipocytes. The results of the bioassay of different HPLC fractions showed stimulation of lipogenesis to different levels compared to the basal and controls (NRBC sample) (Fig. 5.8). However, fractions F3, F4 and F5 stimulated lipogenesis more than the other fractions (near to insulin level) and they had retention times which were similar to that of authentic PI (Fig. 5.7 and 5.8). None of the NRBC fractions stimulated lipogenesis in the rat adipocytes. These findings revealed a similarity between the TLC and HPLC separation of active compounds and confirmed the consistency of the results.

TLC and HPLC did not identify specific active fractions and all fractions obtained showed some synergistic effect with insulin and also stimulated lipogenesis on their own. The next aim was to scale up from TLC to silica gel column chromatography in order to obtain a larger amount of one bioactive material for further characterisation.

5.2.7 Composition and chromatography of PL of IRBC with *P. faciparum* by silica gel column chromatography

Boiled supernatants of *P. falciparum* IRBC culture were extracted as described in Materials and Methods. Aqueous phase and organic phase were collected and tested for their activity in long term cultures of the rat isolated adipocytes. The results showed no stimulation of lipogenesis by the aqueous phase, but there was again activity in the total lipid extract. The active total lipids from different preparations were pooled and were then fractionated on a silica gel column as described in section 2.11.3. The PL classes were separated according to their polarity by the different solvents. The initial non-polar solvent system eluted neutral lipids from the column, which were collected in the first F1 and second F2 fractions. As the polarity of the solvent was increased by increasing the ratio of methanol to chloroform eight fractions were collected F3-F10 (section 2.11.3). TLC of the ten fractions (Fig. 5.9) revealed the presence of different components with the more polar lipids appearing in fractions F7-F10. In order to detect lipid classes in different fractions, TLC plates were stained with specific detection reagents (see below).

In previous experiments the NRBC culture supernatant was extracted as described for the IRBC extraction. During the culturing of NRBC most of erythrocytes remain intact. However, IRBC with ≥10% parasitaemia were ruptured to released the merozoites and other debris. Therefore NRBC were ruptured in an artificial way and total lipid was extracted. The total lipid extract in chloroform was applied to a silica gel column and fractions were eluted with the different solvent systems as described above. The ten fractions obtained were analysed on silica gel G TLC plates, with a general detection reagent to give the results shown in Fig. 5.10.

5.2.8 Detection of phospholipids and glycolipids with specific TLC spray reagents

Chromatography of the different fractions eluted from the silica gel column of total lipids of *P. falciparum* IRBC spent culture on silica gel G TLC plates developed by chloroform/methanol/water (65:25:4 v/v/v) revealed different lipid spots. Fractions F5 to F10 reacted with molybdate reagent (section 2.9.2) and thus, represent phospholipids (Fig. 5.11). The compounds were tentatively identified according to their co-migration on the TLC plates with commercial standards and to their reaction with specific spraying reagents. F5 mostly contained lipid which co-migrated with phosphatidylethanolamine (PE) standard. F6 to F10 contained major materials which co-migrated with PI and PC standards (Fig. 5.11). There was another spot in these fractions which was more polar and migrated lower than authentic PI.

Fractions F1-F4 did not react with molybdate reagent, but reacted with

orcinol-sulphuric acid reagent (section 2.9.2), hence indicating the presence of glycolipids (Fig. 5.12).

Chromatography of the polar lipid fractions of intact NRBC on silica gel G TLC plates developed in the same solvent system mentioned above, showed different lipid spots compare with IRBC lipid extract. Phospholipid spots of the NRBC extract were detected by the molybdate spray reagent (Fig. 5.13) and glycolipids by orcinol-sulphuric acid reagent (Fig. 5.14).

F1 only contained neutral lipid. Glycolipids were detected in F2 to F4. There was apparently more glycolipid spots in the NRBC lipid extract compared to the IRBC lipid extract. However, this experiment was not quantitative, but Fig. 5. 12 and 5.14 reveal some change in the composition of lipids during growth of parasites in the erythrocyte.

5.2.9 Biological activities of F1-F10 from silica gel column chromatography

To test the effect of F1 to F10 for lipogenesis-inducing activity samples were prepared as described in Materials and Methods. The results shown in Fig. 5.15 revealed stimulation of lipogenesis in long term culturing (24 h) in the rat adipocytes in all different fractions. The most active samples were the phospholipid containing fractions. This finding confirmed the results of bioactivity measurements of all fractions obtained by TLC and HPLC methods. Blank regions of the TLC plates were also eluted and bioassayed as controls and they were negative. The total lipid extract

of NRBC did not stimulate lipogenesis in the rat adipocytes.

5.2.10 Analysis of F6-F8 from silica gel column chromatography

Fractions F7 and F8 showed high stimulation of lipogenesis and because there was adequate material in these fractions, they were chosen for further more detailed analysis. The molybdenum spray of the analytical TLC plates showed that these fractions contained phospholipids. These fractions,together with F6, F9 were separately chromatographed on silica gel G TLC plates developed by chloroform/methanol/water (65:25:4, v/v/v). Fraction F6 displayed three component bands (RF,0.22, 0.35, 0.6, respectively) which co-chromatographed with PI (RF, 0.22), PC (RF,0.34) and PE (RF,0.6). The bands of material were eluted from the plate with chloroform/methanol (1:1 v/v) and designated as F6(1), F6(2) and F6(3), respectively. Fractions F7-F9 also showed three components but in these cases they co-chromatography with an unknown (RF,0.1), PI (RF,0.22) and PC (RF,0.35), and have been designated as F7(u), F7(1), F7(2), F8(u), F8(1), F8(2), F9(u), F9(1) and F9(2), respectively. All the samples recovered from the preparative TLC were checked for purity by analytical TLC (Fig. 5.16) and shown to have little or no cross contamination.

The lipid fractions from the intact NRBC were separated by preparative TLC in a similar manner (as above) and the purity of the samples recovered was checked by analytical TLC (Fig. 5.17).

In order to find which of the samples was responsible for the bioactivity, each

individual sample was tested in the rat adipocyte assay. The results shown in Fig. 5.18 demonstrate stimulation of lipogenesis by the three samples obtained from F6-F8 of the *P. falciparum* IRBC. No stimulation has been found with F7(1) and F7(2) of intact NRBC. Fractions from the *P. falciparum* IRBC of different preparations showed similar activity, therefore, F6(1), F7(1) and F8(1) were pooled as were F6(2), F7(2) and F8(2) and these were then used for further analysis. F6(3) co-migrated with PE standard and was kept separately. F7(u) and F8(u) migrated at the same RF (Fig. 5.16) and were also pooled.

5.2.11 Treatment of fraction F7 with *Bacillus cereus* phospholipase C (PLC)

Having shown that all the different fractions eluted from the silica gel column were active and that the most active fractions contained phospholipid which co-migrated with PI-PC standards, we wished to characterise the active molecule(s) present in F7 by more detailed analysis. Enzymatic evidence regarding the nature of the triggering moiety was obtained by digestion of F7 with phospholipase C (PLC) from *B. cereus*. Pure phospholipase C readily hydrolyses PC, PE and phosphatidylserine (PS) with little or no significant hydrolysis of sphingomyelin (SPH), phosphatidylglycerol, cardiolipin and PI (Otnaess *et al.* 1977; Roberts *et al.* 1978).

F7 was incubated with 40 U of PLC at 37°C for 5 h and 24 h as described in Materials and Methods. The results showed that hydrolysis of F7 was complete after 5 h with this enzyme and the same products were present in the 24 h incubations. To

allow time for doing the extraction and TLC preparation of the products of hydrolysis without interruption it was therefore decided to incubate the lipid samples with PLC overnight with gentle shaking at 37°C. The enzymic hydrolysis was terminated after overnight incubation by five minutes boiling. The lipids were extracted with chloroform/methanol (2:1 v/v) for 2h. Both the organic and aqueous phases were collected and used for bioassay. The aqueous phase was dried under N_2 and a known volume of chloroform/methanol (1:1 v/v) added. This preparation was then diluted in PBS and was tested in the rat adipocyte assay for lipogenesis-inducing activity.

There was no measurable activity with the aqueous phase. However, the organic phase containing lipid from hydrolysis of F7 with PLC was bioactive and therefore was chromatographed on analytical silica gel G TLC plates with the solvent system chloroform/ethanol (98.4:1.6 v/v) (section 2.14.2). This solvent system separated cholesterol from 1,2-diacylglycerol (1,2-DAG). Berberine was used to detect the samples on the TLC plate (section 2.9.1) and this revealed six major spots including the material at the origin. The sample bands were eluted with solvent systems as described in section 2.15 and recovered samples used for bioassay. Because we suspected polar lipid in the lower part of the plate which may not elute with diethyl ether, the lower spots on the TLC plate were eluted first with chloroform/methanol (1:1 v/v) then with diethyl ether and combined. There was a blank control preparation from an area of TLC plate on where no sample was loaded. This part was treated as mentioned above. All the purified samples were then tested in the rat adipocyte assay. The component(s) under test were presented to the cells in PBS.

Fig. 5.19 shows that no activity was present in the aqueous phase. Total lipid recovered after extraction of hydrolysed F7 showed enhanced stimulation of lipogenesis on their own in the rat adipocytes when compared to the insulin stimulation level after 24 h incubation. As mentioned above, chromatography of the hydrolysed F7 showed six products. Each product was tested in a long term culture of the rat fat cells and the results are presented in Fig. 5.19. Samples F7(a), F7(d), F7(e) and F7(f) stimulated lipogenesis. However, F7(b), F7(c), (area with no visible sample) revealed little or no stimulation. The rat fat cells showed the highest response to F7(d) which co-migrated with 1,2-DAG standard (1,2-dipalmitoyl-*rac*-glycerol C16:0). The total lipid from intact NRBC and the control preparation from silica gel were tested and they both showed no stimulation of lipogenesis.

The 1,2-DAG sample showed high activity and therefore we wished to know from which of the components of F7 the 1,2-DAG containing sample was derived after treatment with PLC. Therefore, in the next, experiment the samples F7(1) and F7(2) of fraction F7 which co-migrated with PI-PC standards, were incubated with PLC under the same conditions mentioned above for F7. The hydrolysis products recovered after preparative TLC were tested with the rat adipocytes. The results demonstrated in Fig. 5. 20 showed no measurable stimulation of lipogenesis by the aqueous phase recovered from F7(1) and F7(2) after hydrolysis with PLC. However, chromatography of the recovered organic phase of F7(1) after hydrolysis with enzyme showed no visible spots of material upon TLC analysis. Therefore, the TLC plate was divided into two areas, F7(1)a was the material at the origin while F7(1)b was the material recovered from the rest of the plate up to the solvent front. Bioassay showed

activity in sample F7(1)a, but no significant stimulation was detected using F7(1)b. The result apparently demonstrated that PLC did not hydrolyse F7(1) to produce an active component although it seems that some degradation had occurred because the activity recovered was less after PLC treatment (Fig. 5. 20).

Sample F7(2) was also treated with PLC in a similar manner. After extraction and chromatography on a silica gel G TLC plate, three major spots were detected and recovered as F7(2)a, F7(2)b and F7(2)c. Sample F7(2)b co-chromatographed with 1,2-DAG standard and revealed high activity in the bioassay. The bioassay of the other two samples, F7(2)a and F7(2)c showed lower activity than F7(2)b (Fig. 5.20). The F7(2)b sample while referred to the 1,2-DAG sample for the remainder of this Chapter. The fractions F3 and F4, which contained glycolipids, were incubated with PLC but no hydrolysis occurred (Fig. 5.21).

A dose dependent stimulation of lipogenesis was observed with the 1,2-DAG sample. The sample was still active at 1:250 dilution (20 ng/ml) compared to control and basal values (Fig. 5.22).

The fraction F7 of intact NRBC recovered from silica gel column chromatography was treated with PLC and the total lipid extracted and chromatographed as mentioned for F7 of IRBC. The spot which co-migrated with the 1,2-DAG standard, was tested for lipogenesis-inducing activity and no measurable stimulation was found (Fig. 5.22)

To summarize, the results described in this Chapter demonstrate that the TMA activity is related to lipids, particularly the polar lipids. Analysis of the polar lipids with different techniques (TLC, HPLC and silica gel column chromatography) showed that different fractions stimulated lipogenesis and acted synergistically with insulin. Further characterization of the polar lipids showed that materials which co-migrated with PI-PC standards were responsible for high bioactivity. TLC hydrolyses of the appropriate lipid samples with phospholipase C has provided evidence that it may be the 1,2-DAG moiety of the phospholipids which is responsible for the stimulation of the lipogenesis in the rat fat cells.

5.2.12 Synergistic effect between the 1,2-DAG sample *P. falciparum* IRBC and insulin

In this Chapter we found that the 1,2-DAG sample from *P. falciparum* IRBC stimulated lipogenesis. Hence, the study was extended to determine if there was a synergistic effect between the 1,2-DAG sample and insulin. To measure the synergy effect the 1,2-DAG sample was dissolved in a small volume of chloroform and then diluted in PBS (section 2.16). In the first experiment we used rats under standard diet but no synergistic effect was observed. It was thought that perhaps the PBS may not present the active molecules to the fat cells effectively in short term culture. Thus in the next experiment, rats maintained on CRM diet were used to provide fat cells and the 1,2-DAG sample was added to the fat cells in either PBS or a bile salt [sodium taurodeoxycholate (NaTDC)] emulsion (Nichols, 1986). The results showed that there was a synergistic effect of the 1,2-DAG in the presence of both diluents (Fig. 5. 23).

It can be speculated that the problem in the first experiment was due to insensitivity of the rat cells as demonstrated in Chapter three.

5.2.13 Effects of cytochalasin B on the activity of the 1,2-DAG sample on lipogenesis in the rat fat cells

Cytochalasin B specifically inhibits the glucose-transport system of isolated fat-cells (Loten & Jeanrenaud, 1974). In Chapter four we concluded that cytochalasin B abolished the activity of TMA both alone and in synergy with insulin. The studies were then extended to find out the effect of this drug on the activity of the 1,2-DAG sample of *P. falciparum* IRBC. Fig. 5.24 presents results which demonstrate that cytochalasin B (section 2.16) inhibited the synergy effect of the 1,2-DAG sample and insulin. The 1,2-DAG sample of IRBC also stimulated lipogenesis after 24 h incubation at 37°C in the rat adipose cells (Fig. 5.25). Cytochalasin B inhibited this stimulation. The results suggested that the 1,2-DAG sample may have activated the glucose-transport system which can be inhibited by cytochalasin B.

5.2.14 Analysis of the fatty acids from the polar lipids *P. falciparum* IRBC and NRBC by Gas Chromatography (GC)

The analysis of the fatty acid composition of the polar lipids was achieved by gas chromatography. The fatty acids of the samples were converted to the fatty acid methyl esters (FAMES) prior to GC examination.

The polar lipid sample under investigation was transferred to a glass tube with a Teflon insert screw cap and then subjected to transmethylation with H_2SO_4-methanol as described in section 2.12.1. The FAMES were extracted with hexane and a sample (1 μg/μl) injected into a Carbowax capillary GC column as described in section 2.12.2. The results are presented in Fig 5.26 and show that the major fatty acids of the lipids of infected and uninfected red cells were virtually identical.

The results are presented in Fig. 5.26. The major fatty acids were palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1) and linoleic (18:2). Smaller amounts of C_{20} and C_{22} unsaturated fatty acids also evident together with trace of palmitoleic (16:1) and possibly myristic acid. The results showed the compositions of the fatty acid mixtures derived from the NRBC and IRBC were essentially the same.

5.2.15 Molecular species of 1,2-DAG from *P. falciparum* IRBC

A method for the GC analysis of 1,2-DAG released from phospholipids by PLC hydrolysis has been published by Liepkins *et al.* (1993). This method was adapted for the present work to allow analysis and structure determination by using the coupled gas chromatography-mass spectrometry (GC-MS) technique. The samples were first converted to the trimethylsilyl (TMS) ether derivative (see Materials and Methods, section 2.13) to protect the free hydroxyl group on the 1,2-DAG: this provides better stability for the compound at the elevated temperatures of GC-MS operation and usually ensures better peak shape and separation on the GC capillary column. The operating conditions and GC column details for the GC-MS (EI positive

ion) analyses are given in Materials and Methods, section 2.13.

GC-MS analysis of a sample of authentic 1,2-dipalmitoylglycerol TMS ether showed a single sharp peak eluting at 41 minutes. The molecular weight of this DAG TMS ether is 640.9 but a molecular ion $[M]^+$ was not observed. However, an ion at *m/z* 625.9 (relative abundance ~8%) represented an ion due to the loss of a methyl group from the molecular ion $[M-15]^+$ while a more prominent ion at *m/z* 384.9 (relative abundance ~27%) could be assigned to the loss of a palmitic acid moiety.

When the 1,2-DAG TMS ether samples obtained from the NRBC and IRBC were analysed by GC-MS they showed similar GC profiles (Fig. 5.27) and examination of the mass spectra allowed identification of the major components. Each sample had a minor peak at 41.3 minutes which had a mass spectrum closely resembling that of the authentic 1,2-DAG TMS ether above thus identifying it as 1,2-dipalmitoylglycerol. However, the mass spectra of the major peak in each sample showed it to be a mixture. Ions representing $[M-15]^+$ were present at *m/z* 651.9 and *m/z* 649.1. In the case of the former ion this is 26 a.m.u higher than the corresponding ion in the 1,2-dipalmitoylglycerol sample and thus represents the addition of $-CH_2.CH_2-$ (plus 28 a.m.u.) into one of the fatty acid chains together with a double bond (minus 2 a.m.u.). The ion at *m/z* 649.1, being 2 a.m.u. lower must represent a molecular species with one further double bond in a fatty acid chain. There are several candidate fatty acids which in appropriate combinations in DAG could provide molecular species with these molecular weights. However, based upon knowledge of the known major phospholipids in membranes the two most likely

contenders for the identities of these two DAG species are 1-palmitoyl-2-oleoyl-glycerol and 1-palmitoyl-2-linoleoyl-glycerol.

Three minor peaks in the GC-MS analysis of both the NBRC and IRBC samples with retention times longer than the above major components did not give mass spectra which could be identified as 1,2-DAG molecular species. Unfortunately because of base line noise it was also impossible to recognise any compounds of short retention time which could have represented DAG molecular species possessing short chain fatty acids which might conceivably be present and would be potential candidates for high activity in the rat adipocyte assay.

Because of the apparent similarity in the compositions in the 1,2-DAG from NBRC and IRBC and the inability to identify any minor DAG molecular species by GC-MS the alternative analytical procedure of liquid chromatography-mass spectrometry (LC-MS) was tested. This was prompted by the recent publication by Mottram *et al.* (1997) of an improved method for triacylglycerol analysis employing HPLC-Atmospheric Pressure Chemical Ionisation mass spectrometry. The operating conditions for the LC-MS instrument are described in the Materials and Methods, section 2.13.

Examination of the sample of 1,2-dipalmitoylglycerol by LC-MS showed a good symmetrical peak eluting at 5.04 minutes which gave a prominent ion at m/z 551.5 corresponding to loss of water from the molecule with the addition of a proton $[M-H_2O+H]^+$. Analysis of the NBRC and IRBC 1,2-DAG samples produced in each

case two overlapping peaks eluting at 7.16 and 7.67 minutes, respectively. The first peak gave a mass spectrum with an ion at m/z 575.4 which is in accord with the ion $[M_1\text{-}H_2O+H]^+$ arising from 1-palmitoyl-2-oleoyl-glycerol while the second peak gave *m/z* 577.5 $[M_2\text{-}H_2O+H]^+$ representing 1-palmitoyl-2-linoleoyl-glycerol. Thus the LC-MS method of analysis confirmed the identifications of the major DAG species in the NBRC and IRBC samples.

5.3 Discussion

Hypoglycaemia is known to be associated with severe malaria (Brewster *et al.* 1990) and is also associated with increased mortality in patients with cerebral malaria (Molyneux *et al.* 1989). Taylor and colleagues have shown that supernatants from blood stage *P. yoelii* induce hypoglycaemia in mice (Taylor *et al.* 1992a). This putative component (referred as the "toxin") was identified in crude parasite culture supernatants of *P. yoelii*. During the present study, we have also shown that boiled supernatants from *P. falciparum* culture induce lipogenesis on their own and also synergise with insulin *in vitro*. Therefore, characterization of the active component(s) and elucidation of their mechanism of action will increase our understanding of the complications of malaria, particularly hypoglycaemia and may help in the development of anti-disease vaccines and therapy (Playfair *et al.* 1990).

In 1912, experimental investigations suggested that malaria pigment was a factor in the production of malaria paroxysm (Kwiatkowski, 1995). The recent studies suggest that malaria pigment indeed has TNF-inducing properties (Pichyangkul *et al.* 1994). It remains unknown whether the haeme polymer is the cytokine inducing component or whether the haeme polymer is associated with the cytokine inducing components. However, there is no published work on lipogenesis-inducing activity of malaria pigment. In order to find whether the activity of TMA is due to malaria pigment, we collected spent culture of *P. falciparum* (after schizont rupture) and malaria pigment separated from preparations by ultracentrifugation. In the rat adipocyte assay such samples (without pigment) showed a synergistic effect with

insulin and also stimulated lipogenesis on their own. The results were also reproducible, hence we can conclude that malaria pigment is not responsible for the ability of TMA to stimulate lipogenesis and synergise with insulin.

Work on the rodent malaria parasite, *P. yoelii*, by Taylor and co-workers showed that TMA released by this parasite induced hypoglycaemia when given to mice (*in vivo*) and also synergised with insulin *in vitro*. It was obviously important to establish whether this phenomenon occurs with human malaria parasites since rodent models of malaria differ in many respects from disease in man. We investigated the ability of TMA from boiled supernatant of *P. falciparum* to stimulate lipogenesis in the rat isolated fat cells (*in vitro*) in short and long term cultures and the results demonstrated the same synergistic activity in *P. falciparum* IRBC culture supernatant. However, there have been no previous reports on the stimulation of lipogenesis by TMA of *P. yoelii* in isolation in long term fat cell culture (24 h).

During this study, TMA was prepared in different ways by laboratories which worked on murine malaria. They incubated washed parasitized erythrocytes, obtained from mice with more than 50% parasitaemia, in PBS or RPMI 1640 on a roller at 37°C overnight. Next day, the supernatant was collected and boiled for 5 minutes. However, in this situation reinvasion did not occur. Our preparation was more similar to the physiological condition (*in vivo*) and the reinvasion by merozoites occurred and thus allowed the completion of the parasite life cycle. Because the life cycle of the parasite was not completed during overnight incubation of the parasites in RPMI or PBS, the synthesis of active molecules may not be complete and therefore we used

a different method of preparation.

An increasing body of data indicates the existence of toxin(s) whose TNF-inducing activity, and also induction of hypoglycaemia, are associated with a lipid moiety. Evidence for this came initially from the work on *P. yoelii* (Bate *et al.* 1992b; Taylor *et al.* 1992a). They found that TNF-inducing activity and induction of hypoglycaemia were not affected with proteases, but it was destroyed by mixed lipases. Induction of hypoglycaemia *in vivo* can be competitively inhibited by inositol monophosphate or blocked by antiserum against inositol monophosphate, suggesting that the active component contains an inositol phosphate motif (Taylor *et al.* 1992a). This structure is also present in inositol phosphate oligosaccharide(s) from mammalian plasma membranes, which mimic insulin action without being synergistic with the hormone (Saltiel, 1990).

Our preliminary results showed that TMA activity (from boiled supernatant of *P. falciparum*) was resistant to protease digestion but was abolished by lipase which confirms a similarity with TMA of *P. yoelii*. Since molecules were released from parasitized erythrocytes *in vitro*, it could be that similar molecules are released *in vivo* when the schizont ruptures. Hence, we extended our result to find TMA-like activity in the *in vivo*. For this purpose we tested lipogenesis-inducing activity of monkey serum infected with *P. falciparum* on the rat adipocytes and for the first time we demonstrated the presence of TMA-like activity *in vivo* samples. Thus, further analysis of TMA by characterization of this active component(s) was the main objective described in this Chapter.

A variety of techniques were used to characterise the TMA in this work. First of all we chose the Folch extraction method to obtain lipids. The bioassay results of both the aqueous and organic phases showed the effectiveness of the solvent extraction method, as no activity was found in the aqueous phase. In the next step, analysis of the total lipid extract was carried out by TLC. This technique has proved a most effective, rapid and easy to use technique for the separation of complex lipids (phospholipids, glycolipids, etc) as well as neutral lipids. Analysis of the total lipid classes revealed activity in specific fractions (polar lipid classes). Similar results were obtained by bioassay of the polar lipid of the monkey serum infected with *P. falciparum* which established a similarity between TMA *in vitro* and *in vivo*.

The total lipid extract of boiled supernatant was separated by TLC into polar lipid classes with a different solvent system. Because all the different polar lipid classes (phospholipids and glycolipids) showed biological activity in the rat adipocytes assay, we suspected that degradation of an active component during handling, e.g application of lipid sample to the TLC plates, or drying and elution from the plate, may have occurred to give a number of separable products which retained bioactivity. Therefore in order to protect the total lipid extract from degradation, we moved to HPLC and silica gel column chromatography which reduced compound exposure in dry form to the atmosphere and reduced the risk of oxidative degradation.

Analysis of the total lipid extract of *P. falciparum* IRBC and NRBC by HPLC showed a peak with similar retention time to PC standards, but which was larger and wider in the NRBC sample compared to the IRBC material. This may reflect a larger

amount of PC and greater complexity of the fatty acids associated with the PC in the NRBC sample. It should noted that the UV detection at 205 nm is dependent upon the number of double band in the molecule(s) under study. Thus an increase in peak size may indicate a greater proportion of mono, di or tri-unsaturated fatty acids in the PC rather than an increased absolute amount of material.

All the different fractions collected from either HPLC or column chromatography, showed lipogenesis-inducing activity in the rat adipocyte assay. The similarity between these results and the results from the bioassay of different fractions obtained by preparative TLC suggested that the active principle could perhaps reside in a common structure such as a fatty acid moiety which is present esterified in all the lipid fractions. Such a molecule(s) may perhaps be responsible for TMA activity after its release from the parent lipids.

Chemical analysis of the different fractions eluted from a silica gel column has revealed phospholipid and glycolipid classes in a total lipid extract of boiled supernatant of *P. falciparum* IRBC. Treatment of the phospholipids and glycolipids fractions with the enzyme PLC revealed that some phospholipid fractions were sensitive to PLC hydrolysis, however, glycolipid fractions were insensitive.

The molecular mechanisms by which insulin elicits its numerous metabolic responses have been the subject of intense research over the past several decades. Much of this effort has been devoted to the search for a second messenger, a substance produced from the plasma membrane in response to insulin that mediates

some of the intracellular effects of the hormone (Low & Saltiel, 1988). Two molecules have been proposed as second messengers transducing the insulin signal into the target cell. One is an inositolphosphoglycan (IPG) and the other is diacylglycerol (DAG), both deriving from the same plasma membrane glycolipid, which is hydrolysed in response to insulin treatment (Stralfors, 1997). The mechanism by which insulin regulates the cleavage of the glycosyl-phosphatidylinositols (GPI) in the plasma membrane is unclear. It appears that insulin-receptor interaction leads to a rapid, dose-dependent activation of a distinct and selective PLC that hydrolyses this GPI, since both the IPG and 1,2-DAG are produced in response to insulin (Saltiel *et al.* 1987). In rat adipocytes, the stimulation by insulin of glucose metabolism was mimicked by a bacterial phosphatidylinositol (glycan)--specific phospholipase C (IPG-PLC) released substance (Saltiel & sorbara-Cazan, 1987; Suzuki *et al.* 1991). Extracellular blocking of IPG uptake by an excess of simple structural analogues (Stralfors & Alemany, 1990; Machicao *et al.* 1990) or antibodies against IPG (Gaulton, 1991; Romero *et al.* 1990) blocked some of the actions, of insulin such as protein serine/threonine phosphorylation/dephosphorylation, but not insulin stimulation of glucose transport or glucose uptake (Stralfors, 1997).

In plasmodia, GPI serves as a membrane anchor for various proteins including the major merozoite surface proteins, MSP-1 and MSP-2. Schofield and co-workers have strongly argued that the plasmodial form of GPI possesses signal transduction properties and represents the major TNF-inducing activity and also induces hypoglycaemia in mice (Schofield & Hackett, 1993; Schofield *et al.* 1996). Our results revealed that activity of TMA was related to a polar lipid fraction. More

detailed analyses of the polar lipids suggested that the activity came from the glycolipid and phospholipid fractions. It is assumed that hydrolysis of GPI by PLC generates water-soluble IPG and lipophilic DAG which both are insulin second messengers. However, treatment of glycolipid fractions (F3 and F4) from our preparations with PLC did not generate IPG, because a test of the aqueous phase in the rat adipocytes assay did not show stimulation of lipogenesis. However, there is evidence that IPG has an insulin mimicking activity on intact cells (Low & Saltiel, 1988). The absence of lipogenesis-inducing activity from the aqueous phase meant that there was an absence of insulin mimicking substance in the aqueous phase and further suggested that our total lipid extract from boiled supernatant of *P. falciparum* IRBC culture did not contain any malarial GPI. In addition, the boiled supernatants of *P. falciparum* IRBC were extracted with chloroform/methanol (2:1 v/v). Therefore, glycolipids such as GPI with long-chain derivatives of sugars probably would not be extracted by chloroform/methanol (2:1 v/v), although, glycolipids with short-chain (mono or disaccharides) can be extracted. Extraction of GPI needs a more polar solvent system than our extraction system (personal discussion with Dr. J.Goad). Consequently this explanation again suggested the absence of GPI from our preparations.

Silica gel column chromatography of the total lipid extract of *P. falciparum* IRBC revealed that the lipogenesis-inducing activity was co-eluted exclusively with fractions F6, F7, F8 suggesting that these fractions contained phospholipids (PE, PI, PC) which were responsible for the lipogenesis-inducing activity of the TMA in the malaria culture supernatant. Enzymatic treatment of the lipid fractions with PLC

showed that activity of the above fractions mostly related to the 1,2-diacylglycerol (1,2 DAG) moieties of the polar lipids. This 1,2-DAG came from samples which co-migrated with PC standard. However, incubation of samples which co-migrated with a PI marker did not produce 1,2-DAG when treated with PLC. This could be because the lipids containing PI, which can be hydrolysed with bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), are not hydrolysed by PLC. The bioactivities seen in the adipocyte assay may be related to the 1,2-DAG moiety which can be released by PLC (or other hydrolytic enzymes) from the polar lipids. PLC and other lipase are located in cell membranes or closely associated with them. Thus, when the schizonts rupture *in vivo* and release different lipid components into the blood circulation these could be hydrolysed in target tissue to yield derived 1,2-DAG. This could then stimulate cellular processes, possibly synergistically with insulin, and lead to metabolic disturbances within the cells.

Solvent extraction of hydrolysed F7 lipid which was incubated with PLC was done by chloroform/methanol (2:1 v/v). The total lipid extract was chromatographed and fractions found which co-migrated on TLC with 1,2-DAG and 1,3-DAG markers. The 1,3-DAG sample showed lower biological activity than 1,2-DAG sample which fits with previous work by Stralfors. He reported that 1,3-DAG was a poor activator of the glucose transporter (Stralfors, 1988). The 1,2-DAG sample was presented to the fat cells as an emulsion. To enhance the transfer of 1,2-DAG from the emulsion droplets to the plasma membrane of the adipocyte cells in a short term incubation, the 1,2-DAG sample was added either in a bile salt (taurodeoxycholate which had no effect on its own) (Nichols, 1986) or in PBS pH, 7.4. However, the rats under

standard diet were apparently insensitive, and the synergistic effects of neither TMA and insulin (see also Chapter three) nor the 1,2-DAG sample with insulin were observed. However, the fat cells were sensitive to stimulation of lipogenesis in a long term incubation (24 h) by both of these samples. There was a synergistic effect between the 1,2-DAG sample and insulin in the rats under CRM diet. These findings revealed that the molecule(s) which were responsible for activity in isolation and also in promoting the synergistic effect with insulin, were the same.

A prominent effect of insulin that does not appear to be controlled by IPG, is the activation of glucose transport (Kelly *et al.* 1987), but 1,2-DAG, which is generated in parallel with IPG can stimulate glucose transport and may be a second messenger counterpart for control of this membrane transport function. Thus, in order to understand the mechanism of action of the 1,2-DAG sample, the stimulation of glucose uptake by a 1,2-DAG sample of *P. falciparum* IRBC, and also by insulin, was effectively blocked by cytochalasin B an inhibitor of the glucose transporter. The complete inhibition of 1,2-DAG sample stimulated glucose uptake by cytochalasin B, demonstrated that the increased uptake of glucose is mediated by the glucose transporter. It would be interesting to know if the action of the 1,2-DAG sample is on the transporter translocation or on transporter activation of glucose uptake. Insulin rapidly stimulates lipogenesis due to mobilization of glucose transporters from an intracellular location to the plasma membrane (Kitagawa *et al.* 1986) and to enhanced intrinsic activity of the transporters (Gibbs *et al.* 1986). However, stimulation of lipogenesis by the 1,2-DAG sample needs a long time exposure of adipose cells. The mechanism of action of the 1,2-DAG sample on the glucose transporter is not clear,

but it could be that there are two different mechanisms of action for the 1,2-DAG sample and insulin on the glucose transporter.

Analysis of the polar lipids by TLC, HPLC and silica gel column chromatography showed all fractions were biologically active. The presence of a 1,2-DAG moiety in all of the phospholipid classes suggests the possibility that the activity of different samples (F6, F7, F8, F9 and F10 phospholipids) was due to the release of this 1,2-DAG moiety by appropriate lipase activity. Hydrolysis of F7(1)+F8(1) which co-migrated with PI, and also F6(3) (PE) and F7(u)+F8(u)+F9(u) with specific lipases may release the 1,2-DAG which could have a similar structure to the 1,2-DAG sample which was released after digestion of F6(2)+F7(2)+F8(2) (PC) with PLC.

A total lipid extract from a boiled supernatant of NRBC (parallel to IRBC) showed no stimulation of lipogenesis and synergy with insulin in long and short term culture of adipocytes, respectively. In *P. falciparum* culture, when the schizont ruptures merozoites, parasite antigens and erythrocyte debris are released into the culture medium. NRBC were cultured in parallel to IRBC to act as control and most of the erythrocytes remained intact in the culture. Therefore, to have a good control for TMA activity, we extracted total lipids from intact erythrocytes to release components similar to those released when the schizont ruptures. The polar lipids separated from the total lipid extract, and further analysis of the polar lipid fraction F7 of intact NRBC, hydrolysis of F7 with PLC and purification of the 1,2-DAG sample, revealed no stimulation of lipogenesis. Silica gel column chromatography of the total lipid extract of NRBC showed a different composition in the glycolipid part

which could be related to changing the lipid composition during infection, or may be due to red cell lysis and release of components which may not normally be released even after parasitised erythrocytes are ruptured. Bioassay of such samples showed no lipogenesis inducing activity on the rat isolated fat cells. These are evidence for a difference between the molecule(s) responsible for TNF-inducing activity and lipogenesis stimulation. Bate and co-workers reported that TNF-inducing molecules were present in lysates of uninfected erythrocytes, though at a much lower concentration (Bate & Kwiatkowski, 1994a).

The identities of the major components in the FAMES mixture of polar lipids of *P. falciparum* IRBC were confirmed by GC-MS analysis of the samples. It can be seen that the compositions of the fatty acid mixtures derived from the NRBC and IRBC were essentially the same although it is possible that some minor fatty acid components which were present at the limits of detection could have varied between the two samples.

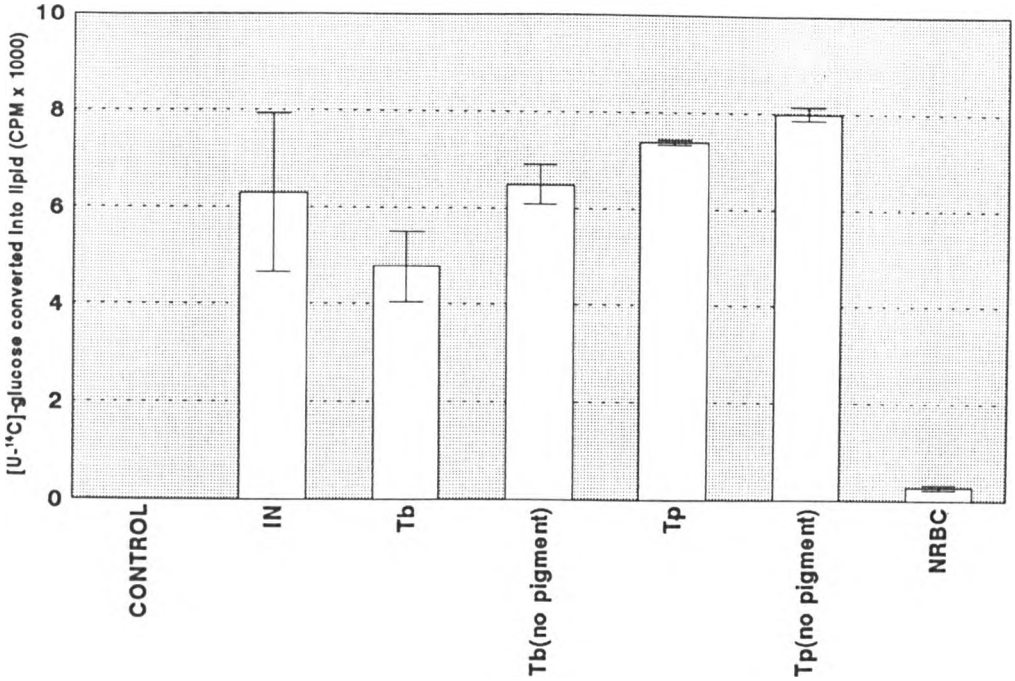
In order to establish that the 1,2-DAG samples recovered from the PLC hydrolysis of the fractions F7 from the NRBC and IRBC did indeed consist of 1,2-DAG they were subjected to analysis by GC-MS and LC-MS. It was hoped that these techniques would not only establish the identity of the major components but might also provide evidence for the structures of any minor 1,2-DAG species which may then provide clues for the identity of the component in the IRBC sample which is most active in the rat adipocyte bioassay. However, again these techniques were not sufficiently sensitive enough to permit the identification of minor components in the

samples and minor peaks did not give identifiable spectra.

It is generally assumed that the toxin of *P. falciparum* (1,2-DAG) is directly synthesized by the parasite, but we can not yet rule out the possibility that it is a modified or over-expressed host structure. However, the results of this Chapter provide further evidence that during the infection some molecular species of phospholipids were changed which give biological activity *in vitro*. These issues can only be resolved by compositional and structural analysis of the purified toxin and also by appropriate techniques.

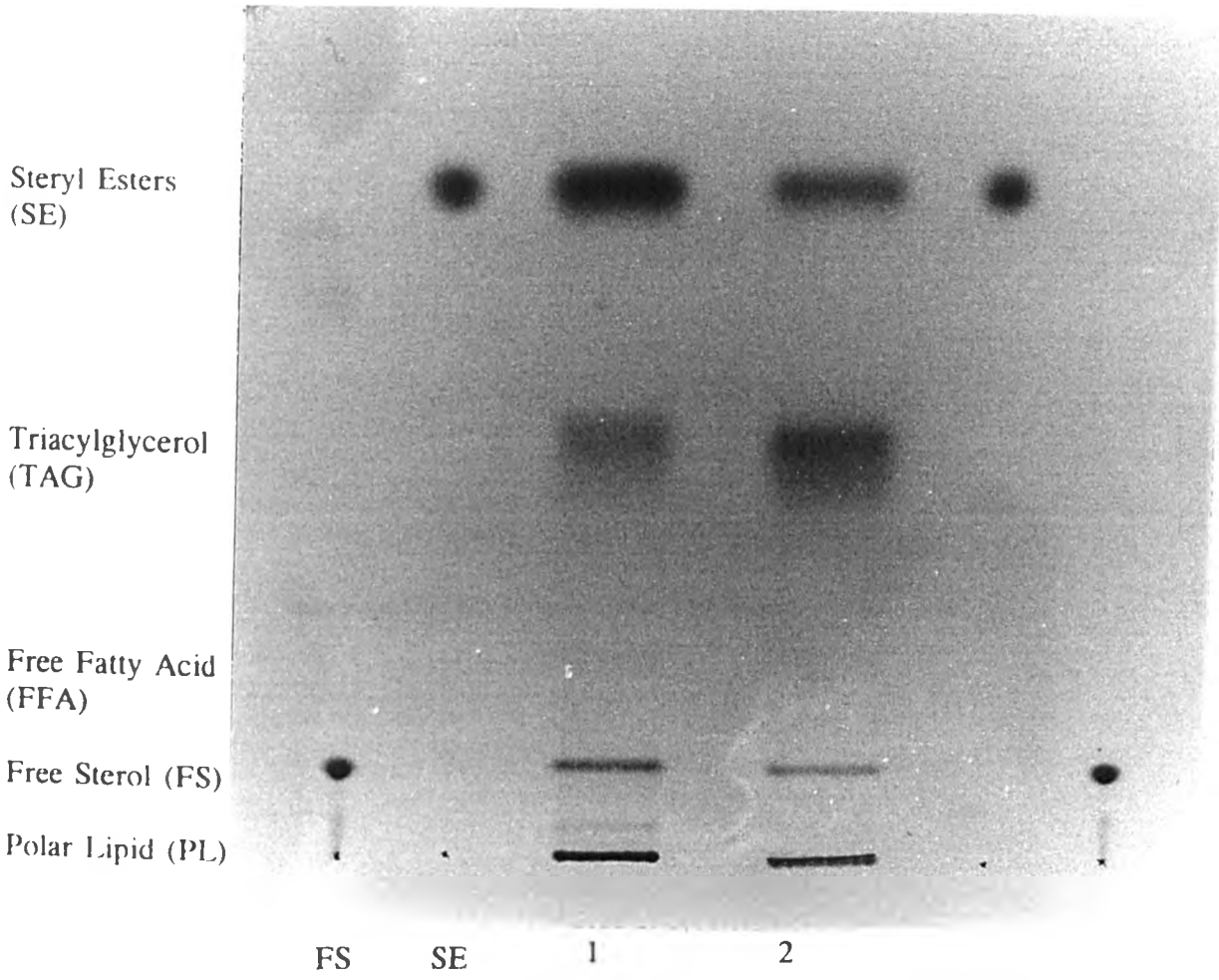
The properties of the TMA on the lipogenic pathway may explain some of the clinical complications of malaria (most important hypoglycaemia) and if so, modification of TMA might provide the basis of an anti-disease vaccine (Playfair *et al.* 1990).

Fig. 5.1 COMPARISON OF THE EFFECTS OF TMA WITH AND WITHOUT PIGMENT ON LIPOGENESIS IN RAT ADIPOCYTES



Schizont stage of *P. falciparum* parasites ($\geq$10% parasitaemia) were cultured for 12-16 h at 37°C to allow infected erythrocyte rupture to occur. Erythrocytes were removed and the cell-free supernatants were prepared as described in section 2.2.1. Supernatants were ultracentrifuged at 20,000 X g for 45 min at 4°C. The supernatants free of pigments were collected and passed through 0.22 μm filter units to remove contaminating particles. Sterilised supernatant was then boiled for 5 min and re-centrifuged. The supernatant was filtered again and kept at 4°C for further bioassay. This figure shows stimulation of lipogenesis in long term culture of the rat fat cells. **Tb** represents boiled supernatant of *P. falciparum* culture *in vitro* which contained malaria pigment. **Tp** shows pronase digested *P. falciparum* spent culture from the same batch of Tb. The samples with no pigment were also from the same batch of Tb. Boiled supernatant of NRBC was used as control and the cells were from the same batch which was used for *P. falciparum* culture and Tb preparation. The results show lipogenesis-inducing activity of TMA was not caused by pigment. The results are the mean ($\pm$ SEM) of two separate experiments performed in duplicate with two different preparations.

Fig. 5.2 TLC analysis of total lipids of NRBC anf *P. falciparum* culture supernatants

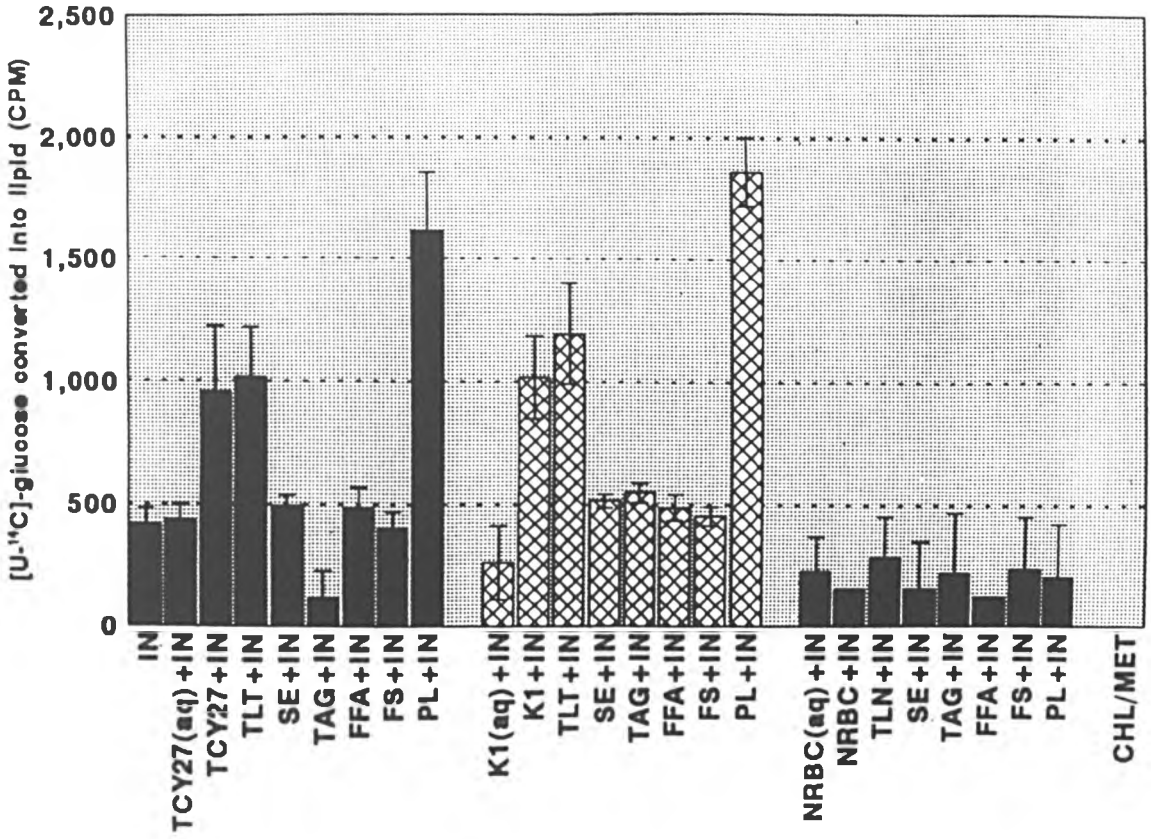


Total lipid was extracted from boiled supernatant of *P. falciparum* and NRBC by chloroform/methanol (2:1 v/v) (Folch *et al.* 1957) for 2 h. The aqueous phase was re-extracted with the same solvent system. The total lipid extracts from first and second extractions were pooled. The solvent was rotary evaporated and redissolved in a known volume of cychlohexane. For thin-layer chromatography (TLC) of total lipids on analytical silica gel G, the plates were first washed at room temperature with diethyl ether, then the plate dried and samples and standards were loaded. The plates were immediately developed in petroleum/diethyl ether/acetic acid (40:10:1 v/v/v). Lipid spots were detected by 20% sulphuric acid (section 2.9.1). There was no major difference between the lipid composition of total lipids of infected red blood cells (IRBC) and normal red blood cells (NRBC).

1 = the total lipid extract of *P. falciparum* culture supernatant

2 = the total lipid extract of NRBC culture supernatant

Fig. 5.3 SYNERGISTIC EFFECT BETWEEN DIFFERENT FRACTIONS OF TOTAL LIPID OF IRBC AND INSULIN



The total lipid extract of boiled supernatant of *P. falciparum* IRBC was prepared and chromatographed as described in Fig. 5.2. Different fractions were eluted from the plate by diethyl ether for SE, TAG, FFA, FS and chloroform/methanol (1:1 v/v) for polar lipid which stayed at the origin. The solvents were evaporated under a stream of nitrogen. The dried lipid was redissolved in known volumes of cychlohexane for neutral lipids and chloroform/methanol (1:1 v/v) for polar lipid. For bioassay all samples were dissolved in PBS and then added to the rat adipocyte culture (final concentration 10 μg/ml). This figure shows the bioassay of different lipid fractions from boiled supernatant of wild isolate CY27 and from a cloned laboratory K1, which are two strains of *P. falciparum*. The results show no synergy in the aqueous phase and also in different lipid fractions of NRBC. However, there was a synergistic effect between total lipids of both *P. falciparum* strains. Further analyses revealed this activity related to the polar lipid (P) fraction of both strains, but not PL of total lipid extract of NRBC. The results represent the means (±SEM) of two separate experiments done in duplicate.

IN=insulin **TCY27 (aq)**= aqueous phase of the lipid extract of boiled supernatant from the wild isolate of *P. falciparum* CY27

TCY27=boiled supernatant of *P. falciparum* before extraction

K1=boiled supernatant from cloned laboratory isolate of *P. falciparum*

TLT= total lipid extract of TCY27 **SE**=steryl esters

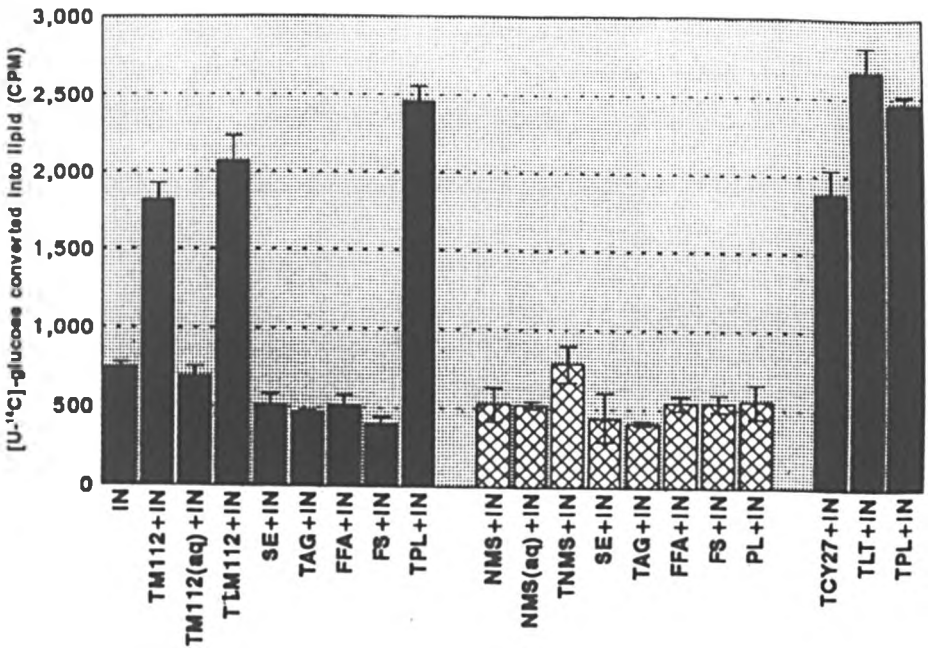
TAG=triacylglycerol **FFA**= free fatty acid **FS**=free sterol

PL=polar lipid **NRBC**=boiled supernatant from normal red blood cells

TNL=total lipid extract of NRBC culture supernatant

CHL/MET=chloroform/methanol (5 μl/ml) in PBS as negative control

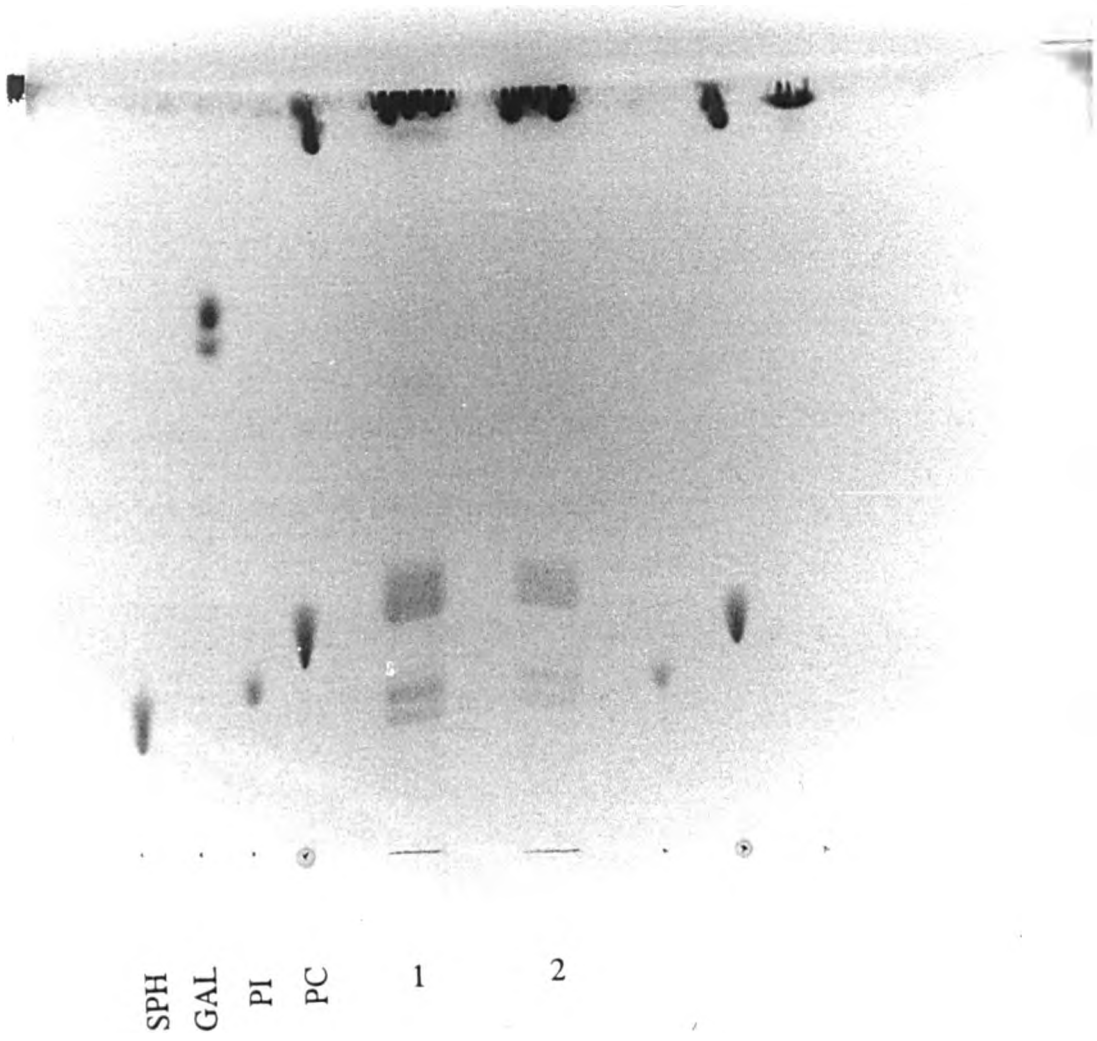
Fig. 5.4 SYNERGISTIC EFFECT BETWEEN LIPID FRACTIONS FROM *P. falciparum* INFECTED MONKEY SERUM AND INSULIN



Monkey serum infected with *P. falciparum* (36% parasitaemia) was extracted and chromatographed as described in Fig. 5.2. The results showed the synergistic effect between the PL lipid fraction and insulin. However, no synergy was identified between the aqueous phase of the extraction of the neutral lipids of monkey serum extraction and insulin. There was a control preparation from NMS which was treated in the same way as infected serum, but no synergy was found with different fractions of lipid from NMS. The results demonstrated similarity between PL activity of total lipid extract of *P. falciparum* IRBC (*in vitro*) and infected monkey serum (*in vivo*). The results shown here are means values (± SEM) duplicate incubation from two different experiments.

IN=insulin
TM112= boiled infected monkey serum with *P. falciparum* (36% parasitaemia)
TM112 (aq)=aqueous phase of TM112 lipid extract
TLM112=Total lipid extract of TM112
SE=steryl esters
TAG=triacylglycerol
FFA= free fatty acid (stearic acid)
FS=free sterol
PL=polar lipid
NMS= boiled normal monkey serum
TCY27= boiled supernatant of *P. falciparum* IRBC culture (*in vitro*)
TLT=total lipid extract of TCY27
TPL= total polar lipid of TCY27

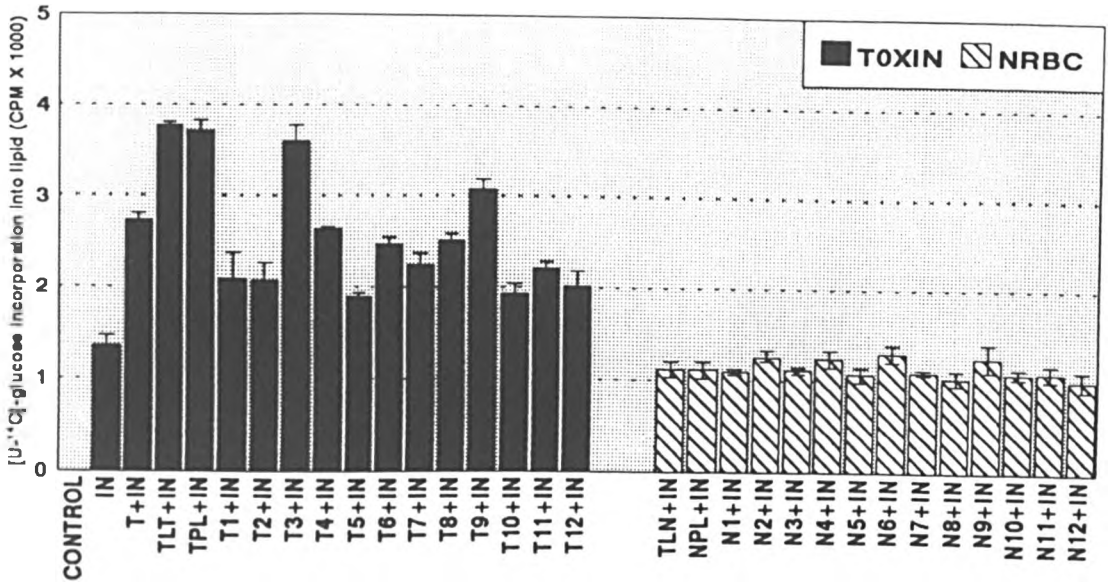
Fig. 5.5 TLC analysis of polar lipids of NRBC and *P. falciparum* culture supernatants



The total lipid extract of a spent culture medium of *P. falciparum* was fractionated in a polar solvent system to separate the polar lipid groups. Sample No. 1 was total lipid extract from spent culture medium of *P. falciparum* and No. 2 was from a NRBC culture supernatant. Chromatography of the polar lipid fraction of both samples was on analytical silica gel G plates developed by chloroform/methanol/water (65:25:4 v/v/v) and it revealed two similar major lipid spots in both samples. These two spots co-migrated with PI and PC standards, respectively. The solvent system also separated the neutral lipid from the polar lipid perfectly. No visible glycolipids were identified in either of the samples.

1 = the total lipid extract of *P. falciparum* culture supernatant
2 = the total lipid extract of NRBC culture supernatant
SPH = sphingomyelin
GAL = galactocerebrosides
PI = phosphatidylinositol
PC = phosphatidylcholine

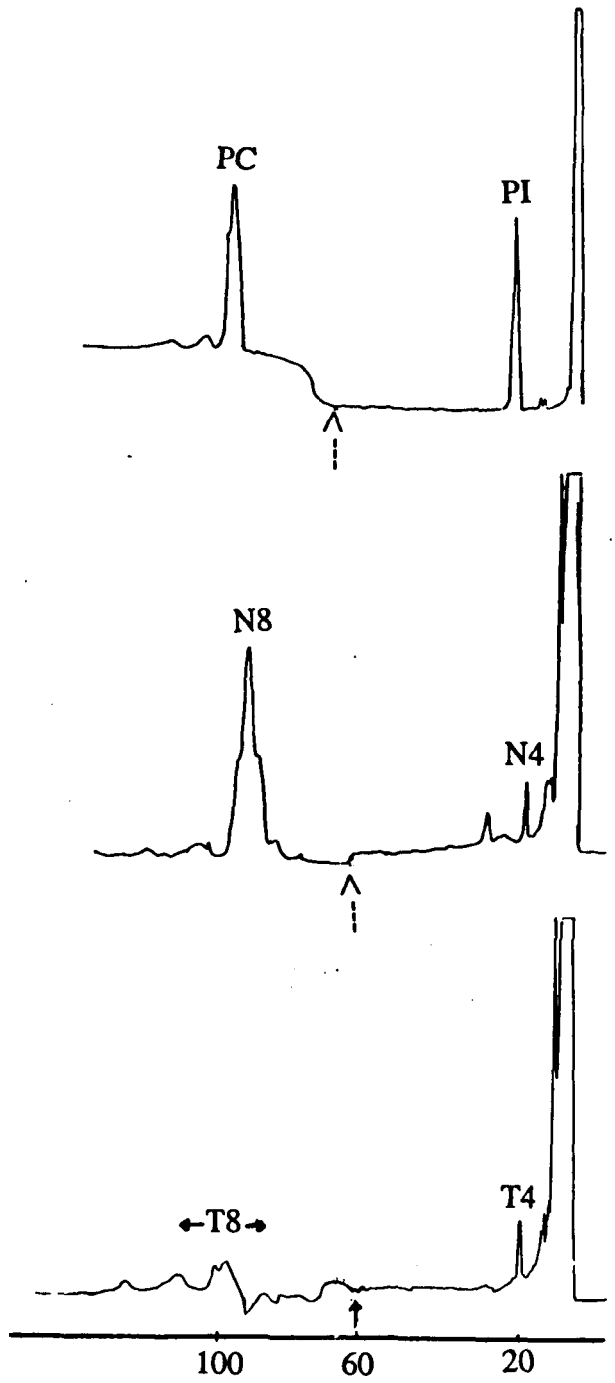
Fig. 5.6 SYNERGISTIC EFFECT BETWEEN DIFFERENT FRACTIONS OBTAINED BY TLC FROM *P.falciparum* IRBC ON LIPOGENESIS IN THE RAT ADIPOCYTES



The total lipid extracts of boiled supernatant of *P. falciparum* IRBC and NRBC cultures were extracted and chromatographed as described in Fig. 5.5. The whole area from the origin to the solvent front was divided into 12 equal fractions. Each fraction was eluted with chloroform/methanol (1:1 v/v) and the solvent was evaporated under nitrogen. Each samples (1 mg) was dissolved in 50 μl of chloroform/methanol (1:1 v/v) (0.1% final concentration in adipocyte culture), and made up with sterile PBS (pH 7.4) to 2ml. This concentration of solvent has been shown to have no effect on glucose metabolism. Diluted samples (50 μl/ml) in PBS were added to the rat adipocytes in culture (final concentration 25 μg/ml). The NRBC supernatant was treated identically to *P. falciparum* IRCB. The solid bars represent synergy between fractions which were prepared from *P. falciparum* IRBC and insulin. The hatched bars demonstrate no synergy between insulin and P classes of NRBC. Values are means ($\pm$ SEM) of four different experiments with two different preparations in duplicate.

IN=insulin
T=boiled supernatant of *P. falciparum* before extraction
TLT=total lipid extract of T
TPL=total polar lipid of T
T1-T12=different fraction of TLT eluted from TLC plate
TLN=total lipid extract of NRBC
NPL=total polar lipids of NRBC
N1-N2= different fraction of NTL eluted from TLC plate

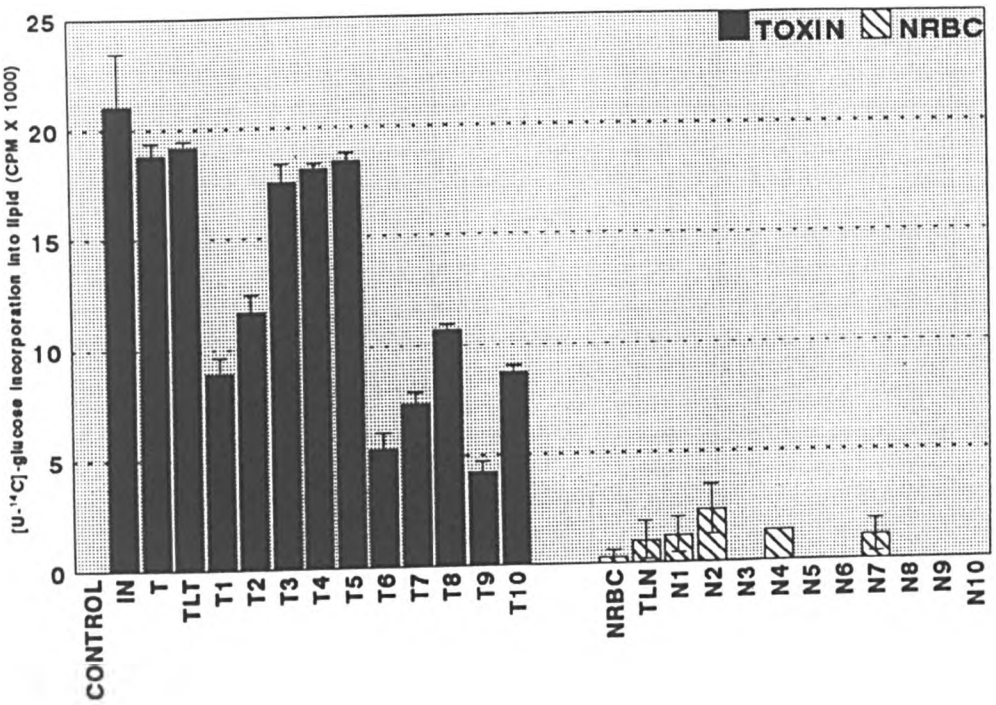
Fig. 5.7 Separation of phospholipid classes by HPLC



A total lipid extract of a boiled supernatant of *P. falciparum* IRBC and a NRBC culture supernatant, containing 10 mg of total lipid, were chromatographed as described in Materials and Methods. The flow rate was 0.5 ml/min at the start and then changed to 1 ml/min as shown by the arrow. Detection was by UV absorption at 205 nm. The analyses showed a similar pattern for both samples and controls except for the PC. There was very wide peak for PC of NRBC but a smaller peak in the IRBC sample.

PI=phosphatidylinositol
PC=phosphatidylcholine
N4=fraction 4 of total lipid extract of NRBC eluted from HPLC column
N8=fraction 8 of total lipid extract NRBC eluted from HPLC column
T4=fraction 4 of total lipid extract *P. falciparum* IRBC eluted from HPLC column
T8=fraction 8 of total lipid extract *P. falciparum* IRBC eluted from HPLC column

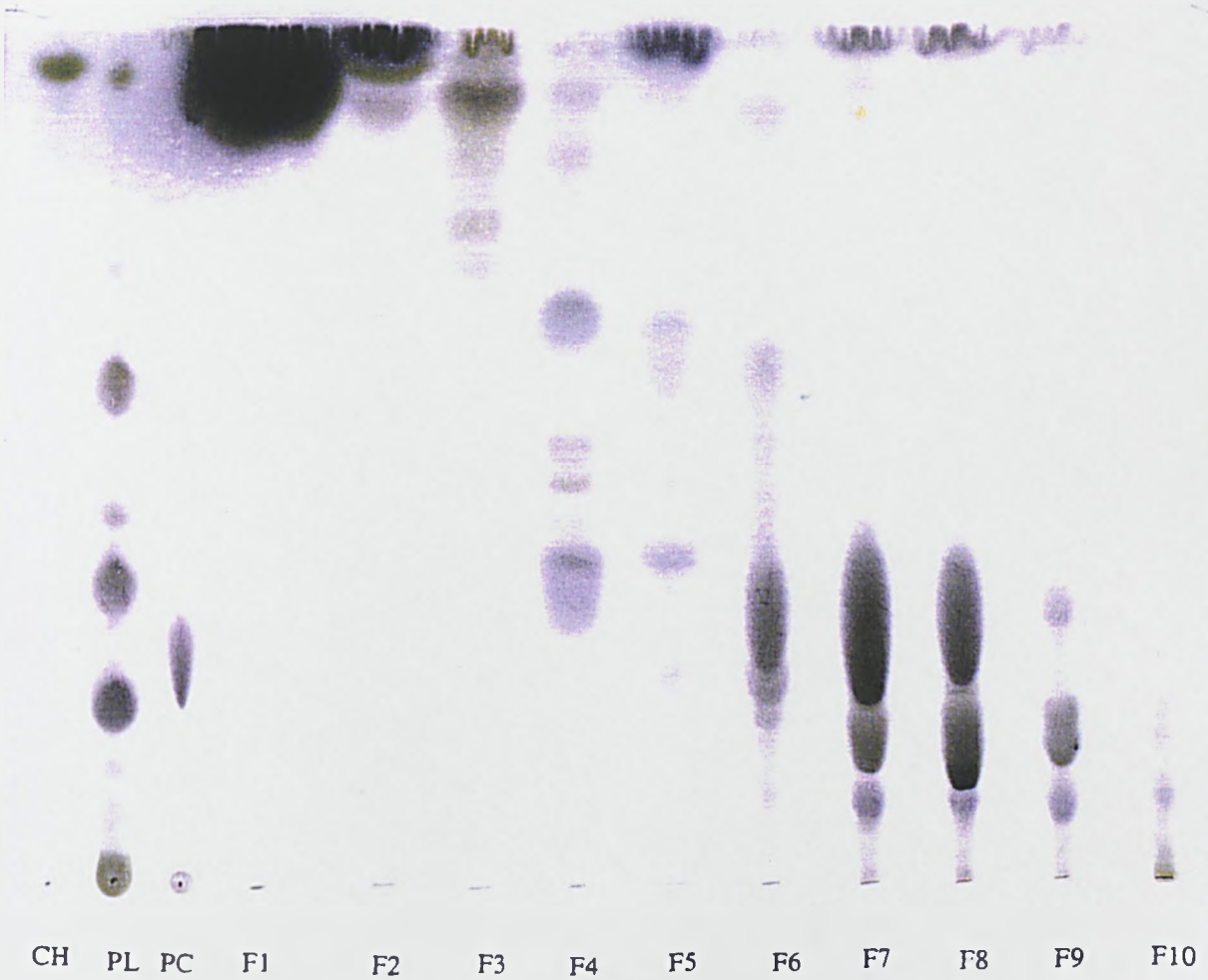
Fig. 5.8 BIOASSAY OF LIPID FRACTIONS OF *P. falciparum* IRBC SEPARATED BY HPLC



Different fractions eluted from the HPLC column (the same conditions as Fig. 5.7) were collected and the solvent evaporated under a stream of N_2 and then stored at -20°C. Each fraction (1 mg) was dissolved in 50 μl chloroform/methanol (1:1 v/v) (0.1% final concentration in adipocyte culture) and diluted with sterile PBS, pH 7.4 (final volume was 2 ml). Diluted samples (50 μl/ml) in PBS were added to the rat adipocytes in culture and incubated for 24 h (final concentration 25 μg/ml). The solid bars demonstrated of lipogenesis stimulation with fractions which were prepared from *P. falciparum* IRBC. The hatched bars revealed stimulation of lipogenesis by NRBC fractions. The results showed no stimulation of lipogenesis by the NRBC fractions. However, there was stimulation by different fractions of *P. falciparum* IRBC. The most highly active fractions were eluted from HPLC with retention times similar to the PI as standard. This data showed a similarity with the results obtained by TLC. Values are means of (±SEM) of four experiments from two different preparations in duplicate.

IN=insulin
T=boiled supernatant from *P. falciparum* culture before extraction
TLT= total lipid extract of T
T1-T10=different fractions of TLT eluted from HPLC column
NRBC= boiled supernatant of normal red blood cells culture before extraction
TLN=total lipid extract of NRBC
N1-N10= different fractions of TLN eluted from HPLC column

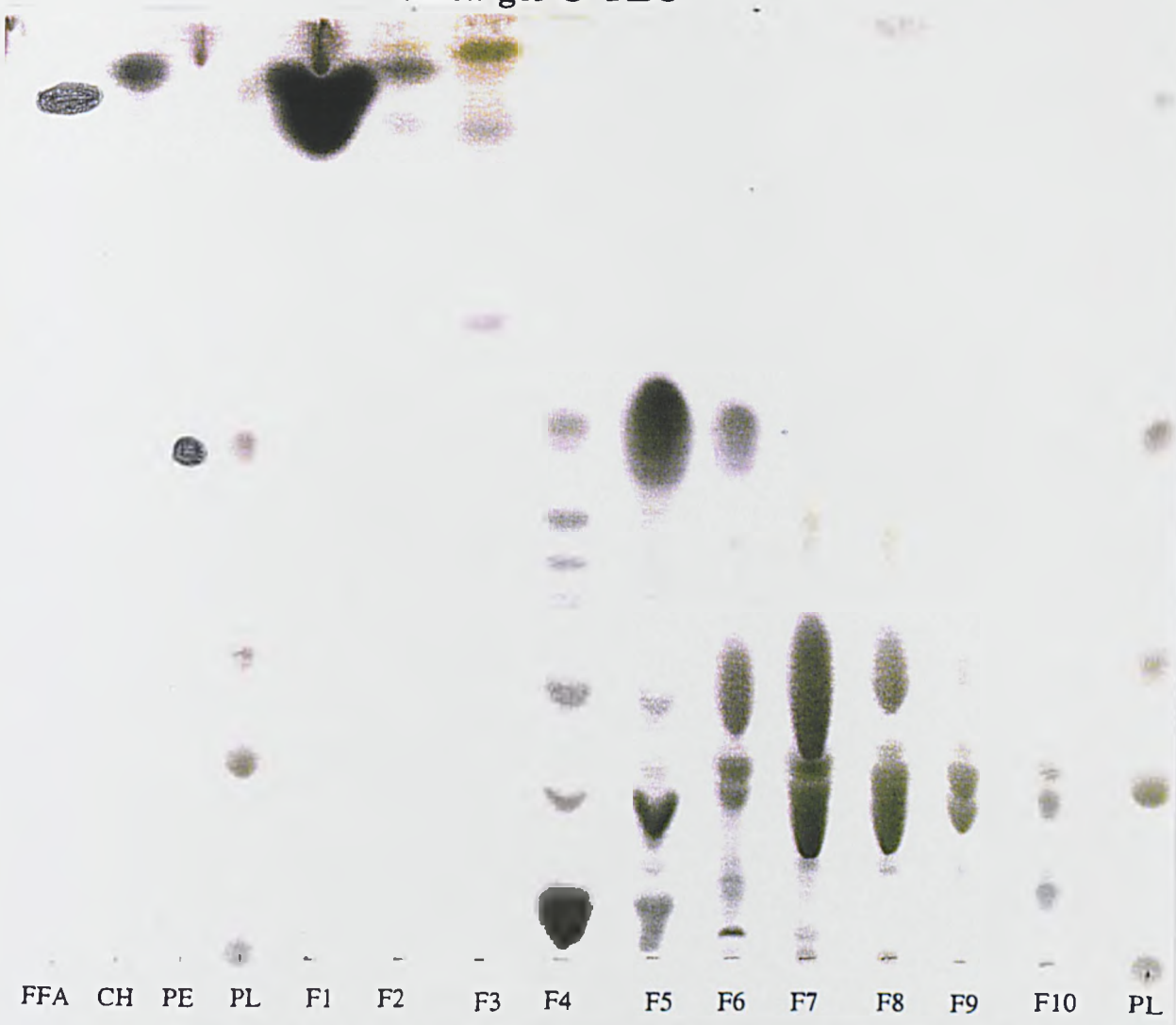
Fig. 5.9 Analysis of *P. falciparum* IRBC lipid fractions by silica gel G TLC



The total lipids (200 mg) from pooled spent culture medium of *P. falciparum* IRBC were fractionated on a column of silica gel as described in Materials and Methods (section 2.11). Fractions (F1-F10) were eluted from the column with different solvent systems comprised of chloroform-methanol mixtures (section 2.11.3). All fractions were analysed on analytical silica gel G TLC plates developed with chloroform/methanol/water (65:25:4 v/v/v) for about 90 minutes. This solvent system gives good separation of the phospholipids into the major phospholipid classes; authentic PI, PC, PE were run as standard phospholipids. The spots were identified by spraying with 20% sulphuric acid and charring at 110°C (section 2.9.1). The results of the TLC analysis revealed that F1 and F2 contained neutral lipids. F3-F10 contained different polar lipids.

PL = mixture of PI, PC and PE as phospholipid standards
PC = phosphatidylcholine
CH = cholesterol

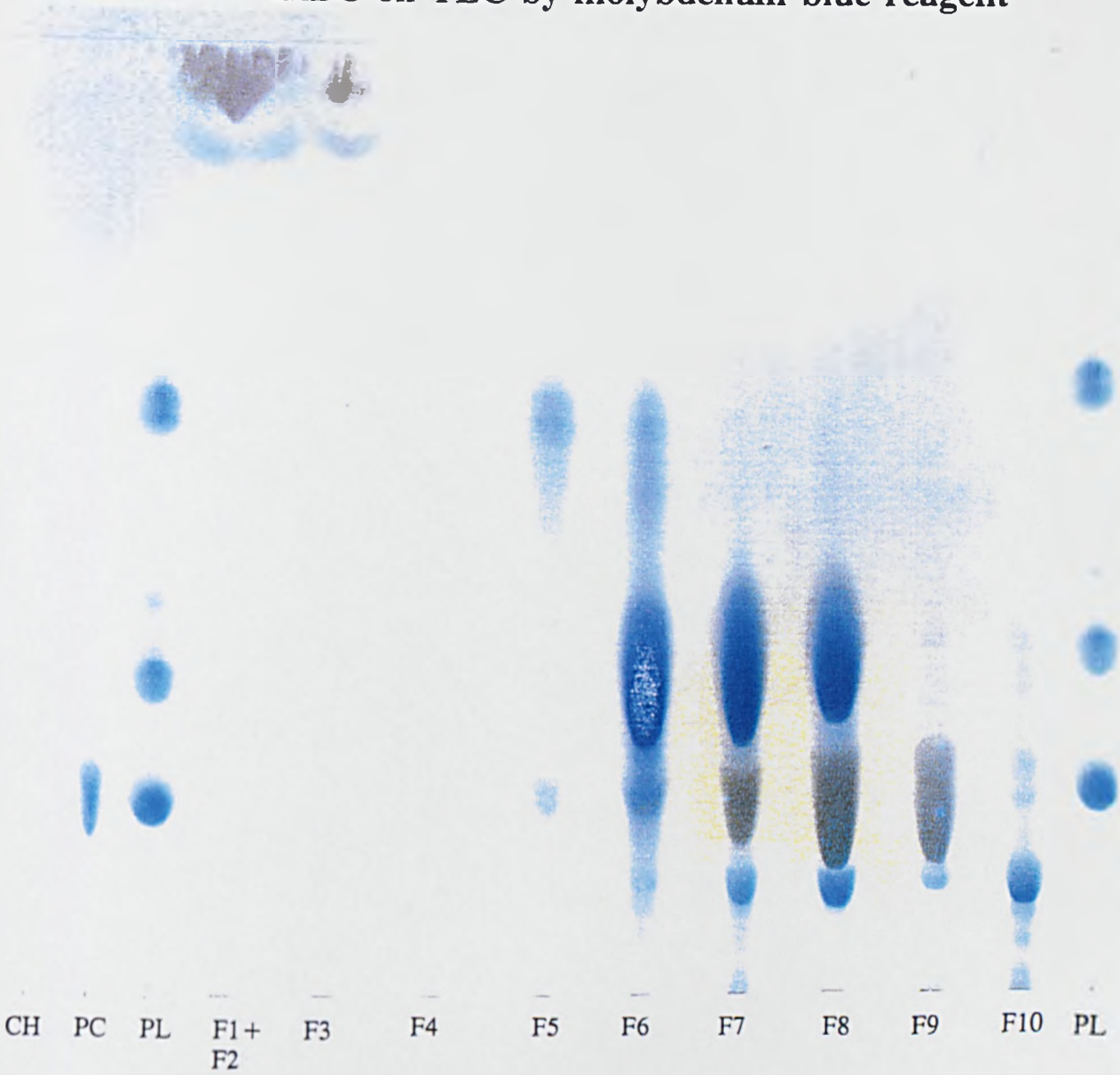
Fig. 5.10 Analysis of NRBC lipid fractions by silica gel G TLC



The total lipid was extracted from freshly obtained NRBC (donated by individual European adult). The total lipid was fractionated on a column of silica gel as described in Materials and Methods (section 2.11). Each fraction eluted from the column was treated identically to the fractions of *P. falciparum* IRBC as mentioned in Fig. 5.9. All fractions were then analysed on analytical silica gel G TLC plates developed by chloroform/methanol/water (65:25:4 v/v/v). F1 and F2 contained neutral lipid which was eluted from the column with chloroform. The remaining fractions (F3-F10) contained different polar lipids which separated according to their polarity.

FFA = free fatty acid
PL = mixture of PI, PC and PE as phospholipid standards
PE = phosphatidylethanolamine
CH = cholesterol

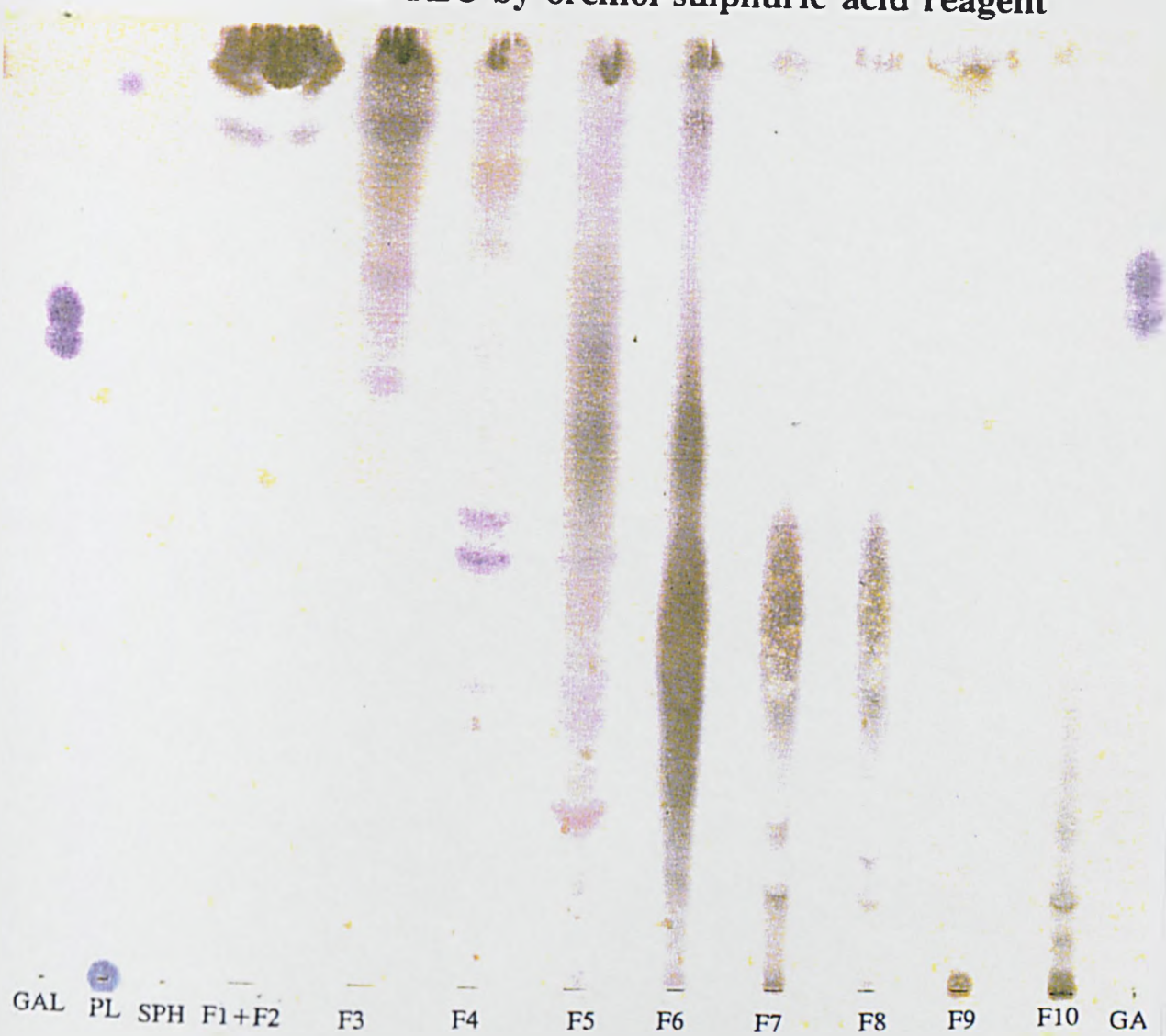
Fig. 5.11 Detection of phospholipid fractions from IRBC on TLC by molybdenum blue reagent



The total lipid (200 mg) from pooled spent culture medium of *P. falciparum* IRBC was fractionated on a column of silica gel as described in Materials and Methods. Each fraction was eluted from the column with different solvent systems (2.11.3). All fractions were chromatographed on the analytical silica gel G TLC plate developed by chloroform/methanol/water (65:25:4 v/v/v). The phospholipids were detected with molybdenum blue (1.3% molybdenum oxide in 4.2 M sulphuric acid) (section 2.9.2). With this spray phospholipids appear as blue-green spots. The results revealed that F5-F10 contained phospholipids. F5 co-migrated with the PE standard. F6-F8 co-chromatographed with PI and PC standards. F9 and F10 contained two major spots one of which co-migrated with PI standard, but the other was located lower than the PI standard. This material is very polar and was mostly eluted with the solvent system methanol/water (9:1 v/v) from the initial silica gel column chromatography.

PL = mixture of PI, PC and PE as phospholipid standards
CH = cholesterol
PC = phosphatidylcholine

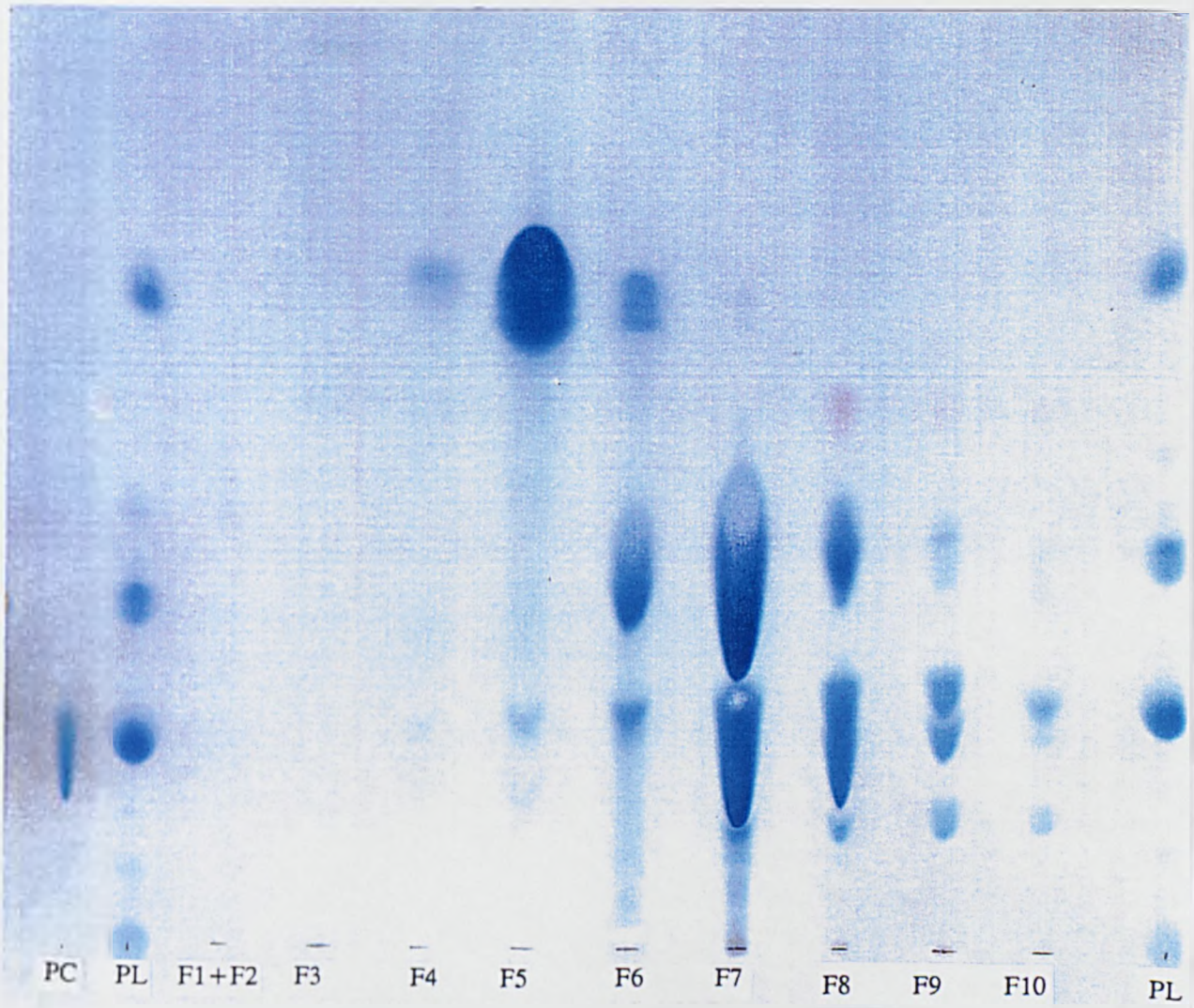
Fig. 5.12 Detection of glycolipid fractions from IRBC on TLC by orcinol-sulphuric acid reagent



The total lipid (200 mg) from pooled spent culture medium of *P. falciparum* IRBC was separated on a column of silica gel as described in Materials and Methods. All fractions were chromatographed on analytical silica gel G TLC developed by chloroform/methanol/water (65:25:4 v/v/v). The glycolipids were detected by orcinol-sulphuric acid reagent (section 2.9.2). Glycolipids appeared as purple-blue spots on a yellowish background. Because this reagent contained 75% sulphuric acid after heating the plates there were some brown spots due to non-specific charring of lipid which reacted with sulphuric acid. The results indicated that F3 and F4 of the total lipid extract of *P. falciparum* IRBC contained glycolipids.

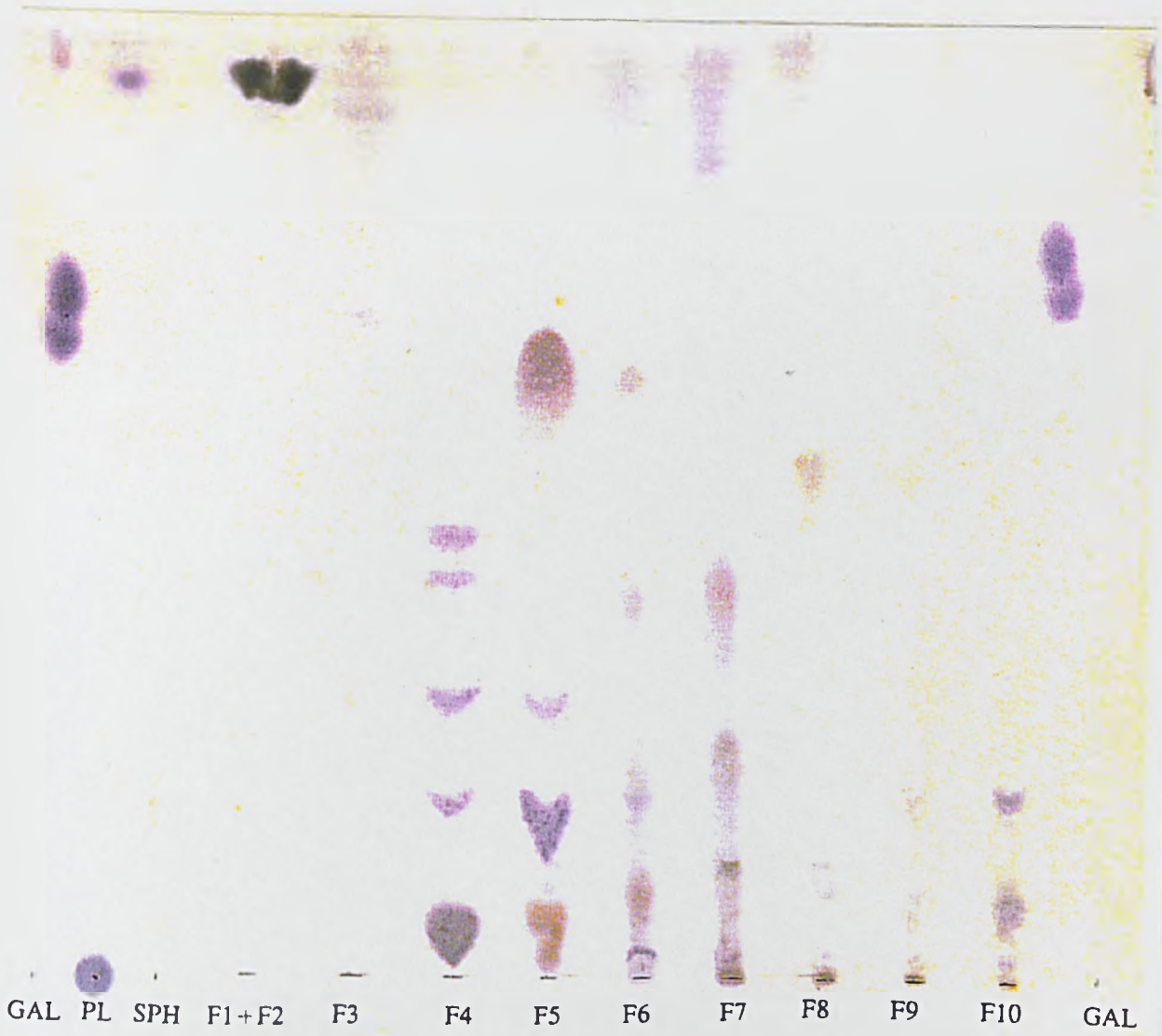
PL = mixture of PI, PC and PE as phospholipid standards
CH = cholesterol
GAL = galactocerebrosides
SPH = sphingomyelin

Fig. 5.13 Detection of phospholipid fractions from NRBC on TLC by molybdenum blue reagent



The total lipid extract from intact NRBC (an individual European adults) was fractionated on a column of silica gel as described in Materials and Methods and then analysed by TLC. Molybdenum blue (1.3% molybdenum oxide in 4.2 M sulphuric acid) revealed that F5-F10 contained phospholipids. With this spray phospholipids appear as blue-green spots. F5 contained material which co-migrated with the PE standard. F6-F8 contained materials which co-migrated with PI and PC standards.
PL = mixture of PI, PC and PE as phospholipid standards
PC = phosphatidylcholine

Fig. 5.14 Detection of glycolipid fractions from NRBC by orcinol-sulphuric acid reagent



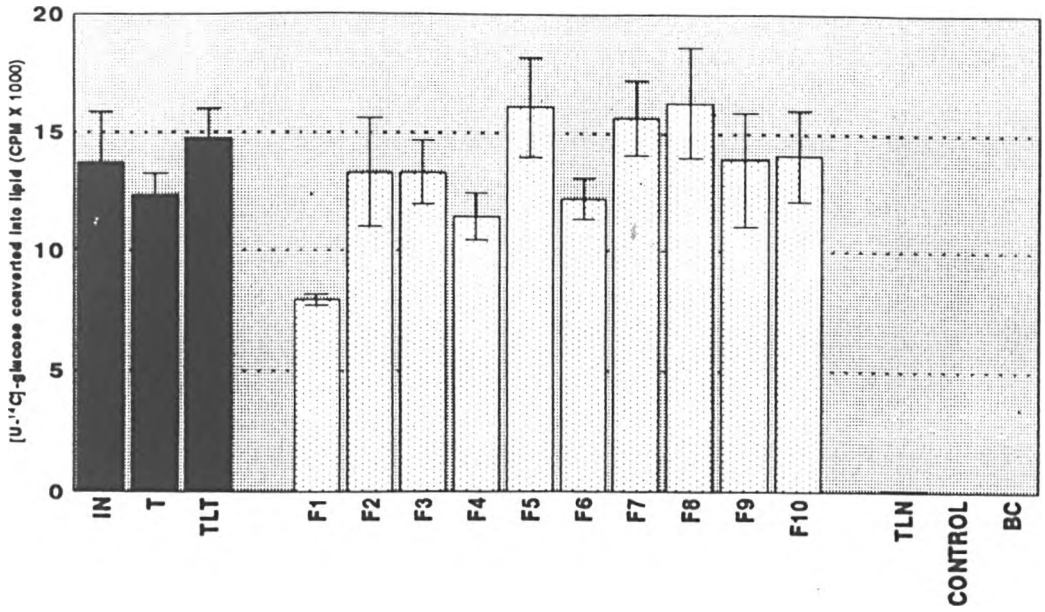
The total lipid from the intact NRBC was fractionated on a column of silica gel as described in Materials and Methods and analysed by TLC. Spraying with orcinol-sulphuric acid reagent revealed glycolipids as purple-blue spots on a yellowish background. The results demonstrated that F3-F5 of the total lipid extract of intact NRBC contained glycolipids. Comparing with Fig. 5.13 there is a change in the glycolipids composition of NRBC during infection with *P. falciparum*.

PL = mixture of PI, PC and PE as phospholipid standards

GAL = galactocerebrosides

SPH = sphingomyelin

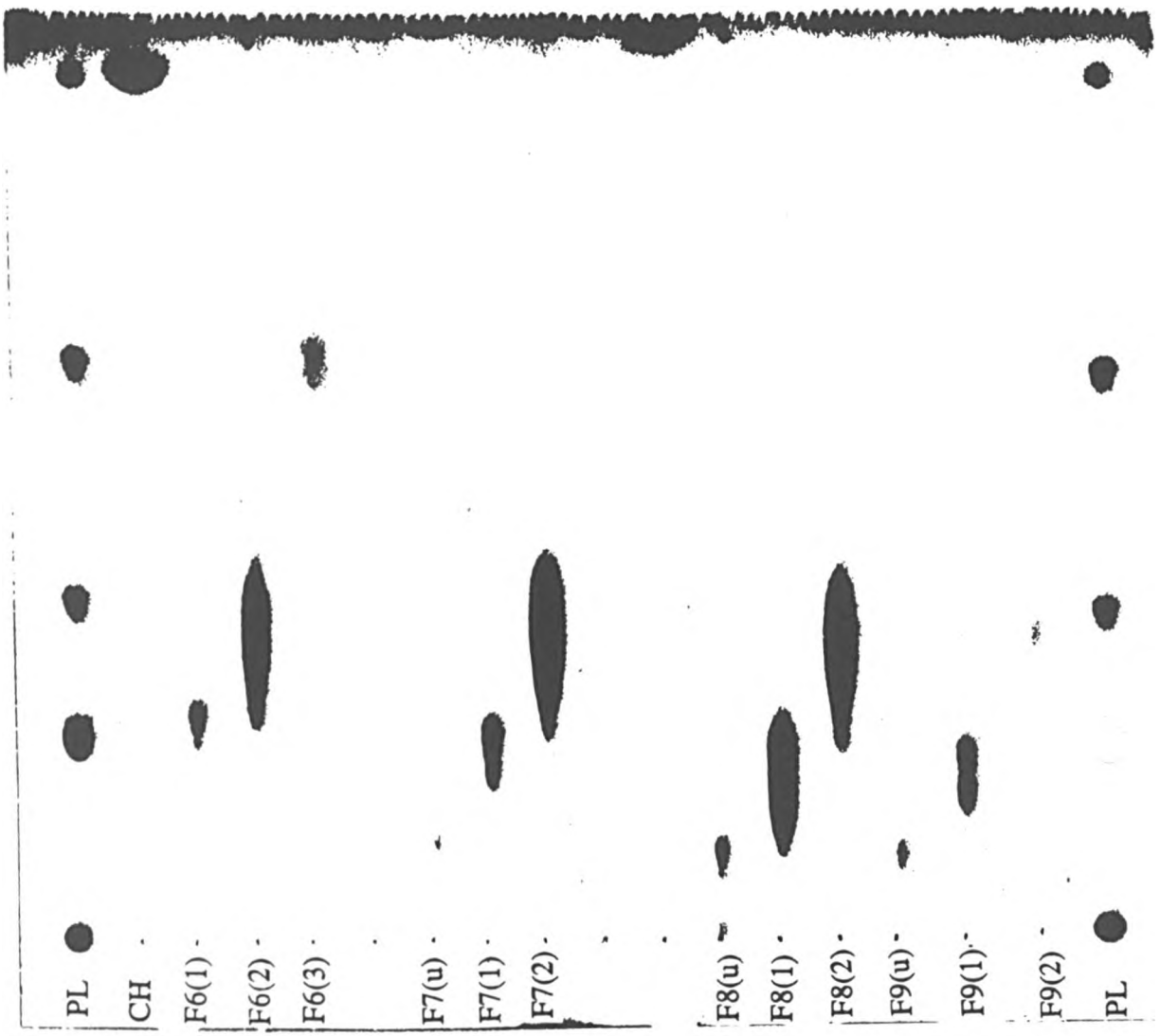
Fig. 5.15 EFFECT OF DIFFERENT FRACTIONS OBTAINED BY SILICA GEL COLUMN CHROMATOGRAPHY OF LIPIDS FROM IRBC ON LIPOGENESIS IN RAT ADIPOCYTES



Different fractions were separated from total lipid extract of *P. falciparum* IRBC. Lipid fractions were dissolved in chloroform/methanol (1:1 v/v) and then diluted in sterile PBS pH 7.4. Each sample (50 μl/ml) after dilution (final concentration of 10 μg/ml) was added to isolated adipocyte culture and then incubated for 24 h. All ten fractions (F1-F10) showed stimulation of lipogenesis in rat adipocytes. However, the total lipid extract of the NRBC supernatant did not stimulate lipogenesis after 24 h incubation. These data confirm the similarity between the bioassay of different fractions obtained from TLC, HPLC and silica gel column chromatography. Values represent the means ($\pm$SEM) of two experiments. The greater activity was observed in F5, F7 and F8.

IN=insulin
T=boiled supernatant of *P. falciparum* culture (*in vitro*)
TLT=total lipid extract of T
F1-F10=different fraction of TTL eluted from the silica gel column chromatography
TLN=total lipid extract of NRBC
CONTROL=incubation of isolated fat cells with only incubation buffer
BC= blank control from an area of plate with no sample

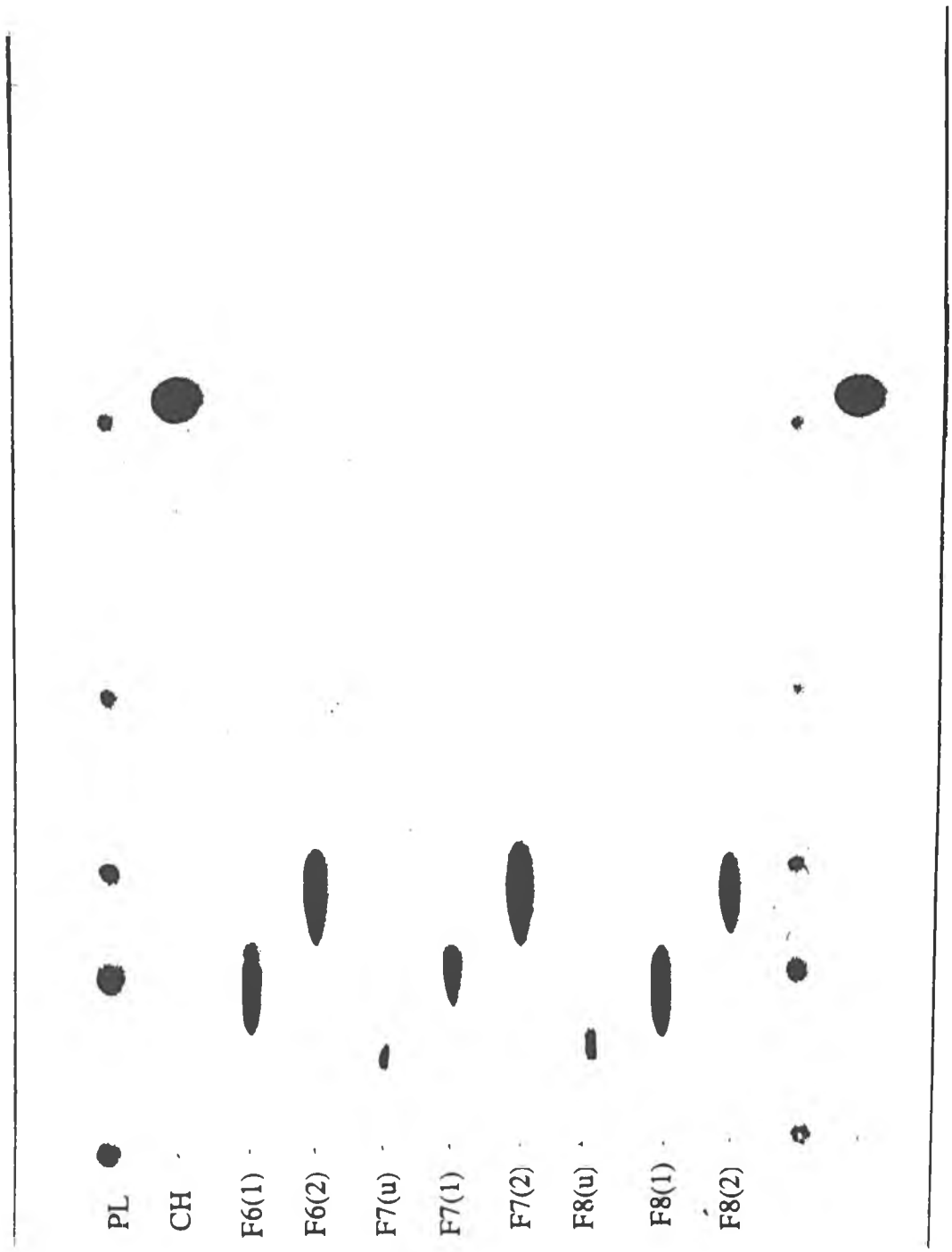
Fig. 5.16 Analysis by TLC of the samples obtained after preparative TLC from the *P. falciparum* IRBC fractions F6-F9



Fractions F6-F9 of the total lipid extract of *P. falciparum* IRBC were eluted and collected separately as described in Materials and Methods. Each fraction was chromatographed on a silica gel G TLC plate and developed in a polar solvent system (chloroform/methanol/water 65:25:4 v/v/v) for 90 minutes. The spots were visualised with the berberine spray (section 2.9.1) and then eluted from the plate with chloroform/methanol (1:1 v/v). The figure shows an analysis of the recovered materials on analytical silica gel TLC developed with chloroform/methanol/water (65:25:4 v/v/v). The results show very little contamination of spots with each other.

PL=mixture of PI, PC and PE as phospholipid standards
CH=cholesterol

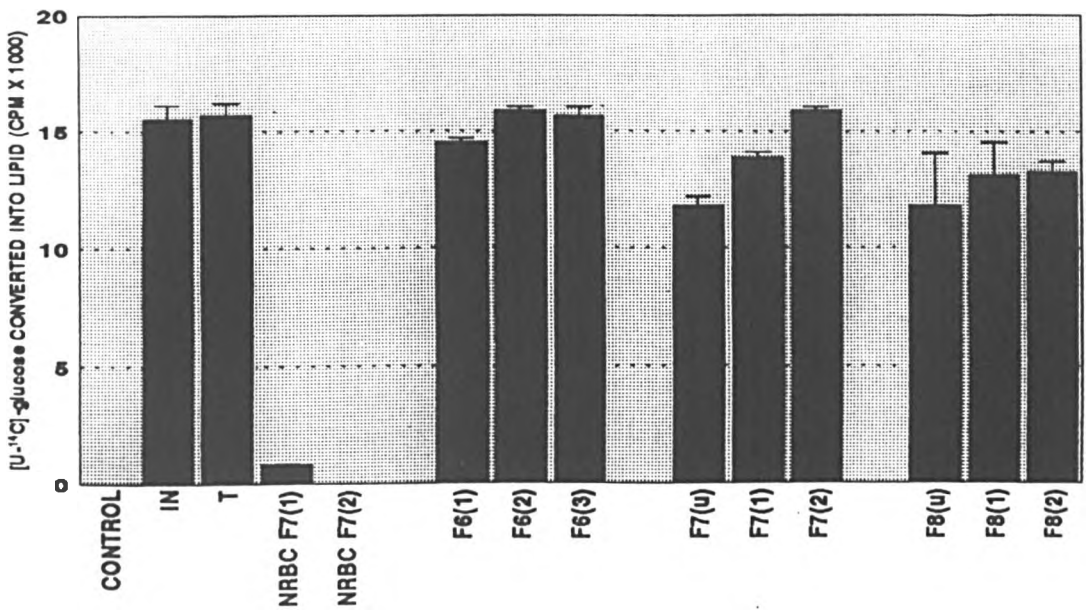
Fig. 5.17 Analytical TLC of the samples purified by preparative TLC fractions F6-F8 of total lipid extract of intact NRBC



Analytical TLC of the samples purified by preparative TLC of fractions F6-F8 of total lipid extract of intact NRBC. The experimental conditions were as described in Fig 5.16.

PL=mixture of PI, PC and PE as phospholipid standards

Fig. 5.18 STIMULATION OF LIPOGENESIS BY DIFFERENT PHOSPHOLIPID FRACTIONS *P. falciparum IRBC*



Fractions F6(1), F6(2) and F6(3) co-migrated with PE, PI and PC standards, respectively. All the lipid samples were diluted in sterile PBS, added to isolated fat cells and incubated for 24 h. All the different lipid samples showed lipogenesis-inducing activity in the rat fat cells. However, no stimulation was observed with F7(2) and F7(3) of the total lipid extract of intact NRBC. These samples co-migrated with PI and PC standards, respectively. The values are means of (± SEM) of two different experiments with two separate preparations.

IN = insulin

T = boiled supernatant of *P.falciparum* culture

NRBC F7(1) = lipid material of intact normal red blood cells which was co-migrated with PI standard

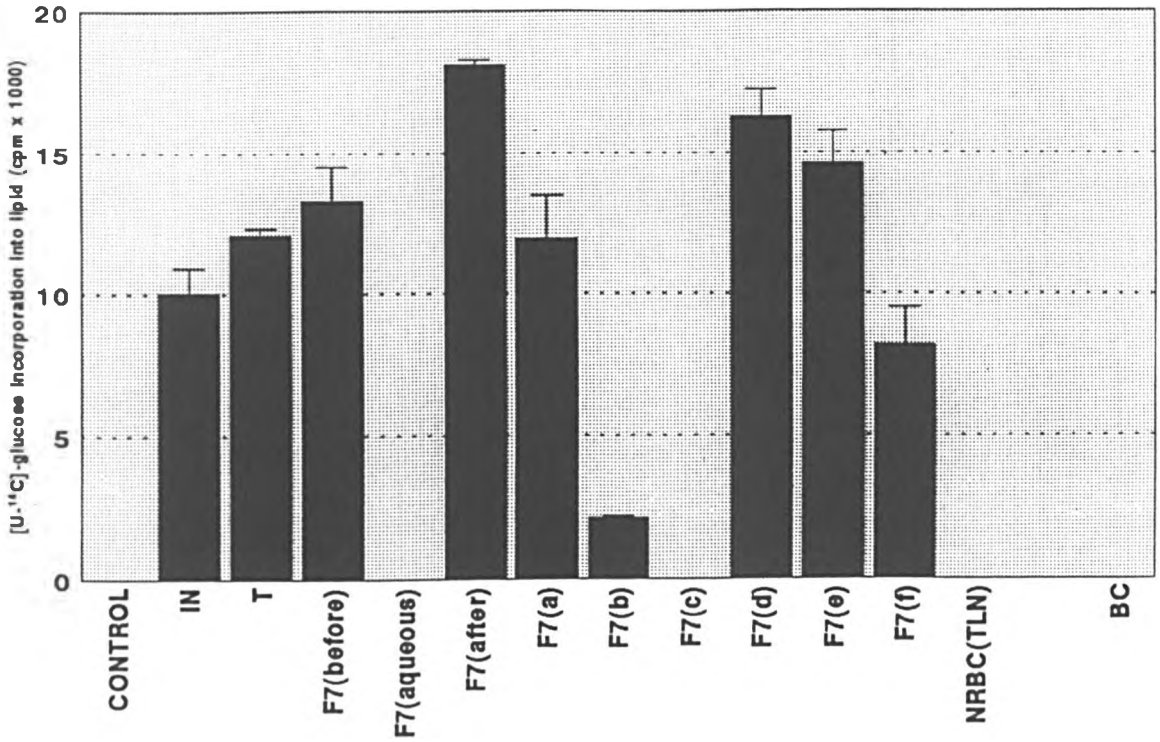
NRBC F7(2) = lipid material of intact normal red blood cells which was co-migrated with PC standard

F6(1), F6(2), F6(3) = lipid material of total lipid extract of *P. falciparum* IRBC, co-migrated with PI, PC and PE standards, respectively.

F7(u), F7(1), F7(2) = lipid material of total lipid extract of *P. falciparum* IRBC, co-migrated with unknown, PI and PC standards, respectively.

F8(u), F8(1), F8(2) = lipid material of total lipid extract of *P. falciparum* IRBC, co-migrated with unknown, PI and PC standards, respectively.

Fig. 5.19 BIOASSAY OF DIFFERENT SAMPLES RECOVERED BY TLC AFTER HYDROLYSIS OF F7 OF P. falciparum IRBC AND NRBC



Fraction F7 (4 mg) from a total lipid extract of *P. falciparum* IRBC and intact NRBC were digested separately with 40 U of phospholipase C (PLC) as described in Material and Methods. After incubation, the lipids were extracted in the organic phase with chloroform/methanol (2:1 v/v). Analysis of the lipid classes was performed by TLC with the solvent system chloroform/ethanol (98.4:1.6 v/v) (section 2.14.2). The lipids were detected with berberine spray and then eluted to give six samples F7(a) to F7(f), and these samples after dilution in sterile PBS pH, 7.4 were presented to the rat isolated fat cells. After 24 h incubation at 37°C, stimulation of lipogenesis was found in the origin material **F7(a)** which was undigested and/or polar material. A small sample which was located above of the origin **F7(b)**, and a large area with no visible sample, **F7(c)**, showed no significant stimulation of lipogenesis. Sample **F7(d)** which co-chromatographed with 1,2-dipalmitoyl-*rac*-glycerol standard showed significant stimulation of lipogenesis in the rat adipocytes. Sample **F7(e)** which co-migrated with 1,3-dipalmitin, showed stimulation of lipogenesis. The last sample **F7(f)** also showed activity. No stimulation was found from incubation of the total lipid extract of intact NRBC. The results are means (± SEM) from two experiments.

IN = insulin

T = boiled supernatant of *P. falciparum* culture

F7(before) = fraction F7 before treatment with PLC

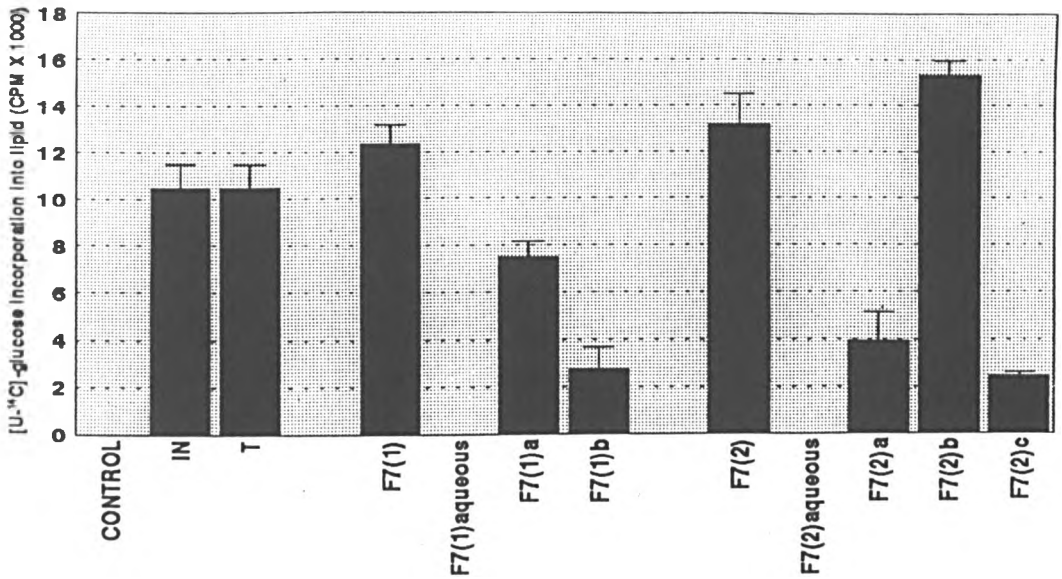
F7(aqueous) = The aqueous phase remaining after digestion of F7 by PLC and extraction with chloroform/methanol (2:1 v/v).

F7(after) = total lipid extract recovered after digestion of F7 with PLC

TLN = total lipid extract of intact NRBC

BC = blank control from an area of plate with no sample

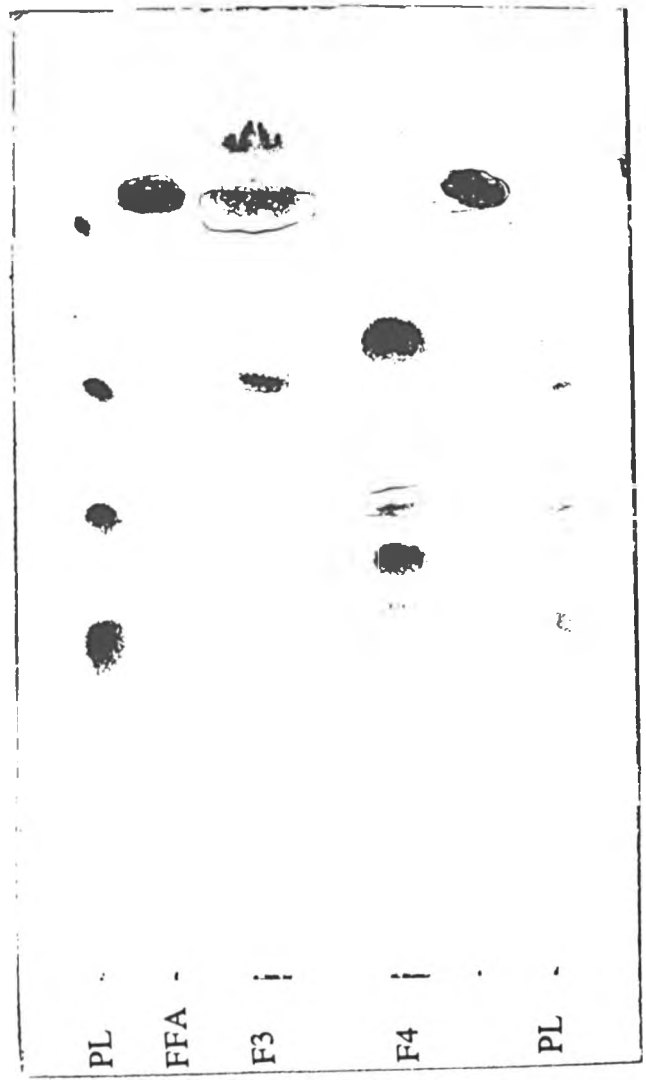
Fig. 5.20 STIMULATION OF LIPOGENESIS BY SAMPLES PREPARED BY PLC HYDROLYSIS OF FRACTIONS F7(1) AND F7(2) FROM *P. falciparum* IRBC



Samples of F7(2) and F7(3) (4mg each) were incubated with PLC as described in Materials and Methods. The reaction was terminated by boiling for 5 minutes and lipids were extracted with chloroform/methanol (2:1 v/v). The aqueous and organic phases were collected and the organic phase was then fractionated by chromatography on a silica gel G TLC plate developed on chloroform/ethanol (98.4/1.6 v/v). No active molecules were found in the aqueous phase. The F7(2) sample was digested by PLC after overnight incubation and the 1,2-DAG moiety released. However, sample F7(1) was not hydrolysed completely. All the different samples were prepared in PBS (see Materials and Methods) and added to isolated fat cells culture. After 24 h incubation, the 1,2-DAG sample stimulated lipogenesis in the rat adiposytes more than the other fractions.

IN = insulin
T = boiled supernatant of *P. falciparum* culture
F7(1) = fraction F7(1) before treatment with PLC
F7(1)aqueous = aqueous phase recovered after digestion of F7(1) by PLC and extraction with chloroform/methanol (2:1 v/v)
F7(1)a = undigested lipids on origin of the preparative TLC
F7(1)b = whole area from origin to solvent front of the TLC plate on which no visible material was observed
F7(2) = fraction F7(2) before treatment with PLC
F7(2) aqueous = aqueous phase recovered after digestion of F7(2) with PLC and extraction with chloroform/methanol (2:1 v/v)
F7(2)a = undigested lipids on origin
F7(2)b = material which co-migrated with 1,2 DAG standard on preparative TLC
F7(2)c = material which had an RF between the 1,2-DAG and the solvent front

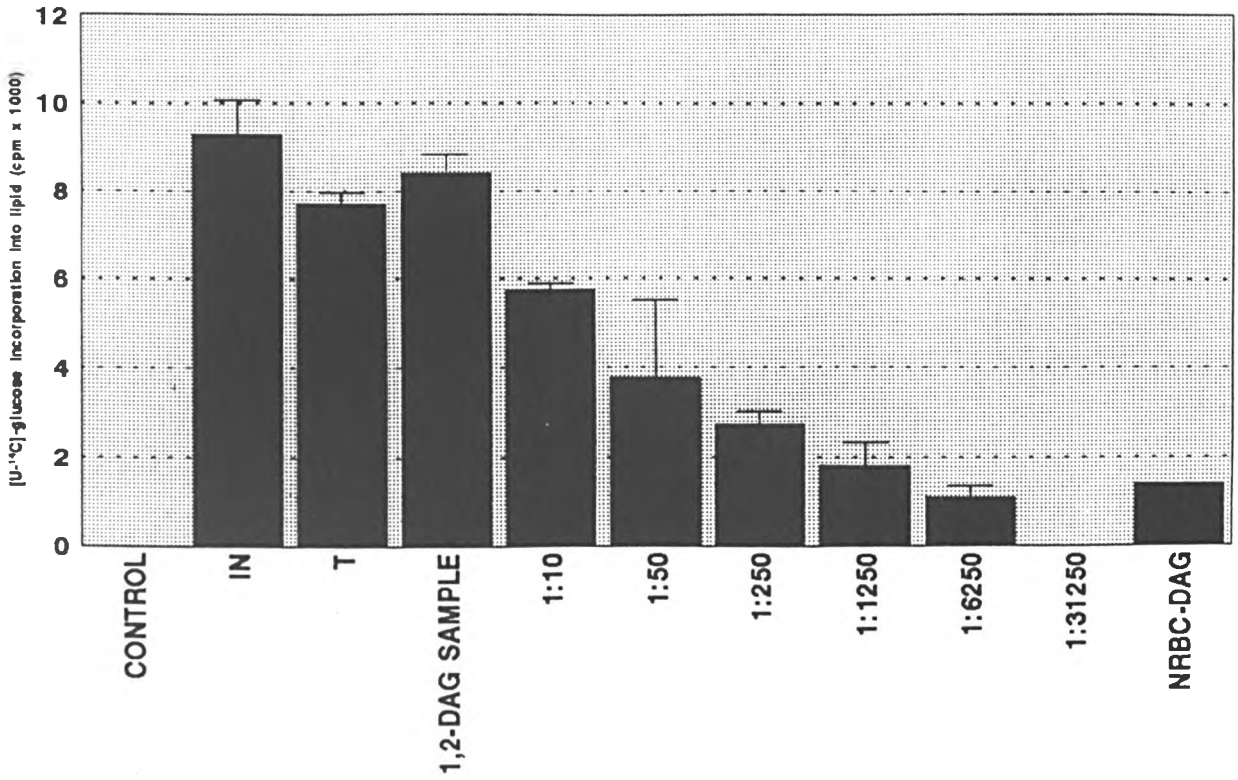
Fig. 5.21 Analysis by TLC of the samples (F3-F4) of *P. falciparum* IRBC obtained after hydrolysis by PLC



Fractions F3 and F4 (4 mg of each) were incubated overnight with 40 U PLC enzyme. The conditions of these incubations were the same as mentioned in Fig. 5.19. Incubation, extraction and chromatography of the lipids on a silica gel TLC plate gave unchanged F3 and F4 which showed the same chromatographic pattern as the starting materials. Both these fractions were shown to contain glycolipids by the orcinol-sulphuric acid reagent.

PL=mixture of PI, PC and PE as phospholipid standards
FFA=free fatty acid standard (stearic acid)

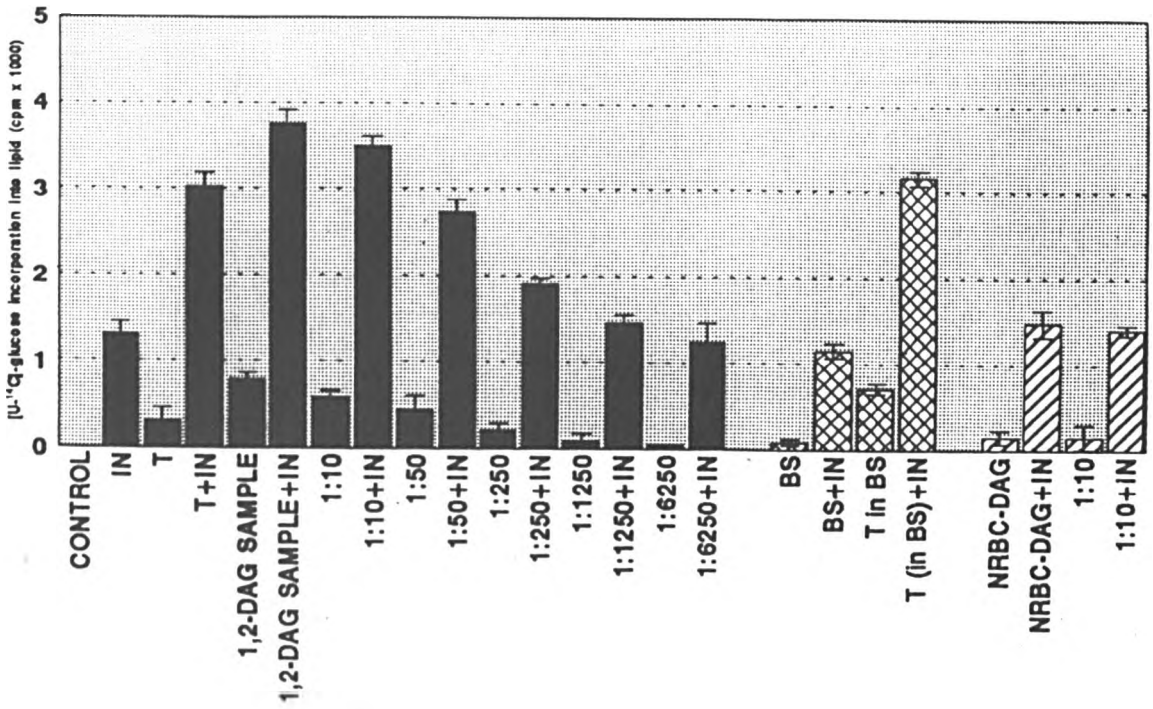
Fig. 5.22 DOSE DEPENDENT STIMULATION OF LIPOGENESIS IN RAT ADIPOCYTES BY THE 1,2-DAG SAMPLE



The 1,2-DAG sample was diluted (log 5) in sterile PBS pH 7.4, and added to isolated fat cells culture. The original concentration was 5 μg/ml of the 1,2-DAG sample. The cells were incubated for 24 h at 37°C and lipogenesis was measured as described in Materials and Methods. The results showed dose dependent stimulation of lipogenesis and the 1,2-DAG sample was active at 20 ng/ml.

IN = insulin
T = boiled supernatant of *P. falciparum* culture
1,2-DAG sample = 1,2-diacylglycerol from F7(2) of *P. falciparum* IRBC (5 μg/ml)
NRBC-DAG = 1,2-DAG from F7(2) of intact NRBC (5 μg/ml)

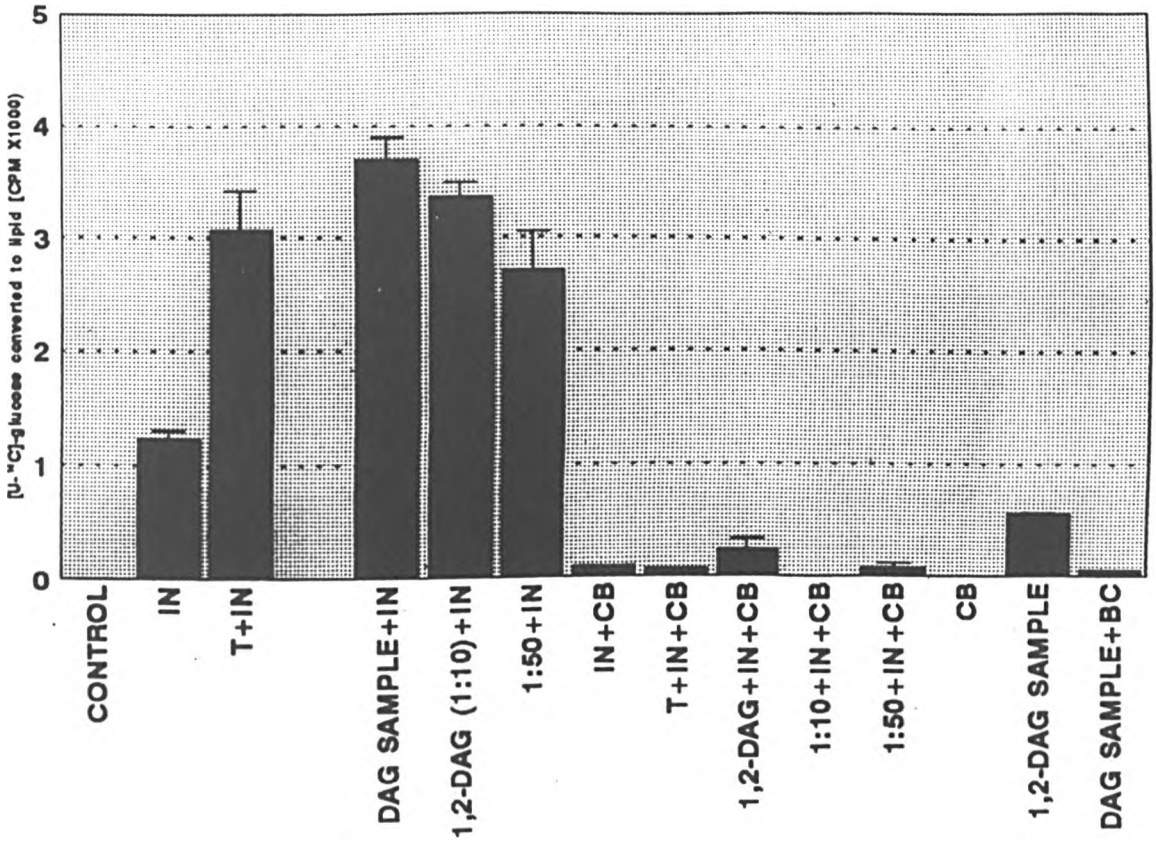
Fig. 5.23 DOSE DEPENDENT SYNERGISTIC EFFECT OF 1,2-DAG SAMPLE AND INSULIN ON LIPOGENESIS



The 1,2-DAG sample was in either sterile PBS pH 7.4, or 2 mM sodium taurodeoxycholate (NaTDC) to provide a series of dilutions (log 5). Each diluted solution (final concentration 5 μg/ml) was added to isolated fat cells and incubated at 37°C for 60 minutes. The reaction was stopped and lipogenesis measured as described in Materials and Methods. The 1,2-DAG sample stimulated lipogenesis alone, but a much greater response was observed with the addition of insulin (1 h incubation). The synergistic effect was dose dependent and both PBS and NaTDC presented the 1,2-DAG sample satisfactorily to the fat cells. There was no synergy between the 1,2-DAG sample of intact NRBC and insulin on lipogenesis. Values are means ($\pm$ SEM) of two experiments in duplicate.

IN = insulin
T = boiled supernatant of *P. falciparum* culture
1,2-DAG sample = 1,2 diacylglycerol containing sample from *P. falciparum* IRBC (5 μg/ml)
NRBC DAG = 1,2-DAG sample of total lipid extract from intact NRBC (5 μg/ml)
BS = a bile salt [sodium taurodeoxycholate (NaTDC)]

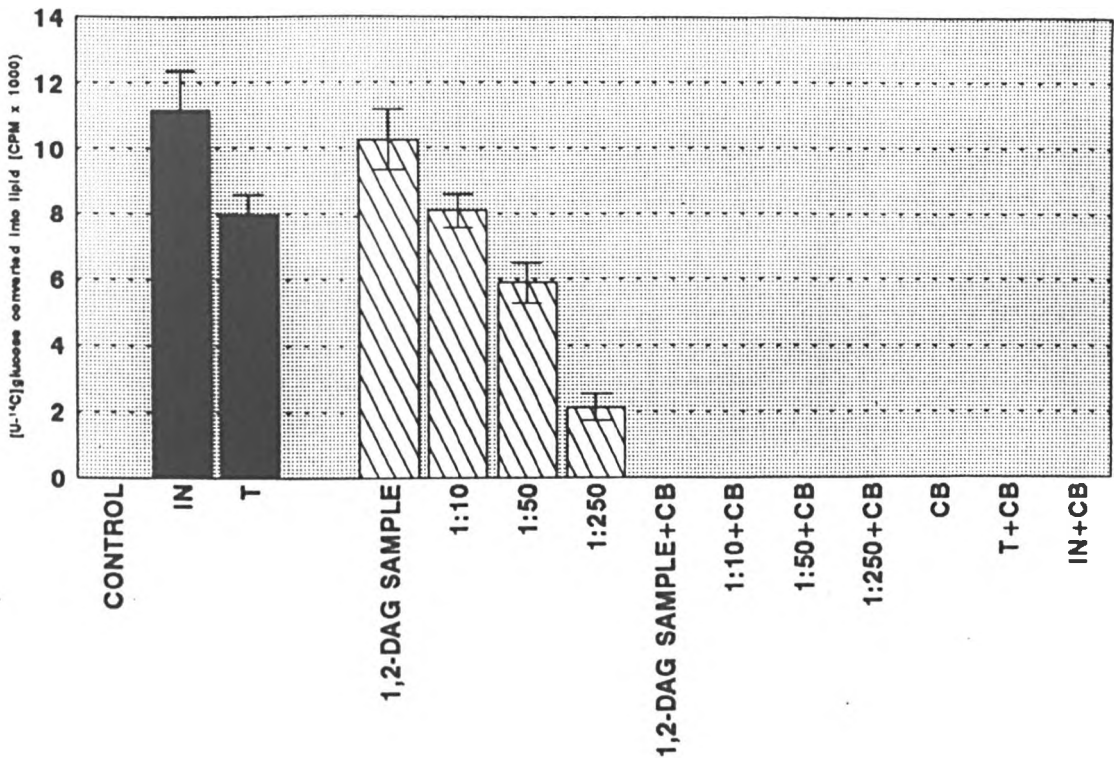
Fig. 5.24 CYTOCHALASIN B INHIBITION OF THE SYNERGISTIC EFFECT BETWEEN 1,2-DAG SAMPLE AND INSULIN.



The rat adipocytes were isolated as described in Materials and Methods. The 1,2-DAG sample (5 μg/ml), 3 x10^{-9} M insulin and 2 μg/ml cytochalasin B were added to the rat isolated fat cells and incubated for 1 h at 37°C (section 2.16). Cytochalasin B was dissolved in 1% dimethyl sulphoxide (DMSO) (see also Materials and Methods); this amount of DMSO did not cause any effect on glucose metabolism in isolated fat cells. The results revealed that there was synergy between the 1,2-DAG sample of *P. falciparum* IRBC in the presence of cytochalasin B. This drug also inhibited the lipogenesis-inducing activity of insulin. The results showed the effect of the 1,2-DAG sample on the glucose transporter which was inhibited by cytochalasin B.

IN = insulin
T = boiled supernatant of *P. falciparum* culture
1,2-DAG sample = 1,2 diacylglycerol containing sample from *P. falciparum* IRBC (5 μg/ml)
CB = cytochalasin B (2 μug/ml)
BS = a bile salt [sodium taurodeoxycholate (NaTDC)]

Fig. 5.25 CYTOCHALASIN B INHIBITION OF LIPOGENESIS STIMULATION BY THE 1,2-DAG SAMPLE FROM IRBC



Cytochalasin B (2 μg/ml) was added to adipocyte cultures in the presence of the 1,2-DAG sample (5 μg/ml). The results revealed inhibition of the 1,2 DAG sample by CB. This data demonstrated the effect of the 1,2-DAG sample on the glucose transporter.

IN = insulin
T = boiled supernatant of *P. falciparum* IRBC culture
1,2-DAG sample = 1,2 diacylglycerol of F7(2) of *P. falciparum* IRBC (5 μg/ml)
CB = cytochalasin B (2 μg/ml)

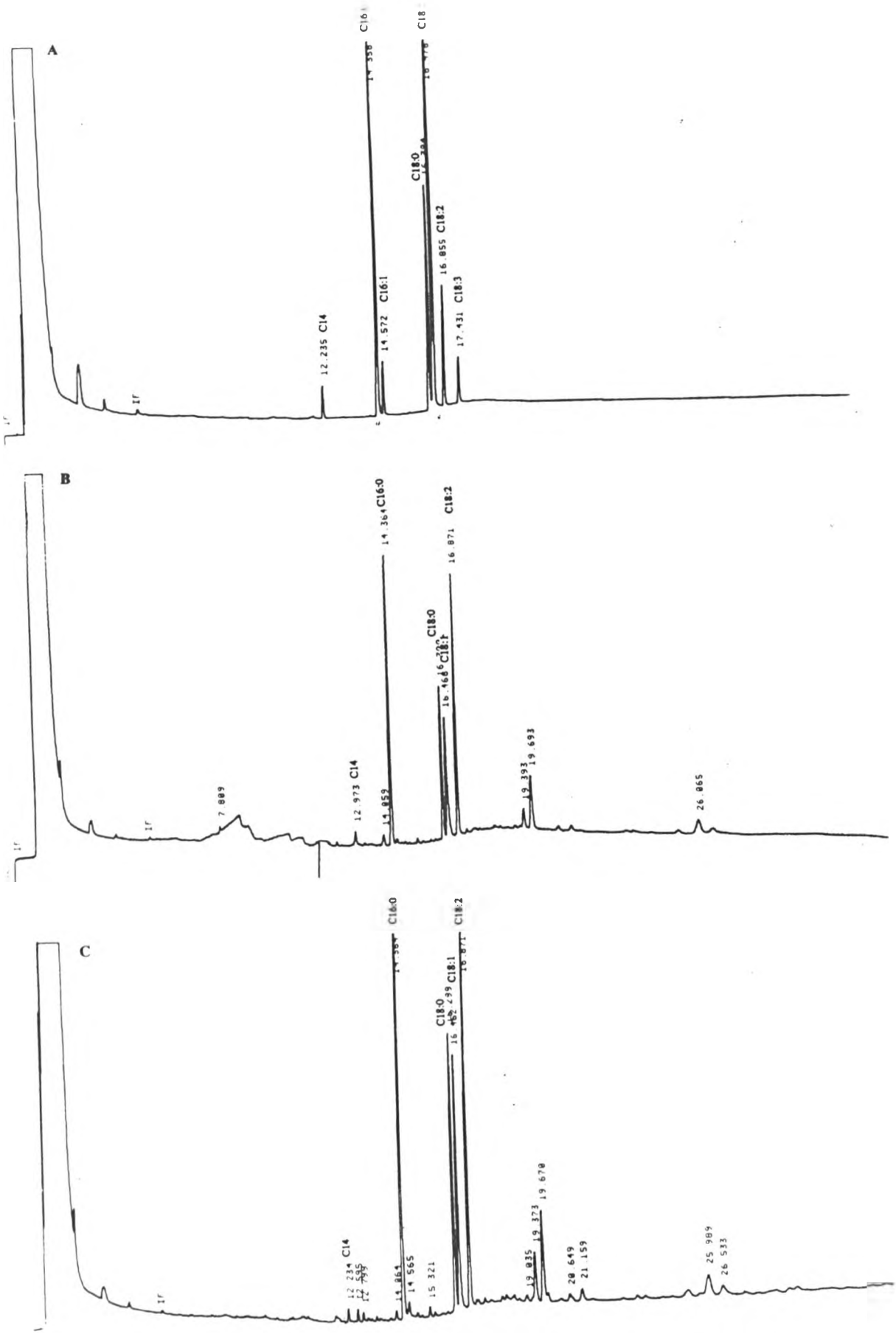


Fig. 5.26 GC analysis of the fatty acid methyl esters (FAMES) derived from the polar lipids of NRBC and IRBC culture supernatants.
A = FAMES standard
B = FAMES from NRBC culture supernatant
C = FAMES from IRBC culture supernatant

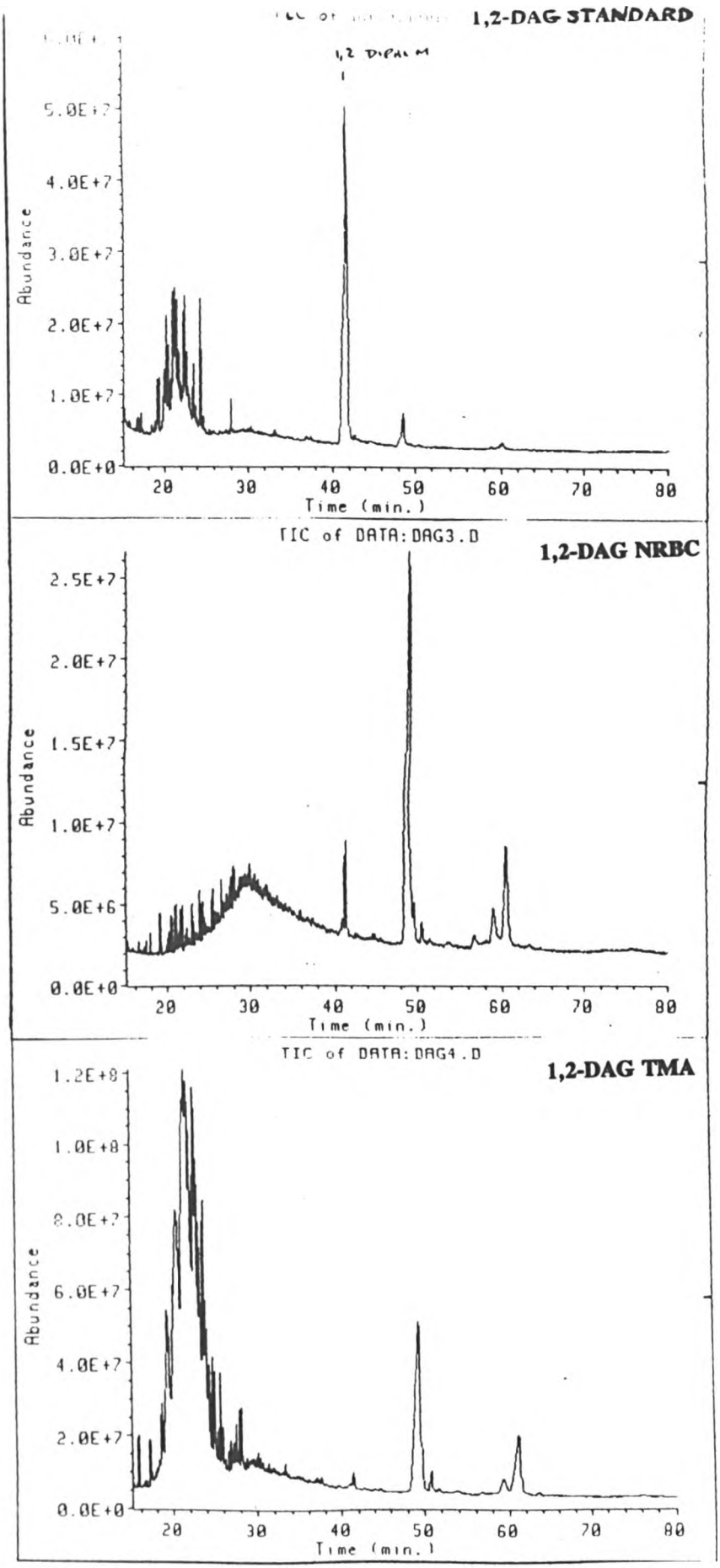


Fig. 5.27 GC-MS analysis of 1,2-DAG derived from the fraction F7 of *P. falciparum* IRBC and NRBC after hydrolysing by PLC. 1,2-dipalmitoyl-*rac*-glycerol was used as standard.

CHAPTER SIX

INHIBITION OF *P. falciparum* TMA WITH MONOCLONAL ANTIBODIES AND MALARIA HYPERIMMUNE SERUM

6.1 Introduction

Plasmodium falciparum malaria is a major health problem in large areas of the tropics. Much remains to be discovered regarding the disease process in malaria and the human immune responses to the parasite. It is not understood why repeated exposure to *P. falciparum* over a number of years appears to be necessary for an effective immunity to develop and even then why immunity functions more in limiting the disease rather than eliminating parasites.

The concept that some symptoms of malaria might be due to a toxin (or toxins) has a long history. In 1939, Sinton felt that immunity to malaria was partly antiparasitic and partly antitoxic (Sinton, 1939). Experienced malariologists have observed that, in endemic areas, immunity to malaria develops over time in two stages: first against the toxic effects of the disease, later against the parasite itself (Sinton, 1939; McGregor *et al.* 1956). The basis of this "anti-toxic immunity" is unknown. The suggestion by Playfair and collaborators that a malaria vaccine might be aimed at neutralizing the toxin rather than eliminating the parasite is a logical extension of this concept (Playfair *et al.* 1990).

In this chapter the possibility of inhibiting *P. falciparum* TMA by anti-plasmodial antibodies was explored by blocking TMA stimulation of lipogenesis in rat adipocytes using patient serum and monoclonal antibodies produced against TMA.

6.2 Results

6.2.1 Inhibition of *P. falciparum* TMA activity by malaria hyperimmune sera

Sera from adult Malawian blood donors previously shown to contain antibody to *P. falciparum* were tested for their ability to inhibit lipogenesis in rat adipocytes. Pool-1, pool-2 and pool-3 (each of them made up from 20 individuals serum sample from donors) were used in this study. All individual serum samples had previously been shown fluorescence on the infected red blood cells with *P. falciparum* (as antigens). Pool-1, pool-2 and pool-3 showed high, medium and low level positivety, respectively, when they screened by IFAT. These three pools are used as standard laboratory samples routinely, in the Immunology Unit of the Liverpool School of Tropical Medicine. Therefore, they contained antibody to *P. falciparum*.

The hyperimmune sera were titrated for their ability to block the lipogenesis by mixing equal volumes of log 10 dilutions of serum with a single concentration from boiled supernatant of *P. falciparum* culture. Normal human serum (European healthy individual blood donor serum) was used as control. Prior to addition to the primary adipocytes, the mixture of TMA and serum was incubated at 4°C overnight.

The results of a titration of the inhibitory effect of antisera against TMA *in vitro* culture of rat adipocytes are illustrated in Fig. 6.1. There was no evidence of reduction in lipogenesis in the presence of normal human serum but all three pools of hyperimmune serum reduced lipogenesis in the rat adipocytes. Pool-1 produced a 100% inhibition at 1:10 dilution, but there was no significant difference between the three pools at other concentrations. These results suggest that anti-TMA antibodies may be present in the serum of hyperimmune adults from endemic countries.

6.2.2 Inhibition of lipogenesis by monoclonal antibodies (McAbs) to TMA

Hybridomas secreting inhibitory antibodies were grown by the fusion of spleen cells from mice immunized with the polar lipid of the total lipid extract of spent culture of malaria parasite *in vitro* or with boiled supernatant of *P. falciparum* culture.

Six fusions were preformed during this study. 841 hybridomas were tested of which 65 were positive by ELISA (Table 6.1). The positive hybridomas were grown up and retested in their blocking activity on lipogenesis in long term culture (24 h) and in synergistic effect with insulin in short term (1-2 h) of the isolated fat cells (24 h) *in vitro*. In total, seven positive hybridomas out of sixty five blocked lipogenesis in the rat adipocytes. Table 6.2 demonstrates isotype of those seven McAbs which blocked lipogenesis in the rat adipocytes.

Cloning was done only after the hybridoma supernatant containing antibodies to TMA had been isotyped and found to contain more than one isotype. If only one

isotype was present, the antibody was considered "monoclonal".

As time did not permit for all the hybridomas to be cloned, only one hybridoma line from fusion SZ-2 was selected for cloning as it was found to have a mixed isotype IgM and IgA. IIIG2, IIB11, IIC2, IIIC8, IF2, IIIG8, IC7, IC9 and ID1 were hybrids which derived from SZ2 VC1 and were positive with ELISA. All these hybrids were found to have a IgM isotype.

Table 6.1 Demonstrated McAbs to TMA *of P. falciparum*

Fusion number	Antigen	Route of immunization	Total number of hybridomas	Positive (%) hybridomas (ELISA)	Positive (%) hybridomas (Adipocytes)	McAbs bloking lipogenesis
SZ-1	PL*	S.C	406	17 (4.2%)	-	-
SZ-2	PL*	S.C	280	28 (10%)	2 (0.7%)	IIIH3, VC1
SZ-3	BS**	I.V	84	11 (13%)	3 (3.6%)	IIIG9, IIID8, IVC9
SZ-4	BS**	I.V	27	2 (7.4%)	-	-
SZ-5	BS**	I.V	24	3 (12.5%)	1 (4.2%)	IB10
SZ-6	BS**	I.V	20	4(20%)	1 (5%)	IIG9
Total			841	65 (7.7%)	7 (0.8%)	

PL* Polar lipids of total lipid extract of boiled supernatant of *P. falciparum*
BS** Boiled supernatant of *P. falciparum*

Table 6.2 Demonstrated antibodies and their relevant isotypes

McAbs	Isotype	Route of immunization
IIID8	IgG2a	I.V
IB10	IgM	I.V
IVC9	IgG1	I.V
IIG9	IgA, IgM	I.V
IIIG9	IgM	I.V
VC1*	IgA, IgM	S.C
IIIH3	IgM	S.C

VC1* All clones of VC1 (IIIG2, IIB11, IIC2, IIIC8, IF2, IIIG8, IC7, IC9 and ID1) were IgM

6.2.2.1 Inhibition of the synergistic effect between insulin and TMA by McAbs

McAbs and controls were incubated overnight at 4°C with a single concentration of boiled supernatant of *P. falciparum* culture before addition to the primary isolated rat adipocytes. 50 μl/ml of each sample was added to the rat adipose cells culture and incubated for 1 h at 37°C. Fig. 6.2 shows the blocking activity of synergy between TMA and insulin by McAbs (Table 1) which were produced against TMA. The results revealed that antibodies blocked TMA activity and reduced the synergy effect significantly compared with controls. The McAb IVC9 (IgG1) from SZ-3 fusion had 96% inhibitory effect upon synergy between TMA and insulin (Table 6.3). Controls were NS-1 culture supernatant, two others antibody raised against *P. falciparum* [PB(IIIB6), PB4(IIB7)] and Normal Mouse Serum (NMS) (1:100 dilution). Fig. 6.2 also demonstrates that controls blocked neither the TMA activity and nor the synergistic effect. Incubation of one McAb with insulin alone showed that this McAb specifically block TMA activity but had no effect on insulin effect. In addition incubation of cells with only antibodies and TMA+antibody showed no effect on lipogenesis. We concluded that these McAbs specifically produced against TMA and that they also inhibited the synergistic effect observed between TMA and insulin.

Table 6.3 Shows % of inhibition of synergy effect between McAbs and insulin by McAbs to TMA.

McAbs+TMA+IN	% of inhibitory effect
IIID8	72.5%
IB10	83%
IVC9	96%
IIG9	76%
VC1	86%
VC1(ID11)	74.5%
VC1(IIB11)	83%
IIIH3	75%
PB4(IIB7)*	0%
PB(IIIB6)*	0%
NS-1*	0%
NMS*	0%

* Represent negative controls

6.2.2.2 Inhibition of TMA activity on lipogenesis using McAbs to TMA

In this experiment McAbs were tested in long term culture of isolated rat adipocytes. McAbs raised against TMA were tested for their ability to block the intrinsic activity of TMA after 24 h.

McAbs and controls were incubated overnight at 4°C with a single concentration of boiled supernatant of *P. falciparum* culture before addition to the primary isolated rat adipocytes. 50 μl/ml of each sample was added to rat adipocyte culture and incubated for 24 h at 37°C. Fig. 6.3 demonstrates that IIIH3, IIIG9 and two cloned of VC1, IC7 and IC9 did not inhibited lipogenesis inducing activity of TMA. However, the rest McAbs inhibited this activity in different levels. IIID8 and IVC9 were two antibodies which had high level blocking activity, 92% and 82%, respectively (Table 6.4). None of controls showed inhibitory activity in this assay.

IIID8 (IgG2a) and IVC9 (IgG1) two McAbs which showed high blocking activity of TMA in synergistic effect in short term culture and the observed TMA effect in long term culture were chosen for titration of antibody. A typical experiment is shown in Fig. 6.4. Both McAbs inhibited TMA activity in a dose dependent manner.

Table. 6.4 % of inhibitory effect by McAbs to TMA in long term culture of rat adipocytes.

McAbs+TMA	% of Blocking activity
IIID8	92%
IB10	62%
IVC9	82%
IIG9	71%
IIIG9	15%
VC1	72%
IIIH3	34%
IIIH3(IIIG2)	72%
VC1(IIB11)	82%
VC1(IIC2)	47%
VC1(IIIC8)	41%
VC1(IF2)	35%
VC1(IIIG8)	41.5%
VC1(IC7)	0%
VC1(IC9)	20%
VC1(ID1)	71%

6.3 Discussion

Asexual erythrocytic parasites cause malaria, the disease associated with plasmodial infection. The goal of vaccines against asexual parasites is the prevention or reduction of disease and death in malaria endemic areas, especially in Africa (Miller *et al.* 1997). An asexual erythrocytic stage vaccine would prevent or reduce morbidity and mortality by eliminating or reducing the parasite load. Furthermore, parasite derived material released from the infected erythrocyte at the time of rupture and release of merozoites is thought to induce the human host to produce cellular factors that contribute to, or exacerbate, the pathogenesis of malaria. By inducing neutralizing antibodies to these parasite products, or by inhibiting cytoadherence of infected erythrocytes implicated in cerebral and other forms of severe malaria, an asexual erythrocytic vaccine would reduce morbidity and mortality (Doolan & Hoffman 1997).

The question is whether advances in the molecular identification of parasite derived material released from infected erythrocyte will provide the basis for an anti-toxic vaccine that prevents their activity and thereby protects against complication of severe *P. falciparum* malaria. The concept of anti-toxic immunity has received support from experimental studies showing that mice immunised with parasite lysates, or with simple chemical structures such as inositol monophosphate or PI, can generate antibodies that inhibit parasite-induced TNF production *in vitro* (Bate *et al.* 1990, 1992d). The result of this chapter was in concordance with previous findings and showed that TMA activity could be inhibited by 1) malaria hyperimmune sera from

endemic area, 2) with McAbs which raised against polar lipid extract of IRBC with *P. falciparum* and, 3) boiled supernatant of *P. falciparum* culture.

Anti-phospholipid antibodies (anti-PL) have been extensively studied in many auto-immune disorders. Interest in the role of anti-PL has been renewed recently in malaria, since the active component of malaria exo-antigen (TMA), capable of inducing TNF-α, has been identified as phospholipid (Bate *et al.* 1992b). Facer and Agiostratidou reported that most malaria patients were positive for antibodies to the anionic phospholipids (PS, PC, and PA) with the exception of PI, where a lower level was found of patients. If antibodies to PI are protective, then individuals with severe (cerebral) malaria might be expected to have lower anti-PI levels (and higher TNF) than patients with uncomplicated disease. This was indeed the case (Facer & Agiostratidou, 1994). Bate and Kwiatkowski (1994b) reported that serum from an European adult infected with *P. falciparum* also inhibited the activity of malaria toxins derived from *P. falciparum*-infected erythrocytes, and that too, was mediated by IgM antibodies which were malaria specific and bound to phosphatidylinositol liposomes. However, the study by Das and co-workers also indicated a significant rise in anti-phosphatidylcholin (anti-PC) in humam *P. falciparum* infections (Das *et al.* 1996).

An explanation for the presence of elevated levels of anti-PL in malaria could be due to the presence of parasite-derived products or malaria-modified host antigens which have specific PL components with antigenic effect. These components may be released when millions of schizonts rupture (from the membrane of infected erythrocytes, from the membrane of parasite or from merozoites) and then they

behave as antigens and stimulate the immune system. Individuals exposed to repeated re-infection can develop a state of protection that prevents the occurrence of clinical symptoms and high parasite burdens.

The essential finding in this chapter was that the active component(s) (TMA) of *P. falciparum* which stimulated lipogenesis in the rat adipocytes was associated with polar lipids, more specifically with the phospholipid classes (see also Chapter five), and this activity was blocked by sera from adult Malawian blood donors. This result suggests that long-lived antibodies against lipogenesis-inducing factor(s) can evolve in a malaria-endemic community, and in that context they may be protective against complications of severe malaria particularly hypoglycaemia. The presence of antibody to TMA in hyperimmune sera may explain why individuals who live in areas where malaria is endemic normally acquire immunity to the symptoms of the disease while their parasitaemia is still high (McGregor *et al.* 1956). The basis of this anti-disease immunity is not yet understood, but it has been argued that it may be due to the development of antibody against toxic malaria antigens. Evidence for a definitive protective role for phospholipid antibodies in anti-disease immunity in human malaria is yet to come. This represents suggestive preliminary evidence.

Using the murine malaria parasite *P. yoelii*, Taylor and co-workers showed that a supernatant of blood stage parasites incubated overnight could induce hypoglycaemia when injected into normal mice (Taylor *et al.* 1992a). Serum from mice immunized by injection of supernatants 10-12 days earlier contained antibody (mainly IgM) which prevented the development of hypoglycaemia 4 h after injection

of TMA to normal mice (Taylor *et al.* 1992a). However, this experiment has been done *in vivo*. There is no report about production of McAb which can block TMA activity *in vitro*. For the first time, we have done immunization by i.v route and boosted every 4-5 days with boiled supernatant or every two weeks with polar lipid (subcutaneous) which could increase the level of antibodies. Isotyping of McAbs revealed IgG1 and IgG2a isotype, two antibodies which blocked TMA by more than 90%. This study concentrated on the ability of McAbs to inhibit stimulation of lipogenesis by McAbs in the rat adipocytes in response to TMA of *P. falciparum* IRBC. We focused on TMA because as it may contribute to the hypoglycaemia in severe malaria. McAbs against TMA of *P. falciparum* provide an essential tool in investigating the molecular pathogenesis of infection and may lead to new forms of treatment for life-treating complications of malaria.

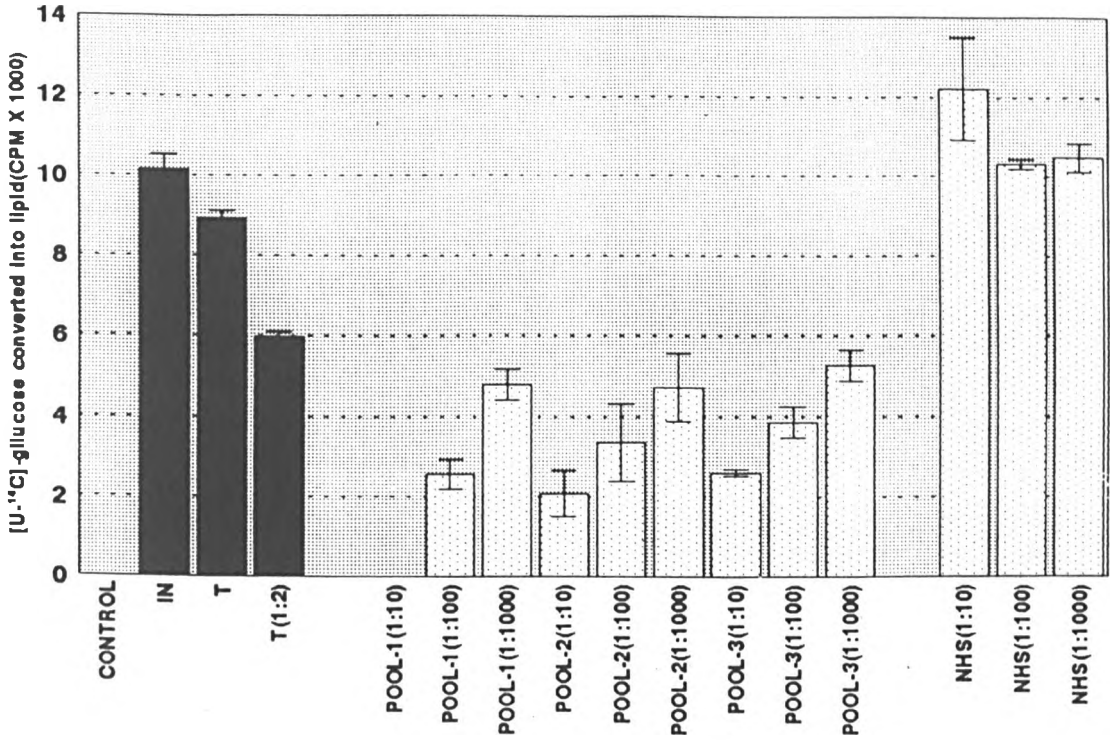
We have identified several McAbs that inhibit both the stimulation of lipogenesis and synergistic effect by of malaria TMA. The inhibitory McAbs were of the IgM and IgG isotypes. Control McAbs to *P.falciparum* were not inhibitory, showing that inhibition observed was specific to TMA of *P. falciparum*. McAbs were raised to malaria TMA with two different antigen preparations. Both of these immunization methods stimulate the production of inhibitory antibodies against TMA. However, when polar lipid extracted from boiled supernatant of *P. falciparum* was used as antigen (subcutaneous immunization) the isotype of antibodies was only IgM, but immunization with boiled supernatant raised both IgM and IgG isotypes. Both McAbs IIID8 (IgG2a) and IVC9 (IgG1) originated from mice immunized with a boiled supernatant of *P. falciparum* culture using an i.v route which inhibited the

synergistic effect by 92%, 82% and lipogenesis stimulation by 72.5%, 96% respectively, which caused dose-dependent inhibition of the lipogenesis.

Another essential finding of this study was the growing of hybridomas in the absence of insulin in the culture medium, as we found the stimulation of lipogenesis in the rat adipocytes by some McAbs which were grown in medium containing insulin. The same is true with some hyperimmune sera and for this reason, sera should be diluted down to a level at which insulin is not effective but, McAbs block the TMA activity.

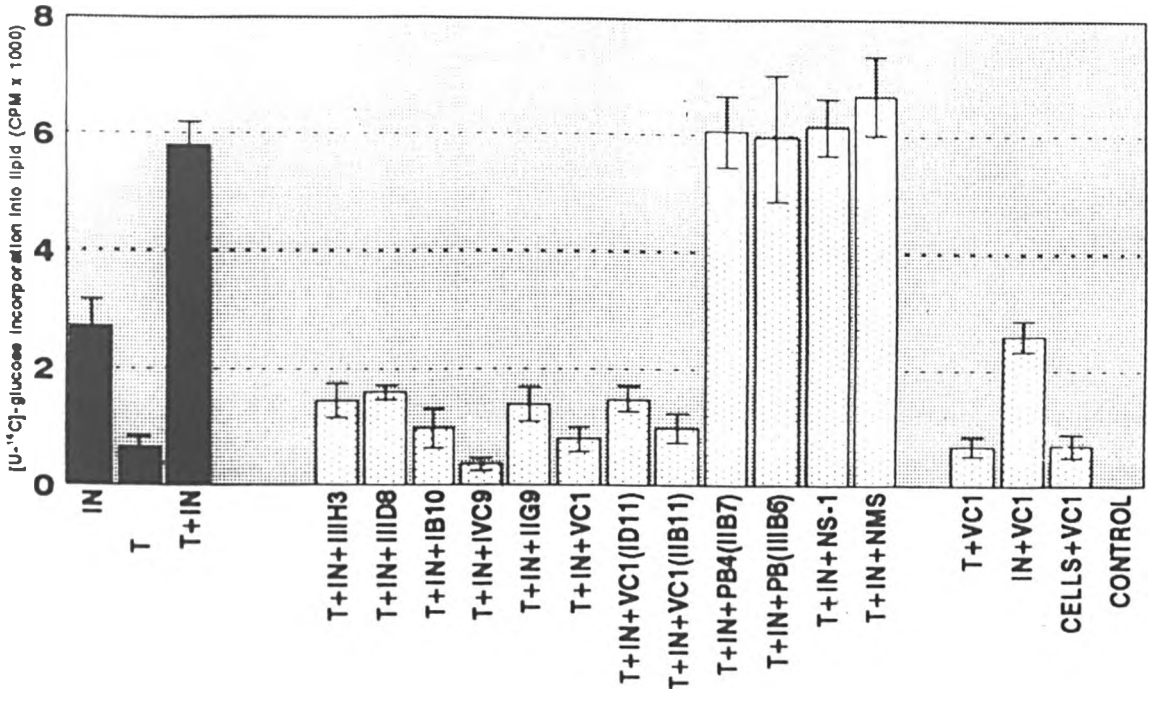
Further work require to test individual hyperimmune serum (who live in malaria endemic area) for their ability to inhibit lipogenesis in rat adipocytes.
It would be also interesting to identify an inhibitory effect of these McAbs to prevent hypoglycaemia *in vivo*. Although PLs are a constituent of normal red cells membranes, we should clarify that these McAbs do not bind to human erythrocytes.

Fig. 6.1 INHIBITION OF TMA ON LIPOGENESIS IN RAT ADIPOCYTES BY MALARIAL HYPERIMMUNE SERUM



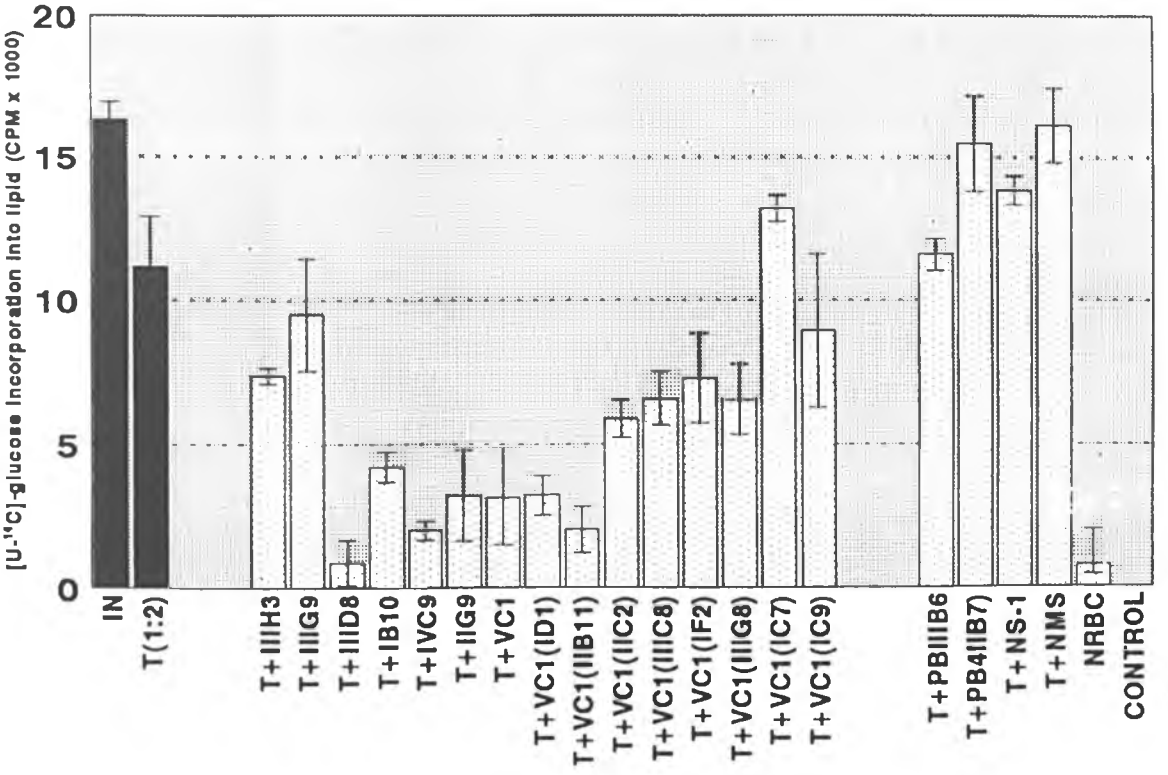
Pool-1, Pool-2 and pool-3 from adult Malawian blood donors containing antibody to *P. falciparum* were used in this experiment. Each pool was a mixture of 20 individual serum samples. The pooled serum samples were incubated with equal volumes of a single concentration of an boiled supernatant of *P. falciparum* culture (1:2) or normal human serum (as control) overnight at 4°C, before addition to the primary rat adipocytes. The cells + agents (insulin, TMA, TMA+antibody and NHS) were incubated for 24 h at 37°C with gentle shaking. Lipogenesis was stopped and measured as described in Materials and Methods. The Results showed the presence of antibodies which blocked TMA activity on lipogenesis in adult Malawian blood donors. Normal human serum (**NHS**) used as a negative control did not inhibit the activity of TMA on lipogenesis. Insulin (**IN**) and boiled supernatant of *P. falciparum* (**T**) were used as positive controls. Because T was incubated with antisera (1:2), we diluted the positive control T (1:2) in KRB. Values are the means of two independent experiments (± SEM) in duplicate.

Fig. 6.2 INHIBITION OF SYNERGISTIC EFFECT BETWEEN INSULIN AND TMA BY MONOCLONAL ANTIBODIES



Six different McAbs and two clone (IgM) were used in this experiment. McAbs were mixed with a single concentration of a boiled *P. falciparum* supernatant (T) and incubated overnight at 4°C. 50 μl/ml each of mixture was added to isolated rat adipocytes in the presence of 3 X 10^{-9} M of insulin and 5 mM [U-^{14}C]-glucose for 1 h at 37°C. Lipogenesis was stopped and measured as described in Materials and Methods. The controls used were two McAbs which were produced against *P. falciparum*, NS-1 culture supernatant and Normal Mouse Serum (**NMS**). We also incubated TMA (**T**) with one of McAb, insulin (**IN**) alone with McAb and isolated cells with antibody as second set of controls. Insulin (**IN**) and IN + T were used as positive controls. Because T was incubated with McAb (1:2), therefore we diluted positive control T 1:2 in KRB. Results showed inhibition of the synergistic effect between TMA and insulin by all McAbs which have been tested. None of controls inhibited the synergistic activity of TMA and IN. Values are the means ($\pm$ SEM) of two experiments in duplicate.

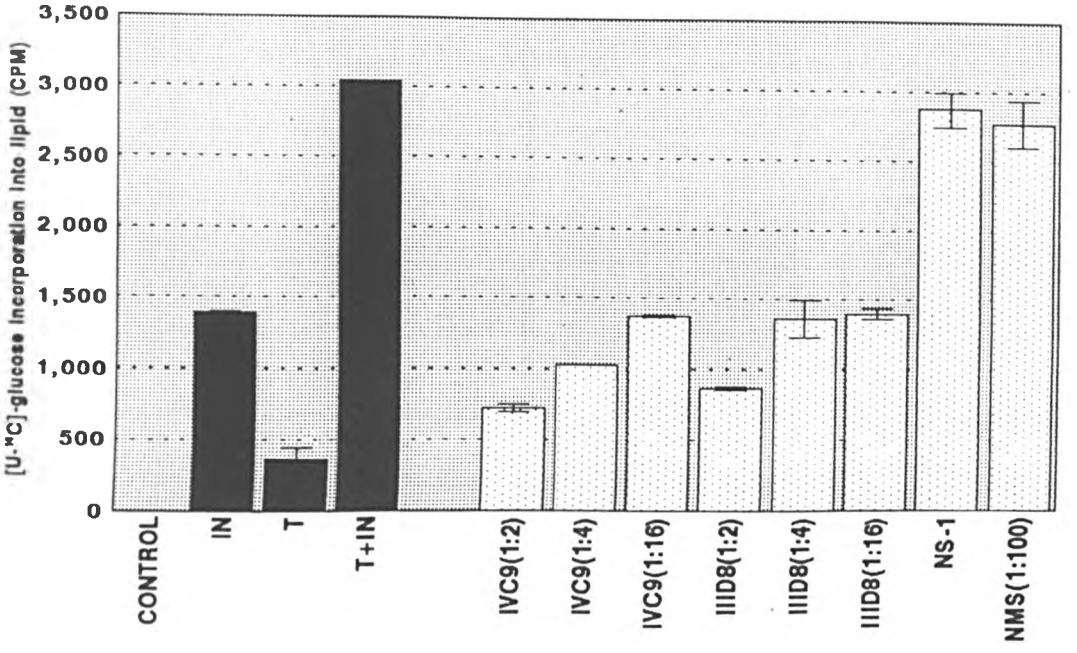
Fig. 6.3 INHIBITION OF TMA ON LIPOGENESIS IN RAT ADIPOCYTES BY MONOCLONAL ANTIBODIES TO TMA



Seven McAbs and the eight clones of VC1 (IgM) were used in this experiment. The conditions of the experiment was as previously described in Fig. 6.2, except that the incubation time was 24 h. The aim of this experiment was to investigate blocking of TMA activity alone by McAbs in long term culture. Most of McAbs inhibited TMA activity. None of controls showed an inhibitory effect. VC1 (IC7) did not block TMA activity compared to controls. Values are means (± SEM) of two experiments in duplicate.

IN=Insulin **T**=TMA
PB(IIB6)=- control **PB4(IIB7)**=- control
NS-1= -control **NMS**=Normal mouse serum

Fig. 6.4 DOSE DEPENDED INHIBITION OF SYNERGY BETWEEN TMA AND INSULIN BY MONOCLONAL ANTIBODIES



McAbs IVC9 (IgG1) and IIID8 (IgG2a) inhibited the synergistic effect of TMA with insulin by 96% and 72.5% respectively. They also inhibited the effect of TMA activity alone by 82% and 92% respectively. These two McAbs were chosen for titration of antibodies. McAb dilutions were mixed with a constant amount of a boiled supernatant of *P. falciparum* culture and incubated overnight at 4°C. 50 µl/ ml of different dilutions of each mixture was added to isolated rat adipocytes in the presence of insulin (3×10^{-9}) for 1 h at 37°C. Lipogenesis was stopped and measured as described in materials and methods. The results demonstrated an inhibitory effect of TMA by these two McAbs in a dose-dependent manner. Figures are means (± SEM) of duplicates.

IN = Insulin T = TMA

NS-1 = -control NMS = Normal mouse serum

CHAPTER SEVEN

MYCOPLASMA CONTAMINATION IN *P. falciparum* CULTURE

7.1 Introduction

In 1898, the French scientists Nocard and Roux, studying pleural fluids of cattle suffering from a disease called pleuropneumonia, discovered organisms that were unlike any other microorganisms then known (Pirie, 1972). The organisms were aerobic and were cultivable only on rich organic media containing animal serum. Because of their obvious relationship to the bovine pleuropneumonia organism, they were known for many years as pleuropneumonia-like organisms, or more simply as PPLOs. Today, they are separated from the true bacteria and have been placed in a new class, Mollicutes, with several families and genera (Buchanana, 1974). These are known informally and collectively as mycoplasma. The mycoplasmas are unique in that they do not possess the distinctive cell wall of true bacteria. They are the smallest of the cellular microorganisms, generally measuring 300-800 nm in diameter.

Mycoplasma, the smallest self-replicating microorganisms, are pathogens capable of causing a wide variety of diseases, including acute respiratory illness, genitourinary tract or joint infections, and autoimmune disorders (Rawadi & Roman, 1996). Mycoplasma, unlike most other pathogenic bacteria, do not invade the tissues or blood stream. Rather, they colonize the cell surfaces of the respiratory and genitourinary tracts, rarely penetrating the cells of the epithelial lining. The resulting

close association between the mycoplasma and host cells is thought to be relevant to other events in pathogenesis. The mycoplasma gain a nutritional advantage from metabolites that are concentrated at the host cell surface; they probably can also utilize lipids and cholesterol contained in host cell membranes. The association of mycoplasma with cell surfaces *in vitro* may be necessary for the organism to obtain the nucleic acid precursors that are required by all mycoplasma (Stanbridge, 1971).

In addition to their pathogenicity, they are also common contaminants of cell cultures (Razin & Barile, 1985). Contamination of cell cultures by mycoplasma was first reported by Robinson and co-workers (Robinson *et al.* 1956), and subsequently, it has been shown that these microorganisms were widespread contaminants of cell cultures. Their presence confounds the interpretation of most cell culture work; their source is often a mystery, and they are difficult to eliminate.

From the first report in 1956 and until 1965, the predominant species found were virtually all human species, particularly *M. hominis* and *M. orale* (Hayflick, 1965). In the next five years, from 1966-1971, another species *M. hyorhinis* has been found in cell cultures (Hayflick, 1965; Purcell *et al.* 1966). In the past years, another predominant species found in over 200 contaminated cell cultures is *Acholeplasma laidlawii*, and occasionally *M. arginini* (Barile & Kern, 1971).

Until recently, there were no reports of mycoplasma contamination of *Plasmodium* cultures (Turrini *et al.* 1997). Mycoplasma do not grow in mammalian erythrocytes, but they could attach to human and guinea-pig erythrocytes (Loomes *et*

al. 1984) by adhering to sialic acid containing proteins (Roberts *et al.* 1989) and glycolipids (Loomes *et al.* 1985).

Turrini and co-workers reported mycoplasma contamination of several strains of *P. falciparum* cultures (Turrini *et al.* 1997). They used polymerase chain reaction (PCR)-based method for mycoplasma detection (Dussurget & roulland-Dussoix, 1994). Their data showed that samples positive for mycoplasma by PCR were also validated by microbiological culturing, which gave positive results for *P. falciparum* cultures and negative results for haemozoin supernatants. They also reported that haemozoin supernatants that originated from a mycoplasma contaminated culture did not contain viable mycoplasma, but contained mycoplasma DNA. The presence of mycoplasma in parasite cultures may easily escape notice, because of their small size (300-800 nm diameter), and the fact that they can pass through 0.1 μm pore-size membrane filters (in most procedures described, medium is filtered through 0.4 or 0.22 μm filter). They do not have a cell wall, so, they are undetectable in smears of malaria cultures stained by Giemsa. Contamination dose not have an inhibitory effect on parasite invasion or maturation. Mycoplasma contamination can be passed on across generations, and persist in spite of freezing in liquid nitrogen and subsequent thawing (Turrini *et al.* 1997).

Because the mycoplasma has no cell wall, antibiotics affecting cell wall synthesis, such as penicillin, are without effect. Eradication of mycoplasma contamination from malaria parasite culture by anti-mycoplasma antibiotics are not easy. Since high doses of antibiotics are toxic to the parasite, but at low

concentrations they may not eliminate mycoplasma.

Mycoplasma contamination may be a source of artefactual results in several ways. Contamination by mycoplasma may mimic or artefactually generate a number of more complex effects due to cell-to-cell interactions. Mycoplasma produce a protein that induces the release of tumor necrosis factor (TNF) from human monocytes (Kostyal *et al.* 1995) and mouse macrophages (Arai *et al.* 1990) and also the induction of interleukin-1 (IL-1), IL-6 and TNF via direct interaction of membrane-bound lipoproteins with human monocytes (Herbelin *et al.* 1994).

A mycoplasma-derived macrophage-activating substance which has been named mycoplasma derived high-molecular-weight material (MDHM), was originally discovered because of its ability to stimulate maturation of cytolytic T-cell precursors to cytolytic effector T cells in thymocyte culture (Quentmeier *et al.* 1990). This molecule with a lipid moiety carrying fatty acids in an ester linkage and a polyol moiety of unknown character activates macrophages to release nitric oxide (NO), TNF-α and IL-1β. MDHM partitions into the Triton X-114 phase or phenol phase on the one hand and is insoluble in chloroform-methanol on the other.

The major unidentified polar lipid (compound X), recently demonstrated in the cell membrane of *Mycoplasma fermentans*, was purified by preparative silica gel column chromatography. Chromatography of the polar lipid fraction of *M. fermentans* cells on silica gel TLC plates developed by chloroform/methanol/water/ (64:25:4 v/v/v) revealed six major lipid spots. Five of the spots which reacted with molybdate

reagent and thus, represent phospholipids are a phosphorous-containing unidentified compound (compound X, Rf=0.04), sphingomyelin (Rf=0.23), phosphatidylcholine (Rf=0.33), phosphatidylglycerol (Rf=0.51) and diphosphatidylglycerol (Rf=0.62). The sixth spot (Rf=0.75) did not react with the molybdate reagent but reacted with anthrone reagent, hence representing a glycolipid. Compound X contained 63-66% of the total lipid phosphorus of *M. fermentans* membranes. Compound X also induced TNF-α secretion from human monocytes (Salman *et al.* 1994).

The objective of this chapter was to identify mycoplasma contamination in *P. falciparum* cultures, and also, if there was any contamination, to check whether TMA originated from infected red blood cells with *P. falciparum* or from mycoplasma. We also aimed to find out if there was any correlation between the TNF-inducing capacity or NO production (both samples contaminated and uncontaminated with mycoplasma) and their ability to mimic the action of insulin or synergize with it in stimulating lipogenesis in the rat adipocytes *in vitro*.

7.2 Results

7.2.1 Detection of mycoplasma contamination by PCR

The polymerase chain reaction (PCR) has been used for the detection of five mycoplasma species (*M. orale, M. hyorhinis, M. fermentans, M. arginini* and *Acholeplasma laidlawii*) that are major cell culture contaminants belonging to the class Mollicutes in *P. falciparum* cultures (section 2.21). Different strains of *P. falciparum* were retrieved as described in section 2.1.8. The parasites have been grown ≥10 % parasitaemia. Spent culture medium of such cultures was collected and were tested for mycoplasma contamination. Four strains (3D7, K1, CY27, CY16) out of five of our cultures gave one PCR product (600 bp) indicating the presence of *M. orale* in *P. falciparum* culture. HB3 was the only mycoplasma-free strain (Fig. 7.1). The cloned laboratory stains K1 and the fresh isolate CY27 have been treated with anti-mycoplasma reagent for further work.

7.2.2 Treatment of *P. falciparum* culture contaminated with *M. orale*

The two contaminated parasite cultures (CY27 and K1 strains) were treated using mycoplasma removal agent (MRA) (section 2.22). following seven days treatment with MRA, parasite culture supernatants were collected and re-tested by PCR. The PCR results showed that *P. falciparum* cultures which treated with MRA were clean of mycoplasma contamination. The parasites were passaged in MRA free medium, until parasitaemia reached ≥10%. The supernatants of ring and schizont

stages of *P. falciparum* contaminated with mycoplasma and treated with MRA (both from same original parasite) was collected and tested by PCR. Fig. 7.2 showed that the treated samples were negative and this indicated MRA was effective to eliminate mycoplasma from *P. falciparum* culture without affecting parasite growth or killing the parasites. The treated and contaminated samples were used in the rat adipocyte assay.

7.2.3 Bioassay of *P. falciparum* supernatant contaminated with *M. orale*

7.2.3.1 Synergistic effect between TMA and insulin

Five different strains of *P. falciparum* were retrieved from liquid nitrogen. It was confirmed that four out of the five strains were positive for mycoplasma contamination by PCR. One wild isolate (CY27) and one cloned laboratory line (K1) were chosen to be treated with MRA. Before treatment, the parasites were split into two flasks with the same parasitaemia. One flask grew as usual and the other one was treated with MRA for seven days (section 2.22). When the parasitaemia reached to $\geq 10\%$ in both the treated and untreated parasite cultures, the supernatant was collected as described in Materials and Methods (section 2.2.1). Both samples were tested by PCR and by bioassay.

TMA from *P. falciparum* culture (contaminated with and without *M. orale*) can act synergistically with insulin *in vitro*. There was no significant difference

between cloned laboratory lines K1 and wild isolates CY27 (contaminated and uncontaminated) in their ability to induce lipogenesis and act in synergy with insulin in the rat adipocyte. This experiment also suggests that the biological active component were specific from *P. falciparum* culture and contamination with *M. orale* was not responsible for the observed TMA activity. HB3 is a strain of *P. falciparum* which was PCR negative when the parasites were retrieved from liquid nitrogen. Boiled supernatant of this strain also showed synergy activity with insulin (Fig. 7.3).

In addition, we synchronised K1 and CY27 strains of *P. falciparum* in culture with and without mycoplasma contamination. Supernatants of both ring and schizont stages were collected (section 2.2.1). All samples were re-tested by PCR, then the preparations were tested by bioassay. Fig. 7.3 shows that TMA activity presented only in supernatants from schizont stages (not from ring stages) and there was no difference in the observed activity on adipocytes between contaminated and uncontaminated samples. This further reinforces the idea that TMA are molecules which are associated with specific stages of the life cycle of malaria parasites.

7.2.3.2 Stimulation of lipogenesis by TMA

The boiled supernatants of five different *P. falciparum* strains which were described in section 7.2.3.1 were used for this experiment. We would also like to extend the result of the observed synergistic effect in short term cultures, to the action of TMA's alone in long term cultures of isolated rat adipocytes *in vitro*.

Fig. 7.4 demonstrates that when used in long term culture, only those components released from the infected red cells with *P. falciparum* (after schizontes ruptured) stimulated lipogenesis on their own, to a level similar to insulin. There was no lipogenesis inducing activity in supernatants which were collected from ring stages of parasites. The results also revealed that, both samples which were prepared from contaminated or uncontaminated cultures with *M. orale* had similar activity on isolated rat adipocytes to stimulate lipogenesis after 24 h incubation. Therefore, we have evidence to suggest that TMA originated from infected red cells with *P. falciparum* and TMA activity was not due to mycoplasma contamination.

7.2.4 Induction of nitric oxide (NO) and TNF-α by TMA from mycoplasma free *P. falciparum* culture

Parasitized erythrocytes from *P. yoelii* stimulate macrophages to secrete TNF-α (Bate *et al.* 1988). The active component (referred as the "toxin") is present in the supernatant of suspensions of cells infected with the rodent malaria parasites incubated overnight (Bate *et al.* 1988) and in the medium of *P. falciparum* cultures (Taverne *et al.* 1990).

P. falciparum boiled supernatants were also tested for their ability to induce TNF-α after incubation with murine macrophages. The results showed that the boiled supernatants (1:10 dilution) which were collected from *P. falciparum* culture contaminated with *M. orale* had TNF-α inducing activity (Fig. 7.5). This experiment also demonstrated that, TMA from *P. falciparum* culture supernatant without

mycoplasma contamination (which were treated with MRA) did not stimulate macrophages to secrete TNF-α. Positive control lipopolysaccharide (LPS) from Gram negative bacteria at 1 μg/ml stimulated TNF-α secretion by mouse macrophages.

The same preparations (as above) were tested for NO production from mouse macrophages. The cells treated with LPS (1 μg/ml) showed high levels of activity whereas samples from parasite cultures with and without mycoplasma did not have NO inducing activity in mouse macrophages (Fig. 7.6).

7.3 Discussion

Mycoplasma infection of mammalian cells in culture is a common occurrence that can affect the results of experimental protocols. Elimination of mycoplasma from cell cultures can be time-consuming, and sometimes unsuccessful.

Several techniques for the detection of mycoplasma contamination of cell cultures have been described (Harasawa *et al.* 1986). PCR is a fast, simple and sensitive test to detect mycoplasmal contamination (Wirth *et al.* 1994). PCR can even detect mycoplasma DNA in samples which do not contain viable mycoplasma.

In the previously described results of our study (Chapter three, four and five) we have shown:

i) the similarity between TMA from *P. falciparum* and *P. yoelii*

ii) TMA from *P. falciparum* were responsible not only of synergy with insulin but

also to act by their own on lipogenesis in long term culture of rat adipose cells

iii) confirmed that the activity of TMA in the lipogenic pathway in the isolated rat adipose cells was associated with polar lipid

iv) results showed that the activity was associated with 1,2 DAG.

During the late stages of this project, (in September 1997) it was reported that mycoplasma contamination of *P. falciparum* culture has affected the work of other groups studying the effect of TMA and other related subjects (Rowe *et al.* 1997, Turrini *et al.* 1997). Although our experiments with ring and schizont stages of boiled supernatant of *P. falciparum* culture showed biological evidence that our suspected molecule(s) was more likely have originated from *P. falciparum* infected red blood cells rather than a contamination, we applied a commercially available PCR kit in order to detect mycoplasma contamination in spent culture medium. In this case we were able to verify whether our cultures were contaminated and/or suspected TMA are derived from *P. falciparum* or originated from mycoplasma.

In order to detect mycoplasma contamination, boiled supernatant of *P. falciparum* was tested by PCR. PCR detected the presence of *M. orale* in the boiled supernatants. Because mycoplasma can only grow on the surface of infected red blood cells to obtain the nucleic acid precursors and when parasites reached schizont stages most of them were ruptured and residual bodies containing mycoplasma would be released into the culture medium. Therefore if there is any mycoplasma contamination in our *P. falciparum* culture, spent culture should contain mycoplasma DNA. PCR detected the presence of *M. orale* in four out of five strains which have been tested.

Since this species is a normal inhabitant of the human oral cavity, the following precautions proved effective in restraining the risk of re-contamination: working alone, wearing surgical mouth masks and sterile gloves; strictly avoiding speaking during culture handling, avoiding cultivation of contaminated and clean strains in the same incubator.

Only one of our laboratory strains, designated HB3, was free from contamination. Parasite cultures were treated with MRA for up to seven days. In comparison with control parasites, cultures treated with MRA grew more slowly and MRA affected the viability of parasites after seven days. So, treatment was stopped after seven days and parasites transferred to MRA free medium containing no MRA. In order to evaluate that MRA had no effect on the biology of the parasite or TMA, HB3 (mycoplasma-free strain) which has not been treated with MRA was used as a control.

The boiled supernatant of HB3 and treated strains CY27, K1, showed activity in the lipogenic pathway in the rat adipocyte assay *in vitro*. This indicates that mycoplasma contamination was not responsible for *P. falciparum* TMA activity and treatment with MRA did not affect TMA activity *in vitro*.

The boiled supernatant from ring stages of parasites culture, even contaminated with *M. orale*, were unable to induce lipogenesis in short and long term cultures of the rat adipose cells *in vitro*. However, if the same parasite culture with the same parasitaemia is grown to the schizont stages and allowed to rupture, this supernatant is then biologically active in synergy with insulin in short term culture

and also shows increased lipogenesis on their own in long term culture in the rat adipocytes. The results represented stage-specific of TMA which is responsible for lipogenesis inducing activity in rat adipocytes and is in concordance with those reported in Chapter three. Specific PCR detection of mycoplasma contamination and application of MRA showed that TMA had not originated from *M. orale*.

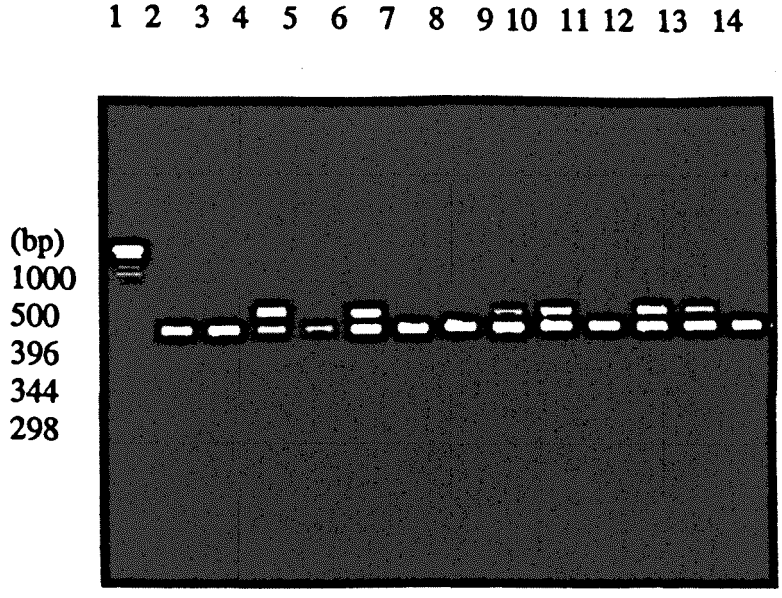
In this study we found that TMA from mycoplasma-free and contaminated *P. falciparum* spent culture did not stimulate murine macrophages to release NO. However, TMA preparations contaminated with *M. orale* (1:10) stimulated macrophages to secrete TNF-α less than LPS. There is also evidence that mycoplasma produce a protein, MDHM and compound X that induce TNF-α release from human monocytes and mouse macrophages (Kostyal *et al.* 1995). Therefore this suggests that TNF-α-inducing activity originated from mycoplasma contamination. However, further work is required to clarify the contamination of mycoplasma and *P. falciparum* in the induction of TNF-α and NO.

To summarize, in the adipocyte assay, we have shown that the active component(s) prepared from boiled supernatants of *P. falciparum* cultures (TMA) did not originate from mycoplasma contamination of *P. falciparum* cultures. In contrast, the active components from mycoplasma-free cultures were not able to induce NO or TNF-α production from murine macrophages. The results of this chapter also indicated that PCR was able to detect mycoplasma DNA in culture supernatant and this assay also is very sensitive (The primer set can detect most mycoplasma infections from 1 μl of cell culture supernatant).

Contamination of mycoplasma in malaria parasites culture could be treated with MRA (5 μg/ml) for up to seven days and the culture remained negative by PCR three months after treatment (Rowe *et al.* 1997).

There is the possibility of very low contamination of *P. falciparum* cultures with *M. orale* which was not detected by PCR, but with regard to the result of stimulation of lipogenesis by molecules which are stage-specific (even in preparations from culture contaminated with *M. orale*), we can conclude that there is no active molecule released from *M. orale* in *P. falciparum* cultures which can stimulate lipogenesis in the rat adipocytes *in vitro*.

Fig. 7.1 PCR products of mycoplasma contamination in malaria culture

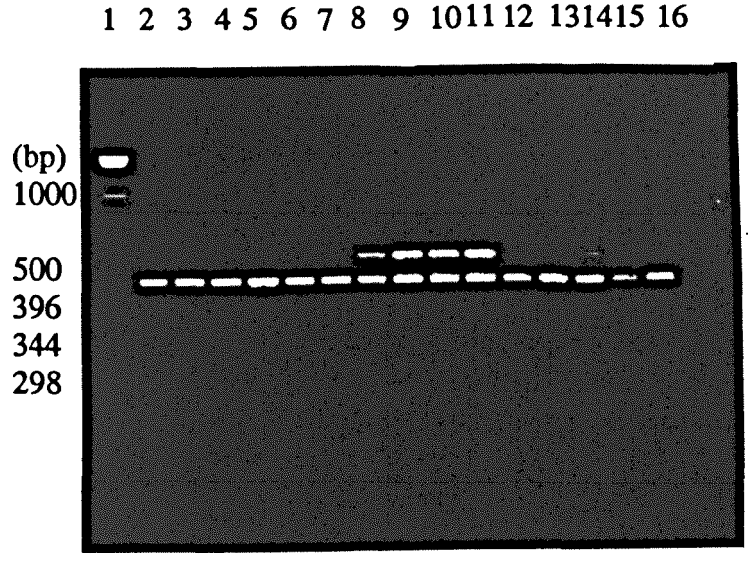


The PCR products were analyzed using standard agarose gel electrophoresis. 2% (w/v) agarose gel designed for the high resolution of 150-700 bp. This ensures good differentiation between PCR products associated with the internal control template and those due to mycoplasma. Five different strains of *P. falciparum* were tested with PCR (HB3, 3D7, K1, CY16, CY27).

Lane 1 molecular weight marker **Lane 2** NRBC supernatant
Lane 3 HB3 **Lane 4** 3D7
Lane 5 CY16 **Lane 6** K1
Lane 7 K1 treated with MRA after five days
Lane 8 K1 treated after seven days
Lane 9 K1 untreated after seven days
Lane 10 CY27 untreated
Lane 11 CY27 treated after seven days
Lane 12 positive control (boiled supernatant of *P. falciparum* contaminated with mycoplasma)
Lane 13 The positive control was inactivated *M. orale* genomic DNA
Lane 14 negative control (UV irradiate water).

Stratagene recommends running samples with the internal control, which serves as an internal control for PCR process. Thus, if there is inhibition of the PCR, the internal control band will not be seen on analysis, which prevents a false negative screening. Analysis of PCR results revealed contamination of *P. falciparum* strains with *M. orale*.

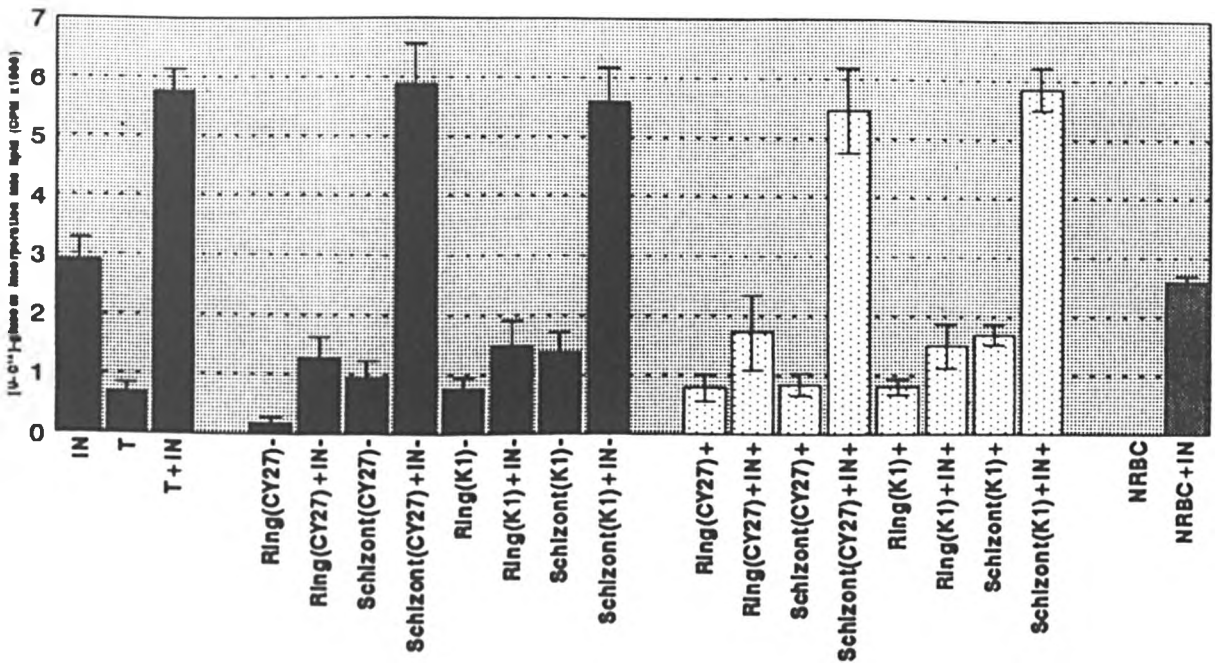
Fig. 7.2 PCR products of treated culture supernatant of *P. falciparum* with MAR



P. falciparum cultures were treated with MRA for seven days and spent culture supernatant was collected. The parasites transferred to fresh medium without MRA and antibiotics. When parasitaemia reached to ≥10% spent culture medium from different stages of parasites were collected and treated as described in section 2.2.1. All samples were tested with PCR (section 2.21) to detect mycoplasma infection in *P. falciparum* cultures. The PCR products were analyzed with 2% agarose gel electrophoresis. 15 μl of the PCR was analyzed.

Lane 1) molecular weight marker **Lane 2)** HB3 ring stages supernatant
Lane 3) HB3 schizont stages supernatant **Lane 4)** CY27 ring stages
Lane 5) CY27 schizont stages **Lane 6)** K1 ring stages
Lane 7) K1 schizont stages **Lane 2** to **7** represented spent culture medium of *P. falciparum* treated with MRA for seven days, then parasites passaged to the medium containing no MRA and antibiotics. When parasitaemia reached to 10% or more supernatants were collected and re-tested with PCR.
Lane 8) CY27 ring stages **Lane 9)** CY27 schizont stages
Lane 10) K1 ring stages **Lane 11)** K1 schizont stages
Lane 8 to **11** demonstrated spent culture medium of *P. falciparum* which have been grown as usual way without any treatment. **Lane 12)** CY27 supernatant
Lane 13) K1 supernatant **Lane 12** and **13** revealed samples from malaria culture which treated for seven days and no passage has been done.
Lane 14) Positive control of *P. falciparum* culture supernatant which was contaminated with *M. orale*.
Lane 15) UV irradiated water as negative control **Lane 16)** NRBC supernatant

Fig. 7.3 EFFECT OF THE TMA FROM SYNCHRONISED CULTURES OF DIFFERENT STAGES OF *P. falciparum* BOTH WITH AND WITHOUT MYCOPLASMA CONTAMINATION (ON LIPOGENESIS)



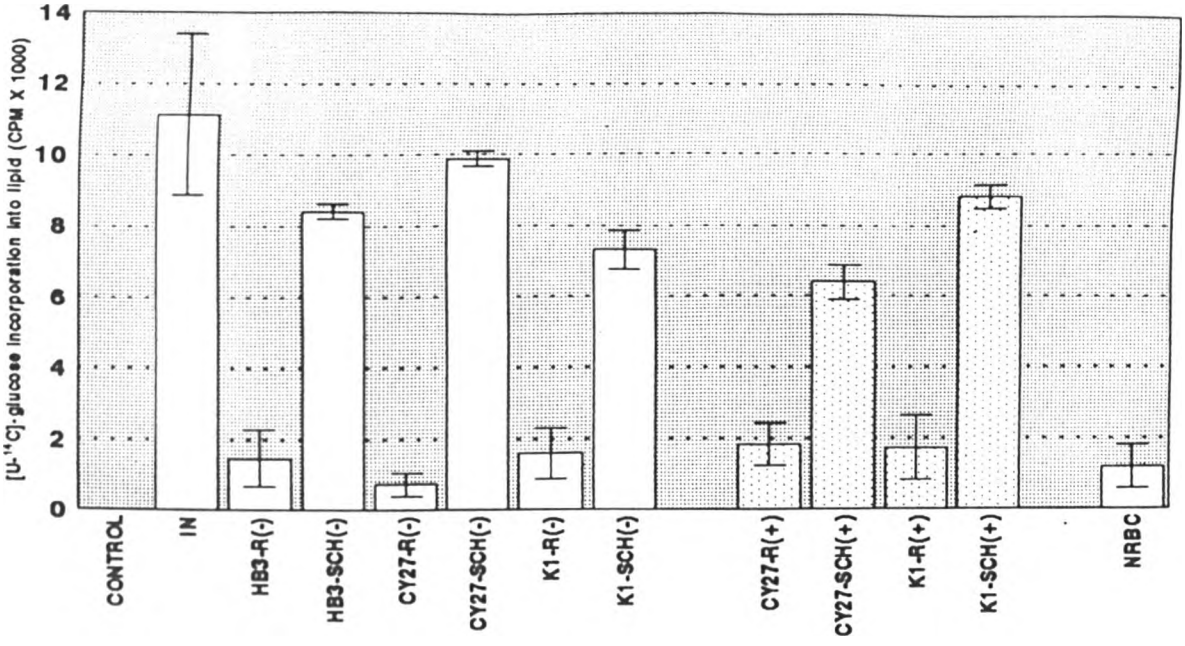
Three different strains of *P. falciparum* were used in this experiment. CY27 and K1 strains were contaminated with *M. orale* and HB3 was mycoplasma free. CY27 and K1 were treated with MRA as described in Materials and Methods. Spent culture medium of *P. falciparum* with and without mycoplasma contamination were collected as described in section 2.2.1. All such samples (treated and untreated with MRA) have been tested with PCR (section 2.21). The solid bars demonstrated supernatants from synchronised culture contaminated with *M. orale*. The hatched bars represented supernatant from synchronised culture mycoplasma-free.

IN = Insulin NRBC = Normal Red Blood Cells

T = boiled supernatant of *P. falciparum* culture

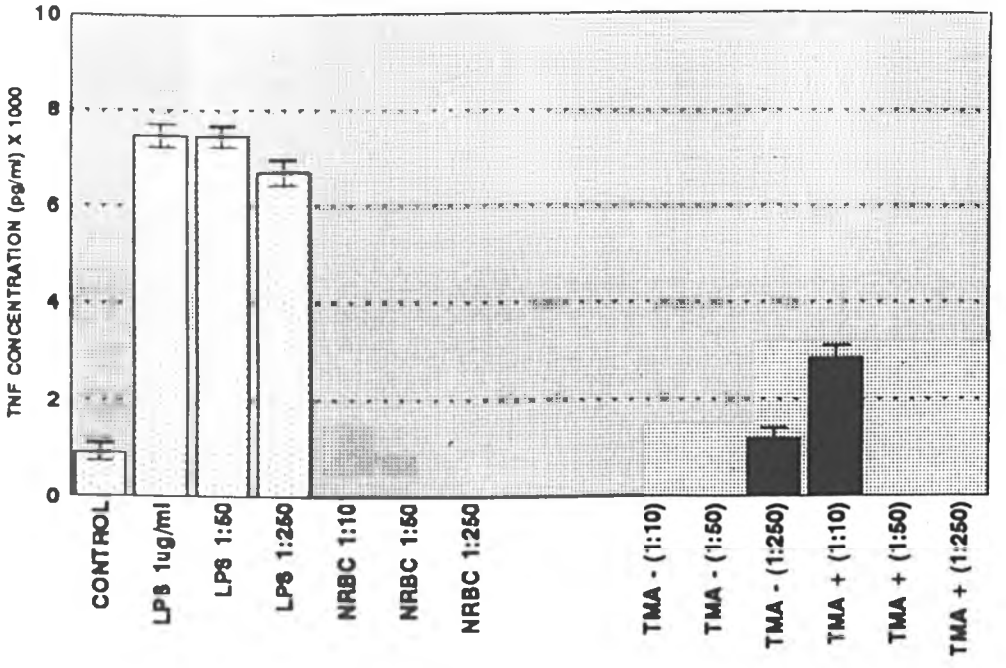
Bioassay of positive and negative samples with PCR showed that boiled supernatant of ring stages (both contaminated and uncontaminated) did not stimulate lipogenesis and no synergy effect with insulin after incubation of samples with the rat fat cells at 37°C for 60 minutes. However, boiled supernatant of the schizont stages ($\geq 10\%$ parasitaemia) of the same population of parasites showed a synergistic effect with insulin (in both contaminated and uncontaminated samples). The results indicated that TMA originated from cells infected with *P. falciparum*. Values are means ($\pm$ SEM) of two experiments in duplicate.

Fig. 7.4 EFFECT OF INSULIN AND TMA FROM CULTURE WITH AND WITHOUT MYCOPLASMA CONTAMINATION ON LIPOGENESIS IN RAT ADIPOCYTES



All samples were prepared as described in Fig. 7.3. Samples positive and negative for mycoplasma by PCR were incubated with the rat fat cells for 24 h at 37°C. The results showed that the supernatants from ring stage parasites (contaminated and uncontaminated with mycoplasma) did not stimulate lipogenesis, however, supernatants from schizont stages of parasites (contaminated and uncontaminated) stimulated lipogenesis on their own in the rat adipocytes assay. Values are means (± SEM) of two experiments in duplicate.

Fig. 7.5 TNF INDUCTION BY TMA OF *P. falciparum* CULTURE INFECTED AND UNINFECTED WITH MYCOPLASMA ON MURINE MACROPHAGES



Murine macrophages were prepared as described in Materials and Methods. The cells were exposed to different dilutions of boiled supernatant of *P. falciparum* or LPS (positive control) for 24 h. The results showed induction of TNF-α by cells. Controls were incubation of murine macrophages with only medium and incubation with NRBC supernatants.

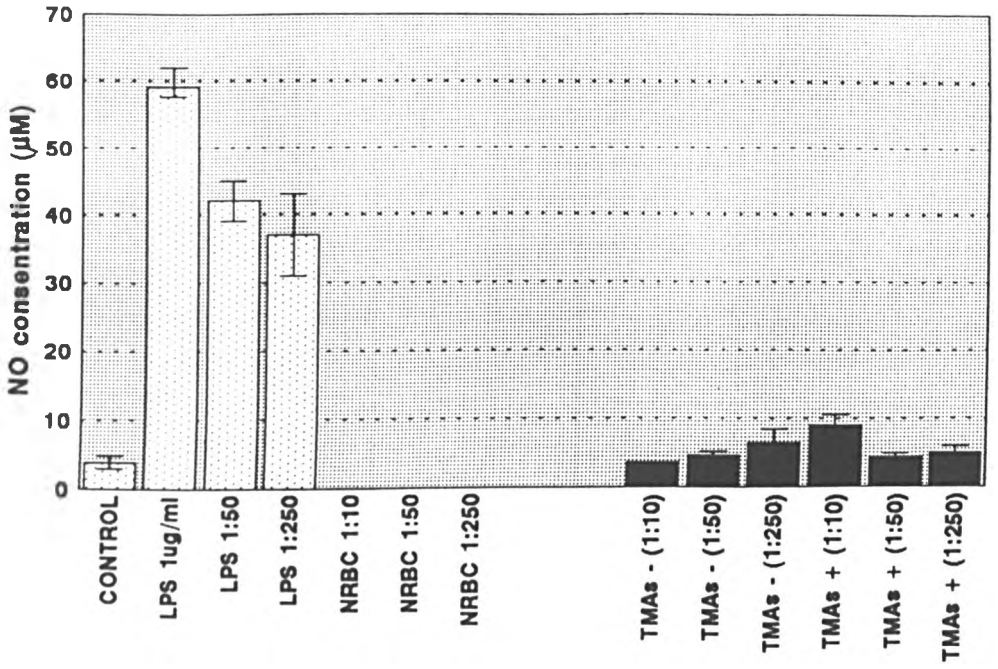
TMA - = demonstrated mycoplasma free supernatant

TMA + = contamination of *P. falciparum* culture supernatant with mycoplasma.

NRRC = Normal Red Blood Cells.

Samples from mycoplasma contaminated but not mycoplasma-free culture supernatant (1:10) were used to stimulate macrophages up to 2000 pg/ml which was roughly half of 1:250 dilution of LPS. So, TMA of *P. falciparum* can stimulate lipogenesis *in vitro*, however can not stimulate mouse macrophages to make TNF-α. This experiment was repeated three times with two different TMA preparations. This Fig. demonstrated typical such experiment. Values are means ($\pm$ SEM) of duplicates.

Fig. 7.6 NO INDUCTION BY TMA OF *P. falciparum* CULTURE SUPERNATANT INFECTED AND UNINFECTED WITH MYCOPLASMA ON MURINE MACROPHAGES



Mouse macrophages were incubated with TMA preparations from *P. falciparum* culture contaminated and uncontaminated with mycoplasma for 24 h at 37°C. The hatched bars represent NO production in control, NRBC supernatant, different dilutions of LPS (positive control) and the solid bars demonstrate after incubation cells with TMA of *P. falciparum* (different dilutions) of positive and negative PCR samples for mycoplasma. There was no induction of NO by TMA of *P. falciparum* (contaminated or uncontaminated), however, LPS stimulated cells to release NO in to the supernatant after 24 h exposure. This experiment was repeated three times with two different preparations. Values are means (± SEM) of duplicate.

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSION

Malaria is a huge and growing problem, especially in Africa. It kills one to two millions children each year and causes disease in a further 500-400 million individuals (Krishna, 1997). *Plasmodium falciparum* is the most prevalent of the four malaria parasites of humans and causes most of the severe and lethal infections affecting young children and nonimmune adults.

In the past 20 years research in malaria has expanded considerably from preventive strategies and development of vaccines to include better understanding of the pathophysiology of disease, both clinically and at the cellular and molecular level.

The occurrence of hypoglycaemia is an important complication of malaria and is of multifactorial causation; depletion of stores largely because of starvation and/or malnutrition, increased tissue metabolism, parasite utilization of glucose, impaired gluconeogenesis and hyperinsulinaemia due to quinine therapy. The mechanisms leading to this and other complications are not well understood and remain an important area for future research.

In severe Falciparum malaria hypoglycaemia may occur in the presence of quinine treatment, due to secretion of insulin, however, hypoglycaemia in the absence of this drug also occurs, indicating a multifactorial origin of the disorder in patients

with malaria (Looareesuwan *et al.* 1985; Mesengi & Yohani, 1984). White and co-workers had been studies large series of severe Falciparum malaria patients and they suggested that hypoglycaemia in patients who did not receive quinine therapy, may be due to another possible factor like endotoxin, which may also stimulate insulin secretion (White *et al.* 1983). The idea that the complications of malaria might be due to a toxin or toxins has a long history. The new interest in malaria toxins (substances released by rupturing schizonts that can injure the host, either directly or by causing immunopathology), which possibly have ability to induce hypoglycaemia, is based on research carried out by Taylor and co-workers (Taylor *et al.* 1992a, 1992b). They have shown that toxic malaria antigen(s) (TMA) released by blood stage *P. yoelii* induce hypoglycaemia in mice (Taylor *et al.* 1992a). TMA from *P. yoelii* can also synergise with insulin both *in vitro* (to enhance lipogenesis) and *in vivo* (to induce hypoglycaemia in mice) (Taylor *et al.* 1992a, 1992b).

The work presented in this thesis was designed to a) to investigate whether TMA (the term "malaria toxin" was used during this work as it has been used in the early literature and refers to substances released by rupturing schizonts) similar to those described for *P. yoelii* would be identified in human malaria *P. falciparum* b) identify and characterize this or these active molecules. To investigate these, a biological assay (adipocyte assay) was used to screen for the presence of *P. falciparum* malaria toxin(s) by inducing lipogenesis in rat adipocytes *in vitro*. The process of lipogenesis is measured by the conversion of [U-^{14}C]-glucose to labelled lipids.

Rat adipocytes are most probably the best system for studying the biochemistry and cell biology of the important physiological response to insulin, including the stimulation of glucose transport (Kublaoui, *et al.* 1995). This is based on the fact that adipocytes respond very well to insulin after isolation, and they can be obtained in large quantities for experiments (Simpson & Cushman, 1986). Therefore, because studies on murine malaria showed TMA has similar activity to insulin in rat adipocytes, it was wise to use the same bioassay to establish whether the same activity is also a feature of the human malaria parasite. The adipocyte assay has been shown to be a valuable tool for the evaluation TMA activity *in vitro*.

The results of this present work strongly suggest that TMA is derived from *P. falciparum* IRBC which has been released following rupture of schizonts into the culture medium *in vitro*. The TMA are capable of inducing lipogenesis alone or acting in synergy with insulin *in vitro*. We also found molecules with similar activity in sera collected from splenectomized monkeys infected with *P. falciparum*. The results confirm the presence of such molecules *in vivo*. There was no sequestration of *P. falciparum* parasites, hence each sample had exact parasitaemia and even a sample with a 0.3% parasitaemia stimulated lipogenesis in the adipocyte assay. Two human samples infected with *P. falciparum* from Malawi have also been tested and compared to normal human serum, there was synergistic effect between preparations from patients and insulin but no synergistic effect was observed from normal human serum with insulin. However, because, the level of parasitaemia and other clinical information were not as clearly defined as in monkey serum more samples would be needed before drawing significant conclusions from human serum.

One of novel findings during this work was the stimulation of lipogenesis by TMA of *P. falciparum* IRBC in long term culture of isolated adipose cells from Wistar rats, which indicated that TMA could not only synergise with insulin, but also have insulin-like activity on its own. There was a difference in time course effect of lipogenesis on adipose cells by insulin and TMA in isolation suggesting that the mechanism of action of TMA and insulin could be different.

A comparison between cloned laboratory lines and fresh isolates of *P. falciparum* suggested that there was no measurable difference between isolates in their ability to induce lipogenesis and synergise with insulin. Taken together the *in vitro* results and work with infected serum of monkeys with *P. vivax, P. falciparum* and *P. brasillianum* (*in vivo*) indicate that the active molecules are conserved between different species of malaria parasites and different strains of *P. falciparum.* However, in our experimental approach, we were not able to prove whether these active molecules are produced by malaria parasites or represent a modification of host components. It is important to note that NRBC components (from NRBC culture supernatant or from lysates of intact NRBC) completely failed to stimulate lipogenesis *in vitro.*

Regarding recent reports on the contamination of *P. falciparum* cultures with mycoplasma (Turrini *et al.* 1997; Rowe *et al.* 1997), we re-examined all our results using treated mycoplasma free parasites and confirmed that TMA inducing-lipogenesis activity was specifically obtained from *P. falciparum* IRBC, and not from mycoplasma. Moreover, TNF-inducing activity of TMA (from cultures with no

mycoplasma contamination) was tested and the results indicate that the molecule(s) which we described during our work, are not capable of inducing TNF or NO production after incubation with murine macrophages.

Kwiatkowski and collaborators reported that TNF as a potent endogenous pyrogen is released by human monocytes in response to schizont rupture (Kwiatkowski *et al.* 1989). They also partially purified the released material which was shown to stimulate macrophages to make TNF. They have extended this work and showed that these same preparations, isolated from parasitized erythrocytes, induce the mouse macrophage to produce inducible nitric oxide (NO). In addition, Taverne and co-workers reported that murine TMA prepared in different ways showed no correlation between the TNF-inducing capacity and induction of hypoglycaemia *in vivo*. The *P. yoelii* TMA preparation was from digesting parasitized erythrocytes with pronase, some from supernatants, some by disrupting the cells by freezing and thawing or by sonication, but all from suspensions containing 10^8 parasitized RBC (toxins) per millilitre first incubated overnight at 37°C. Further investigation into the cause of this hypoglycaemia showed that such extracts can cause the release of insulin *in vivo* (Elased *et al.* 1994) and mimic the action of insulin or synergize with it in stimulating lipogenesis in adipocytes *in vitro* (Taylor *et al.* 1992b). In addition, murine TMA-induced hypoglycaemia may be predominantly a direct effect of the parasite products, as treatment with neutralizing antibodies to TNF does not abrogate hypoglycaemia in mice (Taylor *et al.* 1992a).

In order to draw conclusions from published literature on TNF-induction by

malarial toxin, all groups working with TNF-inducing activity of malaria toxin (*in vitro* and *in vivo*) will need to confirm that the active components are not released by mycoplasma, since mycoplasma produce a protein and a substance [mycoplasma derived high-molecular-weigh material (MDHM)] which activates macrophages to release IL-1, TNF-α and NO (see Chapter seven). This may be true for *in vivo* work since animals under experiment may already have been contaminated with mycoplasma, since mixed infection of mycoplasmas and *P. berghei* was reported by Mercado (1965). He reported that paralytic syndrome associated with an experimental *P. berghei* infection in rats was associated with mixed infection with *Mycoplasma neurolyticum*. There is also report about paralysing syndrome after blood passage in mice, providing a logical means of explaining the interaction between malaria parasite and mycoplasma since normal mice frequently carry latent *M. neurolyticum* (Tully & Rask-Nielsen, 1967). Therefore, all malaria toxin samples which have been prepared from experimental animals will need to be checked for mycoplasma contamination. However, if the active molecules were specific for malaria parasites, it is worth drawing attention to the fact that stimulation of lipogenesis and TNF/NO production may be induced by different components. This idea comes from the following works:

a) Taverne and co-worker results *in vivo* (see above) (Taverne *et al.* 1995),

b) our results *in vitro* (boiled supernatant of *P. falciparum* free of mycoplasma contamination stimulated the lipogenic pathway in isolated rat adipocytes and/or no TNF-inducing capacity with the same TMA preparation) and,

c) based on laboratory and field investigations; which suggest that TNF overproduction plays a key role in the pathogenesis of Falciparum malaria (Grau *et*

al. 1989; Kwiatkowski *et al.* 1990). Studies by Grau and Kwiatkowski showed that hypoglycaemia and hyperparasitaemia were found to correlate with increased TNF concentrations in Malawian and Gambian children, respectively (Grau *et al.* 1989; Kwiatkowski *et al.* 1990). Indeed, the continued presence of high level TNF in the serum of infected patients should counteract the development of hypoglycaemia, since it is known to interfere with the action of insulin, by suppressing insulin-induced phosphorylation of the insulin receptor (Feinstein *et al.* 1993). However, with reference to the studies of Moller and co-workers, it is possible that the TNF measured during various studies by ELISA was already inactive (Moller *et al.* 1991). In addition this may explain why the administration of an anti-TNF McAb to severe Falciparum malaria patients did not reduce severity of disease (Kwiatkowski *et al.* 1993).

Schofield and colleagues have found that a glycosylphosphatidyl inositol (GPI) specific to malaria species is directly capable of inducing effects believed to be involved in malaria pathophysiology. They also reported that incubation of malaria GPI-linked surface proteins and the purified malaria GPI were able to induce a five to six-fold increase in lipogenesis in the rat adipocytes (after 2 h incubation at 37°C), similar to that induced by insulin (Schofield & Hackett, 1993). Although it is not possible to exclude the possibility that molecules unrelated (such as malaria GPI) to the molecules described here (more specifically 1,2-DAG) may also contribute to stimulation of lipogenesis in *in vivo*, however, there is a discrepancy between our data and results which have been reported by Schofield (see above). During our study, none of preparations (boiled supernatant or purified 1,2-DAG of *P. falciparum* IRBC) stimulated lipogenesis similar to insulin level after 2 h incubation with rat adipocytes.

In addition, extraction of GPI (which has long-chain derivatives of sugar) probably would need mixture of chloroform/methanol/water which we have never used.

All together, lipogenesis-inducing activity of TMA of *P. falciparum* IRBC in our study is due to phospholipid or specifically 1,2-DAG derived from phospholipid which can also act synergistically with insulin *in vitro*. In a preliminary study in collaboration with Prof. N. G. Morgan of the Keele University, the boiled supernatants of *P. falciparum* IRBC and NRBC, the polar lipid extracts of boiled supernatants of IRBC and NRBC have been tested for stimulation of insulin secretion from rat islets of Langerhans. The results showed stimulation of insulin secretion by all the above samples, however, samples from *P. falciparum* IRBC stimulated this activity 5 folds more than controls. These data suggest that TMA of *P. falciparum* can also act on other tissues. Further investigations are needed to study the effects of TMA of *P. falciparum* on other tissues such as skeletal muscle and/or, liver. Elucidation of TMA mechanism of action are required to increase our understanding of the complications of malaria and the possibility of looking at anti-toxic immunity, the immune response which would explain why African children become tolerant to malaria many years before developing an immunity to the infection. However, identification of the biochemical nature of this toxin(s) is necessary to avoid autoimmune reactions.

This study has concentrated on the action of TMA of *P. falciparum* IRBC *in vitro* and identification of purified molecule(s) as malaria toxin(s). Therefore, based on the results obtained from *in vitro*, it would be very interesting to test

hypoglycaemia induction of boiled supernatant, polar lipids and 1,2-DAG preparations from *P. falciparum* IRBC *in vivo*. We showed the presence of long-lived antibodies lipogenesis-inducing factor(s) in malaria-endemic community, and in that context they may be protective against severe malaria. However, it is still an open question whether the same antibodies would be beneficial in an individual who has not been previously exposed to natural infection and also to young children in malaria endemic areas. Therefore, the development of quantitative methods to measure anti-TMA in malaria patients (children, adults, patients with complicated and uncomplicated malaria) needs further experimental and epidemiological studies in order to assess the role of the TMA or more specifically the 1,2-DAG and antibody response to them in both clinical response and clinical immunity to infections.

GC-MS and LC-MS were applied to identify structural differences between the 1,2-DAG derived from polar lipids of IRBC and NRBC, but both techniques only identified the major components which were apparently identical in both samples. However, it is possible that the highest lipogenesis activity is due to minor 1,2-DAG component(s) which the above techniques were not sufficiently sensitive to permit the identification of minor components in the samples and minor peaks did not give identifiable spectra. Therefore, the appropriate techniques and more purified samples will be needed to help in the characterization of the active molecule(s)/moieties which may provide the molecular basis for a detailed understanding of the disease process, particularly hypoglycaemia.

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APPENDIX 1

Materials for malaria culture

RPMI

RPMI 1640 powder (Gibco) 10.43 g

$NaHCO_3$ 2 g

Dissolved in 1 L ddH_20 and the pH adjusted to 7.2. Sterile filtered and stored at 4°C.

Culture Medium

RPMI	90 ml
Human serum AB^+	10 ml
HEPES buffer	2.5 ml
Gentamicin	300 μl

Sterile filtered through 0.22 μm filter unite and store at 4°C.

Gentamicin stock

Gentamicin sulphate (Sigma U.K.) 1 g dissolved in 20 ml ddH_20

(50 mg/ml working solution)

Materials for the retrieval and cryopreservation of *P. falciparum* parasite

0.01 M Phosphate buffer

Solution A 1.42 g NaH_2PO_4 (Sigma U.K.) dissolved in 1 L ddH_20

Solution B 0.69 g NaH_2PO_4 (Sigma U.K.) dissolved in 500 ml ddH_2O

Solution A was titrated with solution B to give a pH=7.4

Sorbitol solutions

27% sorbitol 27 g dissolved in 100 ml 0.01 M phosphate buffer

5% sorbitol 5 g dissolved in 100 ml 0.01 M phosphate buffer

Sterile filtered with 0.22 μm filter unit and store at 4°C.

Cryopreservation

Glycerol 285.48 g

Na lactate 7.84 g

KCL 0.186 g

Dissolved in 50 ml ddH_2O. pH adjusted to 7.4 using 0.1 M Na_2HPO_4 (1.38 g/100 ml ddH_2O), increase volume to 500 ml with ddH_2O. Filter sterilise with a 0.22 μm filter and store at 4°C.

Material for synchronization of *P. falciparum* culture

5% sorbitol 5 g sorbitol (Sigma, U.K.) dissolved in 100 ml ddH_2O

Filter through a 0.22 μm filter and store at 4°C.

Material for Rat Adipocyte Assay

Krebs-Ringer Buffer (KRB) solution

0.9% NaCL	100 parts
1.15% KCL	4 parts
1.6% $CaCl_2$	1.5 parts
2.11% KH_2PO_4	1 part
3.82% $MgSO_4$	1 part
1.3% $NaHCO_3$	21 parts

Washing buffer KRB with 1% (W/V) BSA (Sigma,U.K.)

Incubation buffer

KRB	60 ml
BSA (free fatty acid)	0.6 g
0.5 M glucose	0.8 ml
[U-^{14}C]-glucose	50 μl

For primary culture of fat cells, 2.5 mM HEPES solution was added to above buffer. Incubation buffer was sterilized through a 0.22 μl filter and freshly used.

Doles mixture

Isopropanol	40 parts
Hexane	10 parts
0.5 M H_2SO_4	1 part

Buffers for ELISA

Coating buffer

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g

To 1 litre of ddH_2O. The pH adjusted to 9.6 using 0.1 M HCL. The stock solution was stored at 4°C.

Washing and Incubation buffer (0.05% Tween 20)

PBS x1	500 ml
Tween 20	250 μl

Phosphate Buffer Saline (PBS x10)

KCL	10 g
KH_2PO_4	10 g
NaCl	400 g
Na_2HPO_4	57.5 g
ddH_2O	5000 ml

In all experiment X1 of PBS was used.

Buffers for the lipid ELISA assay

Washing and incubation buffer

PBS x 1	90 ml
%10 FCS	10 ml

Buffers for the TNF capture ELISA

Coating buffer	0.1 M carbonate buffer
Washing buffer	0.05% Tween 20 in PBS
Incubation buffer	1% BSA and 0.05% Tween 20 in PBS

APPENDIX 2

Media for Fusion

Media supplements

Stock NCTC medium (Gibco)

NCTC 135 powdered medium	9.52 g
Sodium bicarbonate (BDH)	2 g
ddH_2O	1 L

Filter medium through a 0.22 μm filter and store at -20°C.

Aminopterin (toxic handle with care)

Supplied in vials (Sigma, U.K.) and reconstituted by adding 10 ml of double distilled water through 0.2 μm filter using syringe, which gave a 2 X 10^{-5} M solution.

8-azaguanine (X 100), 10^{-2} M working dilution

8-azaguanine (Sigma, U.K.)	76 mg
Double distilled water	35 ml

Add 1 N NaOH drop to dissolve the azaguanine, adjust to pH 10 with 1 N acetic acid. Add double distilled water to make a 50 ml final sterilise through a 0.22 μm filter and store at -20°C.

This product also available in vials which are reconstituted by adding 10 ml double distilled water through a 0.2 μm filter and stored at -20°C.

Hypoxanthine-Thymidine (HT) (x 100)

Hypoxanthine (Sigma, U.K.)	408 mg
Thymidine (Sigma, U.K.)	114 mg
Double distilled water	300 ml

Adjust to pH 10 using 0.1 M NaOH or 0.1 M HCl and filter through a 0.22 μm filter and store at -20°C in aliquots 1-5 ml each.

Stock OPI (x 100)

Cis-oxaloacetic acid (Sigma, U.K.)	1500 mg
Sodium pyruvate (Sigma, U.K.)	500 mg
Bovine insulin (Sigma, U.K.)	76 mg
Double distilled water	100 ml

Add 1N HCl if needed to dissolve the insulin and then sterilise through a 0.22 μm filter and store aliquots at -20°C.

Stock Iscove's

Supplied by Hyclone Ltd as X1 stock solution ready for use in 500 ml bottles.

Foetal Calf Serum (FCS)

Supplied in 500 ml sterile bottle, and before use the whole bottle was thawed at 37°C, the temperature was then increased to 56°C in water bath and left it for 30 minutes to inactivate the serum, it was then aliquoted into 20 ml and stored at -20°C.

Gentamicin Stock

Supplied in powdered form and prepared as a 50 mg/ml working solution as: 1 g Gentamicin sulphate (Sigma, U.K.) was dissolved in 20 ml double distilled water and filtered through a 0.2 μm filter and stored at 4°C in a sealed tube.

Polyethylene glycol (PEG)

Supplied in vials (Sigma, U.K.) which are reconstituted by heating the vial in a 56°C water bath then 5 ml PBS and 0.5 ml DMSO are added and the mixture is passed through a 0.22 μm filter and aliquoted in 1 ml bijoux, and stored at -20°C.

Media used for fusion

Iscove's + L-glutamine + gentamicin (Isc+g+g or washing medium)

stock Iscove's	90 ml
gentamicin	200 μl
L-glutamine	2 ml

Isc + 10% FCS

stock Iscove's	90 ml
gentamicin	200 μl
L-glutamine	2 ml
FCS	10 ml

Isc + FCS + 8-azaguanine

stock Iscove's	90 ml
gentamicin	200 μl
L-glutamine	2 ml
FCS	10 ml
8-azaguanine	1 ml

OPI-HAT

stock Iscove's	70 ml
gentamicin	200 μl
L-glutamine	2 ml
FCS	20 ml
NCTC	10 ml
OPI	1 ml
HT	1 ml
Aminopterin	1 ml

OPI-HAT

stock Iscove's	70 ml
gentamicin	200 μl
L-glutamine	2 ml
FCS	20 ml
NCTC	10 ml
OPI	1 ml
HT	1 ml

Isc+20% FCS (for hybridomas freezing and recovery)

stock Iscove's	80 ml
gentamicin	200 μl
L-glutamine	2 ml
FCS	20 ml

Isc + 20% FCS + 10% DMSO (for hybridomas freezing)

stock Iscove's	90 ml
gentamicin	200 μl
L-glutamine	2 ml
FCS	20 ml
DMSO	10 ml

Sterilise each medium using a 0.22 μm filter and store at 4°C.

NOTE: All hybriboma cells line were grown in medium with no OPI (to eliminate insulin which can a effect adipocyte assay). The medium was Iscove's+ 10% FCS.

APPENDIX 3

Tissue culture plastic ware

80 cm^2	Nunclon, Life technologies, Glasgow
25cm^2	Nunclon, Life technologies, Glasgow
100 mm^2	Nunclon
35 mm^2	Nunclon
Cryotubes	Nunclon
96 well plates	Costar, High wycomde, Bucks
24 well plates	Nunclon

Filter sterilising units

0.45 μm filter unit	Nalge Company, Rochester, NY, U.S.A
0.22 μm filter unit	Nalgen
Acrodisc syringe filters	Gleman sciences, Northampton

Pipettes

Graduated plastic pipettes (1 ml, 5 ml, 10 ml, 25 ml)	Northern Media supply Ltd, North Humberside
Glass pasteur pipettes	Richaredsons, Leicester

Miscellaneous

15 and 50 ml centrifuge tubes	Sarstedt, Leicester
Micropipette tips	LIP (Equipment and Services Ltd) W.Yorkshire
Syringes and needls	Becton-Dickinson, Dublin
Dissecting instruments	Sarstedt
Vortex	Gallenkamp
Light microscopes	Nikon, Telford, Shropshire
Gilson micropipettes	Anachem, Bedfordshire
Centrifuges	I.E.C., Bedfordshire
pH meter	Pye Unicam, Cambridge
Laminar air flow hood	Nuaire Inc., Minnesota. U.S.A
CO_2 incubator	Flow Laboratories
3% CO_2, 6% O2, 91% N_2	British oxygen company
Water bath	Grant instruments, Cambridge
Magnetic Stirrer	Gallenkamp, Leicester

Solvents

Acetone, Analar grade	BDH	10003 4Q
Chloroform, Analar grade	BDH	100776B
Cyclohexane, Analar grade	BDH	103196W
Diethyl ether	BDH	10094 6B
Ethanol HPLC grade	BDH	15338
Hexane HPLC grade	BDH	152496G
Methanol Analar grade	BDH	101586B
Propan-2-ol HPLC grade	BDH	152526S
Propan-2-ol Analar grade	BDH	K24096872

Petroleum ether, (Banner Fules). Dried over anhydrous $CaCl_2$ and redistilled The 40°C-60°C fraction was collected.

Acids

Glacial acetic acid	BDH	27013 6Q
Hydrochloric acid Analar	BDH	10125 oD
Sulphuric acid Analar	BDH	10276

Reagents

$CaCl_2$ Analar	BDH	10070
KCL	BDH	10198
KH_2PO_4 Analar	BDH	10203
$MgSO_4$ Analar	BDH	101514Y
NaCl Analar	BDH	10241AP
NaHCO3	Sigma	S-8875
Na_2HPO_4	Sigma	S-0876
NaH_2PO_4	Sigma	S-0751
Albumin Bovine Initial fraction	Sigma	A-7030
Aminopterin	Sigma	A-5159
8-Azaguanine	Sigma	A-5284
Berberine hydrochloride	Sigma	B-3251
Bovine Alumin fraction V	Sigma	A-1653
Bovine insulin	Sigma	I-5500
Cocktail T	BDH	145096B
Dimethylsulfoxide (DMSO)	Sigma	D-2650
D-sorbitol	Sigma	S-3889
FCS	GLT*	10106-151
Freund's Ajuvant	Sigma	F-5506
Gentamicin sulphate	Sigma	G-3632
Giemsa	BDH	350865P
Glucose	Sigma	G-8270

[U-^{14}C]-glucose	Amersham GFB96
HEPES buffer(1M solution)	Sigma H0887
Hypoxanthine	Sigma H-9377
Iscove's stock medium	Sigma I-3390
L-Glutamine (200 mM)	Sigma G-7513
NCTC 135 powdered medium	Sigma N-3262
Oxalacetic acid	Sigma O-4126
Polyethylene glycol	Sigma P-7777
RPMI 1640 stock medium	Gelt 51800-019
Silica gel (Keiselgel 60)	Merk TA406731
Silica gel (Keiselgel 60) (70-230 mesh ASTM)	Merk TA367234
Sodium pyruvate	Sigma P-2256
TLC aluminium sheets 20 X 20	Merk 640154510
Tris Analar	BDH 103156X
Trypan blue exclusion dye	Sigma T-8154
Thymidine	Sigma A-1784
Tween 20	Sigma P-1379

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Fixed Formula
Wheat meal, Barley meal, Soya meal, Wheatfeed, Fish meal, Fats and oils, Minerals, Vitamins and trace elements, Molasses

Nutrient		Feed Level	Added Supplement
Proximate analysis			
Dry Matter	%	87.0	
Crude Protein	%	18.0	
Crude Oil	%	2.5	
Crude Fibre	%	4.0	
Nitrogen Free Extract	%	57.0	
Ash	%	5.5	1.7
Energy			
Gross Energy	MJ/kg	15.7	
Minerals			
Calcium	%	0.88	0.39
Total Phosphorus	%	0.70	0.185
Av. Phosphorus	%	0.45	0.185
Sodium Chloride	%	0.78	0.40
Magnesium	%	0.16	0.01
Sodium	%	0.34	0.16
Chloride	%	0.44	0.24
Potassium	%	0.70	
Trace Elements			
Selenium	mg/kg	0.36	0.20
Cobalt	mg/kg	0.46	0.40
Iodine	mg/kg	1.41	1.30
Zinc	mg/kg	95.0	60.0
Manganese	mg/kg	68.0	50.0
Copper	mg/kg	20.0	12.0
Iron	mg/kg	104.0	40.0

Nutrient		Feed Level	Added Supplement
Fatty Acids			
Saturated			
Fatty Acid	%	0.68	
Unsaturated			
Fatty Acid	%	1.37	
Linoleic Acid	%	0.80	
		2.89	
Amino Acids			
Lysine	%	1.00	0.10
Methionine	%	0.30	
Methionine + Cystine	%	0.57	
Threonine	%	0.64	
Tryptophan	%	0.22	
Arginine	%	1.11	
Glycine	%	0.94	
Glycine + Serine	%	1.79	
Histidine	%	0.41	
Isoleucine	%	0.74	
Leucine	%	1.24	
Phenylalanine + Tyrosine	%	1.29	
Valine	%	0.87	
Vitamins			
Vitamin A	iu/kg	12000	12000
Vitamin D3	iu/kg	1500	1500
Vitamin E	iu/kg	104	90
Vitamin K	mg/kg	15	15
Vitamin B1	mg/kg	14	9
Vitamin B2	mg/kg	11	10
Vitamin B6	mg/kg	14	10
Vitamin B12	mcg/kg	20	20
Vitamin C	mg/kg	0	0
Folic Acid	mg/kg	2.5	2.0
Nicotinic Acid	mg/kg	78	30
Pantothenic Acid	mg/kg	26	15
Biotin	mcg/kg	334	200
Choline	mg/kg	1220	224

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NUTRIENTS			SUPPLEMENTATION
Crude Oil	%	2.6	
Crude Protein	%	18.8	
Crude Fibre	%	3.6	
Ash	%	5.9	4.5
N.F.E.	%	59.3	
Urea	%		
Dig. Crude Oil	%	2.3	
Dig. Crude Protein	%	17.0	
Tot. Diet. Fibre	%	12.0	
Pectin	%	1.3	
Hemicellulose	%	6.7	
Cellulose	%	3.2	
Lignin	%	0.9	
Starches	%	45.5	
Sugars	%	4.4	
ENERGY			
Gross Energy	MJ/kg	14.7	
Dig. Energy	MJ/kg	13.1	
Met. Energy	MJ/kg	11.7	
FATTY ACIDS			
Myristoleic Acid	%	0.03	
Palmitoleic Acid	%	0.19	
Oleic Acid	%	0.69	
Linoleic Acid	%	0.60	
Linolenic Acid	%	0.06	
Arachidonic Acid	%	0.15	
Clupanodonic Acid	%	0.03	
Lauric Acid	%	0.02	
Myristic Acid	%	0.10	
Palmitic Acid	%	0.22	
Stearic Acid	%	0.02	
AMINO ACIDS			
Arginine	%	1.31	
Lysine	%	1.02	
Methionine	%	0.31	
Cystine	%	0.31	
Tryptophan	%	0.22	
Histidine	%	0.50	
Threonine	%	0.74	
Isoleucine	%	0.83	
Leucine	%	1.57	
Phenylalanine	%	0.93	
Valine	%	0.94	
Tyrosine	%	0.71	
Taurine	%		
Glycine	%	1.74	
Aspartic Acid	%	1.16	

NUTRIENTS			SUPPLEMENTATION
AMINO ACIDS			
Glutamic Acid	%	3.34	
Proline	%	1.18	
Serine	%	0.85	
Hydroxyproline	%	0.05	
Hydroxylysine	%		
Alanine	%	0.09	
MAJOR MINERALS			
Calcium	%	0.89	0.88
Total Phosphorus	%	0.71	0.46
Phytate Phosphorus	%	0.18	
Available Phosphorus	%	0.53	0.46
Sodium	%	0.30	0.19
Chlorine	%	0.46	0.38
Magnesium	%	0.19	0.17
Potassium	%	0.63	
TRACE MINERALS			
Iron	mg/kg	108.0	32.0
Copper	mg/kg	15.0	7.0
Manganese	mg/kg	76.0	45.0
Zinc	mg/kg	76.0	54.0
Cobalt	µg/kg	453.0	388.0
Iodine	µg/kg	608.0	285.0
Selenium	µg/kg	186.0	
Fluorine	mg/kg	12.0	
VITAMINS			
Retinol	µg/kg	4411.1	3721.0
Vitamin A	iu/kg	14493.0	12279.0
Cholecalciferol	µg/kg	80.5	61.3
Vitamin D$_3$	iu/kg	3225.3	2455.0
α-Tocopherol	mg/kg	94.8	74.4
Vitamin E	mg/kg	104.3	81.8
Vitamin B_1	mg/kg	14.7	10.2
Vitamin B_2	mg/kg	13.7	12.3
Vitamin B_6	mg/kg	18.1	14.3
Vitamin B_{12}	µg/kg	68.3	61.4
Vitamin C	mg/kg	7.03	
Vitamin K_3	mg/kg	185.3	172.0
Folic Acid	mg/kg	4.4	3.0
Nicotinic Acid	mg/kg	72.7	22.9
Pantothenic Acid	mg/kg	24.0	12.2
Choline	mg/kg	1047.8	767.4
Inositol	mg/kg	2502.7	
Biotin	µg/kg	211.7	230.0
p-Aminobenzoic Acid	mg/kg		
β-Carotene	mg/kg	1.39	
PIGMENTS			
Xanthophyll	mg/kg		

Note 1 All values calculated to nominal 10% moisture content
Note 2 Values on left are total calculated value
Note 3 Values on right are amounts added via supplementation
Note 4 1µg Retinol = 3.3 iu Vitamin A activity
Note 5 Total Retinol content includes the Retinol equivalent of Carotene
Note 6 1 µg β-carotene = 1.6 iu Vitamin A activity
Note 7 1 µg Cholecalciferol = 40.0 iu Vitamin D$_3$ activity
Note 8 1 mg α-Tocopherol = 1.1 iu Vitamin activity
Note 9 1 MJ = 239.23 Calories