Induction of TNF by exoantigens of *Plasmodial* species

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ABSTRACT

Parasitized red blood cells (prbc) stimulate activated macrophages to secrete TNF under conditions that exclude the effects of contaminating bacterial Lipopolysaccharide. Molecules stimulating TNF secretion were released into supernatants collected from prbc cultured for 24 hours in PBS, and referred to as **exoantigens**. The characterization and immunological properties of these exoantigens were studied.

Exoantigens were isolated from all species of *Plasmodium* tested, including *P.falciparum.* The active moiety of the exoantigens was resistant to digestion with proteases and nucleases, and to deglycosylation and deamination. Loss of the TNF "triggering activity" was associated with digestion with lipases, deacylation and dephosphorylation, suggesting that the TNF-stimulating moiety is a phospholipid.

Exoantigens are toxic to the point of lethality in sensitized mice, and only supernatants that stimulate TNF *in vitro* are toxic. Mice can be protected by a Mab neutralizing TNF and by factors that were found to reduce the cytotoxicity of r TNF *in vitro.*

Mice immunized with exoantigens are protected against the lethality of injection of exoantigens 12 days later. Antibodies in the serum of such mice can inhibit exoantigen induced TNF secretion *in vitro.* Inhibitory antibodies are only produced in response to injections of supernatants that stimulate TNF *in vitro* and which are toxic *in vivo;* suggesting that the same molecule(s) are being measured in all three assays. Extensive cross reactions between exoantigens from all rodent parasites and *P. falciparum* exist. However there is no evidence of immunological memory, inhibitory antibodies are predominantly IgM, cannot be increased by repeated injection or by the use adjuvants, and can be raised in T cell deficient mice. Immunization with parasite exoantigens protect mice from dying from an otherwise lethal infection of *P.yoelii.* Antibodies raised against these exoantigens might provide the basis of an "anti-toxin" vaccine.

LIST OF PUBLICATIONS

Most of the results in this thesis have been previously published in the following journals;-

1) Bate, C.A.W., Taverne, J., & Playfair, J.H.L. (1988). Malarial parasites induce tumour necrosis factor production by macrophages. *Immunology*. 64. 227-231.

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3) Taverne, J., Bate, C.A.W., & Playfair, J.H.L. (1989). Induction of TNF *in vitro* as a model for the identification of toxic malaria antigens. *Lymphokine Research*. 8. 317-322.

4) Moreno, C., Taverne, J., Mehlert, A., Bate, C.A.W., Brealy, R.J., Maeger, A., Rook, G.A.W., & Playfair, J.H.L. (1989). Lipoarabinomannan from *Mycobacterium tuberculosis* induces the production of tumour necrosis factor from human and murine macrophages. *Clin. exp. Immunol.* 76. 240-245.

5) Taverne, J., Bate, C.A.W., Sarkar, D.A., Maeger, A., Rook, G.A.W., & Playfair, J.H.L. (1990). Human and murine macrophages produce TNF in response to soluble antigens of *Plasmodium falciparum. Parasite. Immunol.* 12. 33-43.

6) Taverne, J., Bate, C.A.W., Kwiatkowski, D., Jakobsen, P.H., & Playfair, J.H.L. (1990). Two soluble antigens of *P.falciparum* induce TNF release from macrophages. *Infect. & Immun.* 58. 2923-2928.

7) Bate, C.A.W., Taverne, J., Dave, A., & Playfair, J.H.L. (1990). Malaria exoantigens induce T-independent antibody that blocks their ability to induce TNF. *Immunology*. 70. 315-320.

8) Taverne, J., Bate, C.A.W., & Playfair, J.H.L. (1990). Malaria exoantigens induce TNF, are toxic, and are blocked by T-independent antibody. *Immunol. Letters.* 25. 207-212.

9) Playfair, J.H.L., Taverne, J., & Bate, C.A.W., & de Souza, J.B. (1990). The malaria vaccine: anti-parasite or anti-disease ? *Immunol. Today.* 11. 25-27.

10) Playfair, J.H.L., Taverne, J., & Bate, C.A.W. (1991). Don't kill the parasite: control the disease. *Acta Leidense.* In press

11) Bate, C.A.W., Taverne, J., Moreno, C., & Playfair, J.H.L. (1991). TNF induction by malaria exoantigens is dependent on phospholipid. *Immunology* In press.

Table of Contents

7.0 Inhibition of exoantigen induced toxicity

8.0 Inhibition of TNF cytotoxicity

9.0 Discussion & Conclusions

10.0 References

List of Figures

2.0 Materials and Methods

3.0 Identification of *Plasmodium* derived macrophage stimulants

3.2.8.b. A comparison of TNF titres obtained from activated macrophages stimulated with *P.yoelii* prbc homogenates at different time intervals

3.2.8.c. TNF titres obtained from activated macrophages stimulated with Triton X-100 lysates from *P.yoelii* infected prbcs and nrbcs

3.2.8.d. Titres of TNF obtained from macrophages by stimulated by supernatants and pellets after *P.yoelii* prbcs were incubated overnight at different temperatures

3.2.9.a.Titrations of the triggering activity released into the supernatant after incubation of prbcs at different densities.

3.2.9.b. Comparison of TNF titres stimulated by LPS and *P.yoelii* exoantigens from macrophages isolated from outbred and C3H/HeJ mice

3.2.9.c. Titrations of LPS in the presence and absence 5 μg/ml of polymyxin B.

3.2.9.d. Titrations of a *P.yoelii* parasite supernatant in the presence and absence of 5 μg/ml polymyxin B

3.2.9.e. TNF titres stimulated by supernatants from *P.yoelii* prbcs incubated in the presence and absence of antibiotics

4.0 Enzymatic and chemical sensitivity of exoantigens

4.2.1.a. Titrations of a parasite supernatant before and after digestion with 100 μg/ml trypsin

4.2.1.b. Titrations of a parasite supernatant before and after digestion with 10 μg/ml papain

4.2.1.c. Titrations of a parasite supernatant before and after digestion with 10 μg/ml pronase E

4.2.1.d. Titrations of an LPS containing rbc supernatant are shown before and after pronase digestion in the presence and absence of polymyxin B

4.2.1.e. Comparative titrations of a supernatant boiled in the presence and absence of 500 μg/ml BSA

4.2.2.a. Titration of a pronase digested supernatant compared with filtrates of the same supernatant after passage through 30 and 10 KD Amicon filters

4.2.2..b. Titrations of a pronase digested supernatant before and after incubation with polymyxin B agarose

4.2.3.a. TNF titres stimulated from macrophages by supernatants before and after digestion with 10 μg/ml DNase and 10 μg/ml RNase

4.2.4.a. Titrations of a parasite supernatant before and after oxidation with sodium periodate

4.2.4.b. Exoantigen activity released into supernatants and extracted from pellets of *P.yoelii* prbcs incubated in the presence and absence of 10 μg/ml tunicamycin

4.2.4.c. TNF titres induced by exoantigens before and after digestion with a mixture of glycosidases

4.2.4.d. TNF titres stimulated from macrophages by exoantigens before and after deglycosylation with TFMS

4.2.5.a. TNF titres stimulated by the aqueous and the non-aqueous phases from a two phase separation of protease digested exoantigens

4.2.5.b. Titrations of a parasite supernatant before and after hydrolysis with mild alkali (deacylation)

4.2.5.c. Pooled TNF titres, expressed as equivalents of LPS μg/ml, stimulated by exoantigen preparations before and after deacylation

4.2.5.d. TNF induction by a parasite supernatant before and after digestion with a mixture of triacyl glycerol lipases and acyl hydrolases

4.2.5. e. Titration of exoantigens before and after digestion with phospholipases

4.2.5.f. TNF titres stimulated by LPS before and after digestion with phospholipase C

4.2.6.a. TNF titres stimulated by a parasite supernatant before and after digestion with alkaline phosphatase

4.2.6.b. TNF titres stimulated by exoantigens before and after dephosphorylation with hydrofluoric acid

4.2.7.a.TNF titres stimulated from macrophages by exoantigens before and after deamination and filtration through a 10 KD Amicon filter

4.2.7.b. Titrations of LPS before and after deamination with nitrous acid

4.2.9.a. Titrations of *P.falciparum* exoantigen from a crude lysate before and after boiling and pronase digestion

4.2.9.b. Titrations of *P.falciparum* exoantigens after deamination deacylation and digestion with phospholipase C.

4.2.9.c. TNF titres stimulated from human monocytes by LPS and exoantigens from *P.falciparum* and *P.yoelii*

5.0 Macrophage activation

5.2.1.a. TNF secretion from IFN-γ activated macrophages in response to parasite exoantigens

5.2.2.a. TNF secretion by macrophages treated with either indomethacin or BW755C and stimulated by *P.yoelii* exoantigens

5.2.2.b. The effects of PGE_2 upon indomethacin activated macrophages stimulated by a constant dilution of *P.yoelii* exoantigens

5.2.3.a. Titrations of pronase-digested supernatant upon macrophages treated with either indomethacin, IFN-γ or both

6.0 Exoantigen induced pathology

6.2.1.a. TNF titres secreted into the serum of *C.parvum* primed mice in response to supernatants prepared from human, rodent prbcs and mouse nrbcs

6.2.2.a.Toxicity of exoantigens from different *Plasmodium* species for sensitized mice

6.2.2.b. Toxicity of cytokines for groups of D-galactosamine sensitized mice

6.2.2.c. Lethality of a sample of protease-digested exoantigens in the presence and absence of D-galactosamine

6.2.2.d. Mortality in sensitized mice injected with digested / treated exoantigen preparations

6.2.3.a. Mortality in groups of control and CD 4+ depleted mice in response to injection of parasite exoantigens

6.2.4.a. Prevention of exoantigen induced toxicity for sensitized mice by prior administration of inhibitors

6.2.4.b. Protection mediated by a neutralizing Mab to TNF in groups of sensitized mice injected with pronase-digested exoantigens

7.0 Inhibition of exoantigen induced toxicity

7.2.1.a. Non-specific protection against LPS or exoantigen induced mortality by 24 hour pretreatment with either reagent

7.2.1.b. Protection against exoantigen induced mortality by 24 hour pretreatment with cytokines

7.2.2.a. Immunization 10 days earlier provides specific protection against mortality in sensitized mice injected with either LPS or parasite supernatants

7.2.2.b. Reduction of TNS titres obtained in serum by immunization with either LPS or parasite supernatants

7.2.3.a. Inhibition of exoantigen-induced TNF secretion by various antisera

7.2.4.a. Primary antiserum against the exoantigens specifically inhibits the amount of TNF released into the serum of mice injected with parasite supernatants

7.2.4.b. Primary antiserum raised against the exoantigens specifically protects mice injected with parasite supernatants

7.2.5.a. Primary antiserum raised against LPS did not inhibit induction of TNF by parasite exoantigens

7.2.5.b. Absorption of primary antiserum against uninfected erythrocytes

7.2.6.a. Inhibition of TNF induction by different parasite preparations by primary antiserum against a parasite supernatant

7.2.7.a. Inhibition of *P.yoelii* exoantigen activity by a 1/100 dilution of antisera raised against variously treated supernatants

7.2.7.b. Inhibition of pronase-digested exoantigens by antisera raised against purified exoantigen preparations

7.2.8.a. Immunoglobulin isotypes responsible for inhibition by primary antisera

7.2.9.a. Time course of the development of inhibitory antiserum

7.2.10.a. Titrations of primary antisera from normal and athymic mice

7.2.10.b. Immunoglobulin isotypes responsible for inhibition by primary antisera from athymic mice

7.2.10.c. Titration of the inhibitory effect of antiserum from CBA/N mice compared with control CBA/Ca mice

7.2.11.a. Inhibition of TNF induction by *P.yoelii* pronase-digested exoantigens by antisera raised against pronase-digested exoantigens from different parasite species

7.2.12.a. Inhibition of TNF induction by exoantigens of *P.falciparum* by antisera raised against exoantigens of rodent parasites
7.2.12.b. Inhibition of TNF induction by a crude *P.falciparum* lysate by antisera raised to a crude lysate and to purified (deaminated) *P.yoelii* exoantigens
7.2.12.c. Inhibition of TNF induction by pronase-digested *P.yoelii* exoantigens by antisera raised against pronase-digested Ag 1 and Ag 7
7.2.12.d. Inhibition of TNF induction by antigen 1 before and after pronase-digestion by primary antisera raised against *P.yoelii* exoantigens
7.2.13.a. TNF titres stimulated by *P.yoelii* prbcs in the presence and absence of a 1/10 dilution of primary antiserum
7.2.14.a. TNF titres stimulated by formaldehyde fixed *P.yoelii* prbcs in the presence and absence of a 1/10 dilution of primary antiserum
7.2.14.b. TNF titres stimulated by fixed *P.yoelii* prbcs before and after deacylation or digestion with phospholipase C
7.2.15.a. Protection against the lethality of infection with *P.yoelii* YM by prior immunization with parasite supernatants

8.0 Inhibition of TNF cytotoxicity

8.2.1.a. Reduction in the cytotoxicity of r TNF by pretreatment of target cells with the cyclo-oxygenase inhibitor indomethacin
8.2.2.a. Reduction in the cytotoxicity of r TNF by pretreatment of target cells with human post exercise serum
8.2.3.a. Protection of target cells by pretreatment with the culture supernatant of dexamethasone-treated macrophages

List of Tables

1.0 Introduction

4.0 Enzymatic and chemical sensitivity of exoantigens

7.0 Inhibition of exoantigen induced toxicity

Abbreviations

Ab.	Antibody
ACTH.	Adrenocorticotrophin
ADCC.	Antibody Dependent Cellular Cytotoxicity
Ag.	Antigen
ARDS.	Adult Respiratory Distress Syndrome
AlOH.	Aluminium Hydroxide
BCG.	Bacille Camette Guerin
BC.	Before Christ
BSA.	Bovine Serum Albumin
CD 4.	T cell marker of MHC class II restriction
CD 8.	T cell marker of MHC class I restriction
CH_3COO Na	Sodium acetate
CIE.	Crossed Immunoelectrophoresis.
CNS.	Central Nervous System
Con A.	Concanavalin A.
CTL.	Cytotoxic T Lymphocytes
CSF.	Colony Stimulating Factor
CSF.	Cerebrospinal fluid
CSP.	Circumsporozoite protein.
DAF.	Decay Accelerating Factor.
DNA.	Deoxyribose Nucleic Acid.
DTH.	Delayed Type Hypersensitivity.
FACS.	Fluoresceine Activated Cell Sorter
FCA.	Freunds Complete Adjuvant.
FCS.	Fetal Calf Serum.
GPI.	Glycosylphosphatidyl inositol.
HIV.	Human Immunodeficiency Virus.
HF.	Hydrofluoric Acid.
ICAM.	Intracellular Adhesion Molecule.
Ig.	Immunoglobulin.
INF.	Interferon.
IL.	Interleukin.
i.p.	intra-peritoneal
i.v.	intravenous
KD.	Kilo Dalton
LAK.	Lymphokine Activated Killer.
LAM.	Lipoarabinomannan.
LBP.	Lipopolysaccharide Binding Protein.
$LD_{50/100}$.	Lethal $Dose_{50/100}$
LDL.	Low Density Lipoprotein.
LFA.	Lymphocyte Function Antigen.
LPG.	Lipophosphoglycan.
LPS.	Lipopolysaccharide.
Lyb.	Mouse cell marker

L929	Mouse fibroblast cell line
Mab.	Monoclonal antibody.
MDP.	Muramyl Dipeptide.
MEM.	Minimum Essential Medium.
MHC.	Major Histocompatability Complex.
MSA.	Merozoite Surface Antigen.
MSH.	Melanocyte Stimulating Hormone
MPL.	Monophosphoryl Lipid A.
$Na\ COOCH_3$	Sodium acetate
$NaIO_4$.	Sodium periodate.
$NaNO_2$.	Sodium nitrite.
NaOH.	Sodium hydroxide
NK.	Natural Killer.
NO.	Nitrous oxide
nrbc.	Normal red blood cell.
O/N.	Overnight.
PAF.	Platelet Activating Factor
PBMC.	Peripheral Blood Mononuclear Cell.
PBS.	Phosphate Buffered Saline.
PG.	Prostaglandin.
PI-G.	Phosphatidyl inositol Glycans.
POMC.	Proopiomelanocortin
prbc.	parasitized red blood cell
r.	Recombinant
RESA.	Ring infected Erythrocyte Surface Antigen
RNA.	Ribose Nucleic Acid.
RNI.	Reactive Nitrogen intermediates
ROI.	Reactive Oxygen Intermediates.
RPMI 1640	Cell culture medium
rbc	red blood cell
rpm.	revolutions per minute.
SRBC	sheep red blood cells
TGF.	Transforming Growth Factor.
Th.	T helper
TI.	T Independent
TFMS.	Trifluoromethanesulphonic Acid.
Thy-1.	T cell marker for mouse cells
TNF.	Tumour Necrosis Factor.
TNS.	Tumour Necrosis Serum
Tris.	Tris (hydroxymethyl) aminomethane
TSST-1.	Toxic Shock Syndrome Toxin-1.
VSG.	Variable Surface Glycoprotein
WHO.	World Health Organization.

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1.0 Introduction

1.1 History

Malaria is an old disease and was originally chronicled in Egyptian papyruses dating from the 16 th century B.C. (Klayman., 1989), one of which noted the association between shivering fits, fever and spleen enlargement. Greek literature also describes the recurrence of fevers at regular intervals. In the 18th century the term "malaria" was coined which derived from the Italian malaria meaning bad-air. Although the disease was thought to be due to poor hygiene; documents from this period also noted an association with marsh insects. Much progress was made in the 1880s: malaria parasites were discovered, first in patients' blood, and then in female *Anopheles* mosquitoes fed on the blood from an infected patient and the mode of transmission was then elucidated.

It was only 300 years ago that most of Europe and Northern America were considered endemic areas; indeed in 1982 the WHO published figures that showed until a few decades ago 72 % of the world population lived within malaria zones. This has now been reduced to 54 %. The potential for malaria epidemics is associated with contact between parasites and non-immune populations. This might come about through the spread of parasites into areas where the disease is unknown or through the introduction of different strains of parasite into new areas. It can also occur with the immigration of non-immunes into malarious areas. If the current theories of global warming turn out to be correct, the spread of habitat for the *Anopheles* mosquito vector, and hence of malarial parasites, could possibly endanger the predominantly immunologically naive population of Europe.

Malaria is one of the most common infectious diseases in the world. It is estimated that there are 200 million clinical cases annually, of which approximately 1-2 million are fatal. The severity of disease and outcome of infection are dependent upon many factors, including the immune status and drug treatment of host. Malaria fatalities generally occur amongst immigrants from non-endemic areas, or children under the age of 5 (Marsh, Otoo &

Greenwood, 1987). In areas of low endemicity, immunity in the indigenous population is low and severe disease can occur in all age groups.

Attempts to control malaria in the past have concentrated upon a combination of the removal of the *Anopheles* vector by reduction of its habitat and through the use of insecticides, and by drug prophylaxis against the parasite. Partial success was achieved, in that the WHO programme to eradicate malaria in the late 50s managed to relieve many temperate regions of malaria. The emergence of resistant strains of both *Anopheles* and *Plasmodium* has led the scientific community to investigate vaccination as an attractive goal.

1.2 Life cycle of *P.falciparum*

The life cycle of *P. falciparum* involves the development of stages in man and in the mosquito. When an infected female *Anopheles* mosquito takes a blood meal it can transmit sporozoites from its salivary gland into the bloodstream of its victim. These are conveyed to the liver and can invade hepatocytes where they multiply and develop into the merozoite stage within the course of 10 - 14 days. Rupture of the hepatocyte releases merozoites into the bloodstream, where they invade erythrocytes, " the erythrocytic stage". Within the erythrocyte, parasites multiply asexually and undergo maturation through the ring, trophozoite and finally the schizont stage. It is at this stage in that the clinical manifestations of malaria occur. When the schizont infected erythrocyte bursts, merozoites are released which can invade new erythrocytes. Some of these merozoites differentiate into the sexual forms, micro and macro gametocytes. Further transmission of the parasite relies upon the uptake of gametocytes by a mosquito in a blood meal, fertilization within the mosquito gut and the production of gametes, a zygote and finally an ookinete. Ookinetes burrow through the endothelium in the mosquito gut and mature into oocysts. From these sporozoites arise, which migrate to the mosquito's salivary gland and are ready to initiate the cycle all over again.

1.3 Rodent malaria

The first *Plasmodium* species isolated from rodents were described by Vincke and Van den Bergh in 1948 and were isolated from Thicket rats (~~*Thamnomys*~~ Grammomys *surdaster*). Other species have been found and in 1974 Killick-Kendrick classified rodent parasites into 4 groups.

(1) *P. berghei*
(2) *P. yoelii*
(3) *P. chabaudi*
(4) *P. vinckei*

Characteristics of rodent parasite life cycles parallel those of parasites infecting man (Landau & Boulard 1978).

Table 1.3.a. Characteristics of *Plasmodium* species infecting rodents.

	P.berghei	*P.vinckei*	*P.yoelii*	*P.chabaudi*
Pre-erythrocytic stage (hours)	50	>60	50	>50
No of Blood merozoites per schizont	6 - 18	6 - 16	6 -18	4 - 10
Asexual cycle (hrs)	22 - 25 asynchronous	24 asynchronous	22 - 25 asynchronous	24 synchronous
Red cell preference	reticulocytes	mature	reticulocytes	mature
Mosquito cycle (days)	14	9 - 10	10 - 13	10 - 1 1

Lethal forms of *P.yoelii* and *P.berghei* grown in outbred or F1 crosses of Balb/C x C57/Bl rapidly kill the host. Lethality is dependent upon the strain of both parasites and its host as in rats *P.berghei* is a non-lethal parasite. However, animal infections are often poor models of the complications of *P.falciparum.*

1.4 Human malaria

Diagnosis of malaria depends upon the presence of symptoms, commonly fever, but this may also be elicited by other pathogens, and the presence of parasites in the blood. The clinical signs of malaria vary between infecting species and strains of parasite. The most common symptoms are periodic fevers, which are associated with the release of merozoites from rupturing red blood cells (Clark & Chaudhri., 1988). Typical progression of the disease includes headaches, nausea with vomiting, fever and rigors. In *P.falciparum* infections can progress into a series of life threatening complications.

Table 1.4.a. Characteristics of *Plasmodium* species infecting humans.

	P.falciparum	*P.vivax*	*P.ovale*	*P. malariae*
Average incubation period (days)	12	13-17	14	28
Bloodstage cycle (hrs)	48	48	50	72
Hypnozoite production	none	yes	yes	none
Mosquito cycle (days)	9	8	12	16

P.falciparum (Malignant tertian malaria)

Untreated infections with *P.falciparum* result in high morbidity and mortality, and cause severe symptoms which include cerebral malaria, hypoglycaemia, pulmonary oedema, circulatory collapse, severe anaemia, renal

and adrenal necrosis. An asynchronous cycle of multiplication is associated with fevers that are either continuous, remittent or irregular. When periodicity of fever does occur it is usually around 36 hours but temperature may remain elevated for 24 hours.

Of the human *Plasmodium* species *P.falciparum* has the shortest duration of infection, the shortest incubation period, the greatest number of merozoites per exo-erythrocytic schizont and results in the highest parasitaemia. The parasitaemia can rise to 40 % as it is not confined by the development stage. *P.falciparum* is found in most malarious areas and predominates in most of Africa, Asia and Oceania. Recrudescence of latent blood stages may occur for up to a year.

P.vivax and *P.ovale* (Benign tertian malaria)

In primary attacks of *P.vivax* and *P. ovale* a rhythm of schizogony occurs after 3-4 days and causes periodic febrile responses at 48 hour intervals. Clinical manifestations of both infections are similar, fatalities are rare and seem to be associated with splenic rupture. *P.vivax* is found in most malarious areas but is rarer than *P.falciparum*. It predominates in Latin America and the Indian subcontinent, whereas *P.ovale* is largely restricted to the African continent. *P.vivax* favours infection of reticulocytes. Relapses, which are characteristic of both infections can occur after significant time periods, and are due to differentiation of parasites into hypnozoites in the liver.

P.malariae (Quartan malaria)

In synchronous infections, *P.malariae* schizonts mature every 72 hours, giving febrile paroxysms every third day. *P. malariae* is spread throughout the world. It is associated with low parasitaemias (0.1 %), invasion of aging erythrocytes and is rarely lethal, fatalities that do occur are attributable to a characteristic renal syndrome. Infection may persist for many years with reappearance of the disease being due to recrudescence of persistent erythrocytic forms, as differentiation into hypnozoites in the liver is not seen.

1.5 Immunity to malaria

Immune responses to *Plasmodium* infections in experimental animals are often complex. Protection should not be seen as mediated by just the proliferation, activation and stimulation of specific cell lineages but also the recruitment, organization and coordination of such cells in immune organs such as the liver and spleen. Comparisons of lethal and non-lethal *P.yoelii* show that the non-lethal strain induces an earlier peak in splenic and hepatic macrophage activation (Taverne, Treagust & Playfair., 1986). Vaccination of mice with bloodstage antigens from P.*yoelii* is associated with increased oxidative capacity in the liver and spleen (Dockrell, Alavi & Playfair., 1986).

1.5.1 Non specific Immunity

Jensen *et al.*, (1983) discovered that sera from infected individuals who had no history of malaria disease contained strongly parasiticidal factors which were not antibody, and which they called "crisis forming factor". A non-specific immunity to malaria in rodents can be induced by agents unrelated to malaria but which have the ability to activate or stimulate macrophages. It has been postulated that protection might be mediated by macrophage derived products. These products are not specifically parasiticidal as they also confer protection against a range of organisms such as *Babesia* (Clark *et al.*, 1976) and ~~tuberculosis~~ mycobacteria (Liew & Cox., 1991). The agents inducing this type of protection can be classified into 2 categories;

a) Whole organisms administered a week or more before infection (BCG, *C.parvum*) (Clark, Allison & Cox., 1976 and Cottrell, Playfair & de Souza., 1977).

b) Purified products derived from microbes (LPS, MDP, Zymosan) and plant mitogens (Con A) given 24 hours prior to infection (Cottrell, Playfair & de Souza., 1977).

Injection of bacterial lipopolysaccharide (LPS) into mice 14-21 days after inoculation with BCG led to the production of factors within the sera of these mice that induced the necrosis of tumours, such sera was named Tumour Necrosis Serum (TNS) (Carswell *et al.*, 1975). TNS was shown to be toxic to *P.yoelii in vitro* (Taverne *et al.*, 1981) and *in vivo* (Clark *et al.*, 1981). *P.falciparum* has also been shown to be susceptible to inactivation by TNS *in vitro* (Wozencraft *et al.*, 1984). The parasiticidal activity of TNS cannot be reproduced by recombinant cytokines and neutralizing anti-cytokine antibodies have not been found to inhibit this activity. Inhibition of *P.falciparum* multiplication by TNS was shown to be associated with the presence of an increased concentration of lipid peroxides in the serum (Rockett *et al.*, 1988).

Resistance to infection may be mediated by what seem to be non-immune mechanisms. In infections with the non-lethal variant of *P.yoelii* resistance correlated with haemopoetic parameters, since an early rise in the percentage of reticulocytes inhibit parasites with a preference for mature red blood cells. Simple transfusion of red blood cells is able to reverse the course of an otherwise lethal infection with *P. vinckei petteri* (Stocker *et al.*, 1984).

Genetic Factors

A number of human genetic factors specifically influencing malaria have been identified. Most of the known factors affect the erythrocytic stage, they either prevent parasite invasion or alter the erythrocytic micro- environment so that it is less favourable to parasite multiplication. *P.vivax* merozoites cannot infect erythrocytes that lack the Duffy blood groups Fy^a and Fy^b. Sickle cell haemoglobin, fetal haemoglobin (HbF), β-thalassaemia and certain forms of G-6-PD deficiency are all factors that are unfavourable to the development of *P.falciparum* within the erythrocyte.

1.5.2 Antibody-mediated Protection

(a) Correlation of antibody with immunity

In man, serum concentrations 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). Maternal anti-malarial antibodies provide protection in the newborn infant but as levels of these antibodies fall the

children become susceptible to the disease. Protective antibodies may act by inhibiting parasite function, inhibiting invasion of erythrocytes, inhibiting sequestration/rosetting or by mediating parasite destruction via complement fixation and promotion of ADCC. However, increased antibody titres may also lead to increased pathology; immune complex formation and hypercomplementaemia are possible consequences of such elevated antibody levels. Most antibody produced during infection is parasite specific but auto-antibodies can be detected to red blood cells, lymphocytes, complement, immunoglobulins and nuclear components (Deans & Cohen., 1983).

(b) Passive transfer of antibody

Passive transfer of purified IgG from immune adults significantly lowered the parasitaemias of a group of children with acute malaria when compared to a control group given IgG depleted serum (Cohen *et al.*, 1961). In rodent models, passive transfer of immune serum can confer protection at different stages of the parasite life cycle. Transfer of immune sera to sporozoites can increase clearance rate and reduce infectivity (Nussenzweig *et al.*, 1969). Protective antiserum has also been raised to merozoites (Diggs & Osler., 1975) and its effect appears to correlate with inhibition of entry into the rbc. Furthermore monoclonal antibodies to blood stage antigens from rodent parasites endow protection (Freeman *et al.*, 1980).

(c) B cell depletion

If antibody plays a major role in defence against infection, depletion of B cells should cause exacerbation of the disease. Mice infected with the normally non-lethal 17 X strain of P.*yoelii* died when they had been previously anti-μ treated (Weinbaum, Evans & Tigelaar., 1976). However μ-suppressed mice are able to control chronic infection of *P. chabaudi* but require antibody to clear the parasitaemia (Grun & Weidanz., 1981).

1.5.3 T - cell mediated Immunity

Some *Plasmodium* antigens such as the 230 KD *P.yoelii* merozoite antigen, are protective in mice (Holder & Freeman., 1981). Although they stimulate antibody production, passive transfer of antiserum does not confer protection, suggesting a non-antibody mediated immunity. In vaccination studies with blood stage antigens from *P.yoelii*, protection correlated with T cell responses, in particular DTH (Playfair *et al.*, 1985).

Cytotoxic T lymphocytes (CTLs), CD 8+ cells have a role in the recognition and destruction of infected MHC class 1 positive cells; in man this mechanism would be restricted to the hepatocytic stage. Helper (CD 4+) T cells produce a variety of cytokines with numerous actions that can modulate immune function.

(a) T cell cytokines activate macrophages to secrete factors responsible for non-specific killing, activate polymorphs for ADCC and enhance NK and LAK cell activity. Furthermore, activated macrophages show increased expression of MHC class II and the subsequent presentation of antigen to T cells.

(b) T cell-derived cytokines offer direct help in the proliferation and differentiation of T and B cells.

(a) T cell transfer

T cells have been demonstrated to confer protection at all stages of the parasite life cycle. Immunity to the hepatocytic stage is dependent upon CD 8+ cells (Romero *et al.*, 1989; Schofield *et al.*, 1987). In contrast, transfer of immune CD 4+ T cell populations confers protection in bloodstage infection with *P.yoelii*. This protection was associated with specific IgG and IgM (Jayawardena *et al.*, 1982) and was not conferred by CD 8+ cells. Cloned CD 4+ T cells generated to *P.chabaudi adami* have been shown to transfer protection which correlates with the secretion of IFN-γ and IL-2. The protection was transient and limited to homologous infection (Brake, Long & Weidanz., 1988).

(b) T cell depletion

In nude mice, the non-lethal variant of *P.yoelii* develops a long lasting chronic infection (Clark, 1974). Depletion of CD 4+ or CD 8+ cells affected the outcome of a *P.chabaudi* infection (Suss *et al.*, 1988): CD 4+ depleted animals died, whereby CD 8+ depleted animals underwent a prolonged infection with slightly raised parasitaemias. In man, the effects of T cell deficiency may become clearer as data accumulate from HIV infected patients.

1.5.4 Problems for vaccination

(a) Stage specificity

As most parasite antigens are restricted to specific stages of the life cycle, immunity is effectively stage specific. Immunity against sporozoite challenge confers no protection against subsequent asexual blood or sexual stages (Nussenzweig *et al.*, 1969).

(b) Species specificity

Ideally, vaccination should be effective against all human species of *Plasmodium*, as frequently two or more *Plasmodium* species are to be found in a patient. *P.falciparum* is seen with *P.vivax* and either *P.malariae* or *P.ovale* in West Africa. The symptoms of *P.falciparum* predominate.

In rodent or primate models, protection to *Plasmodium* can be achieved across some species boundaries (Cox & Voller., 1966; Cox, 1970). *P. yoelii* infections are able to confer protection against subsequent infection with *P.berghei* (Sedagah *et al.*, 1982).

(c) Inter-strain polymorphism

The balance between host immunity and parasite evasion has led to a highly variable antigenic profile. The slow development of natural immunity is consistent with the hypothesis that immunity develops only after exposure to a large number of different parasite strains. Geographically separate strains of *P.falciparum* and *P.vivax* show different resistance profiles to antimalarial drugs and involve different transmission vectors. Moreover there are large differences

in virulence between various isolates of *P.falciparum* when injected into *Aotus* monkeys (Schmidt., 1978).

Isolates from *P.falciparum* in 10 Gambian children showed antigenic diversity when measured by antibody agglutination tests. Serum reacted only with homologous infected cells, in contrast to serum from Gambian adults, which reacted with infected cells from all the children (Marsh & Howard., 1986). Furthermore, many antigens of parasites from different geographical areas show great diversity (Coppel *et al.*, 1984). Surface erythrocyte antigens not only vary in different isolates, but also undergo variation during the course of a natural blood infection with cloned parasites (Mendis *et al.*, 1991).

(d) Immunosuppression

Malarial parasites employ a wide range of mechanisms in order to divert the immune response. The production of large amounts of secreted antigens may have evolved in order to help the parasite evade clearance by antibody dependent cell cytotoxicity. Parasite-derived molecules may suppress immune function. Stimulation of TH-2 cells to secrete IL-4 (Haeffner-Cavaillion *et al.*, 1989), or the adrenals to secrete glucocorticoids, leads to inhibition of macrophage functions such as TNF secretion and MHC class II presentation.

(e) Immune avoidance

P.falciparum is a parasite with a capacity to sequester in deep vasculature during the later stages of its erythrocytic development (Miller, 1969). This sequestration process may benefit the parasite as it prevents passage through the spleen at a time when the parasite is at its most vulnerable to both the specific and non-specific anti *Plasmodium* effector mechanisms assembled there (Wyler *et al.*, 1979). Little is known about where parasites are killed in the host, although the spleen is seen as an important effector organ.

1.5.5 Towards a malaria vaccine

The techniques now exist for the recognition of suitable candidate antigens and perhaps their mass production. There is currently much hope, debate and controversy about the nature of an effective malaria vaccine.

(a) Pre-erythrocytic stage malaria vaccines

The first phase of the parasite life cycle is initiated when an infected mosquito bites and sporozoites are released into the bloodstream. Immunized animals and human volunteers developed high titres of anti-sporozoite antibody when given irradiated sporozoites (Nussenzweig, Vandenberg & Most., 1969). These antibodies neutralize the infectivity of sporozoites *in vitro* and *in vivo*, and appear to be directed towards the circumsporozoite (CS) protein. CS proteins have a single immunodominant epitope, present in multiple repeats, and Mabs generated by sporozoite immunization are directed against this epitope. In *P.falciparum* the CS protein repeat is of four amino acids, asparagine-alanine-asparagine-proline (NANP) and the immunodominant epitope is made up of three consecutive repeats of NANP. Immunization of mice with the synthetic peptide $(NANP)_3$ stimulates antibodies that neutralize sporozoite infectivity (Hollingdale *et al.*, 1984).

There do seem to be grounds for optimism in that the $(NANP)_3$ epitope has been detected in isolates of *P.falciparum* from different areas of the world. However there are problems in that the CS proteins are seen as weak immunogens, T-cell epitopes on the CS proteins are infrequent and polymorphic between strains of *P.falciparum*, recognition of these epitopes is MHC-restricted and in all populations there is a proportion of non-responders (Day & Marsh., 1991). Perhaps it is asking just too much that a vaccine should be able to completely block all sporozoites from reaching the liver, considering the number of sporozoites that may be inoculated. There is recent evidence that CS proteins are expressed upon infected hepatocytes along with MHC class 1 molecules and that direct killing of infected hepatocytes by CTLs is an important part of sporozoite-induced immunity (Targett, 1989).

New culture systems for the production of the hepatic exo-erythrocytic stages of *Plasmodium* in sufficient numbers has allowed analysis of antigens and immune mechanisms to this stage. The importance of CD 8+ T cells (Romero *et al.*, 1989) and the cytokines IFN-γ (Schofield *et al.*, 1987) and TNF–α in immunity to this stage of the parasite life cycle has been shown. There are specific liver stage antigens expressed by *P.falciparum* infected hepatocytes, LSA-1 and LSA-2. Vaccination with LSA-2 protects mice against challenge with *P.berghei* sporozoites and elicits CTLs that kill *P.berghei* exoerythrocytic stages *in vitro* (Hollingdale., 1991).

(b) Asexual blood stage vaccines

There are many candidate antigens from the asexual erythrocytic stage. Indeed parasites at this stage represent the major target of acquired specific immune responses. Infection with *P.falciparum* results in antibody responses against a large number of different blood stage antigens, of which we presume only a few produce protective immune responses. Theoretically, antibodies to antigens present at this stage could work by blocking merozoite invasion of uninfected erythrocytes, by the reduction of prbc density via antibody mediated killing mechanisms and phagocytosis, by inhibition of parasite maturation, or by prevention of mechanisms that possibly cause disease, such as inhibition of parasite sequestration, inhibition of prbc rosetting and the blocking of antigens that stimulate cytokine release.

The mechanisms by which merozoites invade erythrocytes is not yet fully understood, but antigens involved in the invasion process are in theory vaccine candidates. As the glycophorins have been implicated in merozoite binding, several glycophorin-binding proteins have been identified and are the subject of further investigation. The Ring infected Erythrocyte Surface Antigen (RESA) is detected upon membranes of infected erythrocytes shortly after merozoite invasion, and naturally occurring antibodies to RESA are potent inhibitors of merozoite invasion (Wahlin *et al.*, 1985). Furthermore, short sequences from this peptide and sequences from the merozoite surface antigen (MSA) have been incorporated into the vaccine tested by Patarroyo. The MSA-1 is one of the leading vaccine candidates; although this antigen and its breakdown products are considered to show antigenic polymorphism, there are believed to

be conserved epitopes at the anchoring end.

The controversial vaccine {Spf 66} produced by Patarroyo is based upon a combination of 3 peptides from antigens seen on the merozoite surface, including sequences from RESA and MSA-1, bound together with the NANP repeats from the CS proteins, reviewed by Ash, (1991). Initially tested in groups of human volunteers (Patarroyo *et al.*, 1988), it is now claimed that up to 85 % of people given his vaccine are protected. Low responders to the vaccine frequently employed $V_{\beta}3$ in their T cell receptor, which showed homology with parasite proteins. The main arguments against the value of this vaccine seem to be potential variability between batches, composition and doses, safety and a lack of substantiation of the vaccines claims for efficacy in double blind and placebo controlled trials.

One of the new approaches to vaccine development is to identify functional antigens, these being defined as antigens that cannot show extensive polymorphism without loss of function. Parasite aldolase has been identified as one such target (Cox, 1988). It does not exhibit the polymorphism thought to confer species or strain specificity upon other candidate antigens. However there are potential cross-reactions with the host enzyme. There is evidence that parasites must regulate the ionic composition of the host erythrocyte during their maturation. A family of ATPase-like molecules have been found in *P.falciparum* infected prbcs which have this function and are further vaccine candidates (Krishna *et al.*, 1991). Under this category come those, as yet unidentified, antigens that are involved in sequestration and rosette formation of infected erythrocytes (Carlson *et al.*, 1990).

(c) Transmission blocking vaccines

Vaccination to prevent transmission of malaria from the human host to the mosquito vector, has been reviewed by Targett & Sinden (1985). The so-called transmission blocking vaccines would aim to protect populations in endemic areas by reducing the number of infected mosquitoes. Vaccination against sexual stages can suppress gametocyte production in the vertebrate host (L.Winger, personal communication). The extracellular sexual stages, the male and female gametes, the zygote and the ookinete are all potential targets.

Blocking of transmission was first shown by vaccination with attenuated whole macro- or micro-gametes before exposure to infected mosquitoes. Furthermore Mabs raised to either macro- or micro-gametes confer passive immunity and suppress ookinete production (Harte, Rogers & Targett., 1985). A post-fertilization protein with a molecular weight of 25 KD is found upon zygotes and ookinetes and antibodies to this protein prevent parasite development after fertilization. It is believed that these antigens are crucial receptors or ligands that interact during contact of the gametes.

1.6 Malaria as a toxic disease

The idea that the clinical complications of malaria might be due to the presence of toxins was initially suggested by Sinton, (1939) who proposed that natural immunity is partially anti-parasitic and partially anti-toxic. In the Gambia, children develop immunity to the disease of malaria by the ages of 3-5 years, yet retain persistently high parasite levels in the blood. Furthermore, in naive European travellers, clinical complications occur at parasite levels which are much lower than parasitaemias that can be seen in the apparently healthy endogenous population (Kern *et al.*, 1989). These observations suggest what seems to be a bi-phasic immunity to infection, in that an immunity to the clinical manifestations of malaria develops in a period before anti-parasite immunity is acquired.

Interest in malarial toxins was revived by the observation that the symptoms associated with malaria and Gram - negative bacteraemia (septic shock) were similar (Clark *et al.*, 1988). Clark suggested that some toxin like activity might play a role in the production of disease in malaria in a similar manner to the way LPS does in septic shock. The basis of this investigation was to determine if *Plasmodium* species synthesize "LPS-like" molecules that could act as toxins and how they might work.

1.6.1 Measurement of LPS-like activity

The terms endotoxin and lipopolysaccharide (LPS) are used synonymously to describe molecules, which can be extracted from all Gram-negative bacteria, and which have been found to be responsible for much of the toxicity of those infections. Endotoxin levels can be measured by its ability to clot Limulus Amebocyte Lysate (LAL). The use of the LAL test has led to the detection of "Endotoxin-like" activity in the serum of patients infected with *P.falciparum* (Tubbs *et al.*, 1980, Usawattanakul *et al.*, 1985). This LAL reactivity may be a measure of LPS derived from complicating bacterial infections, or of parasite derived molecules with similar activity. Affinity-purified soluble antigens from *P.falciparum* have recently been reported that react with the LAL test (Jakobsen, Baek & Jepsen., 1988). LAL activity shows a positive correlation with the level of parasitaemia and negative correlations with the presence of gram negative bacteria (Usawattanakul *et al.*, 1985).

1.6.2 Biological activities of LPS

LPS has been shown to act on a wide range of mammalian systems (Brade *et al.*, 1988). For example it has been shown;

TO BE MITOGENIC FOR B CELLS
TO ACTIVATE GRANULOCYTES
TO STIMULATE NON SPECIFIC RESISTANCE AGAINST INFECTION
TO STIMULATE MACROPHAGE SECRETION OF ;- IL-1, TNF, CSFS AND ARACHADONATE METABOLITES
TO INDUCE HYPOTHERMIA, FEVER
TO INDUCE A LOCAL SHWARTZMAN REACTION (TISSUE NECROSIS)
TO PROTECT AGAINST IRRADIATION
TO HAVE LETHAL TOXICITY

1.6.3 Biological properties of LPS responsible for toxicity

The toxicity of LPS was shown to be due, not to the bacterial molecules themselves, but rather to a plethora of endogenous factors secreted by the host in response to the toxin. Endotoxin resistant C3H / HeJ mice were made sensitive to LPS by the transfer of 2×10^7 macrophages from an LPS sensitive strain of mouse C3H / HeN (Lehmann, Freudenberg & Galanos., 1987). It is now generally accepted that the endotoxic activities of LPS are mediated by macrophage derived endogenous mediators. In chapter 6 I will present the evidence which suggest that TNF is a principal mediator of LPS toxicity. The toxin LPS reacts in both the LAL test and stimulates TNF from macrophages. However, toxins such as TSST-1 from *Staphylococcus aureus* are negative the LAL test but do stimulate TNF. We decided to use an assay to see if *Plasmodium* derived molecules stimulated TNF secretion from macrophages as the basis for identifying possible malaria toxins.

1.6.4 Discovery of TNF

Carswell *et al.*, (1975) discovered that injection of a normally sub-lethal dose of LPS into mice 14-21 days after inoculation with BCG or *C.parvum* induced the animals to go into shock and die. If the mice were bled 2 hours after injection, the serum, when injected into tumour bearing mice, was found to induce necrosis of the tumours and was subsequently named Tumour Necrosis Serum (TNS). The factor responsible for this tumour necrosis was purified to homogeneity, sequenced and cloned (Wang *et al.*, 1985) and is known as Tumour Necrosis Factor (TNF).

The biology of TNF as a mediator of host responses has been extensively reviewed by Beutler & Cerami., (1989). TNF has a sub-unit molecular weight of 17 KD and shows variable species-specific glycosylation, however individual subunits form an active trimer (Jones, Stuart & Walker., 1989). The mature cytokine consists of 156 and 157 amino acids respectively in the mouse and human forms. A conserved region lies near the N-terminus at which point mutations / deletions can alter activity (Mizuno & Soma., 1988). There is a

similar region in lymphotoxin (TNF-β), a T cell product with a similar *in vivo* and *in vitro* range of biological activities (Kelker *et al.*, 1985). TNF and lymphotoxin genes lie close to one another within the major histocompatibility complex (MHC) (Nedwin *et al.*, 1985), (chromosome 6 in man, and chromosome 17 in the mouse). The principal source of TNF is from macrophages, although there are reports of TNF produced by T cells (Sung *et al.*, 1988), B cells, mast cells (Gordon & Galli., 1990) and NK cells (Djeu *et al.*, 1988); lymphotoxin is primarily synthesized by T cells.

TNF does not exist in a stored form. LPS stimulates increased gene transcription, which is detectable in resting macrophages, by a factor of 3, mRNA levels by a factor of 50 - 100, and secreted protein (undetectable in quiescent cells) by a factor of 10,000. TNF synthesis is controlled at multiple levels; macrophages from C3H/HeJ mice, or macrophages from other mice that had been treated with dexamethasone, when stimulated with LPS produce mRNA, although in reduced amount, but do not produce protein (Beutler *et al.*, 1986). I.V injection of TNF into mice shows that it has a half-life of between 6-20 minutes (Beutler & Cerami., 1987). TNF receptors occur upon a wide variety of cells, (Beutler *et al.*, 1985), receptor binding and endocytosis being responsible for its removal from the serum.

These observations have led to the proposal that TNF may act as a growth factor or hormone under certain conditions. So what is TNF really for if its not a recognition system for malignant cells ? The name TNF is now regarded as a misnomer and the physiological role of the factor is still widely debated. Many believe that its primary role may be as a cytokine modulating the immune response to local infections via its actions upon a range of immune cells and upon basement endothelium.

1.7. Relevance of animal models

Research into any human disease can be approached from two angles, either by the careful measurement of the changes that occur during the onset and duration of the disease or by the use of animal models that mimic that disease. Animal models provide a practical method of testing either theories or therapies that cannot be tested in humans. However those working with animals must be able to justify their use.

The growth and toxicity of parasites in non-natural hosts is dependent upon both parasite and host interactions, unfortunately there is no single "ideal" combination of parasite and animal host that mimics the interactions between man and *P. falciparum*. There are a wide variety of animal models employing different combinations of *Plasmodium* parasites and hosts. This has led to an enormous amount of data, of which, some is almost bound to provide evidence for a favoured theory.

The best we can hope for in mouse models is to identify possible causes of pathology, to propose strategies which could block the production of this pathology, and to show that the effective strategies are not in themselves toxic. Ultimately the "relevance" of any animal model can only be judged retrospectively in that it leads to either a better understanding of the disease or to a new form of therapy for the treatment of human infections.

2.0 Materials and Methods

2.1.1 Mice

Outbred females at least 6 weeks old were used (Tuck No 1, A.Tuck & Sons, Battlesbridge, Essex). Female CBA/N, C3H/HeJ, Balb/c and Balb/c athymic nu/nu mice were obtained from the National Institute of Medical Research, Mill Hill, London. First generation (Balb/C x C57/Bl) F1 hybrids used in vaccination experiments were also obtained from the National Institute of Medical Research.

2.1.2 Parasites

We use the non-lethal *P. yoelii 17X*, its lethal variant *P. yoelii YM*, *P. berghei* Anka, and *P. chabaudi adami*. Mice were infected with 10^4 parasitized erythrocytes i.v. and the parasites kept in passage from frozen stocks every 3 months. Parasitaemias were followed on tail-blood films stained with Giemsa.

2.1.3 Assay for macrophage stimulants

Peritoneal cells were collected from mice given either 1 ml of 4 % thioglycollate (DIFCO) i.p. 3-5 days previously, or 10^7 BCG, (Glaxo, Greenford, Essex) 14-21 days previously, by peritoneal lavage using Hanks balanced salt solution (HBSS) containing 1 unit/ml of heparin and 5 µg/ml polymyxin B (Sigma). Washed cells were suspended in RPMI-1640, 5 % fetal calf serum (FCS) and polymyxin B, counted, and dispensed into 96 well microtitre plates (Nunc, Roskilde, Denmark) in 100 µl volumes at between 5 $x10^5$ -1 $x\ 10^6$ cells/well. Cells were incubated for 2-3 hr at 37°C in an atmosphere of 5 % CO_2 in air to allow macrophages to adhere, and for a further 30 minutes with an added 100µl of indomethacin (Sigma) at 2 µg/ml to activate macrophages. Non-adherent cells were removed using HBSS and the medium was replaced with 200µl of RPMI-1640 containing the test stimulants. Next day, macrophage supernatants were collected and assayed for TNF. A 1/10 dilution was made in 5 % FCS and 1 µg/ml emetine (Sigma), and stored at -20°C in case retitration was needed. Macrophages incubated in LPS or medium alone were always included as positive and negative controls.

In some experiments the results are expressed as equivalents of LPS; these values are calculated from comparison of control LPS and exoantigen dose response curves. The dilution of exoantigen preparation was compared with the concentration of LPS that induced the same amount of TNF release.

Human monocytes

Blood was defibrinated and the mononuclear cells, separated by centrifugation over lymphocyte separation medium (Flow laboratories), were cultured for 2 hours in a flask that had been pretreated with autologous serum. Non-adherent cells were washed off and the monocyte-enriched population detached using EDTA. These cells were resuspended in RPMI-1640 + 5% FCS and dispensed into flat bottomed 96 well microtitre plates at concentrations of $5x10^5$ cells/well. The cells were pretreated with recombinant human interferon-γ (100 units/ml) (Genentech) and indomethacin (1µg/ml) for 3 hours before overnight incubation in serial dilutions of the test samples. The following day supernatants were collected, diluted to an initial 1/10 and assayed for TNF content.

2.1.4 Assay of macrophage activation

Macrophages must receive two signals before they are capable of secreting TNF, an activation signal and a stimulus. In the assay described above the activation signal was constant, which allowed me to measure the strength of the stimulus. In this assay the stimulus was kept constant, allowing the measurement of the activation signal. Peritoneal cells were collected from untreated mice as before. Washed cells were suspended in RPMI-1640, 5 % fetal calf serum (FCS) and polymyxin B, counted, and dispensed into 96 well microtitre plates in 100 µl volumes between $5 x10^5$ -1 x 10^6 cells/wells. To these cells was added 100 µl of the "activator / deactivator" under test, for time periods that varied with the type of activator used. In assays where PGE_2 was used, the plates were incubated under aluminium foil as this compound is light-sensitive. Non-adherent cells were removed using HBSS and the medium was replaced with 200µl of RPMI-1640 containing a constant dose of exoantigens. Next day, macrophage supernatants were collected and assayed for TNF.

Indomethacin and PGE_2 were obtained from Sigma and recombinant mouse IFN-γ (Genentech) was a gift from Dr G.R. Adolf, Ernst-Boehringer Institute, Vienna.

2.1.5 TNF assay

TNF was assayed colorimetrically by its cytotoxicity for L-929 cells (from the European Collection of Animal Cell Cultures, Salisbury.). Samples (serum samples or macrophage supernatants) were initially diluted 1/10 in RPMI-1640, 2 % fetal calf serum and 1μg/ml emetine. Subsequent log 4 dilutions were made from this initial dilution in RPMI-1640 and emetine at 1μg/ml, and duplicates of these were overlaid upon monolayers of the L929 mouse fibroblast tumour line for a minimum of 18 hours. The next day remaining, live, tumour cells were fixed using 100% methanol and stained using 1 % crystal violet (Sigma). The dye was eluted using 100 μl/well 33 % acetic acid and plates were read on an ELISA recorder at 570 nm. An example of primary data is shown (Fig. 2.1.6.a.); cytotoxicity curves are illustrated for macrophage supernatants stimulated by dilutions of exoantigens. In this example each dilution of exoantigen was repeated 3 times, and as each repeat was in duplicate, it was possible to combine results and calculate the standard deviations which are shown.

The TNF content of each macrophage supernatant is calculated by plotting the OD (optical density), which is proportional to cell survival, and therefore inversely proportional to cytotoxicity and to the concentration of TNF, against the dilution of that supernatant. TNF secretion is quantified in two ways: as titre (the log dilution at which 50 % of target cells are destroyed) as shown (Figure. 2.1.6.b.), or as U/ml in the original sample, where one unit is defined as that amount of TNF that destroys 50 % of the cells.

Presentation of Data

Results are expressed as titrations either before or after enzymatic digestion or chemical treatment, or in the presence and absence of antibody, inhibitor or activator. In each case I have chosen to present the results from one such experiment, which does not provide any information on how repeatable these experiments were. However no experiment has been included that was not repeated at least 3 times with the same qualitative result. I have not included data from experiments where different

Figure 2.1.6.a. Primary data showing TNF-mediated cytotoxicity of macrophage supernatants stimulated with dilutions of parasite exoantigens

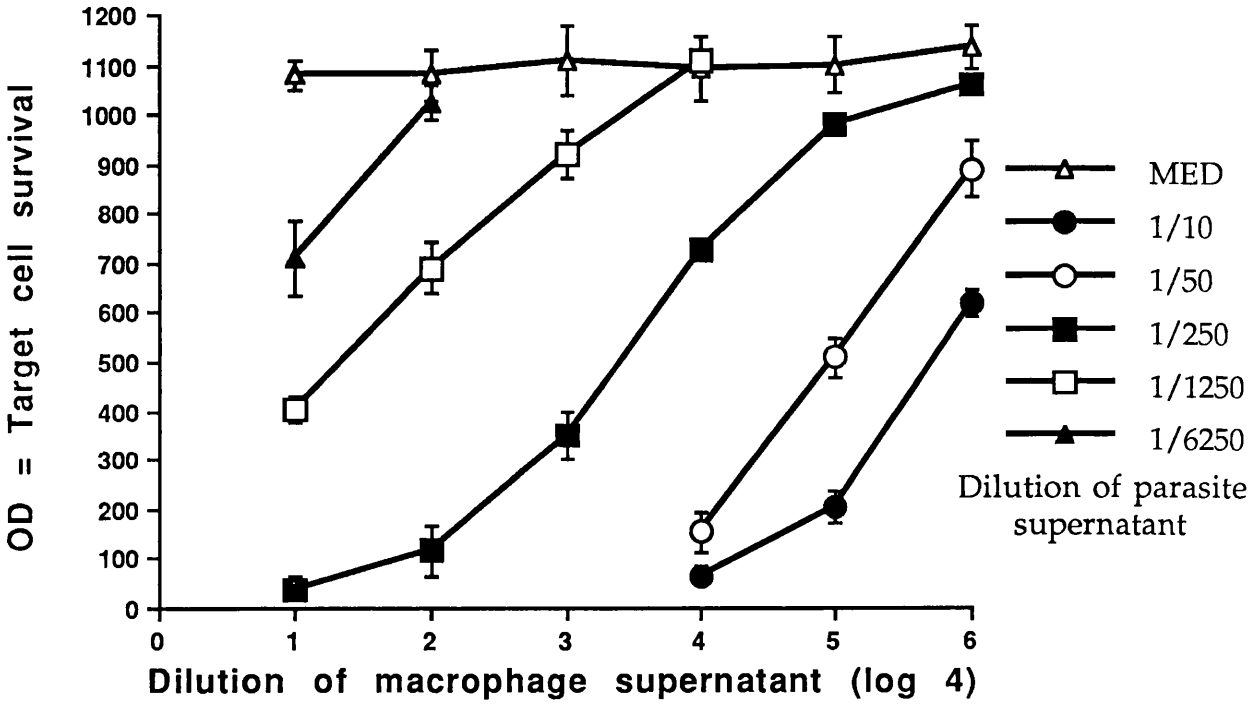
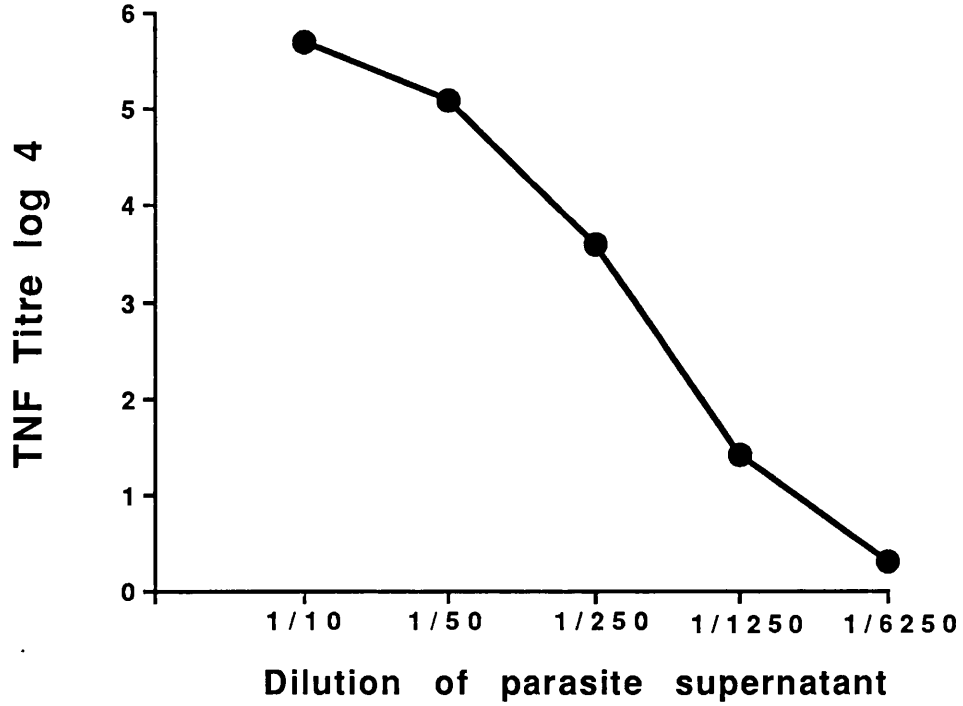


Figure 2.1.6.b. TNF Titres extrapolated from the primary data



preparations gave different results. The limit to the number of macrophages that could be used in any one test did not allow me to repeat titrations within a single experiment. Thus only one well was used per dilution and as macrophage supernatants were titrated in duplicate only there are no error bars. Results from experiments carried out upon different days could not be pooled as there is an inherent variability in macrophage responses which meant, for example, that a positive control (LPS at 1 μg/ml) gave between 5,000 and 20,0000 U/ml of TNF on different occasions.

Assay for TNF in serum

Groups of mice injected with *C.parvum*, 200 μg/mouse (Wellcome Research Foundation, Beckenham. Kent) were given a second injection of either LPS or exoantigens between 9 and 14 days later; 90 minutes after this second injection the mice were bled and their sera assayed individually for TNF on L929 cells.

2.1.6 Toxicity assay in D-galactosamine sensitized mice

Groups of mice were injected i.p. with D-galactosamine (16 mg/mouse, Sigma) as a sensitizing agent. These mice were subsequently injected i.p. with either LPS or preparations of parasite exoantigens 30 minutes later and deaths scored after a further 48 hours. To avoid the use of large numbers of animals groups of 3-5 mice were used and the test was repeated with different samples and the results pooled.

Depletion of CD 4+ T cells: Groups of mice were treated for three consecutive days by injection, first i.v. then i.p. of 400 μg/mouse of a rat monoclonal antibody (YTS 191.1 from Dr. H. Waldmann) against the mouse L3/T4 epitope (Cobbold *et al.*, 1982), before injection of D-galactosamine and parasite exoantigens. This treatment consistently decreased the number of spleen cells staining by FACS analysis from about 40 % to under 1 % (Hutchings & Cooke., 1990).

Inhibitors of exoantigen toxicity: A hamster monoclonal antibody against murine TNF was generously provided by Dr R. D. Schreiber, through the kind offices of Celltech.
Phenoxybenzamine was obtained from the hospital pharmacy.

Indomethacin, Naloxone, Vitamins E & C were obtained from Sigma. Soluble PAF and the PAF antagonist CV3988 were obtained from Novabiochem, Switzerland.

IL-1α, IFN-γ and Mabs to IFN-γ were obtained from Dr A. Heath of this laboratory.

2.1.7 Assay for inhibitors of TNF cytotoxicity

TNF sensitive mouse tumour cells were resuspended in 5 % FCS and dispensed into 96 well microtitre plates in 100µl volumes at 2.5 x 10^4 cells /well. To these were added an equal volume of the test substance in RPMI-1640. The cells were then allowed to adhere and grow overnight. The following day, the medium was removed and serial dilutions of recombinant TNF in 1 µg/ml emetine in RPMI-1640 were overlaid onto the target cells. After a further 24 hours, the surviving cells were fixed and stained and the TNF titres determined.

2.2 Preparation of *Plasmodium* TNF stimulants

2.2.1 Prbcs as a stimulus

Live prbcs: Freshly isolated prbcs, from mice with parasitaemia > 50 % were repeatedly washed before resuspension in RPMI 1640 +/- polymyxin, dilution and incubation with activated macrophages.

Formaldehyde fixed prbcs: Freshly isolated prbcs, from mice with parasitaemia > 50 % were repeatedly washed before incubation in 0.02 % w/v formaldehyde in PBS at 25°C for 30 minutes. Fixed prbcs were pelleted and washed x 3 in sterile PBS + 1 µg/ml polymyxin before dilution and incubation with activated macrophages.

2.2.2 Stimulants collected from supernatants

Soluble antigens from rodent parasites were obtained as previously reported (Bate, Taverne & Playfair., 1988). Parasitized erythrocytes were incubated at 1 x 10^8 prbcs / ml in PBS at 37°C upon rollers. After 24 hours the

remaining cells were removed by centrifugation (10 minutes at 2,000 g) and the supernatants collected, boiled for 5 minutes, centrifuged for 10 minutes at > 5,000 g, filtered and stored at 4°C. After it had been found that the triggering moiety was resistant to digestion with RNases, DNases and proteases it became standard practice to incubate boiled supernatants with these enzymes. This was followed by extensive dialysis, incubation with polymyxin B agarose and filtration through a 0.2 μm millipore filter before further use. In some experiments the medium in which prbcs were incubated was changed from PBS to RPMI 1640 +/- 5 % FCS. In other experiments the culture medium was supplemented with various antibiotics or with tunicamycin (Sigma).

2.2.3 Stimulant extraction from prbcs

Stimulants extracted from pellets: Material pelleted after 24 hour incubation was resuspended in PBS and homogenized using a TRON electronic homogenizer. The resultant homogenate was digested first with a mixture of RNase and DNase, 10 μg/ml for 1 hour at 37°C, and then by pronase E, 10 μg/ml at 37°C overnight. The mixture was boiled to stop the reaction, spun at 5,000 g and the soluble material collected, dialysed, incubated with polymyxin B agarose and filtered.

Stimulants from Triton X-100 lysates: Activity was recovered from the membranes of prbcs extracted with Triton X-100. Prbcs were resuspended in 5μM EDTA, 0. 5 % w/v Triton X-100 and 0.1 M TRIS-HCl at pH 8.0 and homogenized. The subsequent lysate was diluted 1/10 in PBS and digested with a mixture of RNase and DNase, 10 μg/ml for 1 hour at 37°C, followed by pronase E, 10 μg/ml at 37°C overnight, before boiling, dialysis, incubation with polymyxin B agarose and filtration.

Saponin Extraction: Washed prbcs were resuspended in 0.01 % w/v saponin in PBS and incubated at 37°C for 30 minutes. Released parasites were spun out at 2,000 rpm and the supernatant collected and dialysed.

2.2.4 *Plasmodium falciparum* exoantigens

I have had access to preparations from 3 different isolates of *Plasmodium falciparum*, all of which were found to stimulate the production of TNF.

i) A crude lysate obtained from a continuous culture system of *P.falciparum* IGO4 was kindly provided by Dr. D. Kwiatkowski. Purified schizonts were pelleted, resuspended in minimum essential medium (MEM) at 1 x 10^9/ml and incubated at 37°C. After 24 hours the supernatants were collected by centrifugation and boiled.

ii) Soluble antigens from *P.falciparum* Nf 54 obtained from spent medium collected from a continuous flow culture system was kindly provided by Professor G. Targett. The medium was digested with a mixture of DNase and RNase at 10 µg/ml for 1 hour at 37°C, followed by pronase E at 10µg/ml, boiled, centrifuged, concentrated x 10, dialysed, filtered and stored at 4°C.

iii) Affinity purified antigens from *P.falciparum* (F32 Tanzania) culture medium reacting with IgG from clinically immune Liberian adults and identified by CIE, (crossed immuno electrophoresis) were eluted from malaria specific columns (Jakobsen *et al*, 1990) were kindly provided by Dr P. Jakobsen.

2.3 Enzymatic digestion and chemical treatment of exoantigens

2.3.1 Enzymatic Digestions

Digestion with proteinases: Exoantigen preparations in PBS were incubated with either trypsin (Sigma, 100 µg/ml), papain (Sigma, 10 µg/ml), proteinase K (Sigma, 10 µg/ml) or pronase E (Sigma, 10 µg/ml) for between 2 - 24 hours at 37°C. The reactions were terminated by boiling the enzymes and dialysis. Dialysed supernatants were incubated with polymyxin B agarose and filtered.

In each of the following enzymatic digestions the reaction was terminated by a 1/10 dilution in PBS and further digestion with pronase E at 10 μg/ml, 37°C for 18 hours, followed by boiling, dialysis, incubation with polymyxin B agarose and filtration.

Mixed Nucleases: (Sigma) Exoantigen preparations that had been dialysed against H_2O were resuspended in TNE buffer, (TNE = 10 mM TRIS, 10 mM NaCl & 1 mM EDTA) at pH 8. RNase and DNase were added at a final concentration of 10μg/ml and incubated at 37°C for 2 hours.

Mixed glycosidases: (Seikagaku Kogyo Ltd. Tokyo, Japan). A mixture of glycosidases extracted from *Charonia lampas* were used. Exoantigen preparations were diluted in 0.01M CH_3COONa, pH 4 and digested at between 2 mg/ml and 500 μg/ml for between 2-24 hours at 25°C.

Mixed lipases: (Sigma) A mixture of triacyl lipases and acyl hydrolases from *Pseudomonas* were used. Exoantigens were diluted in 0.2 M TRIS, 0.02M $CaCl_2$ and 10 μg/ml BSA at pH 7. Lipases were used at a final concentration of 10 μg/ml at 37°C for between 2 and 18 hours.

Alkaline phosphatase: (Sigma) Extracted from bovine intestinal mucosa. Test samples were diluted in 0.1 M glycine, 0.001 M $MgCl_2$ and 0.001 M $ZnCl_2$ at pH 10.4. Alkaline phosphatase was added to a final concentration of 5U/ml and exoantigens digested at 37°C for between 2 and 18 hours.

Phospholipase D: (Sigma) Extracted from *Streptomyces chromofuscius.* Freeze dried exoantigens were resuspended in 0.02 M $CaCl_2$, 0.2 M TRIS, 0.02 % Triton X-100 at pH 8. To these exoantigens was added phospholipase D at 10 U/ml which was incubated at 37°C for 18 hours.

Phospholipase A_2 : (Sigma) Extracted from bovine pancreas was diluted in 0.1 % DOC, 0.02 mM Hepes and 0.02 M $CaCl_2$, pH 8 at 5 U/ml. Exoantigen preparations were added and incubated at 37°C for 18 hours.

Phospholipase B: (Sigma) Extracted from *Vibrio* species. Exoantigen preparations were resuspended in 0.2 M TRIS, 0.02M $CaCl_2$, and 50 µg/ml BSA at pH 7. Enzymes were added at 10 U/ml at 37°C for 18 hours.

Phospholipase C: (Sigma) Extracted from *Clostridium welchi*. Exoantigens were incubated in 10 mM $CaCl_2$ 1 mM ME, 0.05 % Triton X-100 at pH 7.3 Phospholipase C was added at a final concentration of 10 U/ml and digestion was carried out at 37°C for 18 hours.

2.3.2 Chemical treatment

Oxidation with sodium periodate: Freeze dried supernatants, pellets or lysates were resuspended in 0.05 M $NaIO_4$ (BDH) at 37°C for 2 hours. The reaction was quenched with excess glucose (1.0 M), dialysed, incubated with polymyxin B agarose and filtered before testing.

Deglycosylation: Deglycosylation was carried out to a modification of the method of Edge *et al*, (1981). Lyophilized exoantigens were resuspended in a reaction mixture of 180 µl trifluoromethanesulphonic acid (TFMS) (Sigma) and 20 µl anisole (BDH). The solution was saturated with nitrogen for 1 minute and kept upon ice for 3 hours, the solution was vortexed every 30 minutes. The reaction was stopped either by:

(a) addition of of 4 mls of di-ethyl ether/pyridine (9/1, cooled to - 40°C). The pellet was deposited by centrifugation at 10,000 g and redissolved in PBS, which was neutralized with NaOH and tested. The ether/pyridine soluble material was dried under a stream of N_2, resuspended in PBS, and neutralized.

(b) or dilution in PBS and neutralization with NaOH. The pellet was removed by centrifugation at 5,000 g and the remaining soluble material tested.

Samples from both (a) and (b) were incubated with polymyxin B agarose and filtered before testing.

Deacylation by mild alkaline hydrolysis: Freeze dried supernatants, pellets or lysates were resuspended in 0.05 M NaOH (BDH) and incubated at 56°C for 2 hours before neutralization with dilute HCl. Neutralized samples were either tested directly, tested after dialysis against PBS or extracted into solutions of chloroform, water and methanol and both phases tested. All samples were incubated with polymyxin B agarose and filtered before testing.

Dephosphorylation by hydrofluoric acid: 1-10 mg/ml of lyophilized exoantigens were dissolved in 48% (vol/vol) hydrofluoric acid (BDH), and incubated for 20 hrs at 0°C. The reaction was stopped by neutralization with concentrated NaOH. Neutralized samples were either tested directly, tested after dialysis against PBS, or extracted into solutions of chloroform, water and methanol. Each preparation was subjected to incubation with polymyxin B agarose and filtration before testing.

Deamination by nitrous acid: Lyophilized supernatants were dissolved into a buffer containing 25 mM CH_3 COONa, 160 mM $NaNO_2$ (BDH), at pH 3.5 and incubated for 5 hours at 22°C. The reaction mixture was diluted 1/10 and neutralized with either TRIS or NaOH and pelleted material removed by centrifugation. Neutralized samples were either tested directly, tested after dialysis against PBS, or extracted into solutions of chloroform, water and methanol before testing. Each preparation was subjected to incubation with polymyxin B agarose and filtration before testing. Control lyophilized supernatants were dissolved in the same buffer and pH without the addition of $NaNO_2$.

2.3.3 Preparative Techniques

Freeze drying: An exoantigen preparation (normally predigested with nucleases and proteases and dialysed against H_2O) was frozen to -70°C. The frozen supernatants were placed in a vacuum pump and dried at -50°C. The lyophilized powder was collected after 24 hrs or after the process was complete and resuspended in sterile buffer.

Lipid extraction: The test sample was dialysed against H_2O (except after certain chemical or enzymatic digestions where the generation of an active molecule of < 10 KD was suspected) and mixed with methanol and chloroform in the proportions 1 : 2.5 : 1.25 and homogenized. A further 1.25 volumes of chloroform was added to the sample, followed by a further 1.25 volumes of H_2O and further homogenization. The mixture was left to separate into two phases; on top is the aqueous phase consisting of methanol and water into which the majority of proteins and nucleic acids dissolve. The lower layer consists of chloroform and water into which lipids and other highly hydrophobic molecules dissolve (non-aqueous phase/lipid extract). The aqueous phase was dialysed against PBS before testing. The lipid extract was dried under a stream of nitrogen in a 37°C waterbath, and the residue dissolved in sterile PBS. Delipidated BSA at 20µg/ml was sometimes used to increase the solubility of the lipid extract in PBS.

2.4 Production of Antisera and Vaccination

2.4.1 Production of inhibitory antiserum

Primary antisera: Mice were injected i.p. with 0.2 - 0.5 ml of treated supernatants and bled after different time intervals. Serum from 3 or more mice was collected, pooled, and heat-inactivated at 56°C for 20 minutes. Control serum was collected from groups of mice injected with either PBS or supernatants from uninfected rbcs. To test the ability of antisera to inhibit the induction of TNF by the exoantigens, log dilutions were incubated with macrophages with an equal volume of a constant dose, either of an exoantigen preparation or of LPS. Percentage inhibition was calculated by reference to the control concentration of exoantigens or LPS in medium. The inhibitory titre of antiserum is defined as the reciprocal of the dilution at which that antiserum inhibits 50% of TNF release.

Hyperimmune sera: Made against lethal *P.yoelii* were kindly provided by Mr. J.B. de Souza of this department. They were obtained from mice that had been vaccinated with Triton X-100 lysates of parasitized erythrocytes, injected with saponin as an adjuvant, then infected and bled 5-10 days after recovery (Playfair & de Souza., 1986).

Absorption of antisera: Antiserum diluted to 1/50 was incubated with 1×10^9 uninfected rbcs /ml for 30 minutes at 22°C. Intact cells were removed by centrifugation and the remaining antibodies collected in the supernatant. This absorption was repeated at least 4 times, after which the absorbed serum was titrated in parallel with an unabsorbed control.

Isotype depletions and elution: Antisera were depleted of IgG or IgM on isotype specific columns (Immobilized anti mouse immunoglobulins, Calbiochem). IgG and IgM fractions were then recovered by elution with 0.1 M glycine-HCl buffer at pH 2.5, collected into 1.0 M TRIS buffer at pH 8 and dialysed against PBS before testing.

2.4.2 Vaccination with exoantigens

Mice were injected i.p. with 0.5 ml of crude boiled parasite supernatants. 12 days later they were infected with 1×10^4 *P.yoelii* and the parasitaemias followed by examination of blood films. Control mice were injected with supernatants from uninfected rbcs.

Other reagents

Sigma reagents

Lipopolysaccharide (LPS) (phenol extract of *Escherichia coli* 055:B5)
Polymyxin B sulphate and Polymyxin B agarose.
Tunicamycin.

Amicon filters with cut off points at 30 and 10 KD were obtained from Amicon, Stonehouse, Gloucester.
Saponin and Triton X-100 were obtained from from BDH, Poole Dorset.

3.0 Identification of *Plasmodium* derived macrophage stimulants

3.1 Introduction

It has been suggested that toxins could play a role in the production of disease in malaria, and that such a toxin might be a molecule that stimulates macrophages to secrete TNF. One of the criteria that is required to validate such a hypothesis would be to show that *Plasmodium* parasites stimulate macrophage secretion of TNF; in this chapter I have explored such a possibility. Unless otherwise stated all the following results were obtained using lethal *P.yoelii* infected prbcs.

3.2 Results

3.2.1 TNF secretion by macrophages stimulated by prbcs

Preliminary experiments were designed to see if parasitized erythrocytes co-cultured with activated macrophages could stimulate these cells to secrete TNF. Typical data from one such experiment shows the units of TNF secreted by 5×10^5 BCG activated macrophages incubated with *P. yoelii* prbcs (Fig 3.2.1.a). Parasitized erythrocytes stimulated yields of TNF from macrophages in a dose dependent manner. The maximum amount of TNF secreted was similar to that stimulated by 1 μg/ml of LPS, included as a positive control. Stimulation of macrophages by parasitized erythrocytes was not inhibited by inclusion of 5 μg/ml of polymyxin B in the diluent. Polymyxin B is a small molecular weight peptide antibiotic that binds to the active site of LPS (Lipid A) and inhibits its biological actions.

We wanted to see if the pathogenicity of lethal and non-lethal malaria parasites was related to their ability to trigger macrophages to secrete TNF. All strains of parasite tested, at a density of 5×10^6 infected erythrocytes / well, stimulated the production of TNF from activated macrophages. Exact comparison is not valid as the stage of development, day after infection and parasitaemia would all have been different between the different species in these tests, but approximately equal amounts of TNF were usually observed (Fig 3.2.1.b.). Uninfected erythrocytes from either sheep or from mice incubated with macrophages did not stimulate the production of TNF.

Figure 3.2.1.a. Secretion of TNF by BCG macrophages incubated with P.yoelii prbcs in the presence and absence of 5µg/ml of polymyxin B

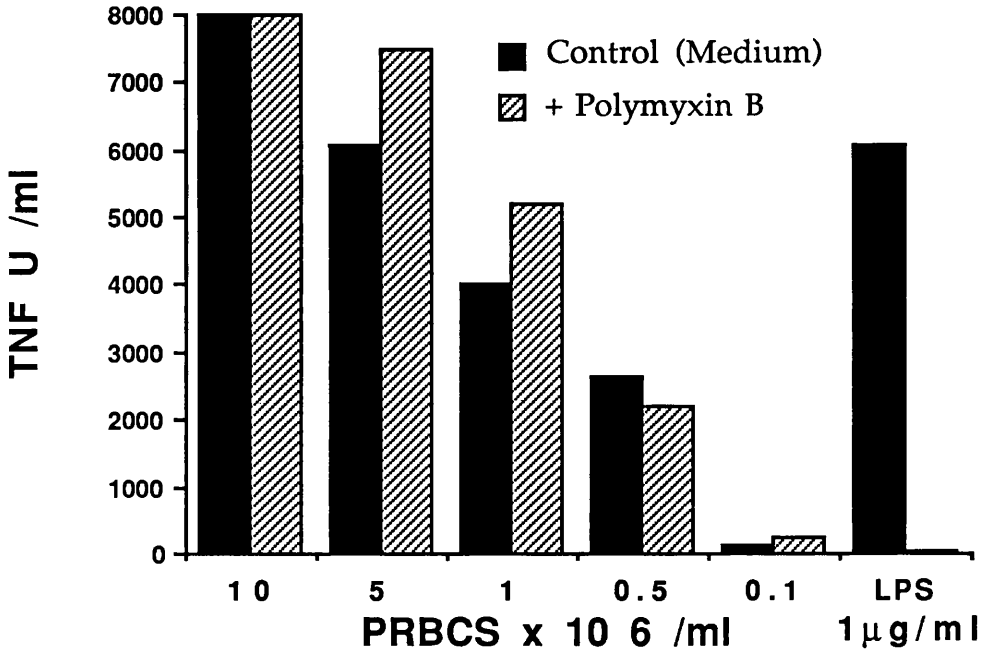
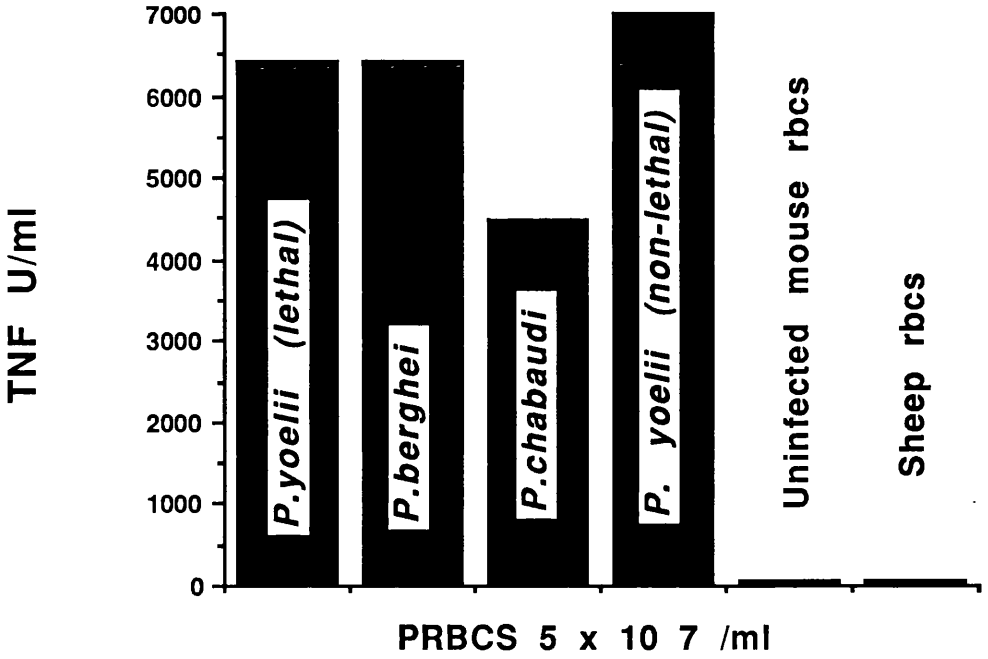


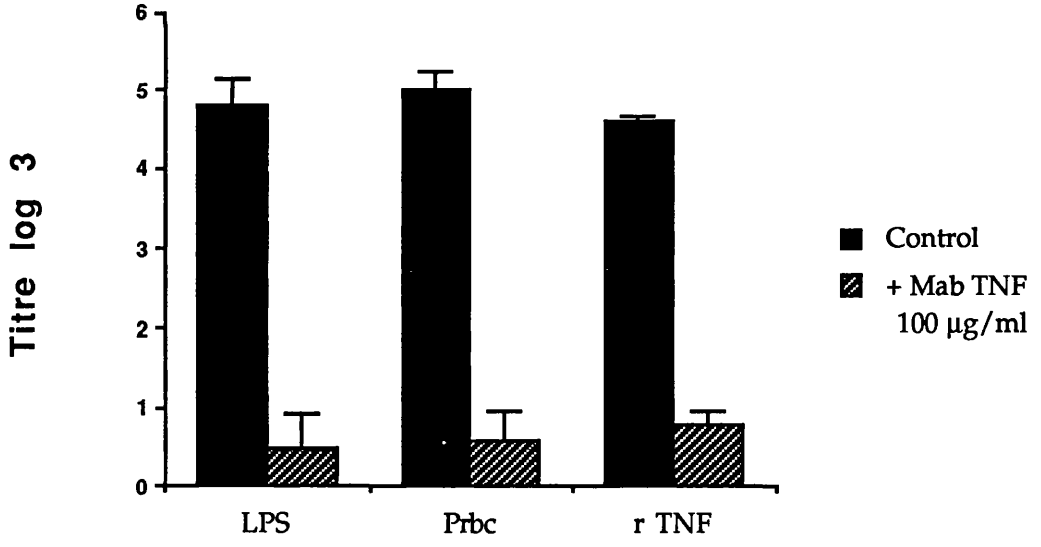
Figure 3.2.1.b. TNF secreted by BCG macrophages after incubation with 5 x 10 7 prbcs/ml infected with different Plasmodium species



3.2.2 Inhibition of L929 cytotoxicity by a Mab to TNF

Both TNF-α and TNF–β (lymphotoxin) are cytotoxic for L929 cells. We wanted to show that the cytotoxicty for L929 cells observed in supernatants from stimulated macrophages was indeed due to TNF–α. The cytotoxicity of supernatants that had been obtained from macrophages stimulated by either LPS or *P.yoelii* infected prbcs as well as the cytotoxic action of recombinant mouse TNF, was blocked by a monoclonal antibody specific for TNF-α (100μg/ml of IgG, Hamster anti-mouse TNF monoclonal) (Fig 3.2.2.a.) which reduced cytotoxicity by at least 4 log dilutions. This showed that the destruction of L929 cells was specifically mediated by TNF-α. The cytotoxicity of macrophage supernatants stimulated by prbcs was consistently inhibited by the inclusion of either monoclonal or polyclonal antisera to TNF.

Figure 3.2.2.a. Cytotoxicity of r TNF, and of macrophage supernatants stimulated by P.yoelii prbcs or LPS titrated in the presence and absence of a Mab to TNF.



3.2.3 Full dose response curve to prbcs

The effect of a wider range of concentrations of prbcs was next studied and the results illustrated in (Fig 3.2.3.a.). These results are from a representative experiment where the lethal variant of *P.yoelii* at a parasitaemia of 46 % was diluted in medium containing polymyxin B and incubated at different densities with $1x10^6$ BCG activated macrophages / well. The bell-shaped curve shown is typical and was reproduced using both the non-lethal *P. yoelii* and *P.berghei*. Although the peaks of stimulation were variable, they generally occurred between ratios of 5:1 and 1:4 prbcs / macrophage. This reduction in the amount of TNF secreted from macrophages incubated with higher ratios of prbcs : macrophage was a constant but unexplained finding.

3.2.4 TNF secretion by macrophages stimulated by fixed prbcs

The observation that fewer than 1 prbc : macrophage still stimulated high levels of TNF suggested that a soluble product might be involved. To investigate this possibility, live and formaldehyde fixed *P. yoelii* infected prbcs were titrated for their ability to stimulate activated macrophages. The TNF levels obtained in one such experiment are compared (Fig 3.2.4 a). Parasitized rbcs that had been fixed with formaldehyde still stimulated TNF secretion, but to a lesser extent than a similar number of untreated prbcs. The differences between live and fixed parasites was variable and in some experiments little difference was seen. This was not due to leakage of formaldehyde from the prbcs poisoning the macrophages, as the same fixed prbcs did not abolish their response to LPS (data not shown).

If the molecules of parasitized erythrocytes that trigger macrophages were not released into the supernatant, then the triggering of macrophages by prbcs might be inhibited by addition of an excess of uninfected erythrocytes. Titres of TNF stimulated by titrations of live prbcs were not altered by culture in a diluent containing excess nrbcs at 1×10^7 /ml (Fig 3.2.4.b.). However nrbcs at 1×10^7 /ml did inhibit the triggering of macrophages by fixed prbcs.

Figure 3.2.3.a. Secretion of TNF by BCG activated macrophages stimulated by different ratios of P.yoelii-infected prbc : macrophage

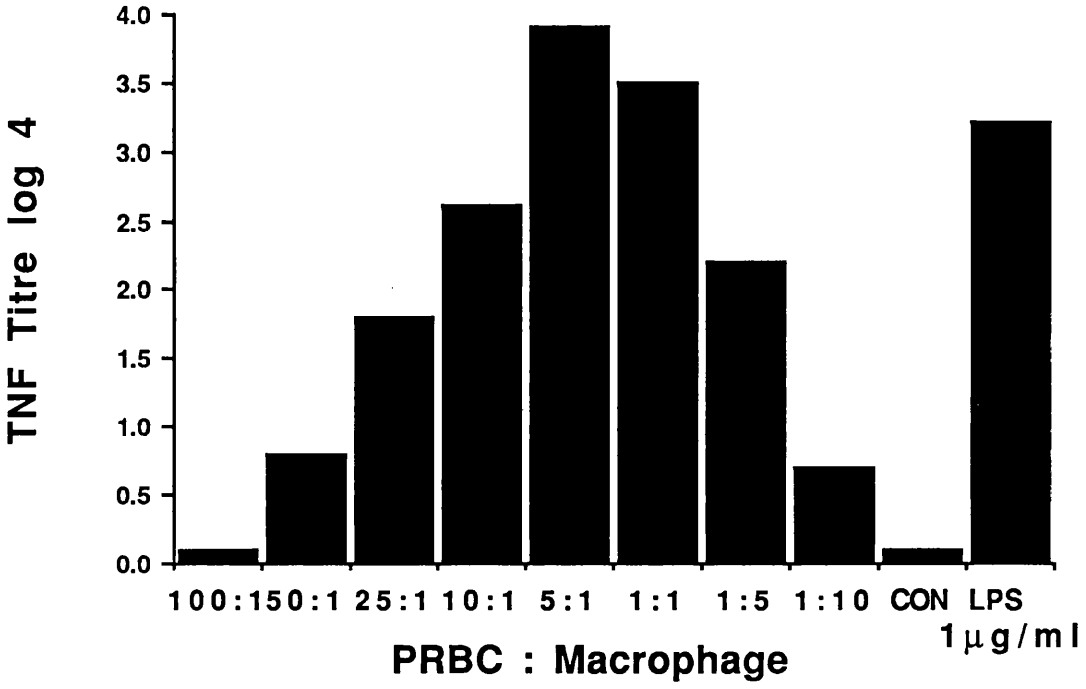


Figure 3.2.4.a. Comparison of amounts of TNF secreted by activated macrophages when incubated with live and fixed P.yoelii-infected prbcs.

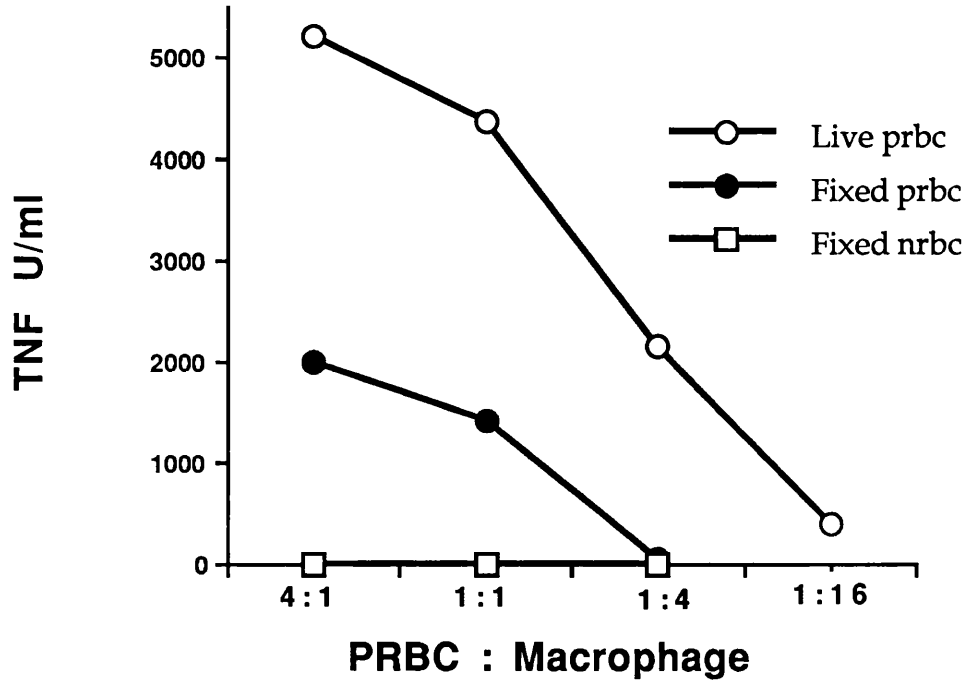


Figure 3.2.4.b. Titrations of TNF obtained when live and fixed prbcs were incubate with activated macrophages in the presence and absence of an excess of nrbcs

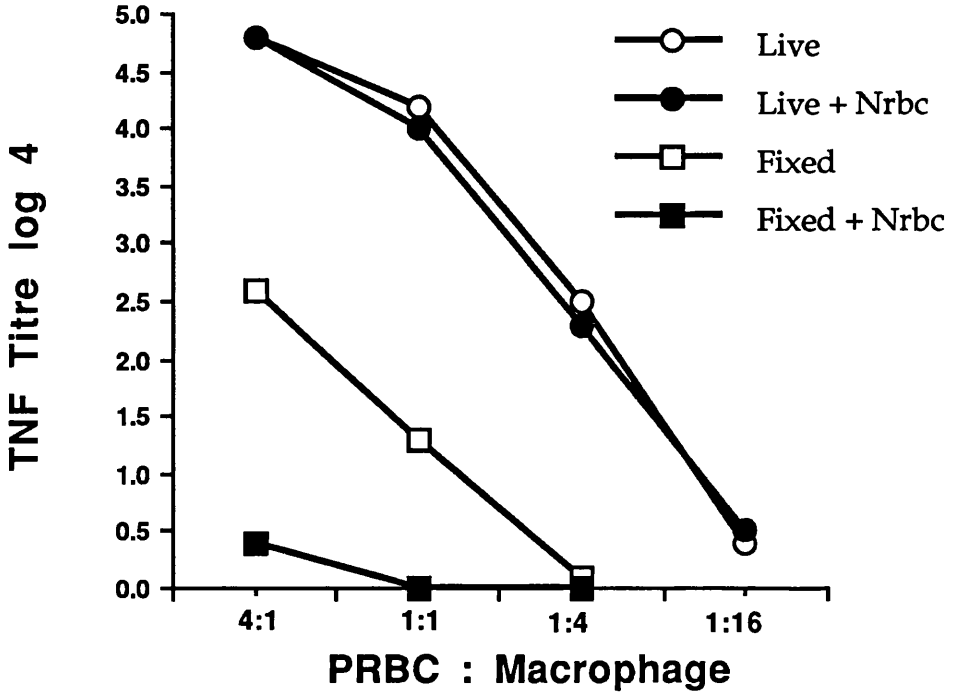
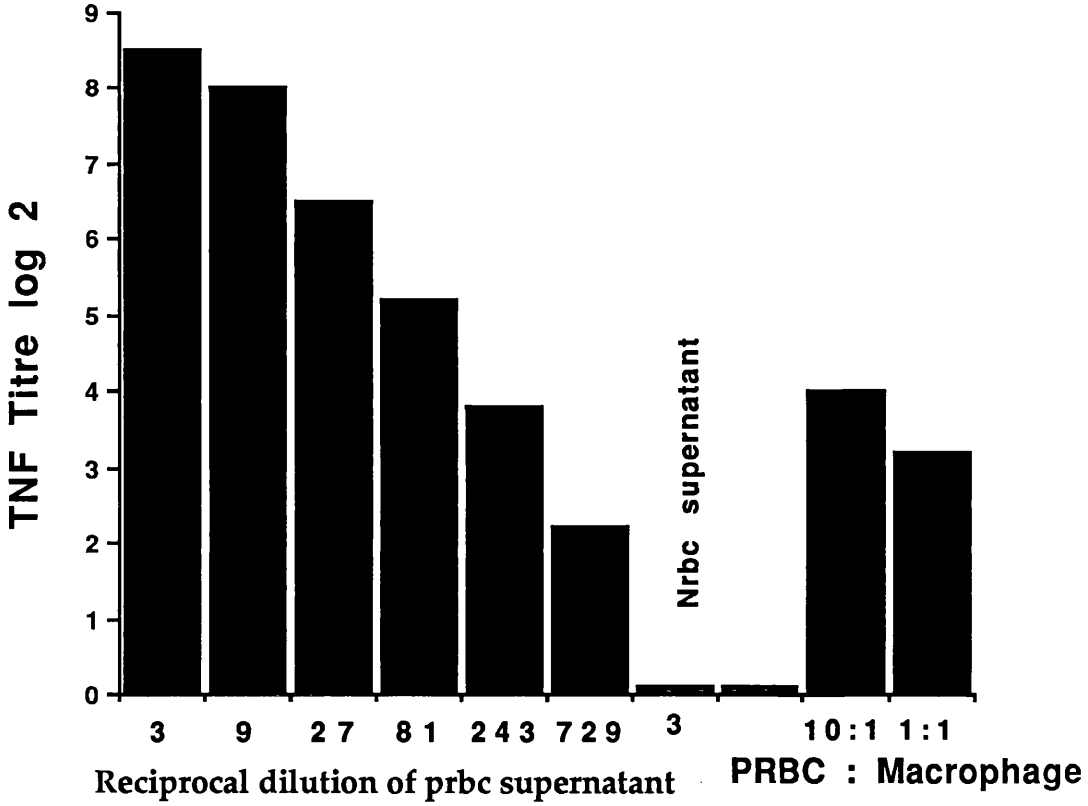


Figure 3.2.5.a. TNF titres obtained from activated macrophages in response to live prbcs and to dilutions of a supernatant from P.yoelii infected prbcs



3.2.5 Molecules stimulating TNF secretion are released from prbcs into the supernatant medium

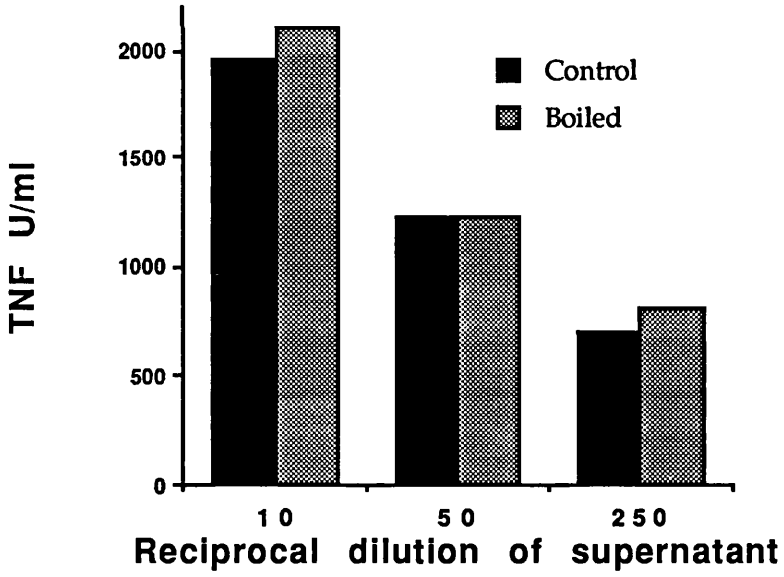
The previous results strongly suggested that molecules capable of stimulating macrophages are released from parasitized erythrocytes. Tests were therefore performed with supernatants collected after 24 hour incubation of prbcs at 1 $x10^8$/ml in PBS. Supernatants were incubated with macrophages at the dilutions shown and the titres of TNF secreted by $5 x10^5$ activated macrophages recorded (Fig. 3.2.5.a.). TNF titres were compared to those stimulated by 2 concentrations of parasitized erythrocytes. Culture supernatants were found to stimulate TNF secretion in equivalent or greater amounts than live prbcs. Control supernatants made from uninfected erythrocytes incubated under parallel conditions did not trigger TNF secretion.

I concluded that some of the molecules responsible for stimulating macrophages were released into the supernatant during overnight incubation, and these are referred to as **exoantigens**, (Chapter 7.0. shows that these molecules are indeed antigenic). This was a fortunate development as it allowed previous preparation of triggering molecules rather than a reliance upon mice with high parasitaemia upon the day of assay. Parasite supernatants that stimulated macrophages could be prepared from prbcs infected with all rodent parasite strains tested.

3.2.6 The triggering moiety is heat stable

The parasite supernatants collected are likely to include a wide range of different constituents. As a preliminary means to purification it was decided to establish the heat-sensitivity of the triggering moiety. Supernatants were boiled for 5 minutes at 100°C, after which significant amounts of material were pelleted upon centrifugation. Titrations of a supernatant in 5μg/ml polymyxin B before and after they had been boiled were compared (Fig 3.2.6.a.): a similar dose response curve was produced by both samples indicating there was not a loss of activity. This experiment has been repeated several times with supernatants from different parasite species, and on no occasion was a significant loss of triggering activity seen. The boiling of supernatants and subsequent removal of denatured material in the pellet is now a matter of routine.

Figure 3.2.6.a. Comparative titrations of the triggering activity present in a supernatant from P.yoelii prbcs before and after boiling.



Separation of the parasite exoantigens was attempted by using a Sephacryl S-200 column, which separates molecules by their molecular weight and by isoelectric focusing on a 110 ml liquid (LKB) column. Under these conditions no purification of the triggering moiety was seen, in that activity was observed over a broad range of fractions.

3.2.7 Conditions for the production of exoantigens

By manipulation of the medium in which the parasites were incubated, parasite survival could be altered and at the same time any correlation with the production of triggering activity could be assessed. In a typical experiment, the triggering activity collected in both (a) the supernatants and (b) pellets from prbcs incubated in RPMI, PBS or RPMI + 5% FCS was measured (Fig 3.2.7.a.). There was triggering activity in both the supernatants and the pellets of prbcs maintained in the different media. However, whereas most of the triggering activity was released into the supernatant during incubation in PBS, it was retained in the pellet when prbcs were incubated in RPMI + 5% FCS. By comparison with a LPS dose-response curve, the activity in such supernatants and pellets were expressed as LPS equivalents (Fig 3.2.7. b.). These results suggest that incubation in different media does not greatly affect the amount of triggering activity produced, and to maximise the yield in the supernatant PBS was henceforth used as routine.

Injection into mice of 0.5 ml of the prbc suspension after 24 hour incubation only initiated an infection when prbcs were cultured in 5 % FCS (data not shown). The results suggest that the production of triggering exoantigens does not depend upon the survival of the parasites. This led to another question: are the exoantigens already present in the prbcs and merely released cells disrupt during the 24 hour incubation period, or is there active synthesis of the exoantigens during that 24 hours ?

Figure 3.2.7.a. A comparison of the triggering activity released into (a) the supernatant and (b) extracted from pellets after overnight incubation of P.yoelii in different media

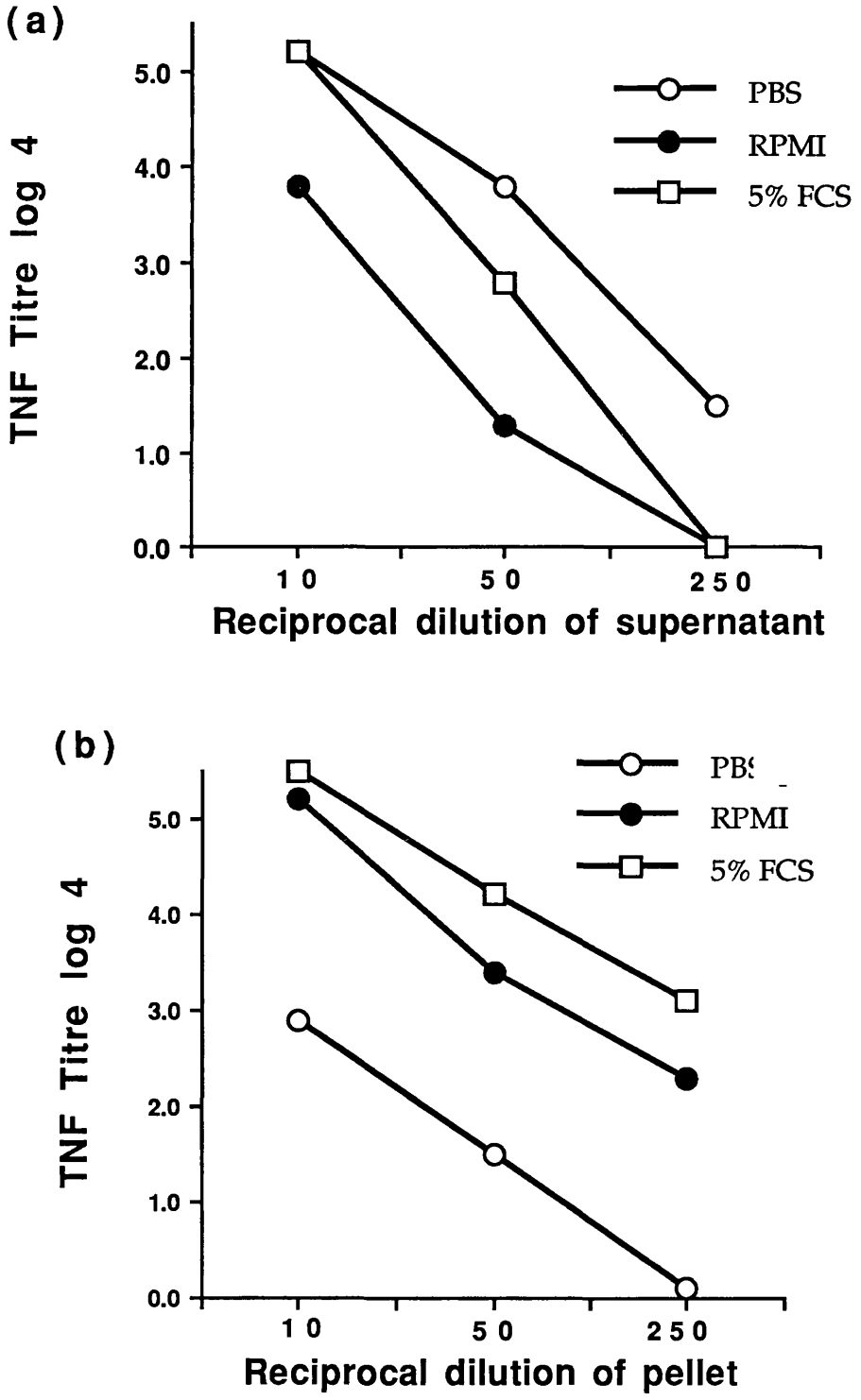


Figure 3.2.7.b. Triggering activity, expressed as equivalents of LPS, extracted from P.yoelii infected prbcs after 24 hr incubation in different culture media

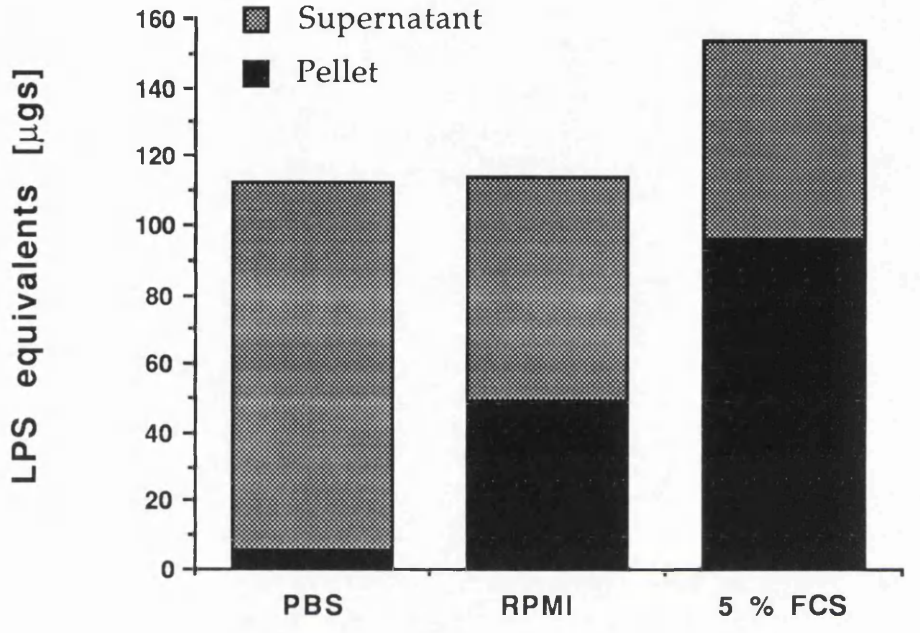
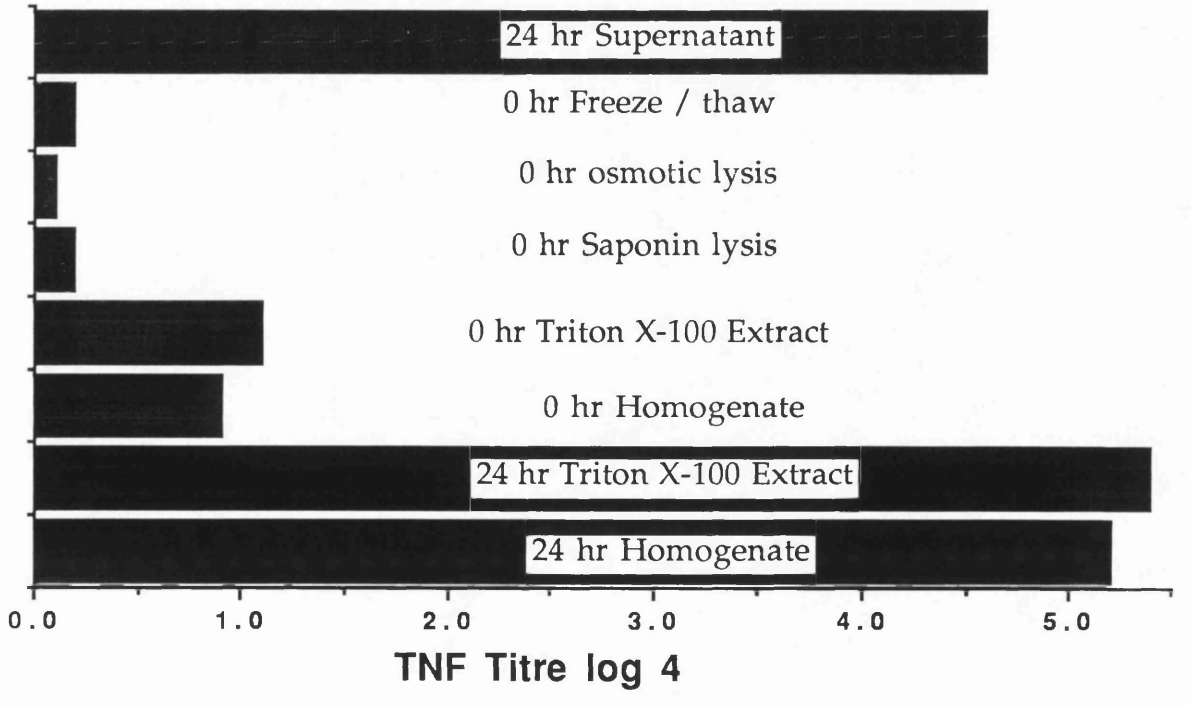


Figure 3.2.8.a. Comparison of TNF titres obtained from activated macrophages stimulated by a 1/10 dilution of treatments of P.yoelii infected prbcs.



3.2.8 Extraction methods for the maximum yield of triggering activity

Freshly isolated prbcs were resuspended at $1x10^8$ /ml under the different conditions. Preparations obtained were diluted 1/10 in culture medium and overlaid onto activated macrophages, 24 hours later these macrophage supernatants were collected and assayed for TNF (Fig 3.2.8.a.). Preparations obtained after osmotic lysis of prbcs by resuspension of prbcs in distilled water or saponin extraction of prbcs induced little TNF. Mechanical disruption and rapid freeze thawing of freshly isolated prbcs also gave rise to preparations with little triggering activity. The homogenization or extraction with Triton X-100, of freshly isolated prbcs also resulted in low triggering activity; however, when prbcs were incubated for 24 hours before homogenization or extraction with Triton X-100 the triggering activity was always greatly enhanced. The results of one typical experiment are shown; this has been repeated 4 times and upon each occasion the same trend was observed.

The simplest interpretation of these results is that the molecule responsible for the triggering activity is not present in large amounts in freshly isolated prbcs, and that during the 24 hr incubation more is synthesized. An alternative hypothesis is that the trigger activity is present upon isolation but not extractable, and that during the 24 hr incubation sufficient cell breakdown or enzymatic degradation occurs to allow its release. Although this has not been disproved, supernatants isolated by either mechanical homogenization (Fig. 3.2.8.b.) or after extraction with Triton X-100 (Fig. 3.2.8.c.) showed an increase in activity after 24 hr incubation. The results of one set of typical experiments are illustrated; they have been repeated for several preparations involving different species of parasites. There is an absolute requirement for cell integrity since if the prbcs were homogenized at T=0, and the homogenate incubated for 24 hrs no increase in activity was seen. In a similar manner there was no increase in triggering activity if prbcs were incubated in the presence of the metabolic inhibitor sodium azide at 0.02 % (data not shown). Uninfected erythrocytes incubated under identical conditions and either homogenized or extracted with Triton X-100 never stimulated TNF secretion from macrophages.

Figure 3.2.8.b. A comparison of TNF titres obtained from activated macrophages stimulated with P.yoelii prbcs homogenates from different time intervals

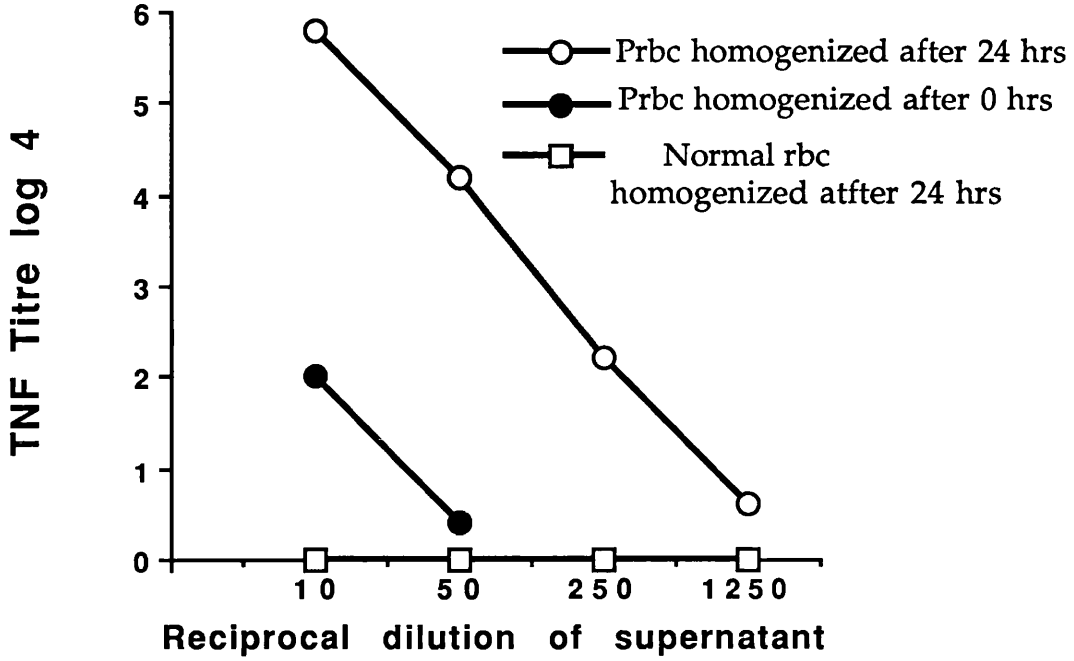
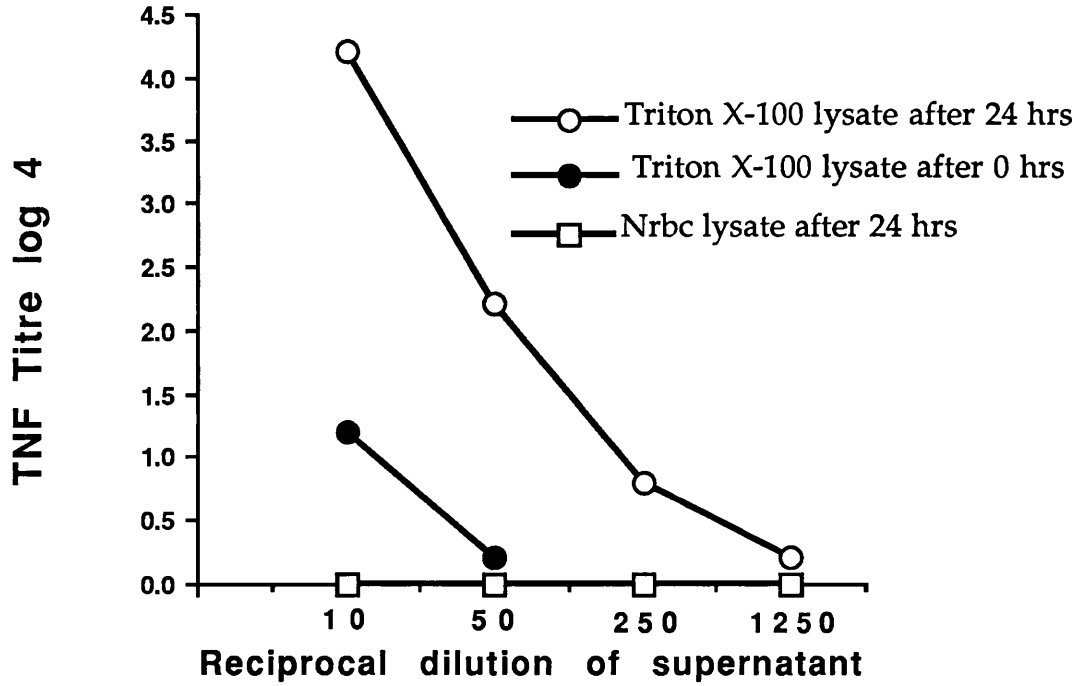


Figure 3.2.8.c. TNF titres obtained from activated macrophages stimulated with Triton X-100 lysates from P.yoelii infected prbcs and nrbcs



Several experiments have shown that the production of exoantigens is temperature dependent. Prbcs were incubated at different temperatures for 24 hours and triggering activity present in the supernatants and from homogenized pellets was then assayed (Fig. 3.2.8.d.). At 37°C triggering activity was released into the supernatant and was found in the pellet; in contrast at 4° C no triggering activity was released into the supernatant, but some was found in homogenized pellets. In each experiment there was always greater triggering activity in the supernatants and pellets when prbcs were incubated at 37°C. This provides further evidence that the "triggering molecules" are synthesized during the 24 hour incubation period and that their synthesis is dependent upon some metabolic activity rather than culture media conditions.

3.2.9 TNF secretion is stimulated by *Plasmodium* derived material and is not due to contaminants

One of the early concerns in this work was to confirm that the molecules triggering TNF release were derived from *Plasmodium* parasites themselves and not from any possible contaminants. If so the amount of triggering activity in a supernatant should show some proportionality to the number of prbcs. Comparison of TNF titres stimulated by a 1/10 dilution of culture supernatants from two species of parasite incubated at different densities shows that this is the case (Fig 3.2.9.a.).

The most likely contaminant known to stimulate macrophages is LPS from Gram -ve bacteria. Titres of TNF from $1x10^6$ resting macrophages, isolated from both outbred and C3H/HeJ mice, a strain of mouse that is hyporesponsive to LPS, stimulated by either LPS or a parasite supernatant are compared (Fig 3.2.9.b). LPS at 1µg/ml stimulated TNF secretion by macrophages from the outbred mice but not those of the C3H/HeJ mice, while a 1/20 dilution of parasite supernatant stimulated TNF secretion from both outbred and C3H/HeJ mouse macrophages. The clear differences between the C3H/HeJ macrophage responses to LPS and to the parasite supernatant suggest that they are separate molecules.

Figure 3.2.8.d. Titres of TNF obtained from activated macrophages stimulated by supernatants and pellets after P.yoelii prbcs were incubated overnight at different temperatures

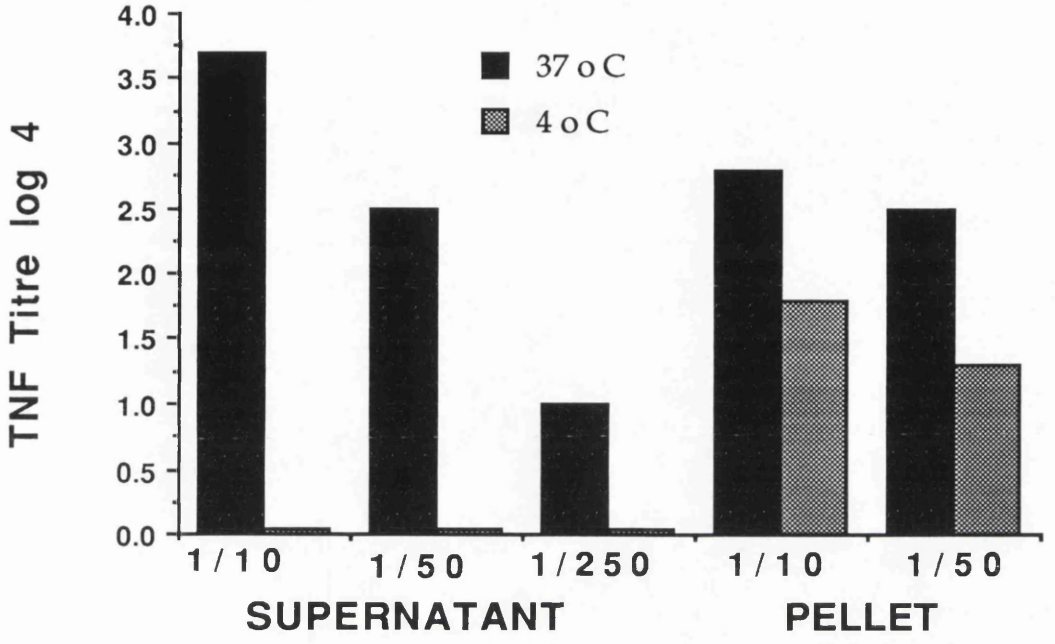


Figure 3.2.9.a. Titrations of the triggering activity released into the supernatant after incubation of prbcs at different densities

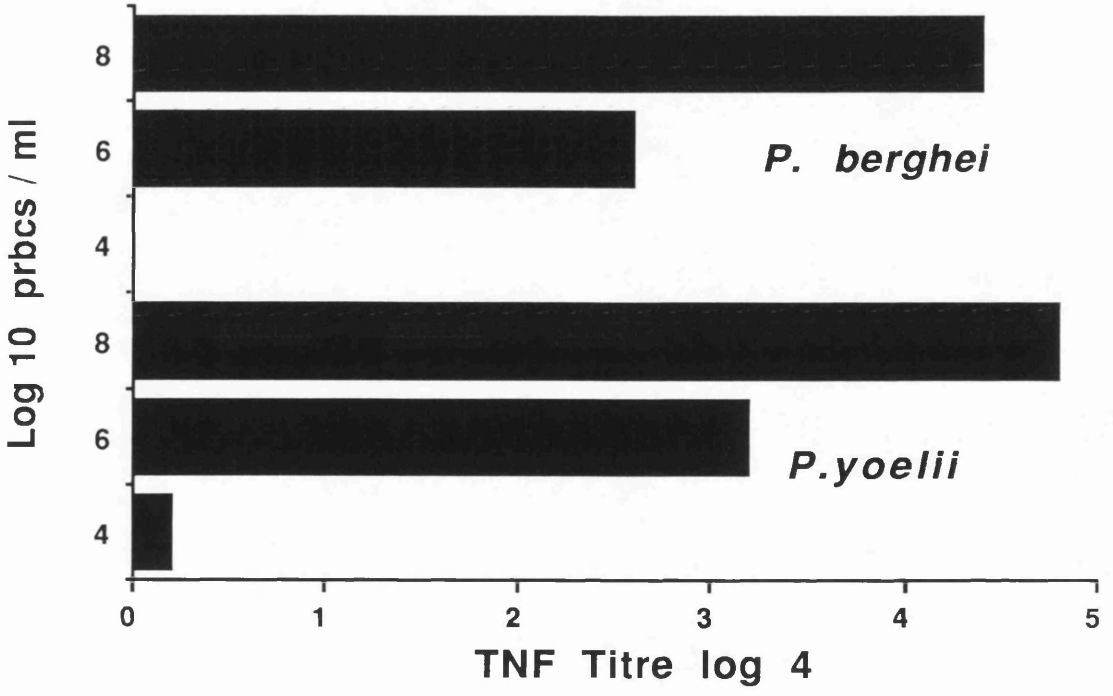


Figure 3.2.9.b. Comparison of TNF titres stimulated by LPS and P.yoelii exoantigens from macrophages isolated from outbred and C3H/HeJ mice

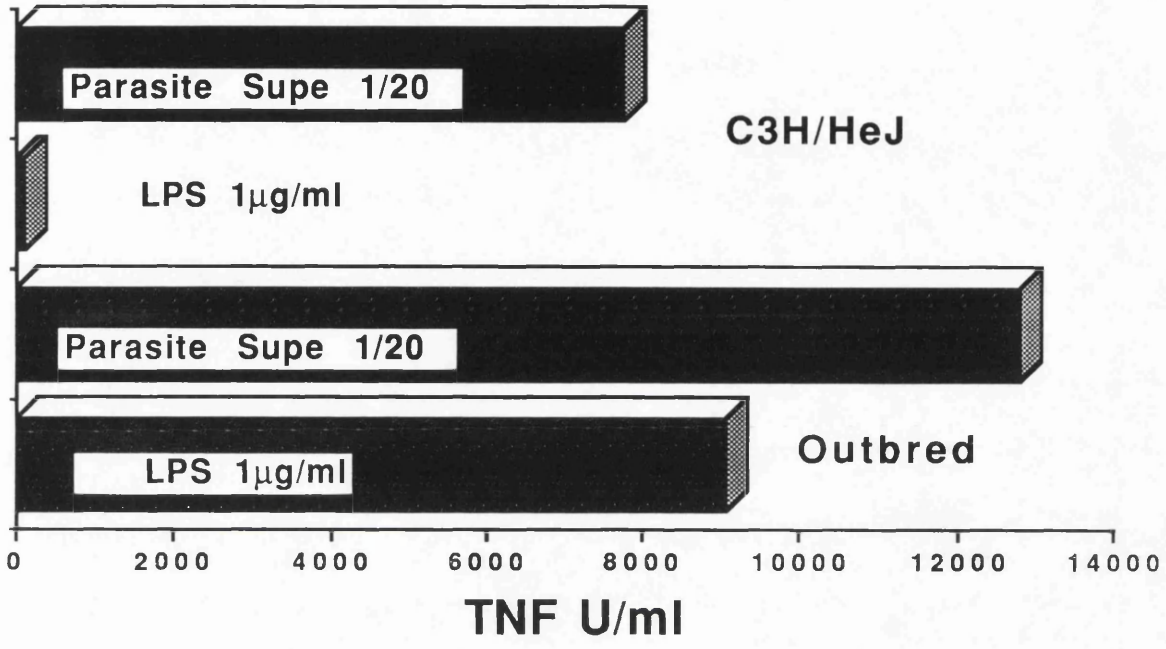
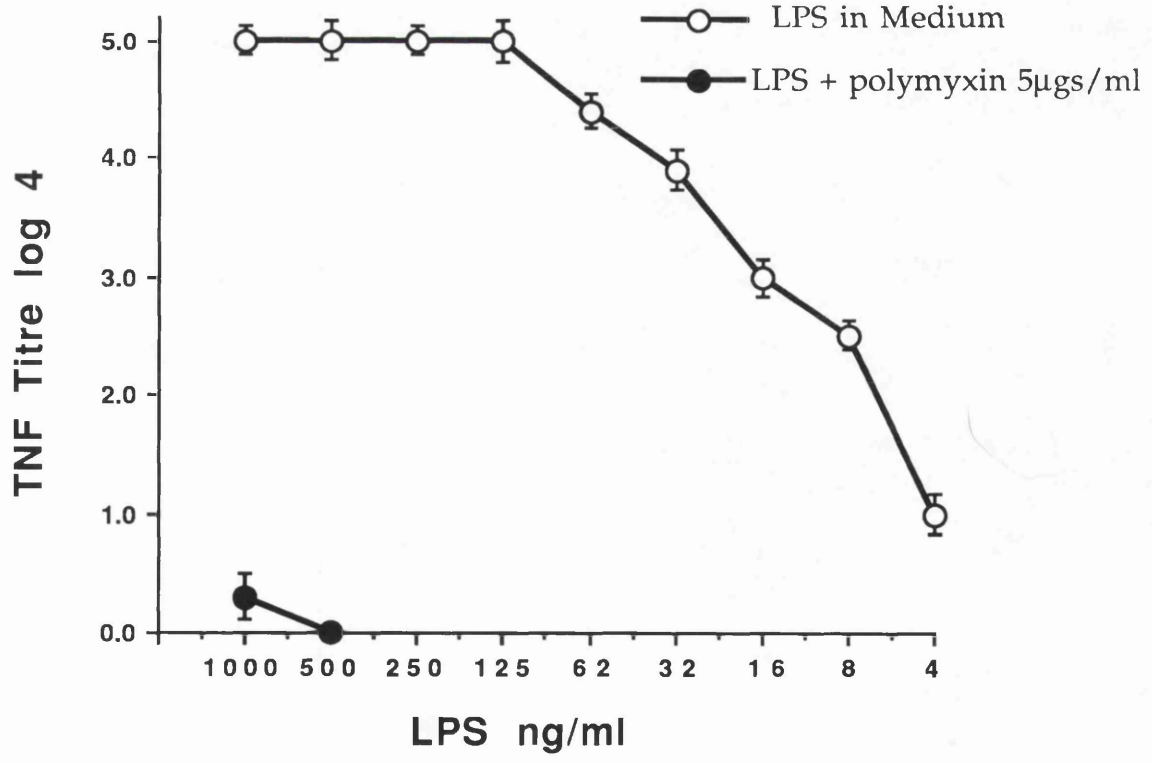


Figure 3.2.9.c. Titrations of LPS in the presence and absence of 5 µg/ml polymyxin B



The antibiotic polymyxin B is a small molecular weight polypeptide that inhibits the biological actions of LPS (Morrison & Jacobs, 1976). TNF titres stimulated from activated macrophages in response to LPS in the presence and absence of polymyxin B at 5 μg/ml were compared (Fig 3.2.9.c.). It can be seen that inclusion of polymyxin B blocked the triggering of macrophages by LPS. In direct contrast, it had no effect upon the amounts of TNF stimulated by parasite supernatants (Fig 3.2.9.d.). To exclude the effects of any contaminating LPS, polymyxin at 5 μg/ml was included in all subsequent dilution media except where LPS was used deliberately.

Although the active moiety in the parasite supernatant was not inhibited by polymyxin B, when a boiled supernatant was passed through a polymyxin B agarose column, the filtrate lost triggering activity (data not shown). From this I conclude that the polymyxin is a ligand for a non-triggering portion of the molecule. Polymyxin B binds the active moiety of LPS (lipid A) but this interaction is not specific and indeed polymyxin has been shown to bind many molecules with a highly cationic nature.

It is possible that the triggering activity in the supernatants was due to other contaminants than LPS. Therefore freshly isolated prbcs were incubated in PBS and PBS supplemented with a cocktail of antibiotics including benzyl penicillin 100 μg/ml, gentamycin 20 μg/ml, fungizone 2 μg/ml and streptomycin 100 μg/ml. After 24 hrs in these media samples were titrated upon activated macrophages and compared. The production and release of exoantigens into the supernatant was not affected by the presence of these antibiotics (Fig 3.2.9.e.).

3.3 Discussion

Erythrocytes infected with asexual rodent parasites stimulated the release of a cytotoxin from activated macrophages. This cytotoxin was inhibited by inclusion of a monoclonal antibody neutralizing TNF-α. Supernatants made from erythrocytes infected with different species of *Plasmodium*, but not uninfected mouse or sheep erythrocytes, stimulate TNF secretion. It is possible however that the molecules with this activity are present in uninfected erythrocytes at levels which do not stimulate TNF secretion. Parasitization might lead to either, an increase in the concentration

Figure 3.2.9.d. Titrations of a P.yoelii parasite supernatant in the presence and absence of 5 μg/ml polymyxin B

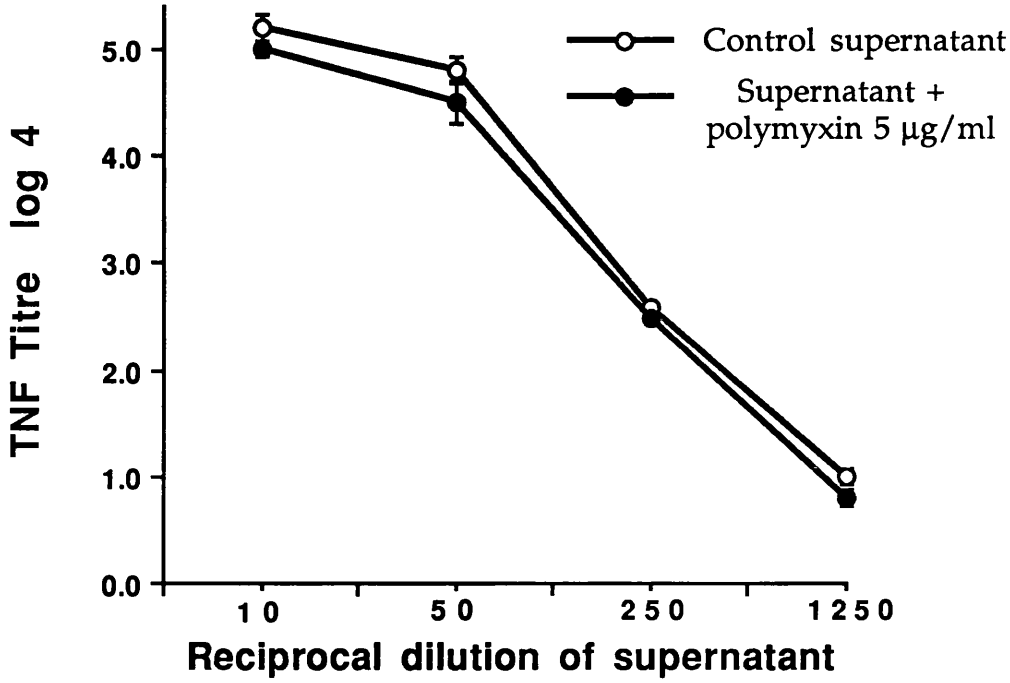
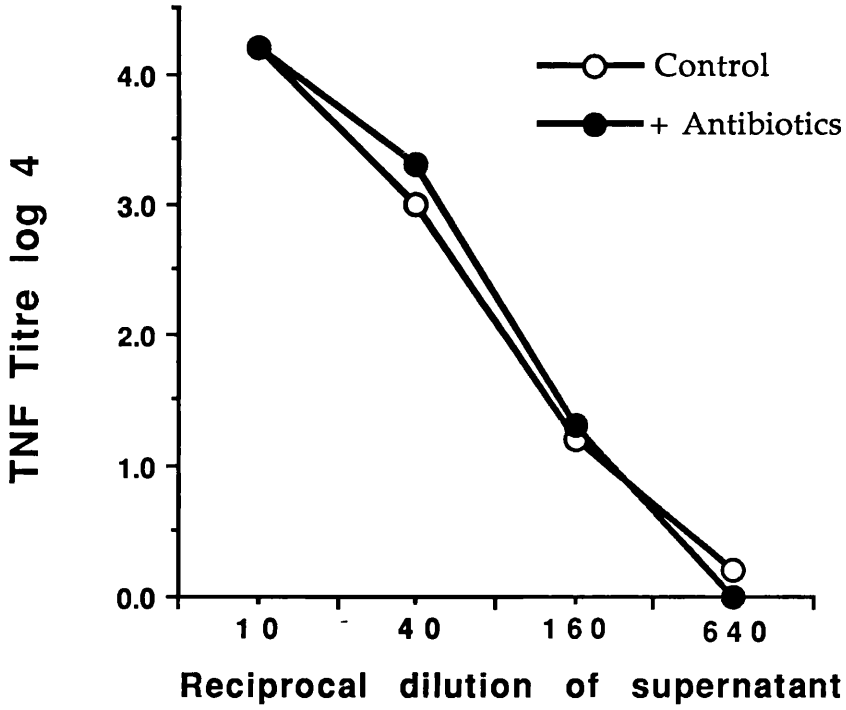


Figure 3.2.9.e. TNF titres stimulated by supernatants from P.yoelii prbcs incubated in the presence and absence of antibiotics



of this molecule, or to expression of molecules, which under normal conditions would be intracellular, on the surface of prbcs.

Formaldehyde fixed prbcs stimulated TNF secretion which indicates that active molecules are expressed in the prbc surface membrane. However, live prbcs stimulate greater amounts of TNF than fixed prbcs, and the full dose response curves generated by prbcs suggested that active molecules were released into the supernatant. Supernatants collected after 24 hr incubation with prbcs contained soluble molecules (which we have called exoantigens) that stimulated macrophages. The release of exoantigens into the media may be either an active secretion or simply result from cell breakdown. Supernatants collected from cultures of 1×10^8 prbcs/ml frequently stimulated similar amounts of TNF as did LPS at concentrations between 0.5 - 2 μg/ml. However, some supernatants did not stimulate TNF secretion. *In vitro* co-culture of synchronized *P.falciparum* prbcs and macrophages, TNF secretion was temporally associated with schizogony (Kwiatkowski *et al.*, 1989).

The source of these exoantigens is unknown; they may be either, modified host molecules or molecules unique to *Plasmodium* metabolic pathways. Exoantigen production does not seem to be dependent upon survival of the parasites, although there is evidence for the synthesis of exoantigens during the culture incubation period. There is always increased triggering activity after 24 hr incubation, regardless of the method of extraction. It could be argued that at the beginning of culture exoantigens were present but not in an extractable form and that enzymatic activity leads to the release of exoantigens. However if prbcs are homogenized at T=0, and left in the homogenate, which will contain active enzymes, no increase in the concentration of exoantigens is seen after a further 24 hours. Exoantigen production appears therefore to require cellular integrity and active metabolism as they are not produced in the presence of sodium azide and the yield is greatly reduced by incubation of parasites at 4°C. The apparently poor correlation with parasite survival indicates that the exoantigens may be produced in response to stress conditions in a similar manner to the heat-shock / stress induced proteins. However, incubation of prbcs under conditions known to stimulate heat shock proteins (40°C for 2 hours) did not induce an increased yield of exoantigen (data not shown).

Non-*Plasmodium* derived contaminants could possibly interfere with this method of measuring exoantigens. However, these experiments show that the major contaminant that would be expected, LPS from Gram -ve bacteria, is not responsible for the triggering of TNF secretion by exoantigens and that the inclusion of antibiotics into the culture medium does not alter their production.

4.0 Enzymatic and chemical sensitivity of exoantigens

4.1 Introduction

Having shown that prbcs were able to stimulate activated macrophages to secrete TNF and that this activity is associated with antigens that are released into culture supernatants, we wished to identify the antigen or antigens responsible for this activity. Similar antigens from other infectious organisms have been identified. Lipopolysaccharide (LPS) is an antigen found on Gram -ve bacteria that stimulates TNF production. There are also antigens that stimulate TNF secretion which are specific for individual species such as OK-432 from *Streptococcus pyogenes.* A small molecular weight peptide, Toxic shock syndrome toxin -1, (TSST-1) isolated from *Staphylococcus aureus* stimulates TNF secretion and can cause shock-like symptoms. A *Mycobacterial* antigen Lipoarabinomannan (LAM) stimulates TNF secretion (Moreno *et al.*, 1989). Unless otherwise stated the results shown are from *P.yoelii* infected prbcs.

4.2 Results

4.2.1 Sensitivity of exoantigens to proteases

As initial attempts to purify the "triggering molecule(s)" by separation by physical methods were not successful it was decided to look at the sensitivities of these molecules to chemical treatment and enzymatic digestion. The activity of boiled supernatants were compared before and after digestion with 100μg / ml trypsin overnight at 37°C. Trypsinized exoantigens stimulated activated macrophages to secrete TNF in a dose-dependent manner (Fig 4.2.1.a.). In the representative experiment illustrated, equivalent dilutions of trypsinized supernatants stimulated more TNF than undigested supernatants. However this was not always the case; out of 11 samples tested, there was an increase in activity in 8 preparations but no change in the remaining 3 after trypsinization. Control supernatants prepared from uninfected erythrocytes and digested with trypsin did not stimulate TNF secretion. Similar treatment of LPS does not alter the amount of TNF released by macrophages stimulated by this molecule (data not shown). Trypsin attacks proteins containing the amino acids lysine and arginine; thus

Figure 4.2.1.a Titrations of a parasite supernatant before and after digestion with 100 µg/ml trypsin

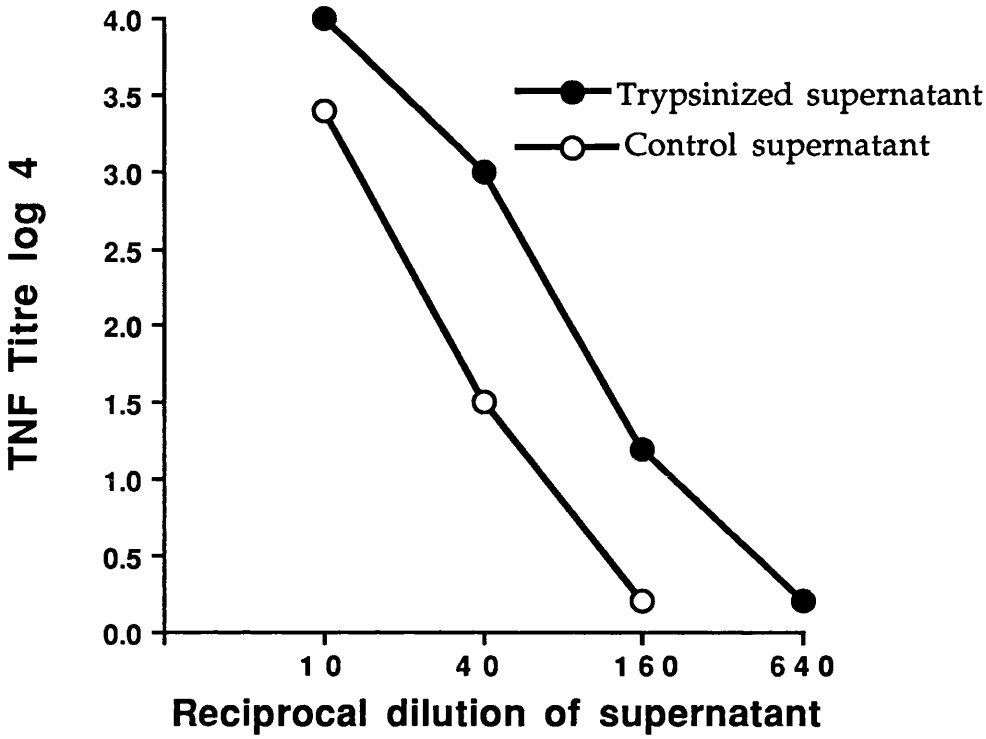
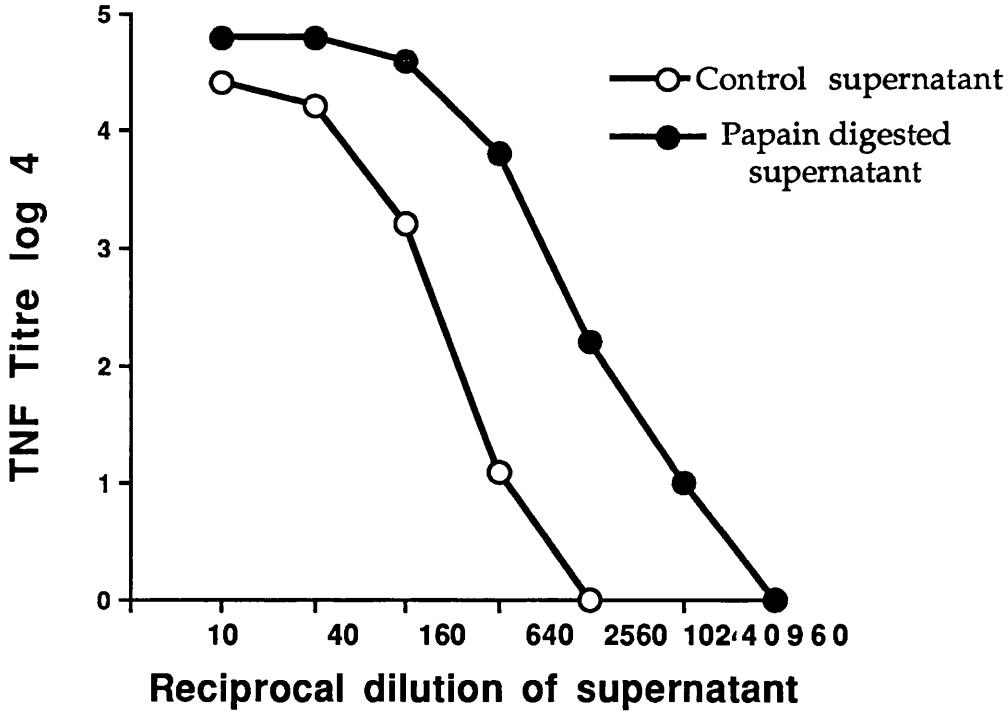


Figure 4.2.1.b. Titrations of a parasite supernatant before and after digestion with 10 µg/ml papain



trypsinization would be expected to break down large 3-dimensional protein structures, but leave small peptides, lipopeptides and glycopeptides intact.

Enzymes with a broader spectrum of activity, such as papain, proteinase K and pronase E would be expected to digest even small peptides down to their constituent amino acids which could then be removed by dialysis. Comparative titrations of boiled supernatants before and after digestion with 10 μg/ml papain (Fig 4.2.1.b.), or with 10 μg/ml pronase E (Fig 4.2.1.c.) are illustrated. Each example shows that digestion with proteases increased the capacity of exoantigens to stimulate TNF secretion; these results were reproducible irrespective of the species of parasite from which the supernatant was produced, or whether exoantigens had been extracted from the pellet. The increased activity was not due to endotoxin contamination of stock enzymes as incubation of enzymes in PBS under the same conditions did not result in preparations that stimulated TNF production. Similarly supernatants and pellets from uninfected mouse erythrocytes digested with proteases, did not stimulate TNF release (data not shown).

Table 4.2.1.a. The effects of digestion with proteases upon protein content and "triggering activity" of a few parasite supernatants. That the enzymes were active is shown by the reduction in protein content after dialysis.

Enzyme treatment		Mean Protein μg/ml		Increase in activity
		Before	After	(fold)
Trypsin, preparation	1	89.5	26	7.1
	2	112	21	5.5
Papain, preparation	3	78.3	4.2	18. 3
	4	94	2	9
Mixed proteases (pronase E / proteinase K)				
preparation	5	94.2	2.2	30.6
	6	82	0	24
	7	78	1	55

Commercial LPS is free of protein, and its TNF inducing activity was unaffected by digestion with 10 μg/ml pronase E (data not shown). However it would not occur in this form *in vivo.* Indeed LPS has been reported to associate with both bacterial proteins and with serum factors

Figure 4.2.1.c Titrations of a parasite supernatant before and after digestion with 10 μg/ml pronase E

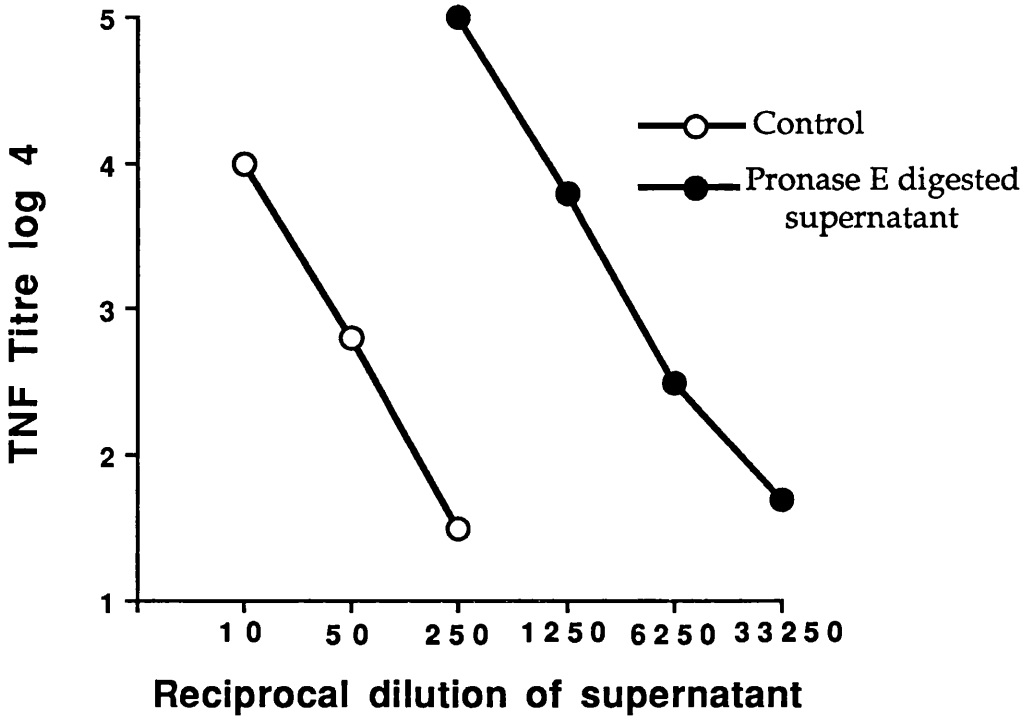
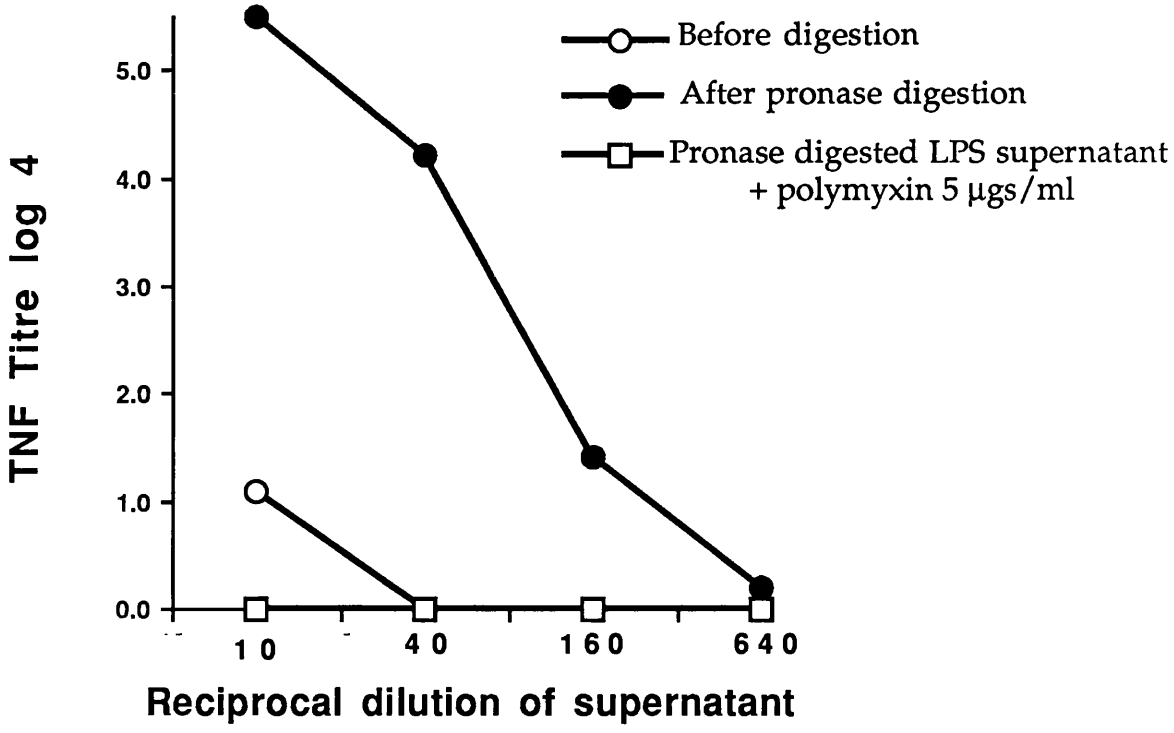


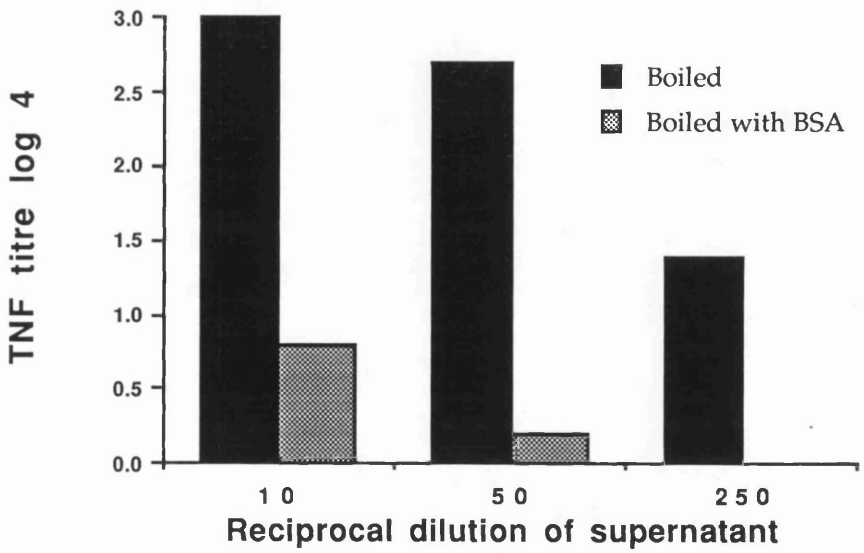
Figure 4.2.1.d, Titrations of an LPS containing rbc supernatant are shown before and after pronase digestion in the presence and absence of polymyxin B



(Schumann *et al.*, 1990) which may alter its biological properties. An "LPS protein complex" was created by injection of 100µg of LPS into a mouse; 5 minutes later this mouse was bled and the whole blood sample incubated at 37°C for 24 hrs. Then the rbcs were spun down and the supernatant collected and boiled. This LPS containing rbc supernatant was digested with 10 µg/ml pronase E, and the same dilutions were tested in the presence and absence of polymyxin B for their ability to induce TNF (Fig 4.2.1.d.). Like the parasite supernatants, this LPS containing supernatant, after pronase digestion, triggered the release of more TNF from macrophages. However, its activity was completely blocked by the addition of polymyxin B so distinguishing it from parasite exoantigens.

It seems clear that the parasite triggering molecules associated with protein, but it is not known if this is by a covalent bond, or due to a non-specific interaction. To test the specificity of this interaction I boiled a pronase-digested parasite supernatant with 500 µg/ml of BSA, and removed denatured protein by centrifugation at 5,000 g. The boiled mixture was then titrated for its ability to induce activated macrophages to secrete TNF and compared with the same supernatant that had been boiled in the absence of additional protein (Fig 4.2.1.e.). The supernatant that had been boiled with BSA stimulated the production of significantly less TNF than the control supernatant. This loss of activity may have been due to the binding of exoantigens to the denatured BSA.

Figure 4.2.1.e. Comparative titrations of a supernatant boiled in the presence and absence of 500 µg/ml BSA



4.2.2 Properties of pronase digested exoantigens

The increase in activity of the parasite supernatants after digestion with proteases suggested that the triggering molecules may be associated with protein which blocks the active site. A non-specific association with protein could possibly explain why single peaks of activity were never found after molecular weight separation or isoelectric focusing. To determine their size, pronase digested exoantigens were spun through 30 and 10 KD Amicon filters. Comparative titrations of the original control and the filtrates were tested (Fig 4.2.2.a.). Pronase digested exoantigens passed through 30, but not 10 KD filters, which suggests that they have a molecular weight between these sizes. Incubation of a pronase digested supernatant with polymyxin B agarose does not lead to the loss of activity seen with undigested supernatants. Titrations of such pronase digested exoantigens before and after incubation with polymyxin B agarose are illustrated (Fig 4.2.2.b.), (Polymyxin B agarose incubation reduces the protein content, as measured by Bio-rad, of a pronase digested supernatant to below measurable levels, > 2 μg/ml).

4.2.3 Sensitivity of exoantigens to nucleases

It has been reported that polynucleic acids injected into tumour bearing mice can induce the necrosis of those tumours (Bloksma *et al.*, 1988) and this might be due to the secretion of TNF from macrophages. To explore the possibility that the triggering exoantigens in a parasite supernatant were nucleic acids, a supernatant was digested with a mixture of RNase 10 μg/ml and DNase 10 μg/ml (Fig 4.2.3.a.). This digested supernatant was titrated in parallel with its control, which had been diluted into the enzyme buffer without the addition of enzymes. A typical result is shown: no significant differences were seen in the TNF titres induced by control and nuclease-digested supernatants or extracts of pellets of preparations from either *P.yoelii* or *P.berghei*.

Figure 4.2.2.a. Titration of a pronase digested supernatant compared with filtrates of the same supernatant passed through 30 and 10 KD Amicon filters

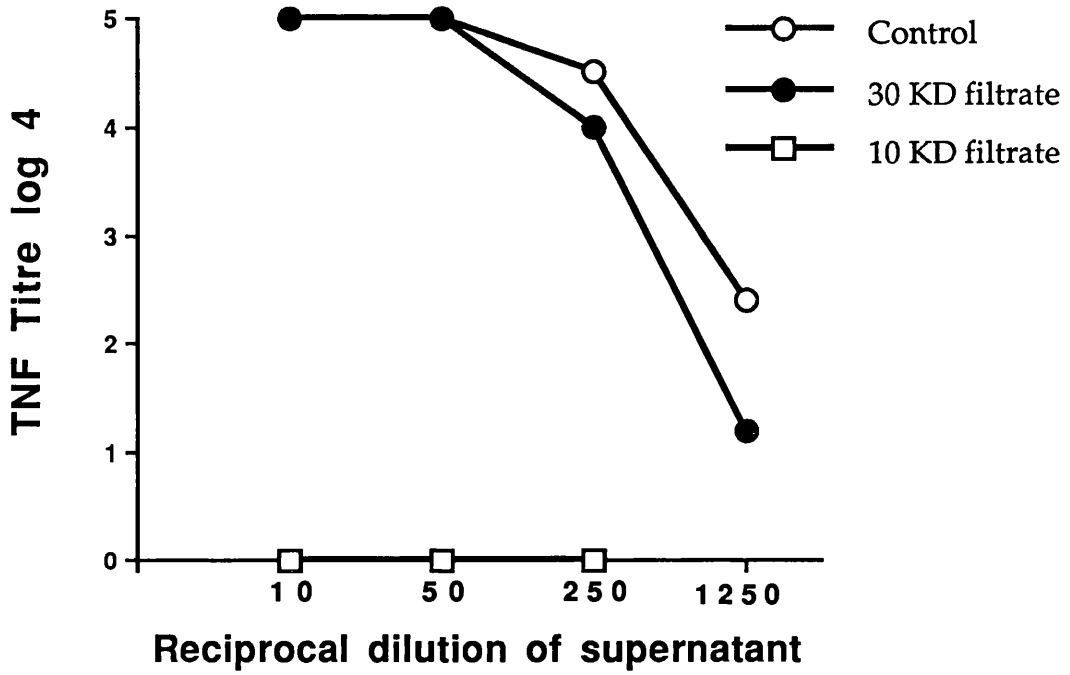


Figure 4.2.2.b. Titrations of a pronase digested parasite supernatant before and after incubation with polymyxin B agarose

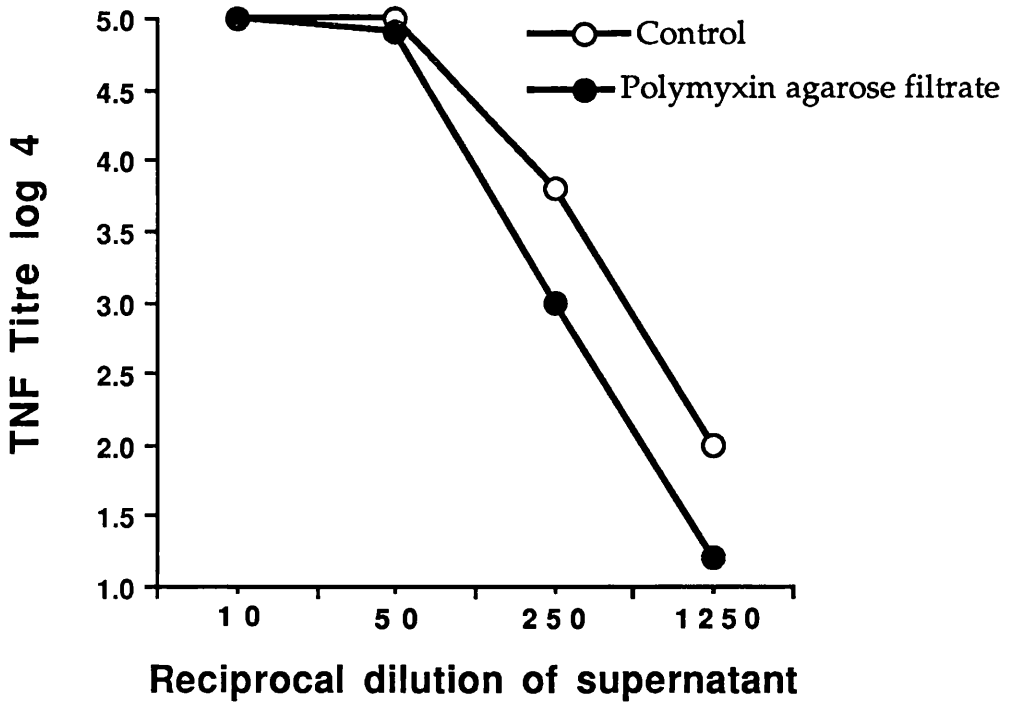


Figure 4.2.3.a. TNF titres stimulated from macrophages by supernatants before and after digestion with 10 μg/ml DNase and 10 μg/ml RNase

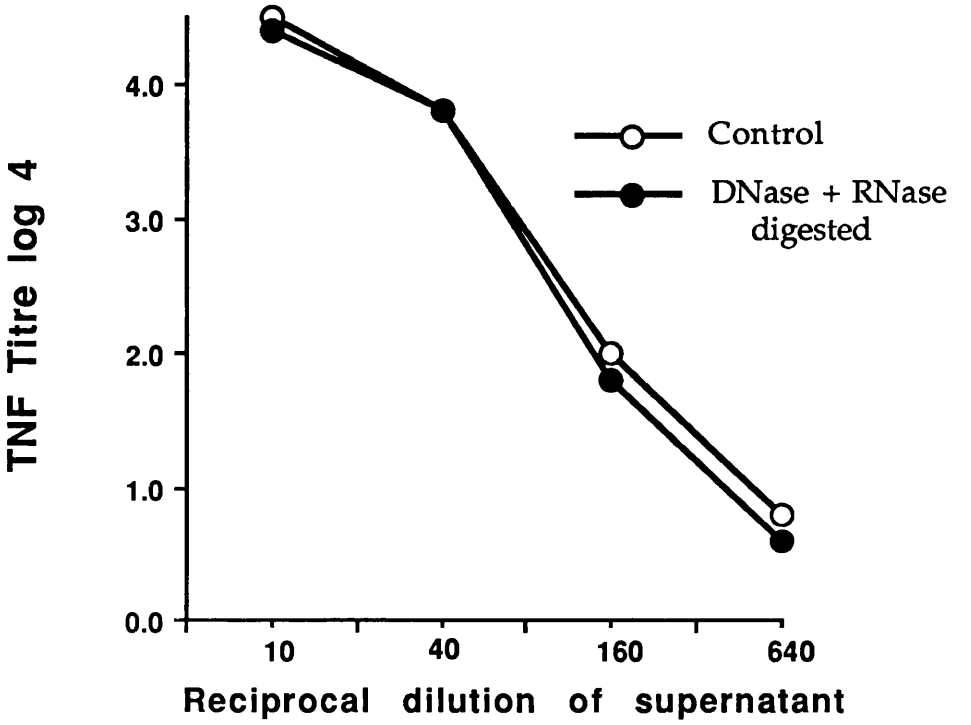
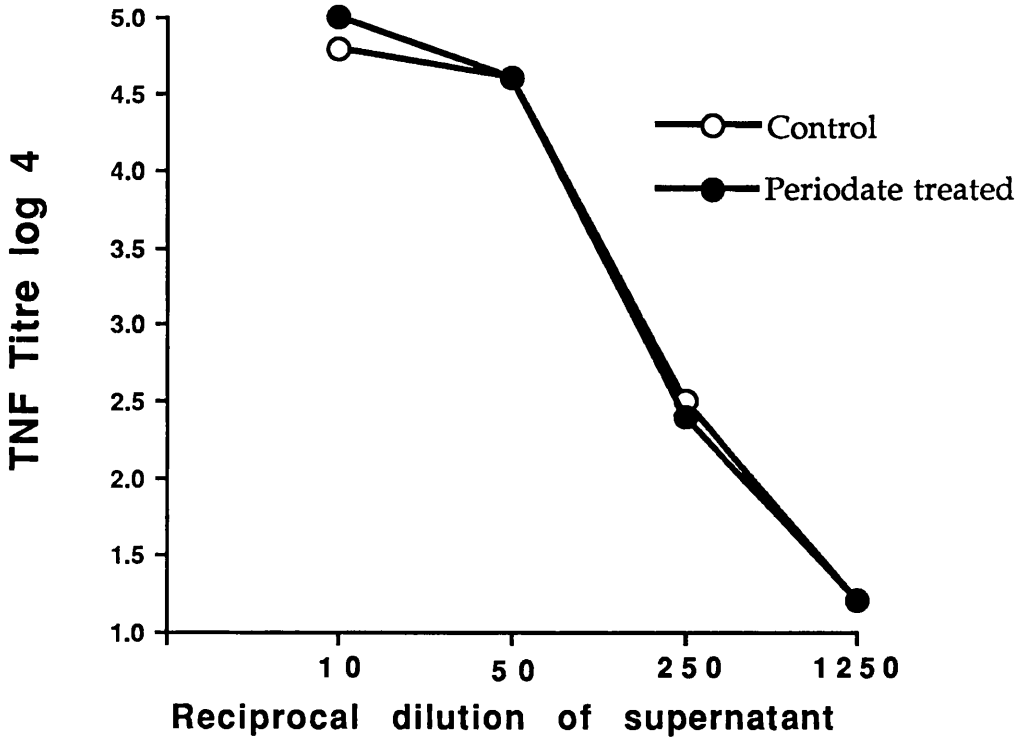


Figure 4.2.4.a. Titrations of a parasite supernatant before and after oxidation with sodium periodate



4.2.4 The role of carbohydrates in the triggering moiety

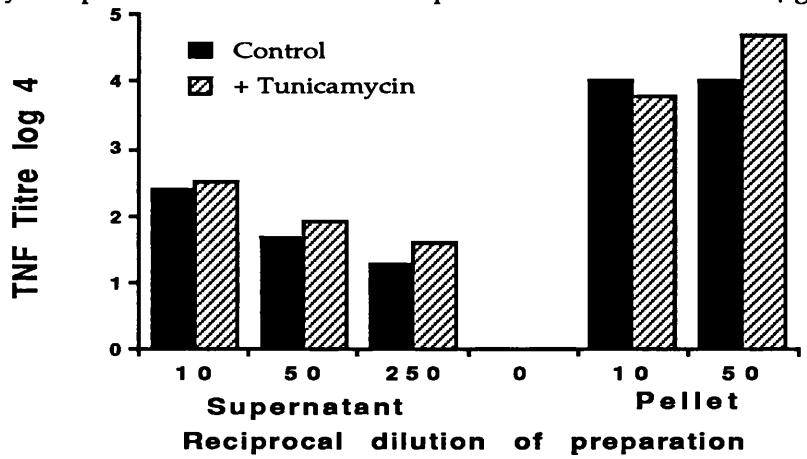
At the time of investigation there was some work that suggested that the triggering exoantigens in the supernatant bound to certain lectin columns, namely that of *Helix pomatia.* As its name suggests, LPS has many saccharide structures associated with lipid A. The antigenicity of some *Plasmodium* antigens seems to be associated with carbohydrate structures (Jakobsen *et al.*, 1987) and in particular terminal galactose residues (Ramasamy & Reese, 1986). My investigation into the possibility that a carbohydrate structure is important in the triggering of macrophages by parasite exoantigens was based upon a three pronged attack;-

1) Chemical or enzymatic cleavage of saccharides.
2) Attempts to inhibit activity by oligosaccharides or monosaccharides.
3) Attempts to inhibit the synthesis of the exoantigens by overnight incubation of parasites in the presence of inhibitors of carbohydrate synthesis.

As a preliminary investigation, a parasite supernatant was incubated with 0.05 M sodium periodate for 2 hours. This causes oxidation of adjacent hydroxy groups on the terminal saccharides. A typical titration of treated and control supernatants is shown; the treated and control supernatants stimulated the production of equal amounts of TNF, suggesting that the triggering moiety is not a terminal carbohydrate (Fig. 4.2.4.a.). The results were reproducible using supernatants prepared from different parasite species and also using pellet homogenates. Although periodate oxidation only denatures the terminal saccharides, it can be argued that it would only be these that could be involved with receptor interaction and hence triggering of macrophages.

The drug tunicamycin has been reported to block incorporation of radiolabelled galactosamine into membrane macromolecules (Udeinya & Van Dyke., 1981) Therefore prbcs were incubated overnight in the presence and absence of 10 μg/ml tunicamycin. After 24 hours supernatants were collected and titrated for their ability to stimulate activated macrophages (Fig. 4.2.4.b.). No differences were seen in the amounts of activity obtained, whether in the supernatant or from the pellet, from control and tunicamycin treated prbcs.

Figure 4.2.4.b. Exoantigen activity released into supernatants or extracted from pellets after P.yoelii prbcs were incubated in the presence and absence of 10 μg/ml tunicamycin



The digestion of *P.falciparum* soluble antigens with a mixture of glycosidases led to a loss of recognition of these antigens by malaria immune sera (Jakobsen *et al.*, 1987). I have tested the sensitivity of the activity of supernatants to a broad range of glycosidases, initially I found that treated supernatants were significantly poorer stimulators of TNF than their controls. However, this appeared to be due to non specific interactions with the large amounts of protein used, as similar losses of activity were obtained when exoantigens were boiled with 500 μg/ml BSA, and the denatured protein then removed by centrifugation. A pronase-digested supernatant digested with the mixture of glycosidases that had caused a reduction in the triggering activity, was therefore redigested with pronase and then titrated. After the supernatant had undergone both treatments, there were no apparent differences in the TNF titres obtained (Fig. 4.2.4.c). This finding was repeatable with different exoantigen preparations: in each case a loss of activity caused by incubation with the mixture of glycosidases was reversed by further digestion with pronase. Furthermore, digestion of exoantigens with a variety of specific enzymes, including α -galactosidase, β-galactosidase and neuraminidase did not alter the amount of TNF they induced macrophages to release (data not shown).

Figure 4.2.4.c. TNF titres induced by exoantigens before and after digestion with a mixture of glycosidases

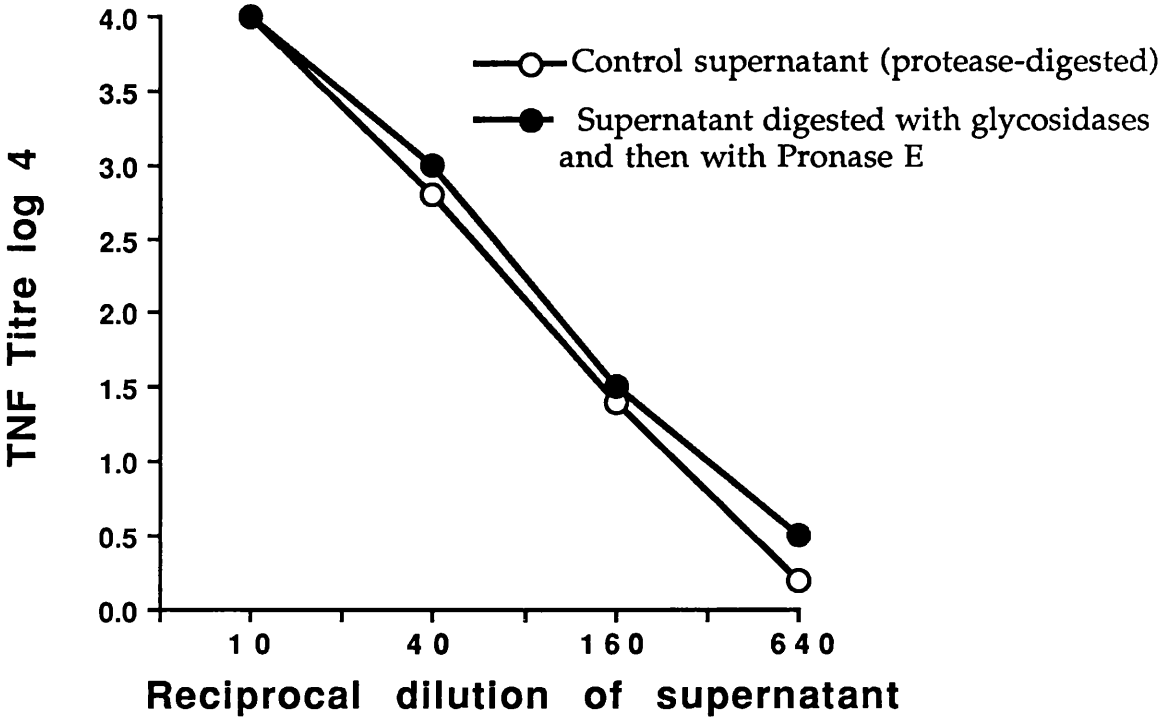
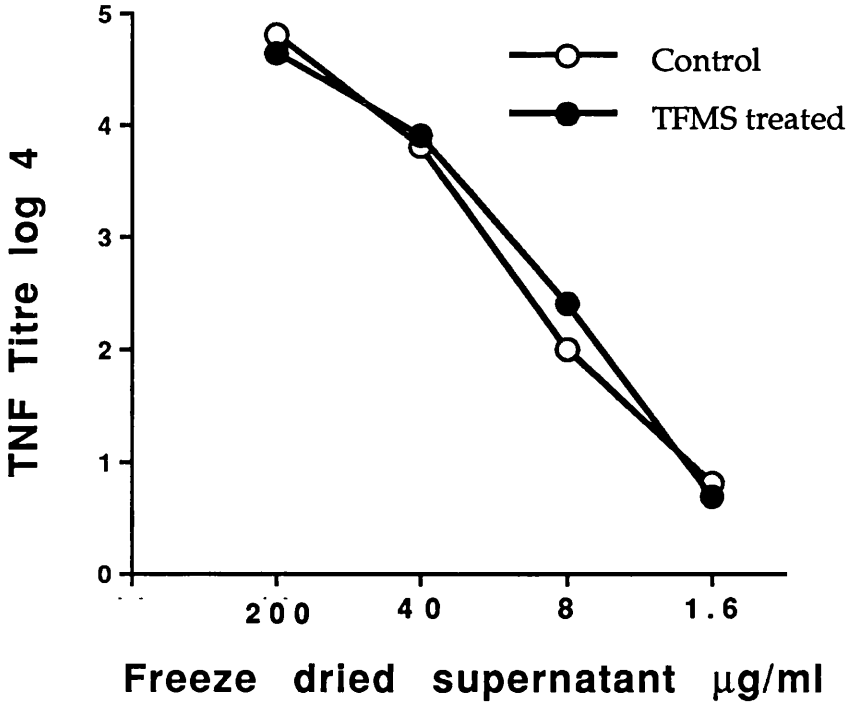


Figure 4.2.4.d. TNF titres stimulated from macrophages by exoantigens before and after "deglycosylation" with TFMS



The compound Trifluoromethanesulphonic acid (TFMS) deglycosylates proteins by cleavage of -o- linked glycosidic bonds (Edge *et al.*, 1981). A pronase E digested lyophilized exoantigen preparation was treated with TFMS and the resulting supernatant was then titrated in the macrophage assay. The results were compared with those from the untreated lyophilized supernatant (Fig. 4.2.4.d.). The results shown are representative of several experiments, each of which clearly showed that the active moiety of parasite exoantigens that trigger macrophages were still intact after deglycosylation. I have assumed that this reaction was complete, but there was no check made upon the carbohydrate content of supernatants before and after TFMS treatment.

The results of the preceding experiments suggest that the active moiety of the parasite exoantigens is not a carbohydrate residue. Incubation of saccharides such as D-galactose, glucosamine, N-acetyl galactose, and α-methyl mannoside in concentrations up to 10 mg/ml did not inhibit of TNF production stimulated by the exoantigens (data not shown) which suggest that the receptor for the exoantigens does not bind these carbohydrates.

4.2.5 The role of lipids in the triggering moiety

Freeze dried pronase-digested exoantigens was separated under a methanol/water / chloroform two phase procedure designed to extract lipids. The aqueous phase, containing most proteins, nucleic acids and saccharides, was dialysed against PBS and tested directly, while the non-aqueous phase, which contains lipids and any hydrophobic glycolipids or lipoproteins, was dried under a stream of N_2 and resuspended in PBS before testing. The resultant fractions obtained from a protease-digested supernatant were titrated for their ability to stimulate activated macrophages and the TNF titres determined (Fig. 4.2.5.a.) The pronase digested exoantigens preferentially separated into the non-aqueous phase and there was no measurable activity in the aqueous phase. The results presented are representative; on no occasion was activity recovered in the aqueous phase, although the extent of retrieval in the non-aqueous phase was variable. These results suggest that the triggering activity of exoantigens is either lipid, or associated with lipid components, but somewhat paradoxically although the triggering exoantigens separated preferentially into a non-aqueous solvent they remained soluble in PBS.

Figure 4.2.5.a. TNF titres stimulated by the aqueous and non-aqueous phases from a two phase separation of protease digested exoantigens.

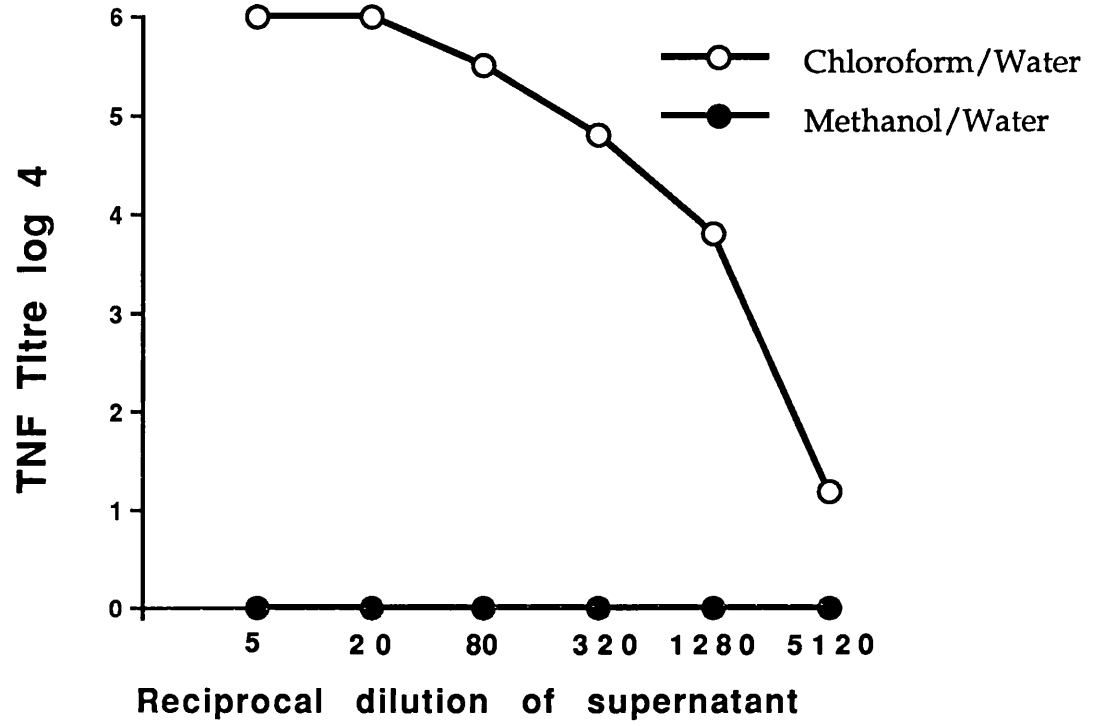
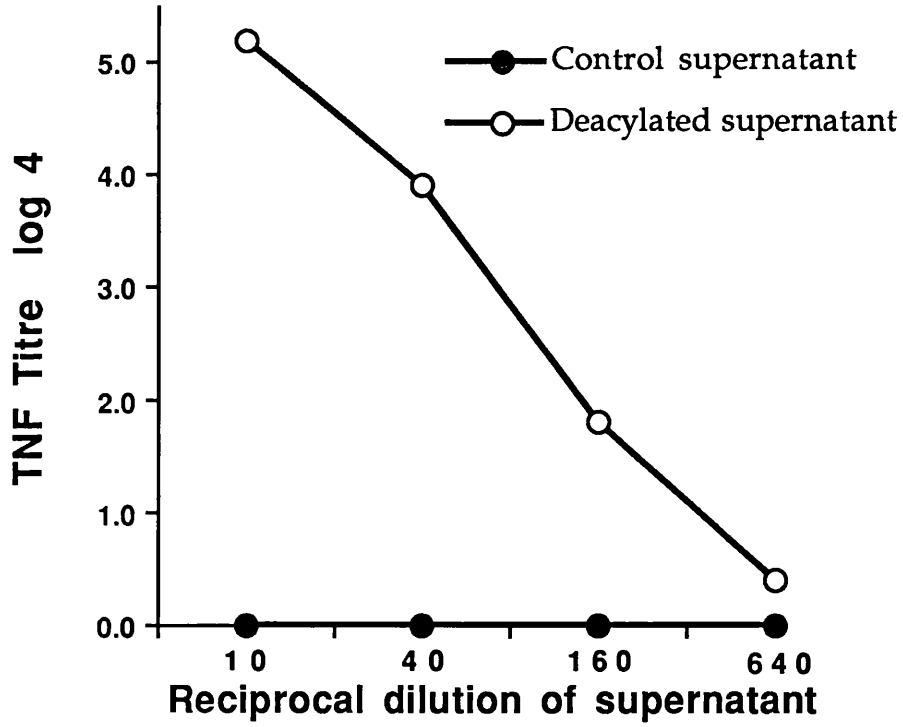


Figure 4.2.5.b. Ttitrations of a parasite supernatant before and after hydrolysis with mild alkali (deacylation)



Most simple lipids are destroyed by treatment with dilute NaOH (0.05M) at 56° C for 2 hours. This procedure, known as deacylation, attacks the ester bonds by which acyl residues are attached to a glycerol backbone. Deacylation of parasite supernatants led to the loss of their ability to trigger macrophages, which is illustrated in (Fig. 4.2.5.b.). These results have also been obtained with exoantigens prepared from Triton X-100 lysates, supernatants and pellets from *P.yoelii* and *P.berghei*. It is postulated that deacylation is the prime mechanism by which LPS is detoxified *in vivo*, and in experiments *in vitro* the capacity of LPS to stimulate TNF secretion was also shown to be abolished (data not shown). The ability of either LPS or parasite exoantigens to stimulate TNF secretion was not affected by control incubations at 56° C for 2 hours in the absence of NaOH. Pooled results from 8 such comparative titrations are shown; the amount of TNF obtained, expressed as equivalents of LPS before and after deacylation (Fig. 4.2.5.c.), clearly demonstrate the loss of functional activity.

Ester bonds can be involved in some saccharide - saccharide bonds and in the composition of some glycoproteins and lipoproteins. To test the hypothesis that the triggering moiety is a lipid, rather than an ester linked carbohydrate or lipoprotein structure, exoantigens were digested with a lipase mixture (triacyl glycerol and acyl hydrolases). Comparative titrations of digested supernatants, and control supernatants that were incubated under the same conditions but without addition of the enzymes, are shown (Fig. 4.2.5.d.). After digestion with the lipases, the exoantigens no longer stimulated TNF secretion. The results of a typical titration are shown; this loss of activity was observed with several exoantigen preparations and with 3 different samples of enzymes.

Further enzymatic evidence of the nature of the triggering moiety was obtained by digestion with specific phospholipases. Pronase digested exoantigens were lyophilized and resuspended in specific buffers at 10 mg/ml and then digested with specific phospholipases. After incubation the exoantigen preparations were diluted 1/10 in PBS, and further digested with pronase, dialysed and titrated in the macrophage assay, and the TNF titres of control and digested preparations compared (Fig. 4.2.5.e.). Exoantigens digested with phospholipases A_2, B and D stimulated TNF secretion to the same extent as control preparations; exoantigens digested with phospholipase C failed to stimulate TNF secretion. The results shown are selected to show

Figure 4.2.5.c. Pooled TNF titres, expressed as equivalents of LPS µg/ml, stimulated by exoantigen preparations before and after deacylation.

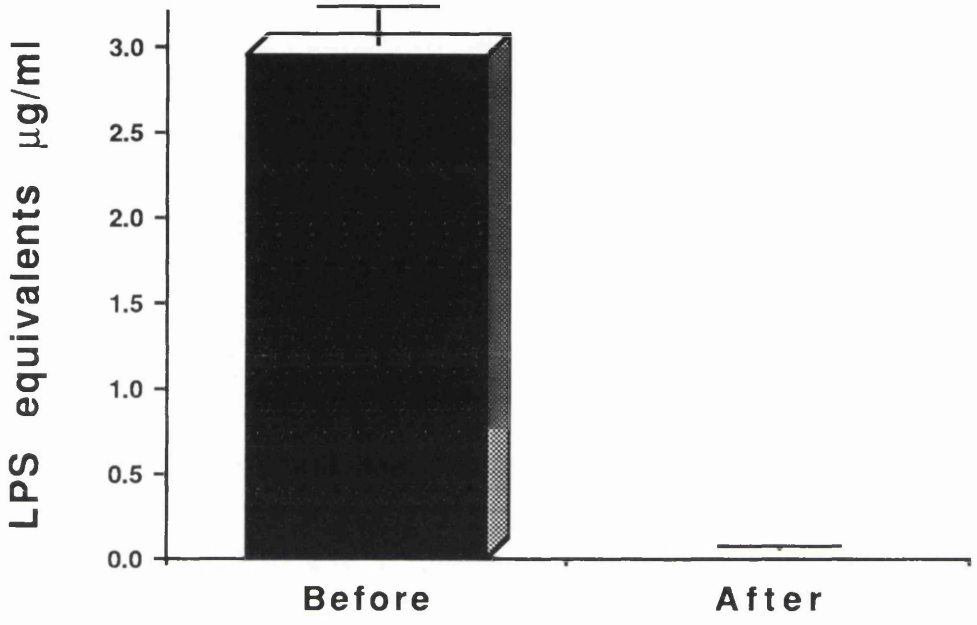


Figure 4.2.5.d. TNF induction by a supernatant before and after digestion with a mixture of triacyl glycerol lipases and acyl hydrolases

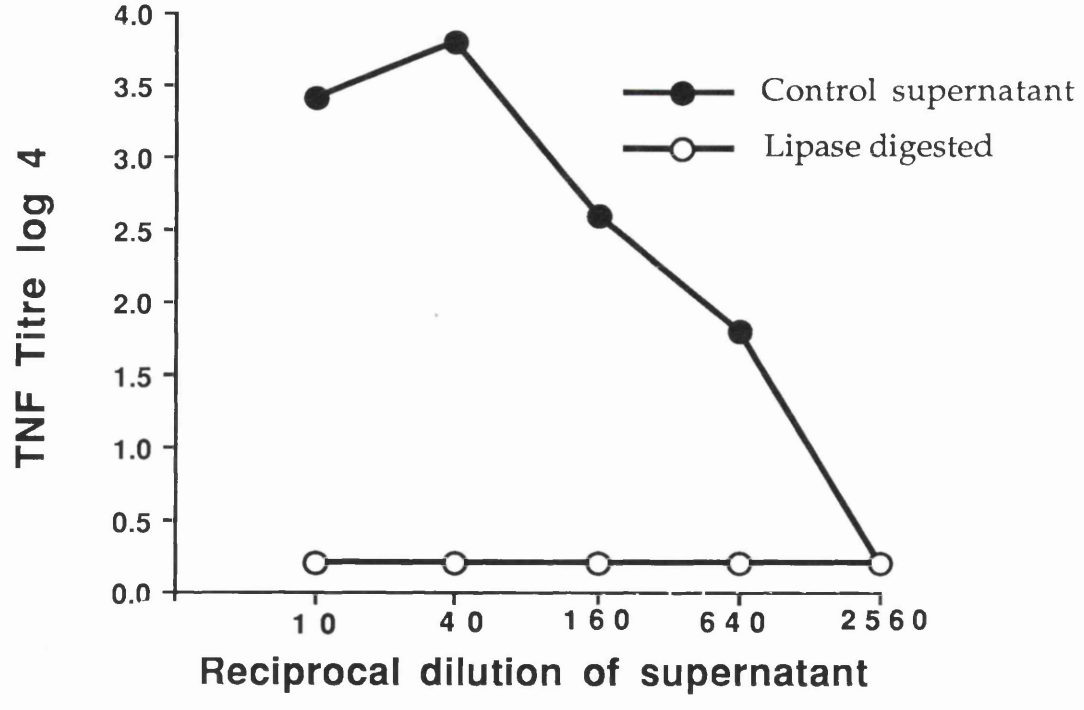


Figure 4.2.5.e. Titration of exoantigens before after digestion with specific phospholipases

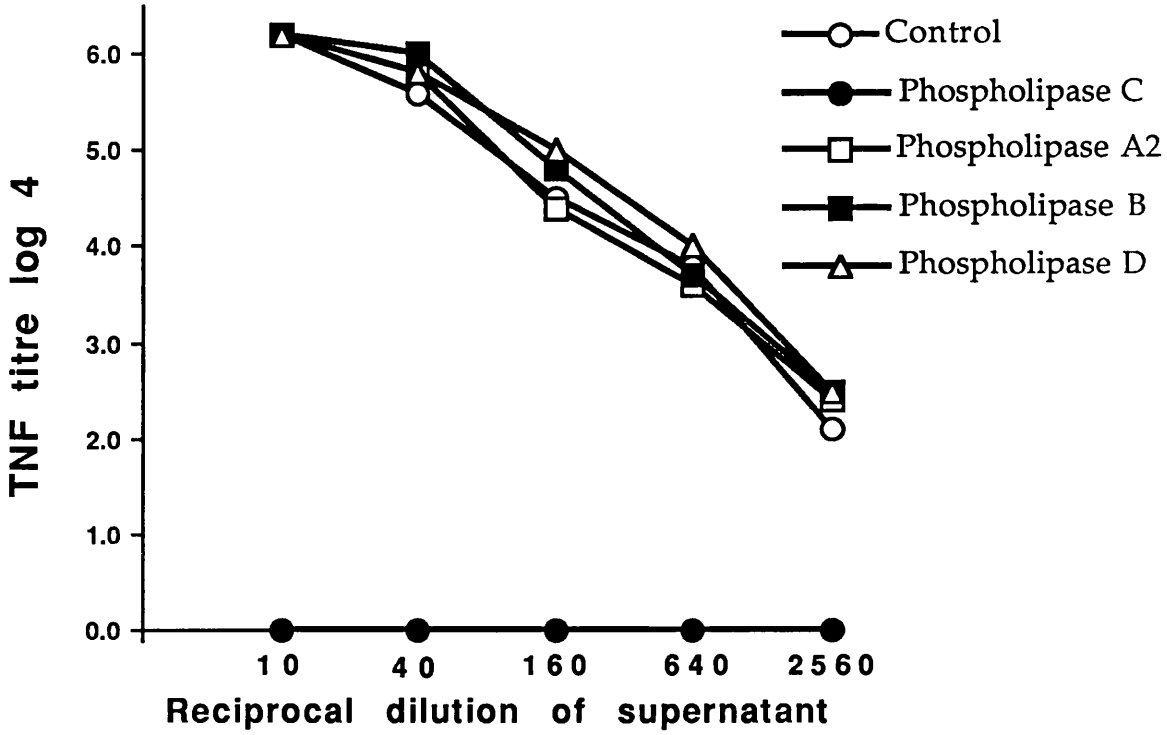
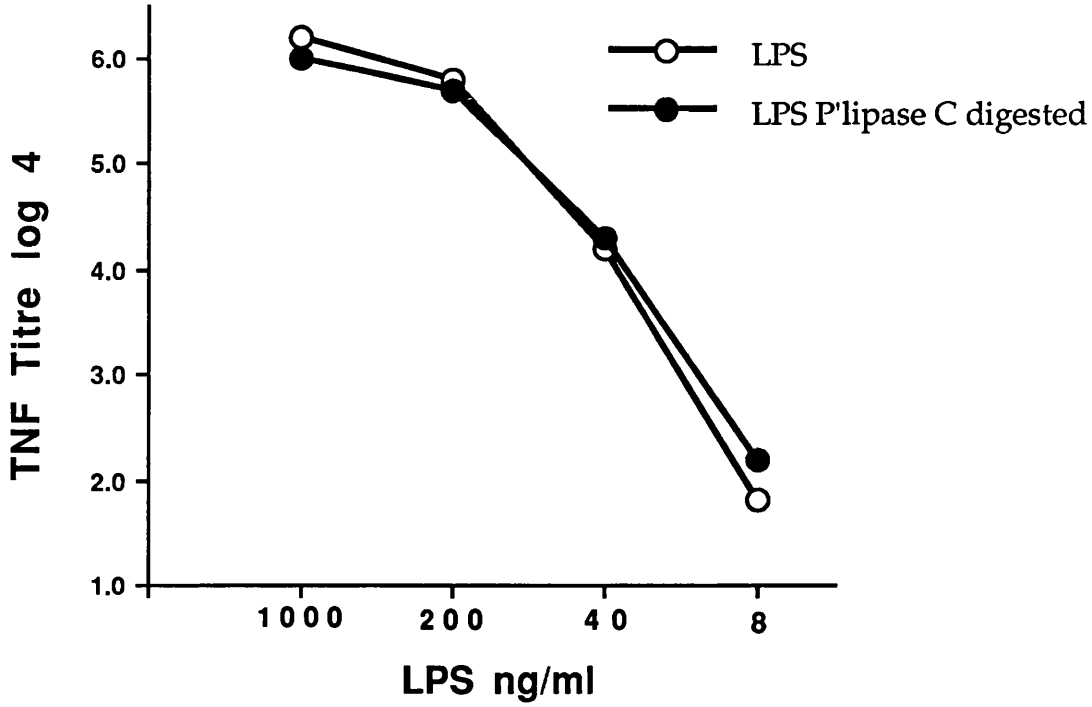


Figure 4.2.5.f. TNF titres stimulated by LPS before and after digestion with phospholipase C



this effect most clearly: out of 6 exoantigen preparations from different parasite species, none stimulated TNF secretion after phospholipase C digestion. As a further control, LPS was digested with phospholipase C, and the TNF titres stimulated by digested and control preparations are shown (Fig. 4.2.5.f.). In contrast to the parasite exoantigens, the activity of LPS remained unaffected by phospholipase C digestion.

4.2.6 The role of phosphate in the triggering moiety

That the triggering exoantigens are sensitive to phospholipase C indicates that the triggering moiety either contains or is closely proximal to a phosphate. If the phosphate is complexed as a mono-ester, such as in he second messenger inositol 1,3,5 tri-phosphate (IP_3), it would act as a substrate for the enzyme alkaline phosphatase. Several pronase-digested supernatants and pellets have been digested with 100 μg/ml alkaline phosphatase and results from one such experiment are shown (Fig 4.2.6.a.). These phosphatase -digested exoantigens triggered macrophages in a dose-dependent manner which did not differ from that of control preparations. It is common to find phosphate diesters which are relatively stable to chemical and enzymatic degradation in biological molecules. Incubation of exoantigens with hydrofluoric acid (HF) was used to break any phosphodiester bonds. Exoantigens that had been lyophilized and resuspended in 46 % w/v hydrofluoric acid for 24 hours were neutralized with concentrated NaOH. The remaining supernatant, or chloroform / water extracts of this material were titrated upon activated macrophages and found to be no longer capable of stimulating TNF secretion (Fig 4.2.6.b.).

4.2.7 The role of nitrogen in the triggering moiety

Nitrous acid at acid pH attacks amines (deaminates), breaking them down to nitrosamines. Treatment of exoantigens with nitrous acid led to precipitation of material which was removed by centrifugation. The activity of the remaining soluble material was titrated and compared with that of control exoantigens that had been incubated under the same conditions but in the absence of nitrous acid (Fig 4.2.7.a.). Deaminated preparations stimulated the production of titres of TNF that were not significantly different from those of the controls. The results from a representative experiment are

Figure 4.2.6.a. TNF titres stimulated by a parasite supernatant before and after digestion with alkaline phosphatase

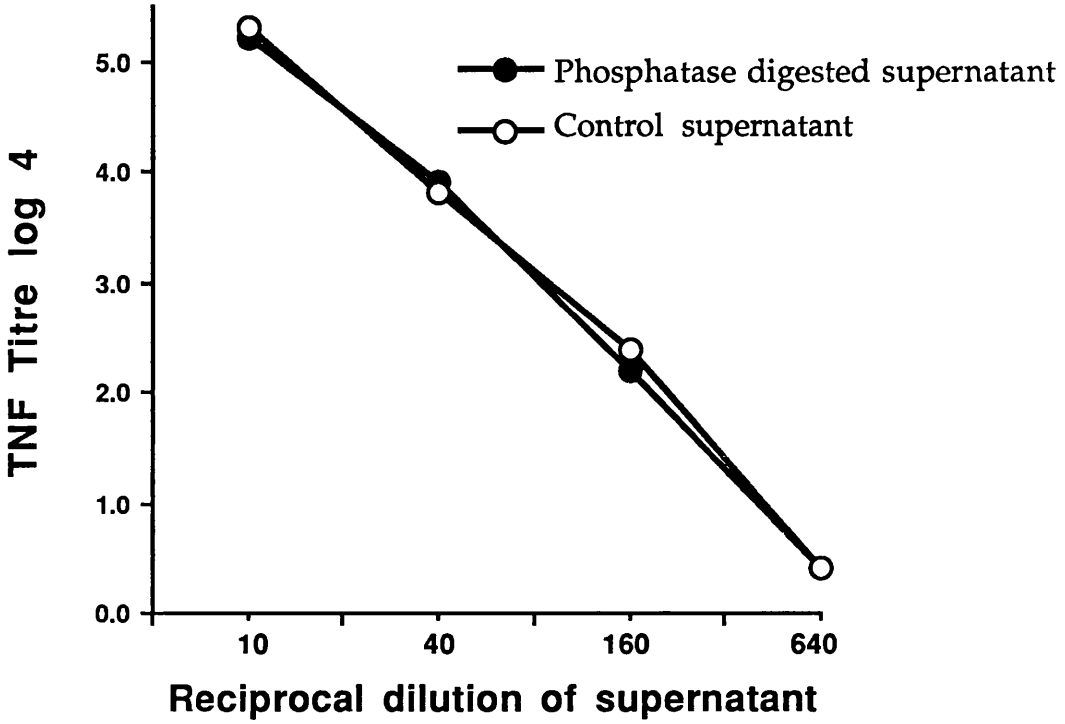


Figure 4.2.6.b. TNF titres stimulated by exoantigens before and after dephosphorylation with hydrofluoric acid

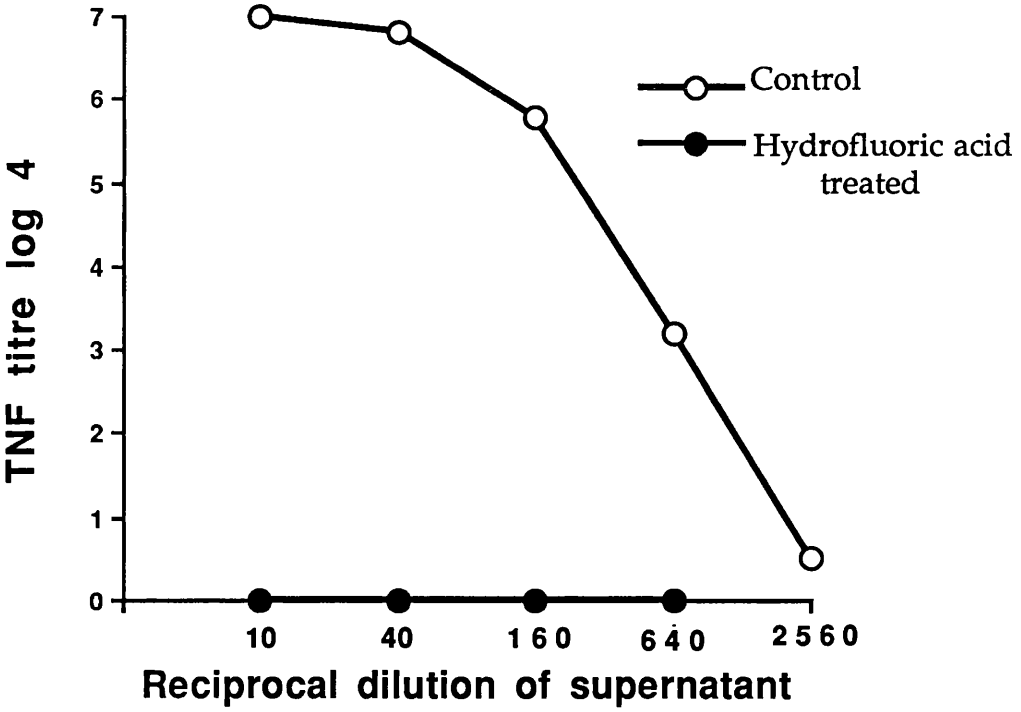


Figure 4.2.7.a. TNF titres stimulated from macrophages by exoantigens before and after deamination and filtration through a 10KD Amicon filter

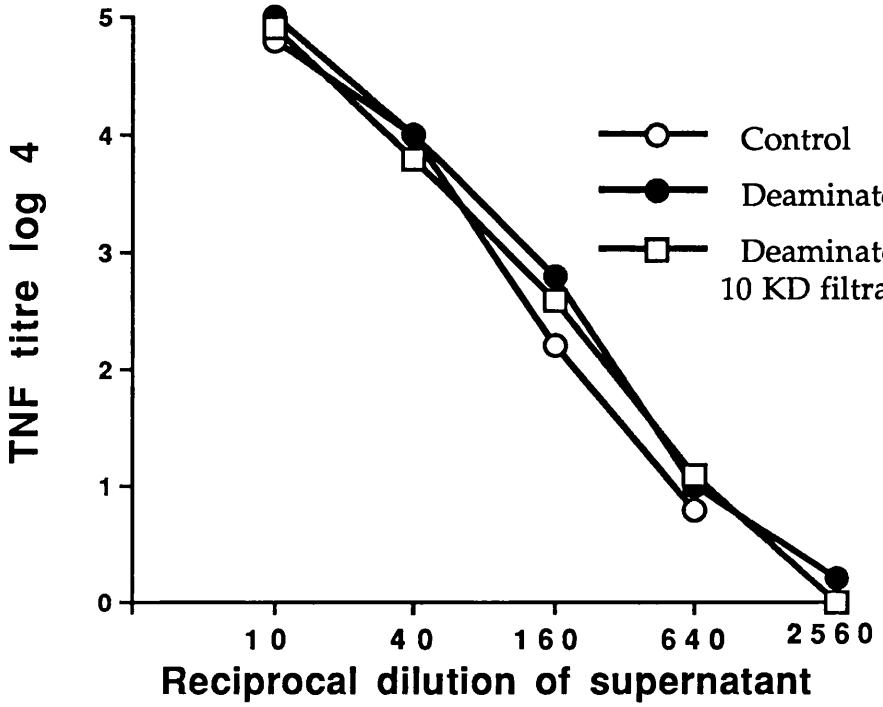
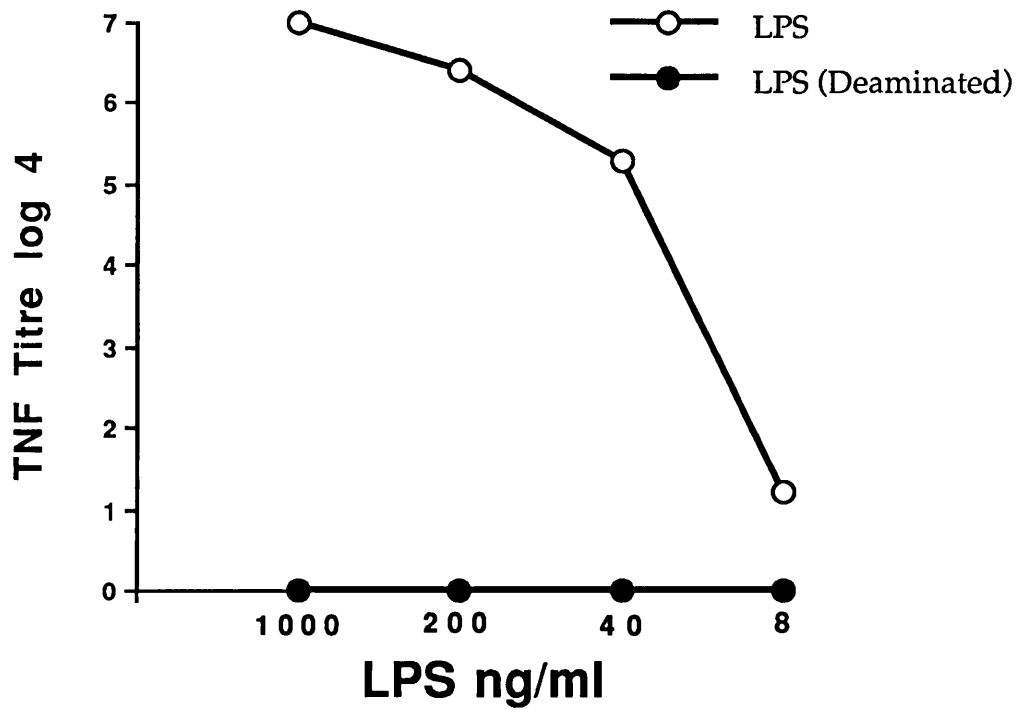


Figure 4.2.7.b. Titrations of LPS before and after deamination with nitrous acid



illustrated; after deamination the activity of either supernatants, lysates or pellet homogenates was always unchanged. Initially, it seemed that deamination had led to the loss of an active moiety, as after dialysis the treated exoantigens failed to stimulate macrophages. However, if the nitrous acid reaction mixture was neutralized rather than dialysed the activity was retained, suggesting that deamination generates an active molecule with a molecular weight of less than 10 KD, the pore size of the dialysis tubing. This possibility was tested by spinning deaminated exoantigens through an Amicon filter with a cut-off point set at 10 KD, and the activity of the 10 KD filtrate was compared with the original material: the capacity to stimulate TNF secretion was found not to be significantly different.

Amine linkages are found extensively in biological molecules and are the cornerstone of proteins, linking amino acids. They are also found in nucleotides, they frequently link saccharides, and can act as links between acyl groups in lipids. Such a linkage is seen in the structure of lipid A and thus deamination would in theory detoxify LPS. LPS was incubated with nitrous acid under the same conditions as above and the resultant products titrated (Fig 4.2.7.b.). In contrast to the parasite exoantigens, nitrous acid deamination of LPS led to the loss of its ability to stimulate macrophages to secrete TNF.

4.2.8 Bioavailability of exoantigens

The loss of activity after exoantigens had been treated with HF, NaOH or digested with either the lipases or with phospholipase C, may not have been due to the loss of the functional moiety but rather to either the generation of a small molecule that was lost upon dialysis or to the purification to a molecule that was not water-soluble. A similar problem is presented by the lipid A molecule of LPS which is insoluble in water unless bound to carrier molecules. Solubilization in PBS might be made possible by the addition of a carrier molecule such as delipidated BSA to the digested exoantigen. However, HF treated, deacylated or lipase digested exoantigens to which had been added delipidated 10 μg/ml BSA still did not trigger macrophages to secrete TNF (data not shown). These exoantigens that had been rendered functionally inactive were tested, either directly after neutralization without dialysis, or after the two-phase extraction procedure, both fractions being tested before dialysis. Only exoantigen preparations that had been deaminated with nitrous acid still triggered the secretion of TNF.

4.2.9 Exoantigens derived from *P.falciparum*

The above findings have been confirmed for different species of rodent parasite. It is however possible that the evolutionary distance between man and rodents means that there are no equivalent molecules in the human parasites. The presence of similar triggering molecules in parasites infecting humans was therefore investigated. The activity of *P.falciparum* exoantigens was resistant to boiling and pronase digestion and was not inhibited by 5μg/ml polymyxin B (Fig 4.2.9.a.). Like preparations from rodent parasites the ability of *P.falciparum* exoantigens to stimulate TNF production was resistant to boiling at 100°C for 5 minutes, was not destroyed by proteases and are active in the presence of polymyxin B. Preparations of pronase-digested *P.falciparum* exoantigens that had been deaminated, deacylated or digested with phospholipase C were also titrated. TNF titres elicited in response to these preparations (Fig 4.2.9.b.) showed that there is functional similarity to rodent exoantigens, in that deaminated preparations still stimulated a response but deacylated or phospholipase C digested exoantigens were inactive.

In collaboration with P. Jakobsen we showed that affinity purified *P. falciparum* antigens also induced the production of TNF, and that two, identified by CIE and designated Ag 1 and Ag 7 consistently triggered TNF secretion. Both these antigens retained activity when pronase digested, however the antigens could be separated functionally by their reactions with polymyxin B and malaria specific antiserum (Taverne *et al.*, 1990).

It is possible that rodent macrophages react to parasite exoantigens that do not stimulate human cells. However, human adherent peripheral blood mononuclear cells (PBMCs) that had been activated with indomethacin, secreted TNF in response to exoantigens derived from both human and rodent parasites diluted in 5 μg/ml polymyxin B (Fig 4.2.9.c.). TNF levels stimulated by exoantigens were roughly equivalent to those stimulated by 1μg/ml LPS.

Figure 4.2.9.a. Titrations of P.falciparum exoantigens from a crude lysate before and after boiling and pronase digestion.

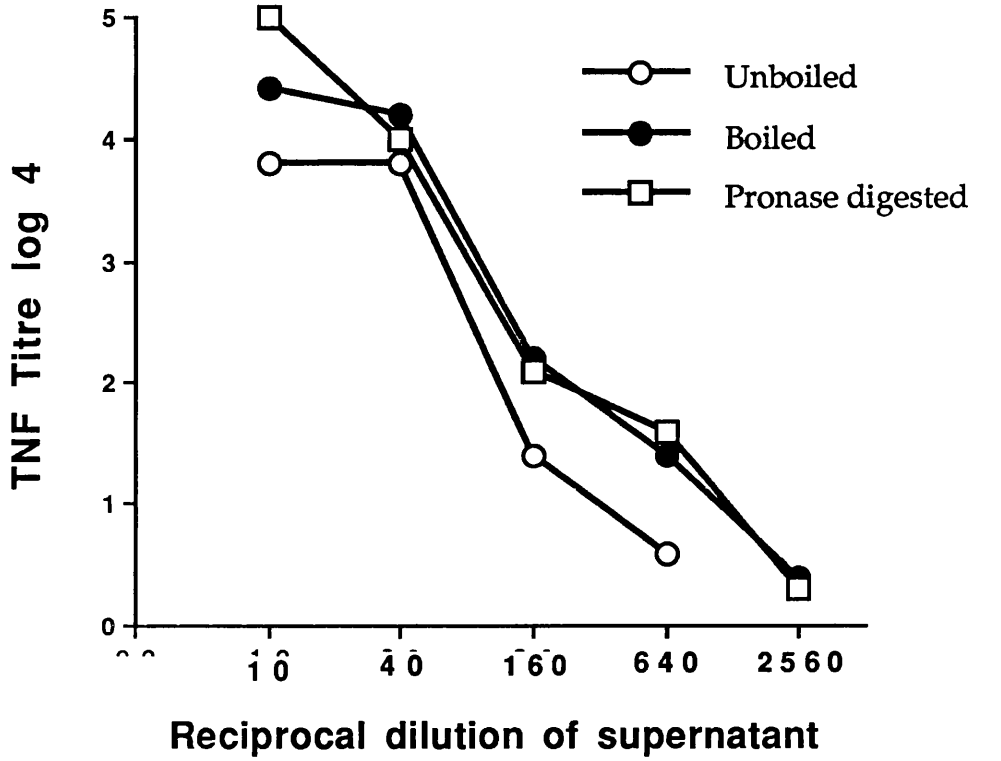


Figure 4.2.9.b. Titrations of P.falciparum exoantigens after deamination, deacylation and digestion with Phospholipase C

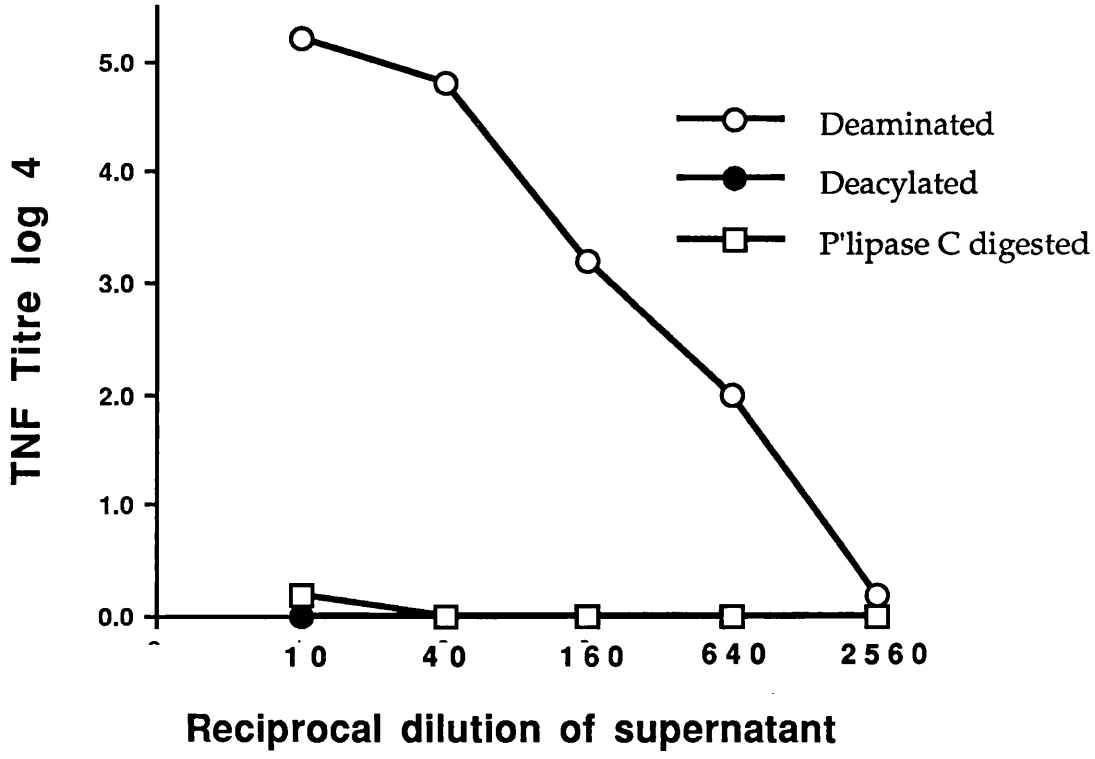
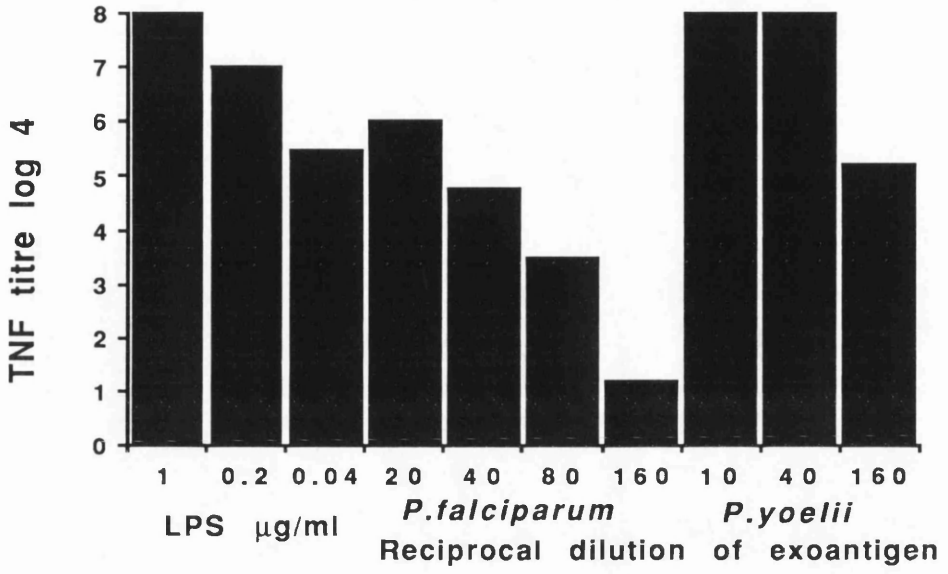


Figure 4.2.9.c. TNF titres stimulated from human monocytes by LPS and exoantigens from P.falciparum and P.yoelii



4.3 Discussion

There are a number infectious organisms which stimulate TNF production and for many of these the molecules responsible have been well characterized. In the previous chapter I have shown that prbcs stimulate TNF secretion and this can, at least in part, be attributed to the release of soluble molecules which have been called exoantigens. In this chapter I have described my attempts to characterize the physical and chemical nature of these exoantigens.

The active moiety of the exoantigens resists protease digestion, unlike TSST-1 of *Staphylococcus aureus*, and therefore is not a protein. However the exoantigens seem to be associated with proteins, either parasite or host-derived, which inhibit some of their activity. It is known that other macrophage stimulants such as LPS, bind to a variety of proteins which can modulate its actions. There are reports of LPS binding to a host protein which enhances its ability to stimulate TNF secretion, for example binding to LBP, a 60 KD glycoprotein (Schumann *et al.*, 1990) but also of its binding to a

lipoprotein found in the serum which inhibits its activity (Cavallion *et al.*, 1990). Exoantigens can also bind in a non-specific manner to serum proteins as there was loss of activity after they were boiled with BSA.

Digestion of crude supernatants with a mixture of DNase and RNase does not alter the activity of the exoantigens responsible for triggering macrophages to secrete TNF. In a similar manner I investigated the possible involvement of carbohydrate in the active moiety of these exoantigens. Conclusive proof is not available but most of the evidence is against the active moiety being a carbohydrate structure, since the triggering activity was not affected by periodate oxidation, incubation with a wide range of glycosidases or by deglycosylation with TFMS. Deamination with nitrous acid, a treatment which destroys N-linkages, generates an active small molecular weight molecule of <10 KD.

Pronase-digested exoantigens separate preferentially into the non-aqueous phase of a two phase extraction procedure, indicating that they are at least associated with lipid structures. The loss of activity seen when exoantigens were either deacylated, or more specifically digested with lipases is highly indicative that the active moiety is, like LPS, lipid in nature. Further digestions with specific phospholipases showed that the triggering moiety is sensitive to phospholipase C. The loss of activity seen was probably due to the destruction of an active moiety rather than to the generation of a small molecular weight molecule that passed through the dialysis membrane, or simply a loss of bioavailability.

The presence of phosphate in the exoantigen is suggested by its sensitivity to phospholipase C, and was confirmed by the destruction of the active moiety by treatment with hydrofluoric acid. However, it was not destroyed by digestion with alkaline phosphatase, indicating that the phosphate is probably in the form of a diester.

Exoantigens can be extracted from different parasite species including from the human parasite *P.falciparum*. The method of extraction or the species from which the exoantigens were obtained does not seem to affect their properties in that all preparations tested showed the same chemical and enzymatic sensitivities. Exoantigens synthesized by *P.falciparum* stimulate activated human monocytes to secrete TNF (Taverne *et al.*, 1990; Picot *et al.*,

1990). The apparent similarity in the biochemical nature of triggering exoantigens from human and rodent parasites suggests that rodent parasites provide a viable working system for investigating the nature of the exoantigens isolated from the human parasite (Taverne *et al.*, 1990).

The parasite exoantigens share certain features with other TNF inducing molecules, for example, the active moiety of LPS, lipid A. Lipid A is also insensitive to proteases, nucleases, glycosidases and sodium periodate oxidation. It also separates into non-aqueous media and can be detoxified by deacylation. However LPS, in direct contrast to the exoantigens, is insensitive to phospholipase C digestion and is detoxified by nitrous acid deamination. Table 4.3.a. summarizes the differences between LPS and parasite exoantigens.

Table 4.3.a. Differences between the TNF-inducing activity of parasite exoantigens and of bacterial endotoxin

	Parasite exoantigens	LPS
Inhibited by polymyxin B	-	+
Destroyed by nitrous acid deamination	-	+
Destroyed by phospholipase C	+	-
Stimulates macrophages from C3H/HeJ mice	+	-

Exoantigens share similar properties with molecules termed glycosylphosphatidyl inositols (GPIs). These GPIs have stimulated interest as they function to anchor many antigens to membranes and have also been implicated in cell signalling. Amongst the molecules known to contain this type of anchor are normal constituents of host cells such as decay accelerating factor (DAF) and Thy-1 and antigens such as variable surface glycoprotein (VSG) from trypanosomes, lipophosphoglycan (LPG) from *Leishmania* and merozoite surface antigen (MSA-1) from *P.falciparum*. Cleavage of GPI anchors has been proposed as a mediator of receptor activated signal transduction mechanism (Ferguson., 1988). Samples of MSA-1 and LPG, complete with an intact GPI tail, were tested on mouse macrophages and found not to stimulate TNF production (data not shown), although this may

be a quantitative rather than a qualitative distinction. A group of molecules called phosphatidylinositol-glycans (PI-Gs), which are stimulated by insulin, and activate insulin-dependent enzymes have been identified. It has been proposed that insulin may function by stimulating phospholipase C cleavage of a PI-G precursor which releases diacylglycerol and a glycan-inositol phosphate with functional activity (Alemany *et al.*, 1987). Such molecules show similar biochemical and enzymatic sensitivities to our parasite exoantigens.

Intra erythrocytic parasitization by *Plasmodium* species leads to a considerable increase in the phospholipid content of erythrocytes which may be increased by 600 % during a 24 hour cycle (Holz., 1977). It is believed that the parasite satisfies most of this requirement by the breakdown of host fatty acids and assembly of new phospholipids. In synchronous *P.falciparum* infections the biosynthesis of phospholipids is practically non-existent in the ring stage and occurs essentially only when the parasite is mature (Vial *et al.*, 1989). This correlates with recent data that indicate that molecules stimulating TNF release are also associated with schizogony (Kwiatkowski *et al.*, 1989). Furthermore, prbcs show a transverse destabilization of the lipid bilayer and are prone to lose phospholipid asymmetry (Maguire *et al.*, 1991). This might lead to the presence of acidic phospholipids in the outer leaflet of the parasitized erythrocyte membrane; expression of such acidic phospholipids is associated with enhanced phagocytosis and adherence to endothelium (Maguire *et al.*, 1991). As phospholipid metabolism is a crucial process for the growth of *Plasmodium* this area is thought to represent a target for chemotherapeutic intervention (Vial *et al.*, 1984).

Although the triggering moiety seems to be essentially lipid, exoantigens are soluble in saline. Unless the triggering of macrophages is mediated by lipid micelles, the active lipid moiety must be complexed to molecules that give it water solubility. It is interesting to note that several glycolipids produced in a stage-specific manner appear to be identical between species and strains of *P.falciparum* and *P.knowlesi* (Sherwood *et al.*, 1986). Characterization of the triggering moiety and the associated structures that give this moiety water solubility is the the ultimate goal.

5.0 Macrophage Activation

5.1 Introduction

The term macrophage activation is only really useful when the function for which macrophages are activated is defined. For example, IFN-γ activates macrophages by increasing their secretion of TNF in response to LPS (Collart *et al*, 1986) and upregulating MHC class II expression, but it has no effect on the secretion of lysozyme and it reduces parasite killing by peripheral blood monocytes (Jones *et al.*, 1989). In this study activation is defined in terms of TNF secretion.

The effects of "activators" depend upon the population and maturity of the cells on which they act. It is believed that morphological and functional heterogeneity of macrophages is due to variable states of maturation and activation. Different macrophage populations respond differently to combinations of activating agents and triggers. For example IFN-γ and indomethacin synergize to activate macrophages to secrete more TNF in response to LPS or parasite supernatants; however if tumour cells are used to trigger macrophages, IFN-γ inhibits the state of activation achieved by indomethacin (unpublished data).

Both the production of TNF and protection against malaria can occur in mice whose macrophages has been activated with BCG (Clark, Allison, & Cox., 1976) or *C.parvum*. The mechanisms underlying this activation are poorly understood; indeed this state of activation may be achieved via different routes, as in mice infected with BCG it has been shown to be T cell independent, while in contrast *C.parvum*-induced activation showed a requirement for T cells (Mannel *et al.*, 1979). Activation *in vivo* may be due to a direct effect upon individual cells, such as IL-2 causes *in vitro* (Nedwin *et al.*, 1985) or it may also result from an increase in the number of macrophages at the relevant site, induced by cytokines such as IL-3 and IL-5.

Macrophage activation is frequently measured by adding a constant amount of one stimulus, such as LPS, to cells and measuring the amount of products secreted, for example TNF or IL-1. The T-cell product IFN-γ has been shown to activate macrophages for a wide range of activities, including the

secretion of TNF, IL-1, GMCSF and ROI, and the upregulation of MHC class II molecules. However, other T cell-derived cytokines can also inhibit secretion of some monokines; for example, IL-4 has been shown to inhibit macrophage secretion of IL-1 (Haeffner-Cavallion *et al.*, 1989) and TNF (Reynaud *et al.*, 1989) by human monocytes, in response to LPS.

Macrophages have a wide range of autocrine control mechanisms, predominant amongst which is the autoregulatory role of prostaglandins and leucotrienes. Thus LPS stimulation of macrophages results in secretion of arachidonate metabolites, in particular PGE_2 and PGI_2, which are thought to mediate an inhibitory feedback mechanism (Kunkel *et al.*, 1986). Macrophages incubated with indomethacin, which inhibits the formation of prostaglandins, secrete more TNF. IFN-γ is also believed to alter arachidonate metabolism. Secreted monokines may set up a positive feedback loop. IL-1 stimulation of monocytes can induce both its own secretion (Dinarello *et al.*, 1987) and secretion of TNF (Shparber & Nathan., 1986). In a similar manner, TNF is able to enhance its own production and that of IL-1 (Dinarello *et al.*, 1986). Cytokines such as TGF-β and IL-6, produced by a broad range of cells, inhibit LPS stimulated TNF secretion by human monocytes *in vitro* (Chantry *et al.*, 1989).

Further regulation comes from the interplay between the immune system and both neural and hormonal systems. Somatotropin has been shown to activate peripheral blood and alveolar macrophages to secrete ROIs (Edwards *et al.*, 1988). Neurotransmitters, in particular acetyl choline, sympathomimetic amines and the encephalins / endorphins have not been shown to alter macrophage function directly. The inhibition of macrophage secretion by cortisone is well established and this seems to be the prime mediator of the diurnal rhythm seen in macrophage secretory responses (Aabe *et al.*, 1987). Unless otherwise stated all the following results were obtained using *P.yoelii* infected prbcs.

5.2 Results

5.2.1 Activation of macrophages by IFN-γ

Macrophages secrete only small amounts of TNF, even in the presence of high doses of stimulants, unless previously elicited or activated; IFN-γ is the most widely studied of macrophage activators. Macrophages incubated with different concentrations of recombinant IFN-γ for 3 hours before removal of the medium and stimulation with a 1/10 dilution of a pronase-digested supernatant secreted increased amounts of TNF in a dose related manner (Fig 5.2.1.a.). As the concentration of stimulant was constant, differences in the amount of TNF secreted can be attributed to the state of activation of the macrophages. This is a representative experiment; increased titres of TNF were also secreted after IFN-γ treatment in response to LPS and to exoantigens that had not been protease treated. The changes induced by IFN-γ that might have led to the increased secretion of TNF, include upregulation of macrophage receptors and the inhibition of prostaglandin formation.

5.2.2 The role of arachidonate metabolites in macrophage activation

To test the hypothesis that arachidonate metabolites also regulate TNF secretion by macrophages when stimulated by the parasite exoantigens, macrophages were pretreated with either indomethacin, (an inhibitor of cyclo-oxygenase which inhibits prostaglandin formation) and BW755C, (an inhibitor of lipo-oxygenase which prevents the generation of leucotrienes). The effects of a 1 hour preincubation of unelicited macrophages with dilutions of indomethacin or BW755C on TNF responses to *P.yoelii* exoantigens are illustrated (Fig 5.2.2.a.). Both drugs caused a dose-dependent increased secretion of TNF in the presence of a constant dilution of the exoantigens. These results suggest that stimulated macrophages synthesize prostaglandins and leucotrienes which inhibit further secretion of TNF. Indeed, indomethacin treated macrophages stimulated by exoantigens were directly inhibited by the addition of exogenous PGE_2 (Fig 5.2.2.b.). The assays shown are representative of several such assays all of which showed that indomethacin and BW755C pretreatment of macrophages increased TNF

Figure 5.2.1.a. TNF secretion by IFN-g activated macrophages in response to P.yoelii exoantigens

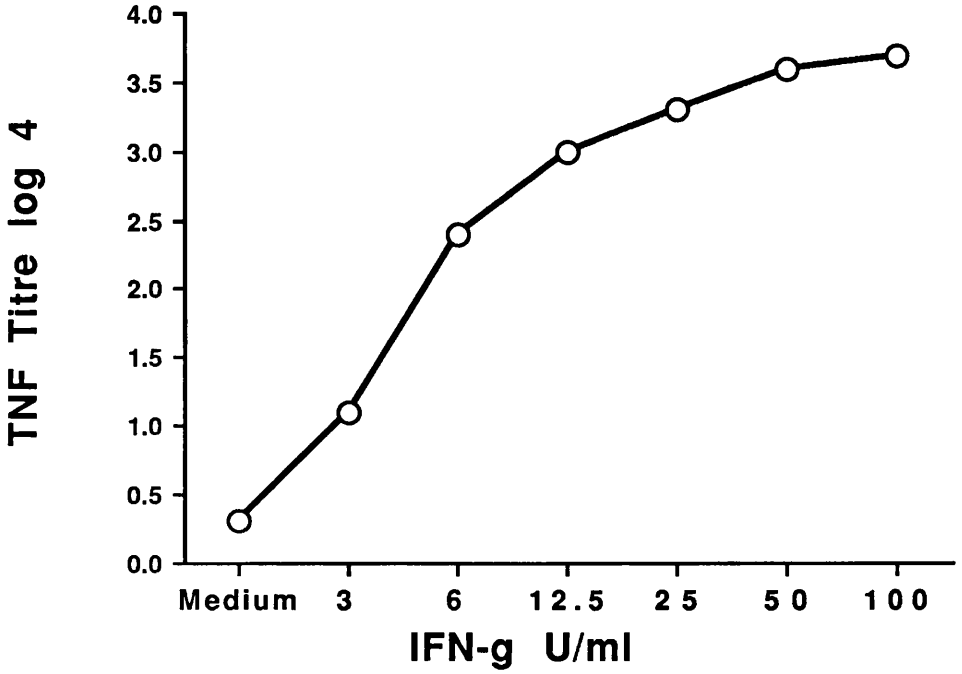


Figure 5.2.2.a. TNF secretion by macrophages treated with either indomethacin or BW755C and stimulated by P.yoelii exoantigens

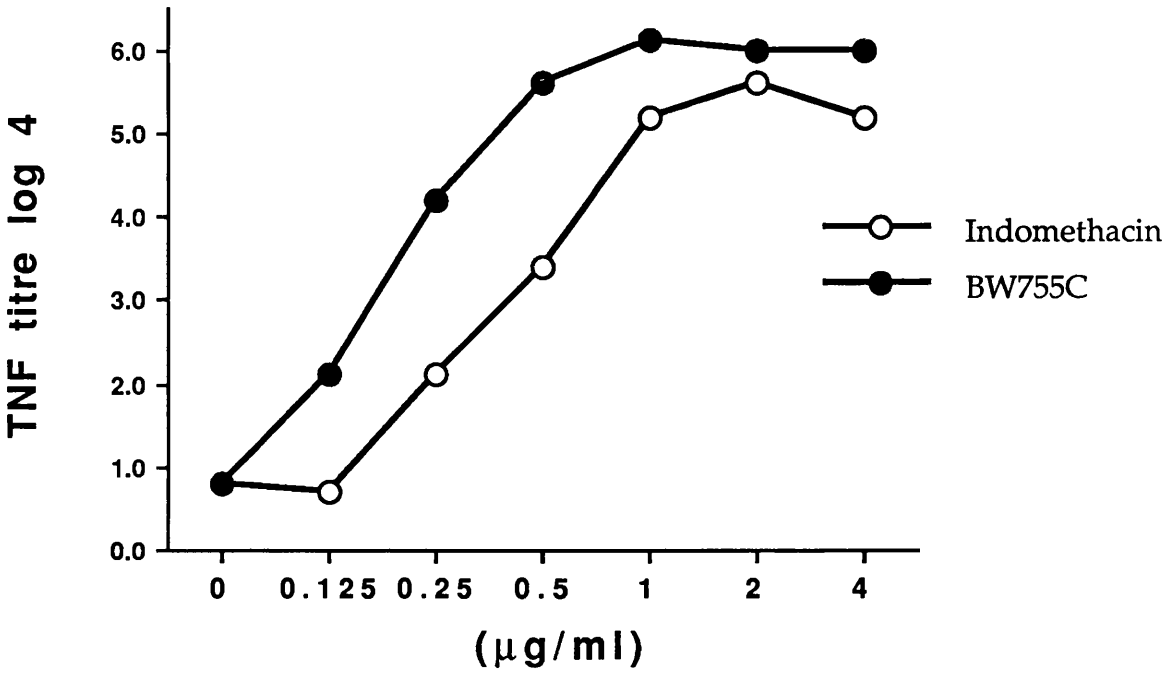


Figure 5.2.2.b. The effects of PGE 2 upon indomethacin treated macrophages stimulated by a constant dilution of P.yoelii exoantigens

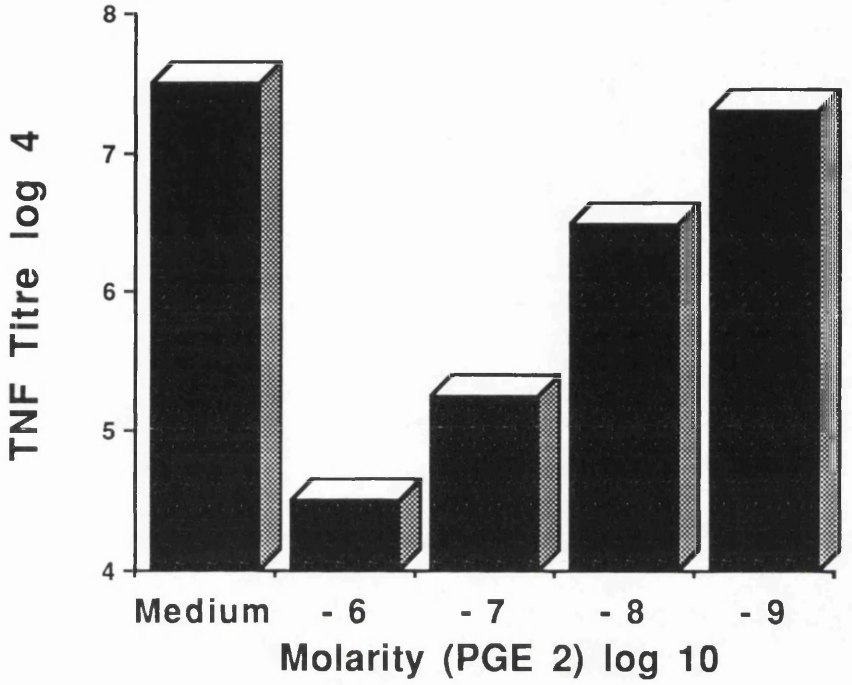
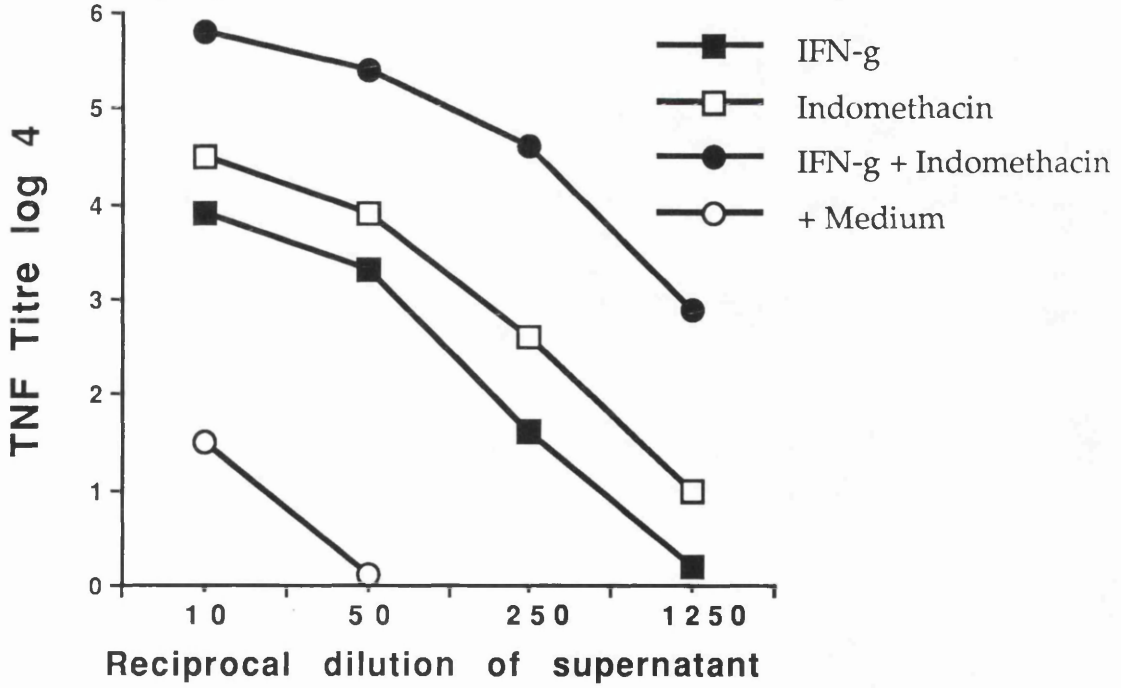


Figure 5.2.3.a. Titrations of a pronase-digested supernatant upon macrophages treated with either indomethacin, IFN-g or both.



titres and that PGE_2 inhibited TNF secretion in a dose-dependent manner.

5.2.3. Synergy between IFN-γ and Indomethacin

During an infection there is likely to be either local or circulating IFN-γ secreted by T-cells. Lipomodulin is an endogenous protein with inhibits phospholipase A_2 and thus has similar actions to indomethacin. Macrophages incubated in either culture medium, IFN-γ 100 U/ml, indomethacin 1 μg/ml or both agents were stimulated by varying concentrations of pronase-digested exoantigens and TNF titres recorded (Fig 5.2.3.a.). As before TNF secretion was enhanced by both indomethacin and IFN-γ and it was increased even further by the combination of both together.

5.2.3 Discussion

The activation of macrophages *in vitro* does not necessarily reflect what is happening during infection, as many of the effects of cytokines are only seen at certain concentrations and in synergy with other cytokines. Unknown factors, different cytokines acting either directly or in synergy with other cytokines and acting upon cells at different stages of maturation result macrophage populations with highly variable states of activation.

Plasmodium infections can activate populations of macrophages in at least two ways, by the recruitment of new macrophages (*P.yoelii* evokes a 10 fold accumulation of macrophages in the liver and spleen) (Lee, Crocker & Gordon., 1986) and by the activation of individual cells. There is evidence that parasites may directly activate macrophages, as only those macrophages in contact with parasites (blood monocytes, bone marrow, liver and spleen) become activated, but not those in the skin or peritoneal cavity (Lee, Crocker & Gordon., 1986).

Macrophage activation in infections of *P.berghei* can be independent of T cells (Lelchuck, Dockrell & Playfair., 1983). However T-cell stimulation does occur during infection and T-cell derived cytokines are likely to affect at least some macrophage functions. It has been shown that macrophages in the spleens and livers of mice infected with *P. yoelii* (Taverne,Treagust & Playfair., 1986) and mononuclear cells from human patients are activated to secrete more TNF (Kwiatkowski *et al.*, 1989).

6.0 Immunopathology of malaria

6.1 Introduction

6.1.1 Malaria pathology

Protozoan parasites, even at very low densities, cause a wide range of tissue and cellular disruption far distant from the site of infection. There is renewed interest in the proposition that the "inappropriate", or excessive stimulation of cytokine production by parasites may cause some of the symptoms and pathology of malaria. Although I have concentrated upon the idea that much of the disease of *Plasmodium* infection might be due to excess cytokine production, I am aware that there are many other possible causes of disease and I have explored some of these with respect to the pathology of cerebral malaria.

The initial signs of the onset of malaria are a general malaise, fatigue, myalgia, headache and fever, which are in no way distinctive of malaria. The development of synchronous infections may give rise to characteristic malarial paroxysms, which have three stages: a cold stage with rigor and shaking chills, a hot stage (high fever) and a sweating stage. As the disease progresses the clinical picture might include a number of organ dysfunctions which are reviewed below.

Hepatic Dysfunction

Some impairment of hepatic function is common in malaria (White & Warrell., 1983) and is usually found in fatal cases. Several abnormalities have been described but none are specific to malaria. In experimental simian malaria, hepatic pathology can be reversed by α-blockade (Sitprija., 1971), suggesting that hyperactivity of the sympathetic nervous system might be involved in the pathogenesis. Reduced hepatic function may lead to some of the other symptoms associated with malaria, such as hypoglycaemia and lactic acidosis.

Hypoglycaemia and lactic acidosis

Hypoglycaemia and lactic acidosis commonly coexist, are frequently seen in the terminal phases of several animal malarias and are recognized as important risk factors in *P.falciparum* infection (Warrell, Molyneux & Beales.,

1990). Hypoglycaemia may be the result of both a reduced supply of glucose, due to hyperinsulinaemia and reduced hepatic gluconeogenesis and also an increased demand for glucose due to the increased metabolic requirements of both the parasite and the febrile host. Insulin has been detected during *P.falciparum* infection at levels even higher than those found in insulinomas. This could be due to parasite toxins as injection of LPS into mice can result in elevated levels of insulin in the serum (Michie *et al.*, 1988) and which might be mediated by macrophage-derived cytokines (Welsh *et al.*, 1989). Measurements in quinine treated infections are unreliable as the drug stimulates insulin secretion.

Raised levels of serum lactate are a common feature of severe malaria (Warrell, Molyneux & Beales., 1990), and can be caused by both parasite and host anaerobic glycolysis, or reduced hepatic clearance. Lactate production is likely to be accentuated in tissues where mature parasites are adherent, and may cause local hypoglycaemia and lactic acidosis at the endothelial interface without changes occurring in the general circulation.

Pulmonary oedema

Pulmonary oedema is a potentially lethal manifestation of *P.falciparum* malaria and is seen in approximately 30 % of cerebral malaria cases. Sequestration of prbcs is not considered to be of importance in mediating this event as in the lungs it is 10 fold less than in the cerebral vasculature. At autopsy, the lungs are congested and oedematous and there is often distortion of capillary endothelial cells, increased capillary permeability and cardiac dysfunction (Warrell, Molyneux & Beales., 1990). Systemic mediators, parasite-derived toxins, inflammatory products and central nervous system hyperactivity have all been proposed as possible causes of pathology.

Renal Dysfunction

In *P.falciparum* malaria renal dysfunction may result from acute renal tubular necrosis; however it is usually transient and improves with control of the infection. Blackwater fever may occur in some patients with high parasitaemia and is thought to be caused by haemolysis exceeding the binding capacity of haptoglobins and albumin, resulting in the appearance of haemoglobin and related pigments in the urine (Dukes *et al.*, 1968).

Renal dysfunction may be due to reduced cortical perfusion, which is common to both nephrotoxic and ischaemic renal failure but is not specific for malaria. Histopathological studies in *P.falciparum* malaria have revealed glomerular abnormalities such as mesangial and endothelial cell proliferation. Suggested causes of these abnormalities include jaundice, sympathetic nervous hyperactivity (Sitprija., 1978), sequestration of prbcs (Warrell, Molyneux & Beales., 1990) and circulating endotoxin (Usawattankul *et al.*, 1985).

Haematological abnormalities

Haematological abnormalities include anaemia, thrombocytopenia and disseminated intravascular coagulation. There is extensive haemolysis which precipitates a fall in haematocrit, but the destruction of parasitized erythrocytes cannot completely explain either the rate at which anaemia develops or the degree of anaemia. In murine malaria, accelerated antibody-independent phagocytosis of both parasitized and uninfected red cells is seen. Dyserythropoeisis is common in bone marrow samples obtained from acute *P.falciparum* infection. The role of autoimmune Coombs-positive haemolysis is still debatable. Erythrocytic maturation abnormalities often persist for weeks after infection has been cleared. Thrombocytopenia and disseminated intravascular coagulation are frequently found in *P.falciparum* malaria although their contribution to the pathogenesis of infection is unclear.

Algid malaria

This often fatal manifestation of severe *P.falciparum* describes a rapid development of hypotension, with impaired peripheral perfusion and loss of consciousness. It has been suggested that this complication is directly attributable to concurrent bacterial infection, the state of which algid malaria closely resembles. The syndrome is characterized by cardiovascular dysfunction, and massive vasoconstriction with severe hypotension similar to acute adrenal insufficiency is seen (Brooks *et al.*, 1967). Patients have pale, cold and clammy skin and rapid shallow breathing. In *P.knowlesi*-infected *Rhesus* monkeys peripheral perfusion is reduced, a symptom which can be reversed by pretreatment with an α-antagonist (Skirrow *et al.*, 1964). Vasoconstriction is greatest at the time of schizogony which suggests association with the release of a *Plasmodium*-derived toxin, rather than the

involvement of LPS in the pathogenesis.

Gastrointestinal abnormalities

Anorexia, nausea and vomiting and abdominal pain are common in severe *P.falciparum*, and persistent diarrhoea may lead to dehydration. The intestinal mucosa may be oedematous and intestinal capillaries may be filled with parasitized erythrocytes. Visceral vasoconstriction is common (Maegraith & Fletcher., 1972) and can lead to upper gastrointestinal bleeding, a potentially lethal complication. Gastrointestinal abnormalities might predispose the patient to superinfection with Gram-negative bacteria.

Pregnancy

Pregnancy is associated with increased frequency of clinical episodes and higher rates of parasitaemia. Falciparum malaria in pregnancy carries a high mortality for both the mother and fetus (Bray & Anderson., 1979). It is not clear if this is due to the natural immune depression that occurs during pregnancy or to the enhancement of parasite multiplication by the placenta. The placenta seems to be a site for preferential parasite development and, as in other vascular beds, microcirculatory obstruction and nutrient competition may be important factors that lead to fetal death.

Cardiac dysfunction

Direct abnormalities of cardiac function in malaria such as arrhythmias are rare. Tachycardia is often seen, with a pulse rate frequently raised above 110/minute; common symptoms such as cyanosis of the lips and extremities are also seen. Low and fluctuating blood pressure has been reported, but these may simply be secondary responses in order to maintain homeostasis to metabolic acidosis, hypotension and hypoxaemia.

Cerebral Malaria

The most severe clinical complication of *P.falciparum* infection is the cerebral syndrome, which according to some reports accounts for 10 % of all admitted cases and 80 % of fatalities (WHO Malaria Action Programme, 1986). The condition is invariably fatal if untreated and is still associated with a 20 % mortality in treated patients. Typically, patients' initial complaints include severe headache, drowsiness and mental confusion. If not rapidly treated, the patient becomes delirious and suffers hallucinations, finally lapsing into a coma. Neurological findings include contracted or unequal

pupils, exaggerated tendon reflexes, muscular twitching and convulsions. The condition of cerebral malaria has been excellently reviewed (Phillips & Warrell, 1986; Warrell *et al.*, 1990) and is briefly summarized here.

Coma resulting from blockage of the cerebral microcirculation

Early theories of the pathophysiology of cerebral malaria were based upon post mortem observations that cerebral capillaries and venules contained large numbers of parasitized erythrocytes. It was proposed that these lead to the obstruction of the microcirculation and local hypoxia and ischaemia within the brain. Parasitized erythrocytes have difficulty passing through capillary beds; potential causes of this may be either reduced erythrocyte deformability, cytoadherence of infected cells or disseminated intravascular coagulation. Infection of erythrocytes with *P.falciparum* causes reduced deformability which is related to the stage of parasite development. However, it is unlikely that this alone can account for the severity of malaria as *P.vivax* causes greater enlargement of the infected erythrocyte and yet does not induce the cerebral syndrome; blockage does not occur at the site of minimum cross sectional area (i.e the capillary) and so cannot explain the preferential deposition in cerebral capillaries.

An essential pathophysiological feature of severe *P.falciparum* malaria is the sequestration of erythrocytes; which is greatest in the brain (MacPherson *et al.*, 1985) and is due to the cytoadherence of *P.falciparum* infected erythrocytes to the endothelium. The possible consequences of cytoadherence include obstruction of the microcirculation and interference with gas and substrate exchange. In the brain, local abnormalities in energy metabolism and neurotransmitter synthesis might be sufficient to cause coma.

Cytoadherence results from the specific binding of molecules on the parasitized erythrocyte to the endothelium. The expression of the adhesin on the parasitized erythrocyte surface depends on the stage of parasite development and the presence of the spleen. In splenectomized *P.falciparum*-infected squirrel monkeys, cytoadherence *in vitro* and sequestration *in vivo* are significantly reduced (David *et al.*, 1983), and splenectomized patients have an abundance of trophozoites and schizonts in their blood (Garnham., 1970). Antigens that might be involved in sequestration as parasite derived adhesins are the histidine rich proteins

found in "knobs" and a high molecular weight protein designated Pf EMP (Howard., 1987). It is trypsin sensitive and appears to be strain specific. Several ligands expressed upon the endothelium surface might mediate binding of prbcs including platelet glycoprotein (CD36), thrombospondin and ICAM-1; the latter has been shown to bind *P.falciparum* isolates (Berendt *et al.*, 1989).

Patients with cerebral malaria show normal cerebral haemodynamics and cerebrovascular resistance, and there are no significant differences in cerebral blood flow when patients became rousable (White *et al.*, 1984). Indeed the remarkable observation that survivors have complete neurological recovery (Warrell, Molyneux & Beales., 1990) argues against total microcirculatory obstruction, as the ischaemia would be expected to lead to permanent neuron damage. Furthermore, although cytoadherence occurs in *P.falciparum* infected *Aotus* species, it is not associated with pathology (Clark & Chaudhri., 1988).

The sequestration of prbcs to cerebral vasculature does not adequately explain either cerebral malaria, or any of the other pathological consequences of malaria. While I would not wish to suggest that all the pathology of malaria is a result of excess cytokine production, I would like to present the argument that cytokines, and in particular TNF, could cause some of the disease seen in malaria.

A murine model of cerebral malaria

CBA/Ca mice infected with *P. berghei* ANKA, die with neurological symptoms at low parasitaemias and moderate anaemia. Several of the clinical and histopathological changes are similar to those seen in patients with cerebral malaria (Grau *et al.*, 1989), although sequestration of infected erythrocytes is not a feature of this infection. Early deaths and the associated histopathological abnormalities were inhibited by a TNF neutralizing rabbit anti-mouse antibody. Mortality was also prevented by regimens that reduced the capacity of mice to produce TNF. Thus thymectomized and athymic nu/nu mice did not develop neurological lesions; nor did groups of mice depleted of CD 4+ helper/inducer T-cells. It was suggested that this was due to the lack of T cell derived macrophage activators; thus polyclonal and monoclonal antibodies against IFN- γ as well as combined treatment with neutralizing antibodies to both IL-3 and GM-CSF

also protect mice.

There were marked increases in serum TNF levels in mice at the time when neurological signs were evident. Regimens that inhibited the neurological symptoms and early deaths also inhibited the rise in serum TNF. Elevated serum TNF levels were not found in resistant mice, athymic mice, mice depleted of their CD4+ cells or mice treated with the monoclonal antibodies mentioned above.

6.1.2 Identification of cytokines that might induce toxicity

I have argued that much of the pathology seen in malaria might be caused by toxins with some functional similarities to LPS. Furthermore, these toxins might be molecules which stimulate excess production of endogenous factors such as cytokines. In the following pages I will outline the evidence suggesting that TNF is involved in the production of pathology, and briefly review the possible roles of other factors.

(a) Correlation between serum levels of TNF and severity of disease

In plasma from mice infected with *P.vinckei,* TNF levels show an exponential rise when tissue damage begins (Clark *et al.*, 1988). In the murine model of cerebral malaria described above, neurological dysfunction is associated with elevated serum TNF (Grau *et al.*, 1987). In 31 out of 32 patients with *P.falciparum* malaria, TNF levels in the serum were raised, and patients with complicated malaria had significantly higher levels than those without complications (Kern *et al.*, 1989). In Malawian children infected with *P.falciparum,* mean levels of TNF in the serum were significantly higher in those who subsequently died than in those that survived (Grau *et al.*, 1989). Furthermore, a comparison of 400 cases of malaria in the Gambia showed that there was a statistical correlation between elevated levels of both TNF and IL-1 in the serum, cerebral malaria and fatal outcome (Kwiatkowski., 1990).

Interpretation of the significance of raised TNF levels is complex as there are several confounding factors:

a) The toxicity of TNF may be regulated by the presence of native TNF inhibitors and interactions with other cytokines.

b) Individual differences in sensitivity to the toxicity of TNF may exist.

c) The release of TNF is likely to be intermittent, and TNF may be cleared from the serum long before its clinical effects are apparent.

Injections of LPS into mice stimulate peak titres of TNF in the serum 1-2 hours later, while the first signs of pathology become apparent only after 8 hours (Mathison, Wolfson & Ulevitch., 1988). In some infections, such as bacteraemic shock in baboons, a massive transient spike of circulating TNF (20,000 pg/ml) was found before the terminal phase of the illness, at which point TNF levels were undetectable (Tracey *et al.*, 1987; Hesse *et al.*, 1988). In malaria it is important to appreciate that the elapsed time since the last wave of schizogony, rather than the severity of illness at the time of sampling, will influence how much TNF can be detected in an individual serum sample.

(b) TNF can induce toxicity in animal models

The production of recombinant cytokines has made investigation of their effects possible. However, not all observed effects that follow administration of a particular cytokine can be directly attributed to that recombinant cytokine itself. For example, recombinant cytokines produced in bacterial vectors might be contaminated with LPS. Furthermore, many mediators stimulate the synthesis of other cytokines.

TNF is a monokine which can mimic the whole spectrum of disease seen in malaria and not just some specific aspects. Headaches, fever, nausea and vomiting, hypotension and myalgias are common side effects of TNF therapy in cancer patients (Spriggs *et al.*, 1987). It might be argued however, that their reactions differ from those of normal healthy people or of patients with malaria. The toxicity of high dosages of cytokines injected into animals has been extensively reviewed by Clark & Chaudhri, (1988). The most common clinical feature of malaria in man is fever, which can be reproduced in animal models by injection of TNF (Kettlehut *et al.*, 1987). Raised serum TNF levels are associated with hyperinsulinaemia (Michie *et al.*, 1988) and r TNF reduced hepatic gluconeogenesis (Cianco *et al.*, 1987). Injection of r TNF also caused lactic acidosis, renal and adrenal necrosis, pulmonary oedema (Tracey *et al.*, 1986) and fetal death (Clark & Chaudhri., 1988). Several features of the anaemia of malaria, such as dyserthropoeisis and erythrophagocytosis, can be induced by administration of r TNF (Miller *et al.*, 1989).

(c) Pathology can be blocked by specific inhibitors of TNF

In most cases inhibitors specific for individual mediators are monoclonal or polyclonal antibodies or pharmacological products. There are also reports of the existence of endogenous cytokine inhibitors: an inhibitor of TNF has been found in serum from patients suffering from sarcoidosis, tuberculosis and Crohns disease (Foley *et al.*,1990).

Passive immunization by a neutralizing Mab to TNF was shown to protect mice against the mortality caused by injections of LPS (Tracey *et al.*, 1987; Mathison *et al.*, 1988), and neutralizing antibodies to TNF protected baboons infused with a lethal dose of bacteria (Tracey *et al.*, 1988). Neutralizing polyclonal antiserum against TNF inhibits the cerebral syndrome induced by *P. berghei* in CBA/Ca mice (Grau *et al.*, 1989). Preliminary trials in both septic shock and malaria of a monoclonal antibody that neutralizes the cytotoxicity of human TNF are in progress.

6.1.3 Other cytokines that might induce toxicity

Some of the effects of toxins like LPS that can be mimicked by injection of TNF may also be mediated by other host molecules; for example, the fever caused by LPS can be induced by injections either of TNF or IL-1. Moreover, some of the effects of injected TNF may occur because TNF initiates the release of a cascade of cytokines. Although mice pretreated with a neutralizing antibody to TNF before injection of LPS did not show the customary rise in CSF levels, this antibody did not inhibit the LPS induced increase in either cortisone or IFN-γ, or the subsequent hypoglycemia (Vogel & Havell., 1990).

As toxins such as LPS are able to stimulate the synthesis of cytokines other than TNF, which might prove toxic, the roles of such cytokines has been investigated. "Potential toxins" induced by LPS include TNF, IL-1, CSFs, IFNs, prostaglandins and leucotrienes, PAF, endorphins, insulin, ACTH, endogenous glucocorticoids, the catecholamines (adrenaline and noradrenaline), complement and the acute phase proteins.

Complement activation

Endotoxins activate both the classical and the alternative pathways of complement *in vitro*, thus liberating the anaphylatoxins C3a and C5a. These molecules are potent inducers of vasodilation and smooth muscle contraction and are potential mediators of the hypotension. Studies in man however, concluded that determination of complement levels served no prognostic value in shock (Shatney *et al.*, 1985).

Arachidonic acid metabolites

Endotoxins stimulate the production of prostaglandins and leucotrienes by macrophages and elevated levels are detectable in post endotoxin serum. Inhibitors of arachidonate release, such as indomethacin, can inhibit the deleterious effects of endotoxin, which would suggest that toxic prostaglandins were present; however exogenous prostaglandins alone cannot reproduce all the symptoms of endotoxicity. The role of prostaglandins may well be as secondary mediators, stimulated by either TNF or IL-1. For instance indomethacin can inhibit the fever seen in response to both TNF (Kettlehut *et al.*, 1988) and IL-1 (Dinarello *et al.*, 1986).

Platelet Activating Factor (PAF)

PAF has been implicated in the pathogenesis of endotoxicity. It is found in post endotoxin serum and specific PAF antagonists are able to partially block the hypotension seen in response to LPS (Issekutz & Szpejda., 1986). Like prostaglandins, the role of PAF is probably secondary to TNF. TNF stimulates human monocytes and endothelial cells to secrete PAF (Camussi *et al.*, 1987), and in rats TNF-induced bowel lesions could be inhibited by a PAF antagonist (Sun & Hsueh., 1988).

Interleukin-1 (IL-1)

LPS is a potent stimulator of IL-1 and on a molar scale IL-1 is the most potent pyrogen (Flad *et al.*, 1989); elevated serum levels are found during septic shock (Waage *et al.*, 1989) and infection with *P.falciparum* (Kwiatkowski., 1990). Recombinant IL-1 β injected into rabbits at 5 mg/kg induces a shock-like state (Okusawa *et al.*, 1988).

There is no obvious homology in amino acid sequence between IL-1 and TNF and although structural similarities exist, the receptors for the monokines are distinct. TNF and IL-1 show great functional overlap reviewed by Le & Vileck., (1987). *In vivo* the effects of these monokines are difficult to separate as macrophage triggers often induce both. The situation is further complicated by the fact that IL-1 can stimulate macrophages to secrete TNF (Dinarello *et al.*, 1986) and TNF can induce endothelial cells to secrete IL-1 (Nawroth *et al.*, 1986). The lethality of LPS in rabbits has been inhibited by an interleukin 1 inhibitor (Ohlsson *et al.*, 1990).

Lymphotoxin

Lymphotoxin and TNF have around 30 % homology at the amino acid level and interact with the same receptor, although the activity of the two can be differentiated by neutralising monoclonal antibodies. It would seem likely that lymphotoxin would also be a candidate for the role of an endogenous toxin.

Natural Opiates (Encephalins and Endorphins) and ACTH

These small molecular weight molecules were originally discovered as neurotransmitters and are now recognized to be modulators of the immune system as well. LPS can induce the synthesis of ACTH and of endorphins (Harbour-McMenamin *et al.*, 1985). Similarities exist between the clinical manifestations of opiate abuse and some of the symptoms of malaria: primarily nausea and vomiting, hypotension and significantly altered psychological states, including coma.

The Interferons, α,β and γ

Endotoxins induce the production of α,β (Maehara & Ho., 1977) and of γ IFN (Le *et al.*, 1986). IFN α and β are stimulated directly from B lymphocytes and macrophages but IFN- γ secretion by T-cells is complex and has a requirement for adherent cells and IL-2 (Matsumura & Nakano., 1988). In clinical trials, IFN-α induced an influenza-like toxic reaction, characterized by fever, chills, myalgias, anorexia, fatigue, headache, nausea and vomiting. Continued treatment caused tachyphylaxis and the severity of symptoms was gradually reduced. Rarer side effects include persistent heamatological, hepatic, gastrointestinal and neurological abnormalities. Neurological toxicity includes neuropathies, somnolence, lethargy, malaise, stupor and

psychosis (Dinarello *et al.*, 1984). In the generalized Shwartzman reaction, a dose of IFN- γ can replace the second injection of TNF, and accordingly anti-IFN-γ prevents the reaction (Flad *et al.*, 1989).

Synergy between cytokines responsible for toxicity

Although TNF may be of primary importance in the induction of pathology, synergy with one or more other cytokines can affect the toxicological outcome. Cytokines secreted by T-cells might be of importance in mediating TNF induced toxicity. T-cell products can increase the numbers of macrophages in immune organs and increase their capacity to secrete TNF, however T-cell products might also increase target cell sensitivity to TNF. Athymic mice which lack the capacity to produce IFN- γ do not suffer the cerebral dysfunction of *P.berghei* infections (Grau *et al.*, 1989). IFN- γ has been found in the serum of patients infected with *P.falciparum* (Rhodes-Feuillete *et al.*, 1985) although levels do not correlate with disease severity (Kern *et al.*, 1989). IFN- γ has been shown to increase the expression of TNF receptors on cells (Ruggerio *et al.*, 1986), and to increase sensitivity the of L929 cells to TNF cytotoxicity (Taverne & Bate unpublished data). Interleukin -1 is another possible mediator of pathology associated with LPS-like toxins. IL-1 increases the toxicity of TNF for mice (Waage & Esperik., 1988) and more so for mice infected with *P.vinckei* (I.A. Clark., personal communication.)

6.2 Results

6.2.1 TNF release *in vivo*

Groups of mice primed with *C.parvum* and injected with LPS, 7-14 days later, secrete large amounts of TNF into the serum 1-2 hours after injection (Carswell *et al.*, 1975). In the previous chapters I have described how parasite-derived molecules stimulate macrophages to secrete TNF *in vitro*, and the logical next step was to confirm that these molecules could also stimulate TNF release *in vivo*. Supernatants prepared from cultures of *P.yoelii* or *P.falciparum* prbcs and uninfected nrbcs were injected into groups of mice which had been primed with *C.parvum* 10 days earlier; 90 minutes later the mice were bled and their serum was then assayed for TNF. Titres of TNF stimulated by different preparations are shown (Fig 6.2.1.a.). Preparations from all the species of *Plasmodium* tested stimulated the production of similar amounts of TNF, which were of the same order of magnitude as titres stimulated by 10μg/mouse of LPS (data not shown). Control supernatants made from uninfected rbcs did not stimulate the release of TNF into the serum.

6.2.2 Toxicity of exoantigens for sensitized mice

Prior administration of D-galactosamine increases the sensitivity of mice to the toxic effects of LPS by approximately 100,000 fold (Lehmann *et al.*, 1987). Parasite exoantigens stimulate the production of TNF *in vitro* and *in vivo*, and TNF has been shown to be the prime mediator of toxicity in the D-galactosamine model. The injection of exoantigens into D-galactosamine sensitized mice caused the deaths of those mice in a dose-dependent manner. Groups of 3 - 5 mice were generally used and the tests repeated with different samples; pooled results from several experiments are shown (Fig 6.2.2.a.). Supernatants prepared from erythrocytes infected with different species of *Plasmodium*, including preparations from *P.falciparum*, proved lethal when injected into D-galactosamine sensitized mice.

Figure 6.2.1.a. TNF titres secreted into the serum of C.parvum primed mice in response to supernatants prepared from human and rodent prbcs and mouse nrbcs

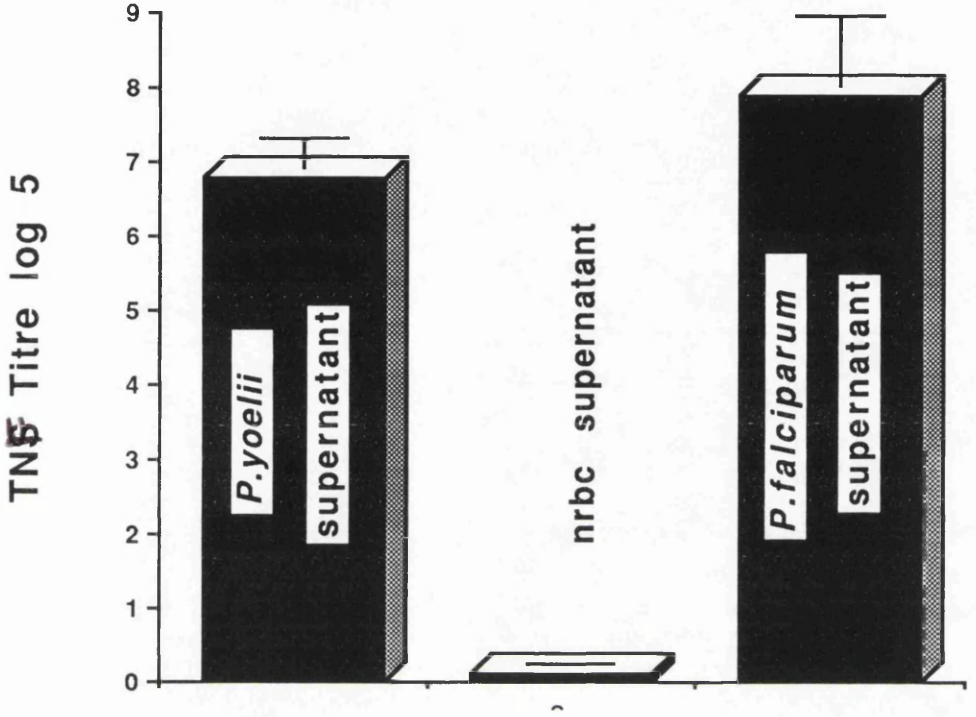
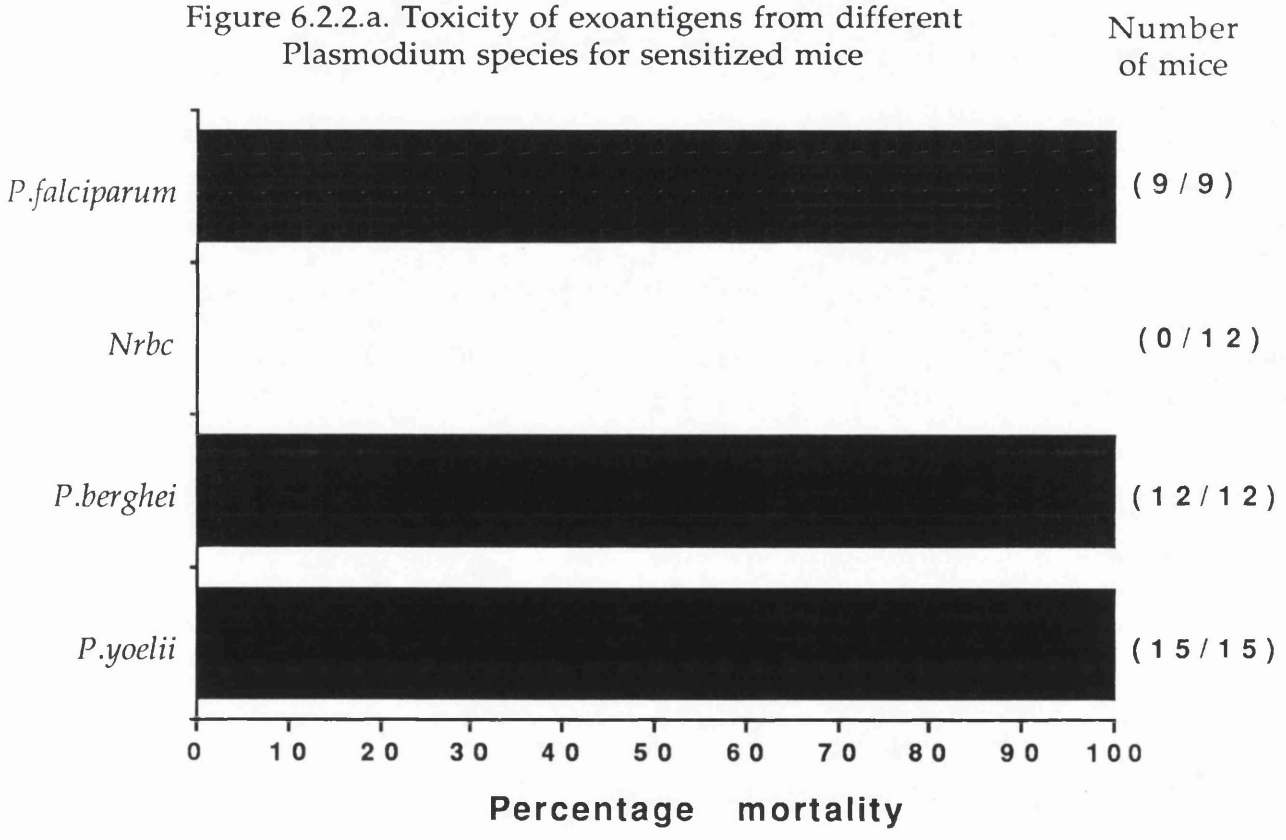


Figure 6.2.2.a. Toxicity of exoantigens from different Plasmodium species for sensitized mice



D-galactosamine alone or control supernatants made from uninfected rbcs were not toxic. Mice were bled 1-2 hours after injection of exoantigen and their sera collected and assayed for TNF. There was no differences between the TNF titres from mice treated with D-galactosamine and from untreated mice (data not shown). This confirms that D-galactosamine sensitization to toxins is mediated by increased sensitivity to TNF rather than to the release of more TNF.

It was previously reported that D-galactosamine enhances mouse susceptibility to the toxic effects of TNF (Wallach *et al.*, 1988). Administration of cytokines in the presence of D-galactosamine showed that mice were killed by TNF (1 μg/mouse) and PAF (100 μg/mouse) but not IFN-γ ($1x10^8$ U / mouse) or IL-1α ($1x10^7$ U/mouse) (Fig 6.2.2.b.). The mortality of mice injected with PAF was not affected by prior administration of D-galactosamine (data not shown). TNF was the only cytokine tested the toxicity of which was increased by the presence of D-galactosamine. It is possible, however that D-galactosamine also sensitizes mice to the toxicity of IFN-γ, and IL-1α, but this was not seen at the concentrations tested.

A supernatant that had been digested with pronase E was titrated in groups of untreated and D-galactosamine sensitized mice (Fig 6.2.2.c.). Some preparations of pronase-digested exoantigens, including the sample shown, killed mice in the absence of D-galactosamine. The toxicity of such a supernatant was greater than that of comparable undigested preparations (data not shown); this could be due either to an increased capacity to stimulate the production of cytokines or altered biokinetics. However as such protease-digested exoantigens bind BSA *in vitro* I would assume that after injection, they rapidly associate with plasma proteins. A control supernatant made from uninfected rbcs and digested with pronase E did not kill mice.

I wanted to know if the exoantigens stimulating the production of TNF *in vitro* were the same as those that were toxic to sensitized mice. As they have not yet been purified, it was decided to test various digested and treated supernatants, and homogenates to compare their activity in the two systems. Comparable dilutions of *P.yoelii* supernatants that had undergone various biochemical, enzymatic or purification procedures were therefore injected into D-galactosamine-sensitized mice and mortality scored after 48 hours. The pooled results of several such experiments show that control

Figure 6.2.2.b. Toxicity of cytokines for groups of D-galactosamine sensitized mice

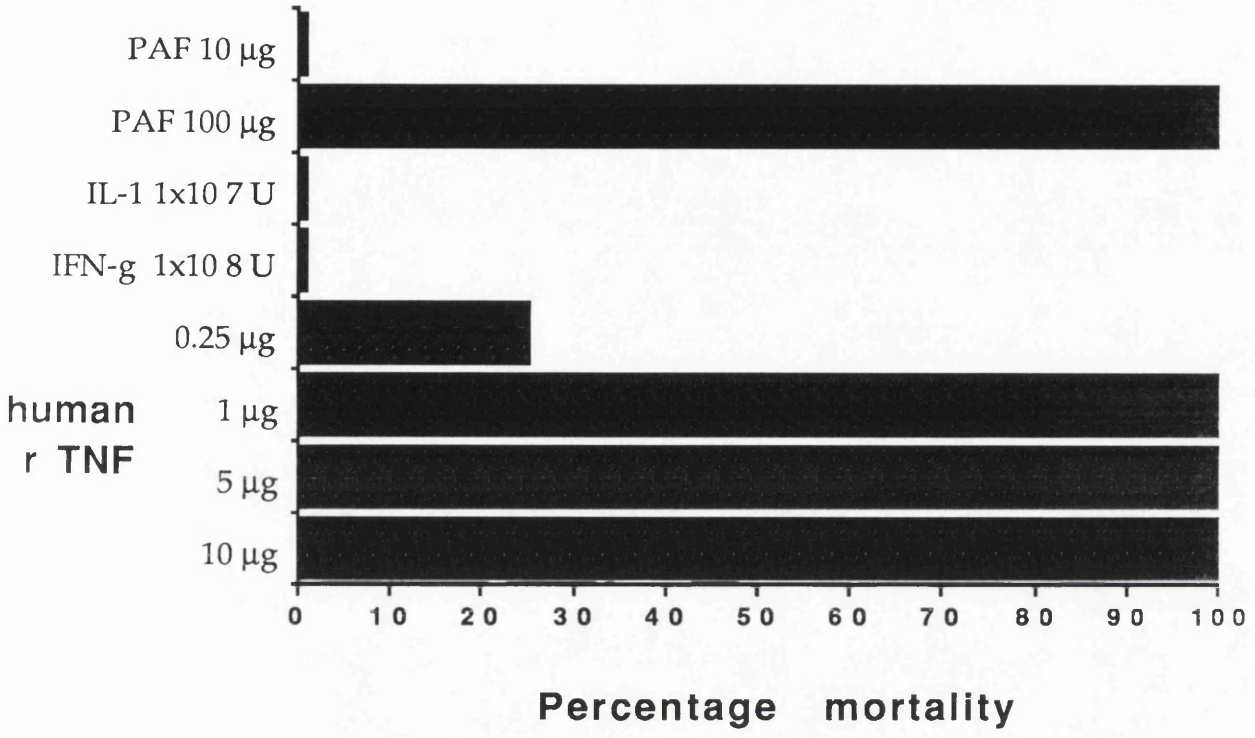
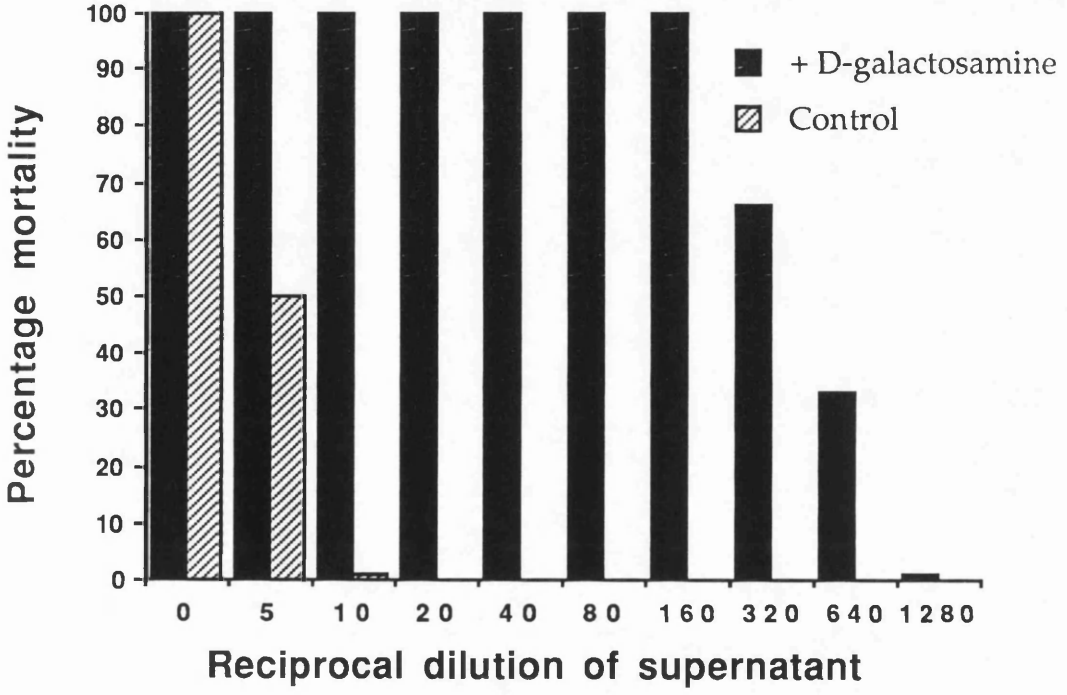


Figure 6.2.2.c. Lethality of a sample of protease treated exoantigens in the presence and absence of D-galactosamine



boiled supernatants and supernatants that had been digested with pronase E retained toxicity (Fig 6.2.2.d.). Pronase-digested supernatants that had undergone further treatment with nitrous acid (deamination) or TFMS (deglycosylation) were also toxic to mice. However, pronase-digested supernatants that had then been digested with mixed lipases or deacylated did not kill D-galactosamine sensitized mice. Further investigation using a 1/10 dilution of a pronase-digested supernatant used as a control, showed that the supernatant killed mice after digestion with phospholipase A_2, but lost activity after digestion with phospholipase C. The direct correlation between the sensitivities of supernatants inducing toxicity *in vivo* and the capacity to stimulate TNF secretion *in vitro* suggests that similar molecules are responsible for both activities.

6.2.3 T-cells in exoantigen-induced toxicity

If the mortality of D-galactosamine sensitized mice was affected by T-cell products, then depletion of T-helper (CD 4 +) cells should protect the mice. Mice were depleted of their T-helper cells by a series of injections of a rat anti-mouse CD 4+ antibody. Twenty four hours later the mice were sensitized with D-galactosamine and injected with exoantigen, and deaths within 48 hours scored. The percentage mortality in groups of mice depleted of their T-helper cells was found to be no different than that of control mice (Figure. 6.2.3.a.). Apparently, the toxicity induced in sensitized mice does not require the presence of functional T-helper cells, which would imply that T-cell derived cytokines such as IFN-γ are not necessary to produce pathology in this case.

6.2.4 Inhibition of toxicity

As described above injection of a parasite supernatant into D-galactosamine sensitized mice kills the mice. Methods of preventing this mortality by prior administration of various drugs and inhibitors have been investigated. Mice were protected when pretreated 30 minutes earlier with either 100μg Mab neutralizing TNF, 500 μg phenoxybenzamine, 100 μg indomethacin or 24 hours earlier with 1 ml of human post-exercise serum. Pretreatment 30 minutes earlier with 20 μg of the PAF antagonist, CV3988, 1 mg of a Mab to IFN-γ, 500μg of naloxone, an endorphin / morphine receptor

Figure 6.2.2.d. Mortality in sensitized mice injected with digested / treated exoantigen preparations

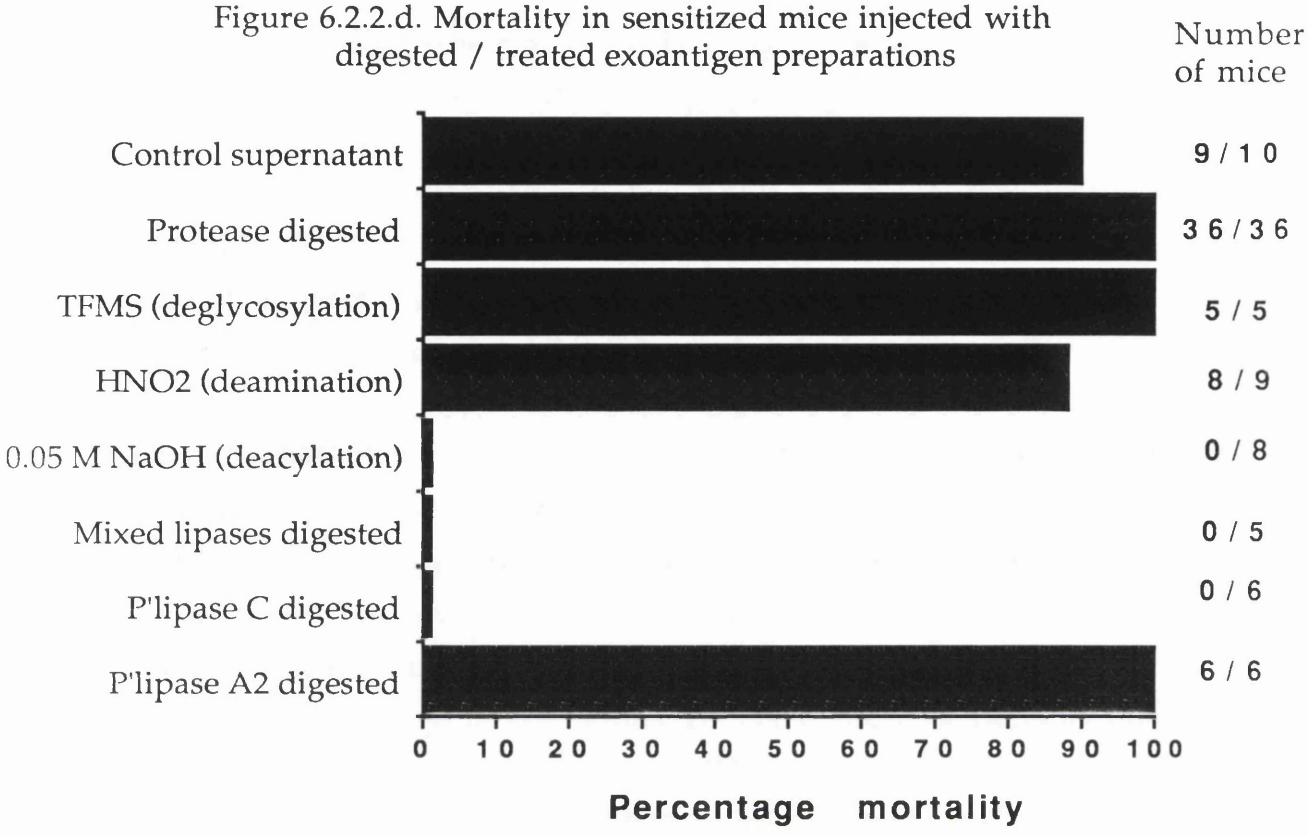
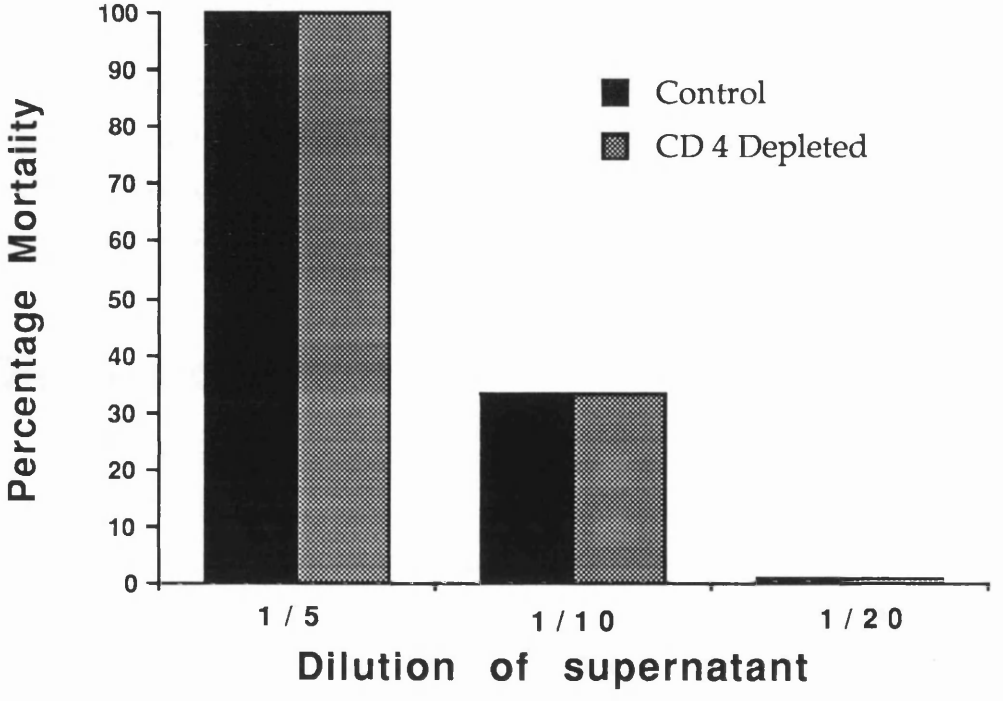


Figure 6.2.3.a. Mortality in groups of control and CD 4 + depleted mice in response to injection of parasite exoantigens



antagonist, 20 mg of vitamin C, an anti-oxidant or 24 hours earlier with 1 ml of normal human serum was not protective (Fig 6.2.4.a.).

A possible explanation for these results is that there are other toxic molecules besides TNF, including perhaps prostaglandins and catecholamines. Alternatively the protective regimens might inhibit steps in a cascade, of which TNF is the initiator. There are reports that indomethacin inhibits the toxicity of r TNF *in vivo* (Kettlehut *et al.*, 1987) and its cytotoxicity *in vitro* (Matthews *et al.*, 1987). In a similar assay to the latter the cytotoxicity of r TNF for L929 cells was inhibited by preincubation of the cells with 5 % post-exercise serum (Chapter 8). The drug phenoxybenzamine blocked the toxicity of supernatants *in vivo* but has no effect on either the cytotoxicity of TNF *in vitro* or on the stimulation of TNF production by parasite exoantigens (10 - 0.01 μg/ml, data not shown). Phenoxybenzamine blocks α_2 receptors, which respond to either adrenaline or nor-adrenaline, the serum levels of which are raised in response to LPS. The necrosis of a Meth A sarcoma induced by injections of either TNS or LPS can be partially inhibited by phenoxybenzamine (Bloksma *et al.*, 1982). It is believed that necrosis of tumours may be mediated by the interruption of blood flow within the tumour which is mediated by sympathetic neurons secreting nor-adrenaline.

The potency of the protection mediated by the Mab neutralizing TNF was tested using a pronase-digested supernatant which still killed 50 % of a group of sensitized mice at a 1/500 dilution. Even undiluted pronase-digested supernatant did not kill mice when they had been pretreated with this antibody (Fig 6.2.4.b.). Titres of TNF secreted into the serum of mice primed with *C.parvum* and injected with a pronase-digested supernatant 10 days later were reduced from 47,765 U/ml in mice pretreated with a control antibody to only 795 U/ml in mice pretreated with the Mab to TNF.

6.3 Discussion

Exoantigens prepared from different parasites, including the human parasite *P.falciparum* stimulate the release of TNF *in vivo* and are toxic to the point of lethality in sensitized mice. There are no obvious differences between species of *Plasmodium* in this system that might explain why some parasites are lethal for mice and others are not. There is a

Figure 6.2.4.a. Prevention of exoantigen induced toxicity for sensitized mice by prior administration of inhibitors

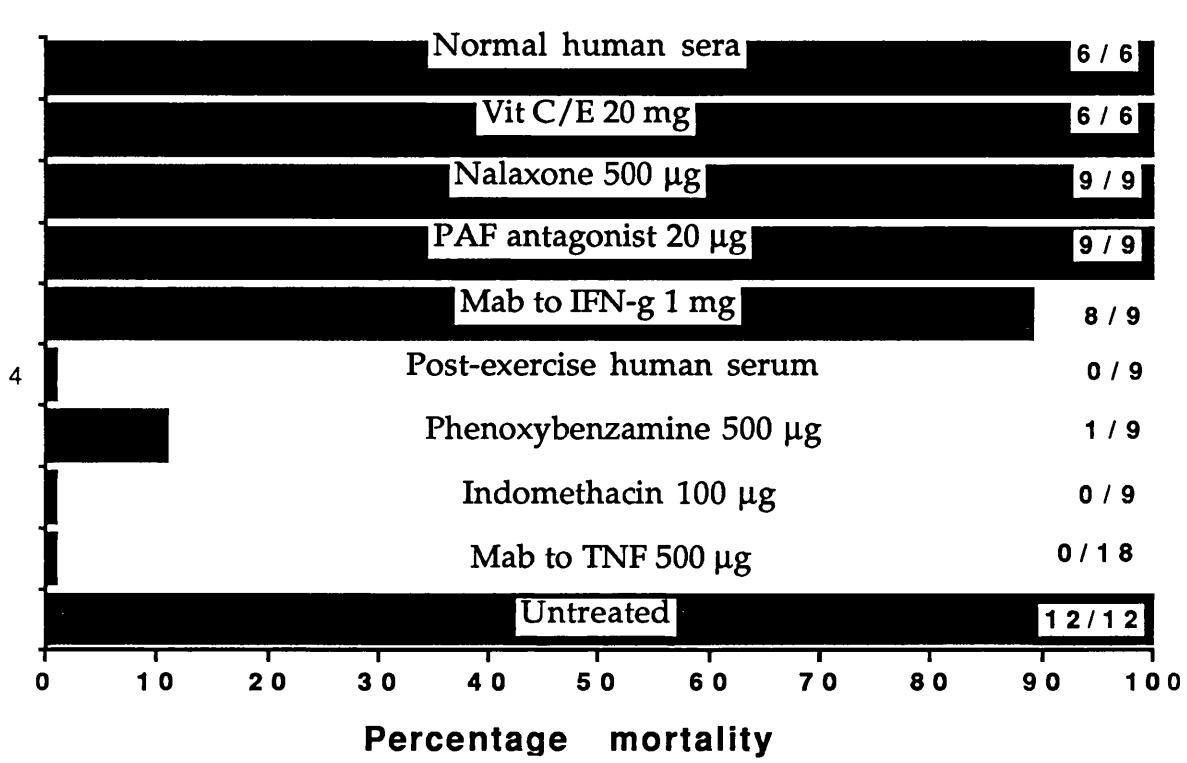
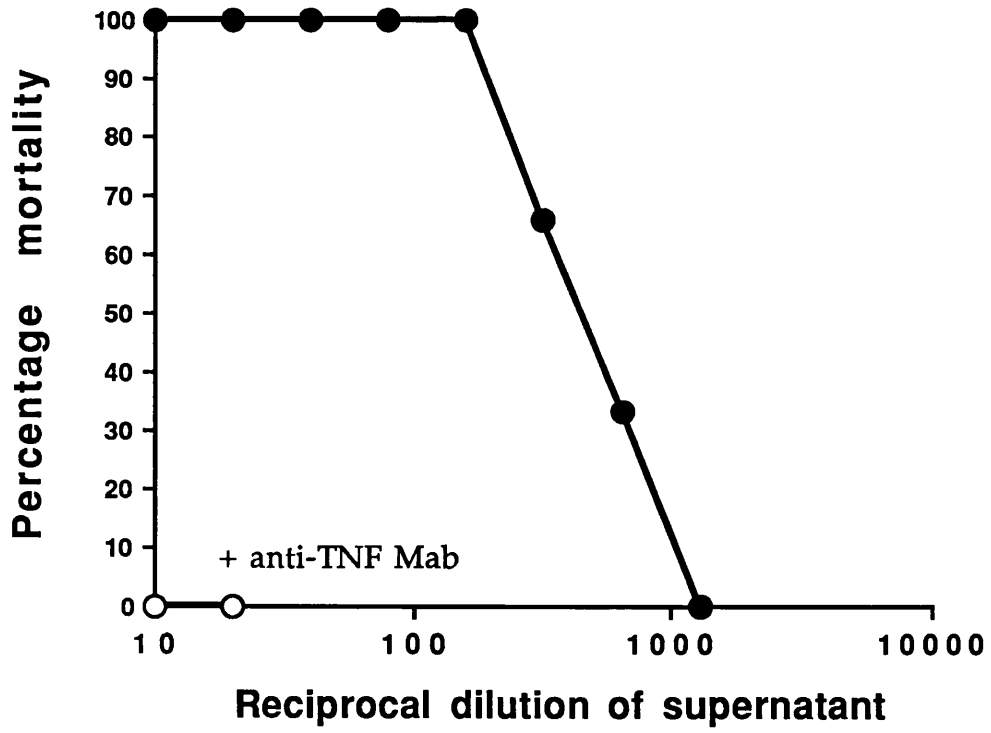


Figure 6.2.4.b. Protection mediated by a neutralizing Mab to TNF in groups of sensitized mice injected with pronase-digested exoantigens



correlation between the toxicity of supernatants *in vivo* and their ability to stimulate the production of TNF *in vitro*, which strongly suggests that the two responses are mediated by a common moiety. If such a moiety exists, then it could have other functions apart from the stimulation of TNF secretion. I have investigated some factors that might contribute to the toxicity of exoantigens for mice pretreated with D-galactosamine. The injection of low doses of r TNF killed sensitized mice. Mice were also killed by PAF, but only with a high dose, 100 μg/mouse, and mice were not sensitized to the cytokine by prior administration of D-galactosamine. IL-1α or IFN-γ did not kill the mice at the doses tested.

There may be other mediators however, which although not lethal on their own are still important in the pathological process. Depletion of functional T-helper cells was found not to alter the toxicity of the exoantigens, which suggests that T-cell derived products are not important in this system. Exoantigen-induced toxicity was completely inhibited by a monoclonal antibody that neutralizes TNF-α. Pretreatment of mice with neutralizing antibodies to IFN-γ, high doses of the antioxidant Vitamin C and the PAF antagonist CV3988 do not affect the toxicity of exoantigens. These results suggest that in D-galactosamine sensitized mice parasite exoantigens mediate toxicity through their ability to stimulate TNF production.

Indomethacin, phenoxybenzamine and unspecified factors in human post exercise serum also protected mice. Indomethacin activated macrophages secrete more TNF and therefore pretreatment of mice with indomethacin would be expected to enhance toxicity. However, prostaglandin formation is an essential part in the signal transduction pathway of L929 cells stimulated by TNF which causes their destruction, indomethacin treated L929 cells are less sensitive to the cytotoxicity of r TNF than untreated controls (chapter 8). Indomethacin has been shown to inhibit the development of fever (Kettlehut *et al.*, 1987), the cytotoxicity of TNF *in vitro* (Matthews *et al.*, 1987) and toxicity of TNF in mice (Okusawa *et al.*, 1988).

Mice were also protected against exoantigen-induced toxicity by 24 hour pretreatment ~~of mice~~ with human post-exercise serum. *In vitro* this serum also inhibited both the secretion of TNF by activated macrophages (data not shown) and the cytotoxicity of r TNF for target cells (Chapter 8).

Preliminary results suggest that the inhibitor is resistant to proteases. Cytokine inhibitors might be an important mechanism for inhibiting the toxicity of some infections, however the factors governing the release of these molecules are still to be discovered.

Phenoxybenzamine also protected against the toxicity of exoantigens for mice without altering either TNF secretion or the cytotoxicity of r TNF *in vitro*. It is not known whether parasite exoantigens stimulate the release of catecholamines which are toxic, or if catecholamine release is secondary to TNF production, or if catecholamines sensitize target cells to the effects of TNF. The inhibition of exoantigen-induced toxicity by neurotransmitter antagonists implies that exoantigens may affect the central nervous system.

Some workers have interpreted the neuropathological findings of cerebral malaria as resulting from a hyperactivity of the CNS (Toro & Roman., 1978). Indeed, abnormal neural innervation has been implicated in many of the organ dysfunctions seen in malaria. Cerebral malaria rarely induces irreversible neuron damage in patients that recover (D. Kwiatkowski, personal communication). Coma caused by either hypoglycaemia or neurotransmitter imbalance by pharmacological overdose rarely causes permanent neuron damage. It is possible that coma in malaria is due to the alteration of neurotransmitter levels by the exoantigens, either as a direct effect upon neurons or through the actions of cytokines.

Many physiological responses to endotoxins have now been shown to be mediated through the CNS. Thus, LPS induced fever is inhibited by pretreatment of mice with the neurotoxic agent 6 OH dopamine, which destroys dopaminergic neurons (Ovadia *et al.*, 1989). Furthermore, chlorpromazine (a dopamine receptor antagonist) which acts on the CNS has been reported to inhibit the pathology of experimental endotoxicity (Bertini *et al.*, 1989).

If coma is a consequence of the actions of parasite-derived toxins, is it mediated by cytokines ? Injections of TNF can induce cerebral symptoms in mice (G, Grau, personal communication). Infusion of r TNF into human patients resulted in altered neurological function, however, once treatment ceased, neurological function returned to normal (Sherman *et al.*, 1988). TNF and IL-1 can directly affect whole body metabolism, either directly or through the elevation of body temperature which alters metabolic requirements in

non-immunological tissues. As these two monokines can exert such a powerful effect upon metabolism, it is reasonable to assume that coordination with the hormonal and nervous systems occurs. As it has long been accepted that hormonal and neural responses are integrated, is it not to be expected that both systems should interact with the immune system as well ? How these systems overlap and regulate one another remains largely unknown, but there are clues that suggest a mode of action. Firstly, TNF and IL-1 both interact with the pituitary and the hypothalamus, the cerebral areas that control homeostasis. Secondly, both monokines alter basal hormone levels, including those of insulin and cortisone, and cortisone inhibits macrophage secretion of TNF and IL-1 thus mediating inhibitory feedback (Aabe *et al.*, 1987).

TNF and IL-1 both bind to receptors in the pituitary and the hypothalamus and can induce fever (Woloski *et al.*, 1985). In rabbits both TNF and IL-1 are somnogenic, and IL-1β is a potent hyperalgesic in rats (Ferreira *et al.*, 1988). Intra-cerebral injections of TNF and IL-1 cause fever and the appearance of acute phase proteins in the serum at much lower doses (1/100th) than peripherally administered cytokines (Turchick *et al.*, 1980). Furthermore intra-cerebral injections of the neurotransmitter α-melanocyte-stimulating hormone (MSH) inhibit the development of fever in response to peripherally administered TNF and IL-1 (Robertson, Dostal & Daynes., 1988). Both TNF and IL-1 have been shown to stimulate mRNA expression of pituitary pro-opiomelanocortin (POMC), a precursor for a variety of hormones such as ACTH, β-endorphin, the encephalins and MSH peptides (Brown, Smith & Blalock., 1987), which could act as an inhibitory feedback mechanism.

Hypothalamic concentrations of ACTH and β-endorphin are increased during cytokine-induced fever (Murphy *et al.*, 1983). Endorphins are natural opiates and hyperstimulation of their receptors, as in morphine overdose, can lead to coma. Morphine-induced coma can be reversed by the administration of naloxone; however in my experiments, naloxone did not protect mice. Cytokines can be produced in the cerebral microenvironment; thus immortalized murine microglial clones from embryonic brain have been shown to secrete TNF, IL-1 and IL-6 (Mori *et al.*, 1989), and there is some evidence that IL-1 plays a role as a neurotransmitter (Bernton *et al.*, 1987;

Breder *et al.*, 1988).

Other cytokines or factors may also affect the CNS. In clinical trials of IFN-α, an influenza-like toxic reaction is seen. IFN-α is intrinsically pyrogenic and fever is a commonly observed side effect (Dinarello *et al.*, 1984). Rarer side effects include the production of other neurological abnormalities, somnolence, lethargy, malaise, stupor and psychosis. Thus, although TNF and IL-1 affect CNS function, it remains to be seen if this can lead to the cerebral malaria and under what conditions this might occur.

Cytokines might be involved in the pathogenesis of cerebral malaria through their ability to activate human endothelial cells. IL-1, TNF and IFN-γ can upregulate adhesion molecules on the endotheilum, including ICAM-1 (Pober *et al.*, 1986), which has been implicated as a receptor involved in sequestration. Presumably reduced expression of such ligands would reduce the the number of prbcs that sequester. If sequestration contributes to the pathogenesis of cerebral malaria, then such a reduction might reduce the severity of cerebral malaria.

The D-galactosamine system used here appears to be a valid method for the identification of toxins such as LPS from Gram -ve bacteria and parasite exoantigens in malaria. This model is selective for toxins on the basis of their ability to induce TNF secretion, but other modes of toxicity may be mediated by different *Plasmodium* antigens. Indeed, a monoclonal antibody that neutralizes TNF and protects mice against the toxicity of exoantigens does not protect mice against infection with the lethal variant of *P.yoelii*, which suggests that TNF is not the only toxic factor produced during infection. Antigens that have a direct effect upon cells, such as the pertussis and cholera toxins, would not be recognized in such a model. Indeed we cannot be sure that the toxins identified in any particular model are of general importance during infection. The ultimate test of these questions would be to see if neutralizing antibodies directed against the candidate toxin inhibit the pathology of infection.

7.0 Inhibition of exoantigen induced toxicity

7.1 Introduction

Many distinguished malariologists have remarked that in endemic areas a form of "anti-toxic" immunity develops several years before an anti-parasitic immunity. Children in endemic areas develop resistance to the serious toxic aspects of malaria several years before their parasitaemias start to fall. The basis of this "anti-toxic immunity" is unknown; possible explanations include age differences in the sensitivity of endothelial cells to prbc sequestration, the development of antibodies that block sequestration and tolerance to cytokines including TNF. If molecules which stimulate excessive TNF release during malaria behave, in effect, like toxins, then antibodies to these molecules could possibly mediate "anti-toxic" immunity. In this chapter I explore the possibility that toxicity can be inhibited by antibodies that block exoantigen stimulation of monokines and how these exoantigens might therefore be modified for use as vaccines. Unless otherwise stated parasite supernatants are from *P.yoelii* prbcs.

7.2. Results

7.2.1 Protection against toxicity

Low doses of parasite supernatants injected 24 hours earlier protected groups of D-galactosamine-sensitized mice against what would otherwise have been a lethal dose of exoantigens. This desensitization to the toxicity of parasite exoantigens was not specific, since it inhibited the toxicity of LPS as well as that of parasite exoantigens. Injections of either 1 ml of a 1/20 dilution of a supernatant or 1 μg/mouse of LPS protected groups of mice given a second injection of either 1 μg/mouse LPS or a 1/10 dilution of supernatant 24 hours later (Fig 7.2.1.a.). Studies with LPS had shown that r TNF can also mediate this desensitization and that the refractory period lasted for 48 hours (Wallach *et al.*, 1988). To see if the desensitization to exoantigens was mediated by cytokines, various recombinant cytokines were injected into groups of 3-5 mice 24 hours before D-galactosamine sensitization and injection of a 1/5 dilution of supernatant. Protection occurred in those mice

Figure 7.2.1.a. Non-specific protection against LPS or exoantigen induced mortality by 24 hour pretreatment with either reagent

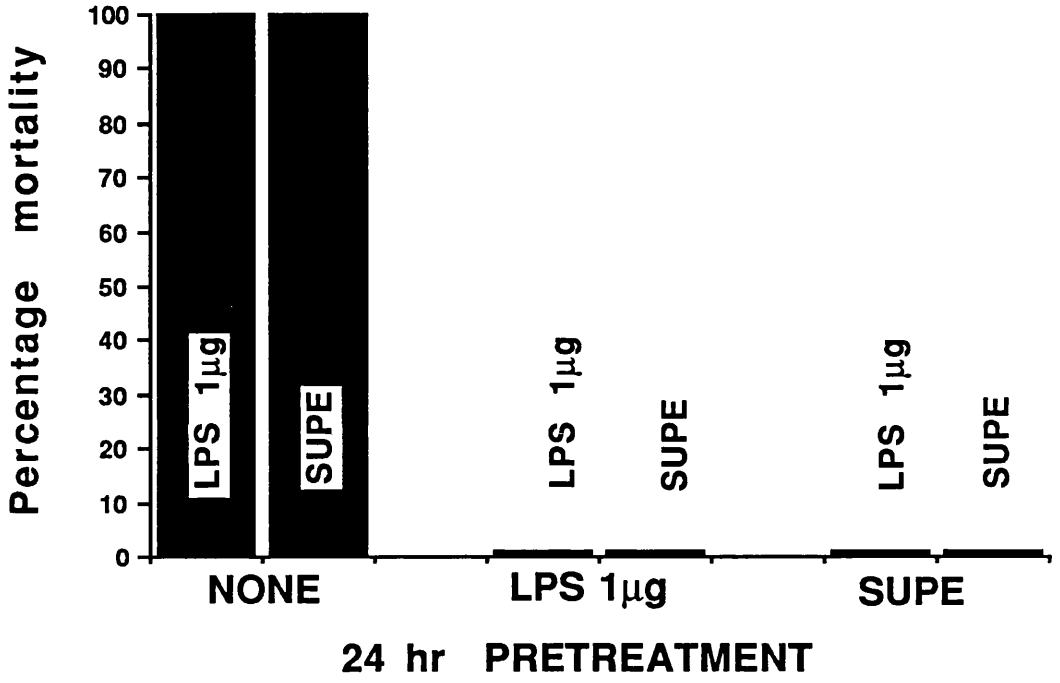
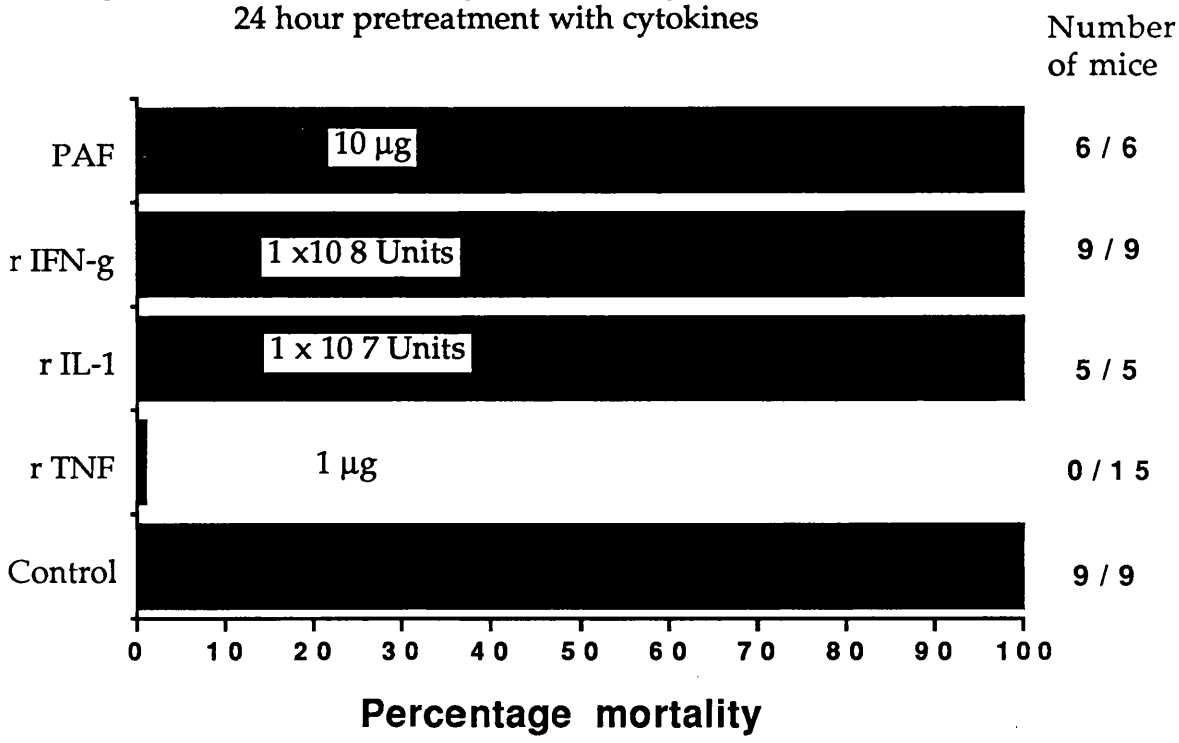


Figure 7.2.1.b. Protection against exoantigen induced mortality by 24 hour pretreatment with cytokines



that had received 1 μg of r TNF 24 hours earlier. The administration of 1×10^8 U of r IFN–γ, 1×10^7 U of r IL-1, or 10 μg PAF did not affect subsequent mortality mice injected with exoantigens (Fig 7.2.1.b.).

7.2.2 Specific inhibition of exoantigen toxicity by injection of exoantigens 10 days earlier

In contrast to this non-specific inhibition (tolerance), mice injected 10 days earlier with exoantigens were protected against challenge with parasite supernatants but were killed when challenged with LPS. Mice immunized with 10 μg/mouse of LPS were afforded some protection against reinjection of LPS 10 days later, (mortality was reduced from 100 to 75 %), but not against the mortality of parasite supernatants (Fig 7.2.2.a.). There was a clear difference in the protection mediated by immunization with supernatants compared with immunization with LPS, which was consistently seen 9-12 days after the initial injection. Immunization with exoantigens protected mice against challenge with exoantigens from different *Plasmodium* species but not against challenge with LPS. Immunization with a control supernatant made from uninfected rbcs did not alter the toxicity for mice of either parasite supernatants or of LPS.

To see if immunization also reduced the ability of mice to produce TNF, groups of mice immunized either with supernatants or 10 μg/mouse of LPS and injected 10 days later with supernatants or LPS were bled 1-2 hours later and sera were assayed for TNF (Fig 7.2.2.b.). The experiment shown is rather unusual in that unprimed mice gave high yields of TNF responses to both stimulants. However, pretreatment with LPS significantly reduced the titres obtained in response to LPS but not in response to supernatants. In contrast, immunization with supernatants inhibited titres obtained in response to injection with supernatants, but also acted to prime the mice to give enhanced secretion of TNF in response to LPS. This experiment has been repeated 5 times, and although control titres have been greatly variable, the salient features of the experiment remain the same in that pretreatment with the supernatants invariably led a reduction in the amount of TNF obtained in response to the homologous supernatant and enhancement in the amount of TNF obtained in response to LPS. Supernatants prepared from nrbcs given to another group of mice did not alter the titres of TNF produced

Figure 7.2.2.a. Immunization 10 days earlier provides specific protection against mortality in sensitized mice injected with either LPS or parasite supernatants

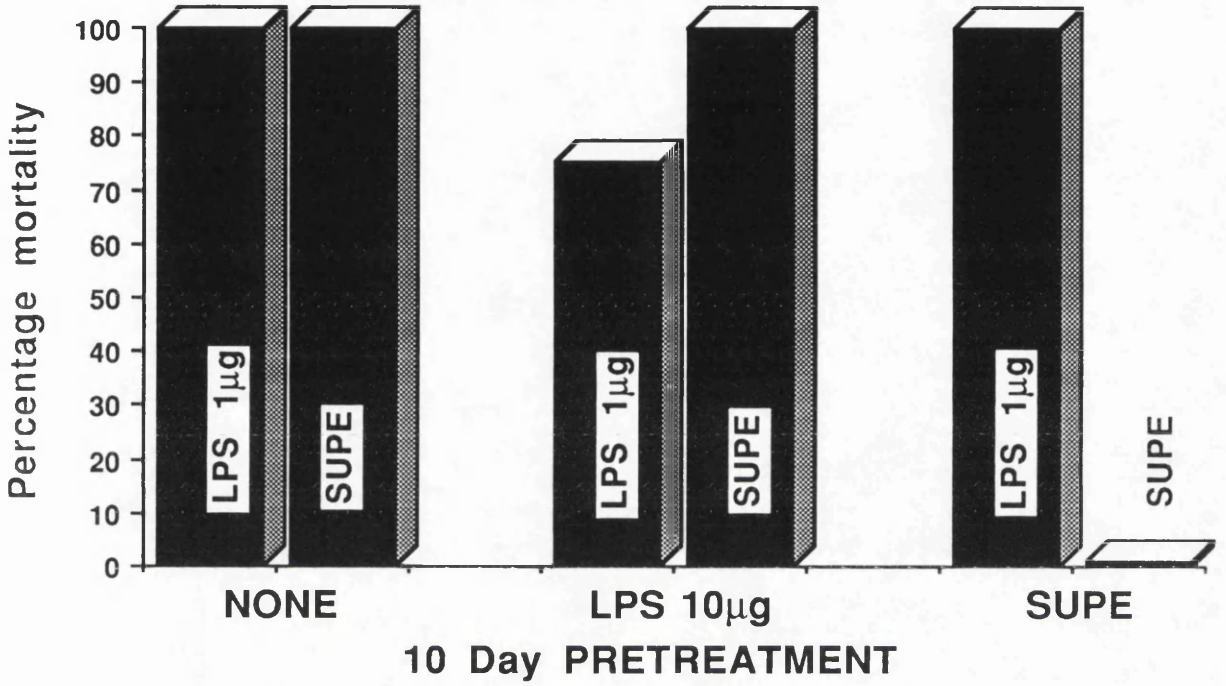
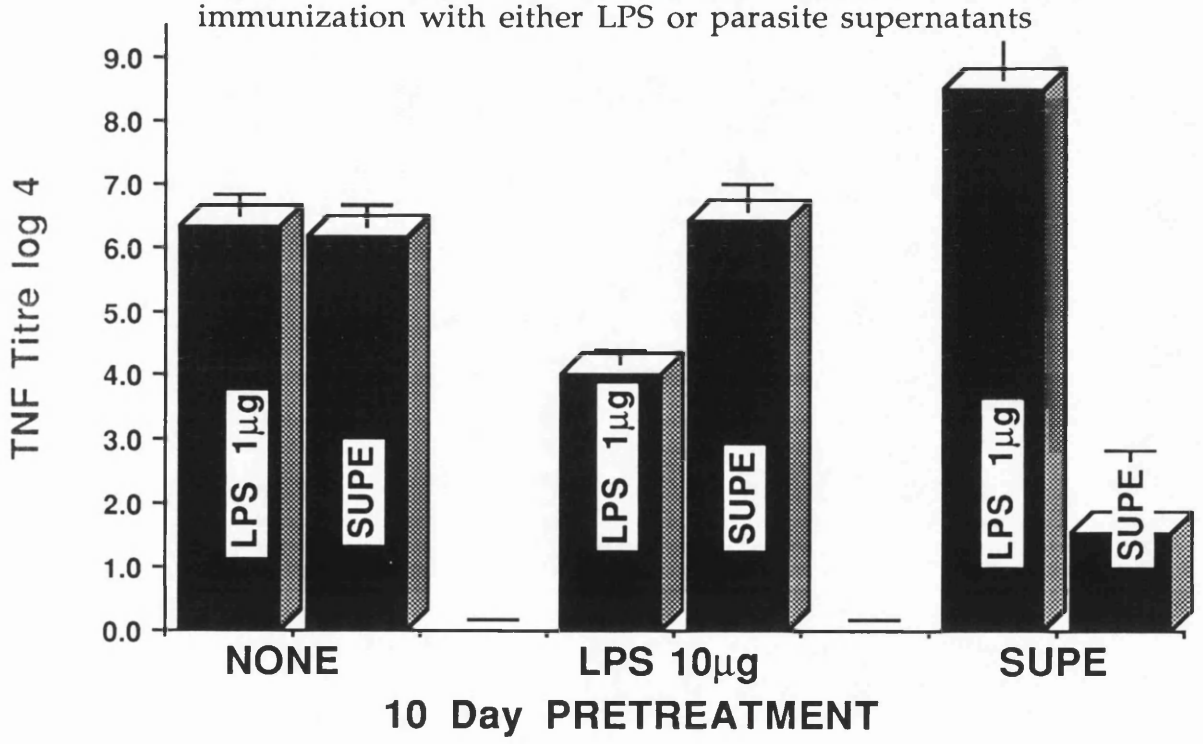


Figure 7.2.2.b. Reduction of TNF titres obtained in serum by immunization with either LPS or parasite supernatants

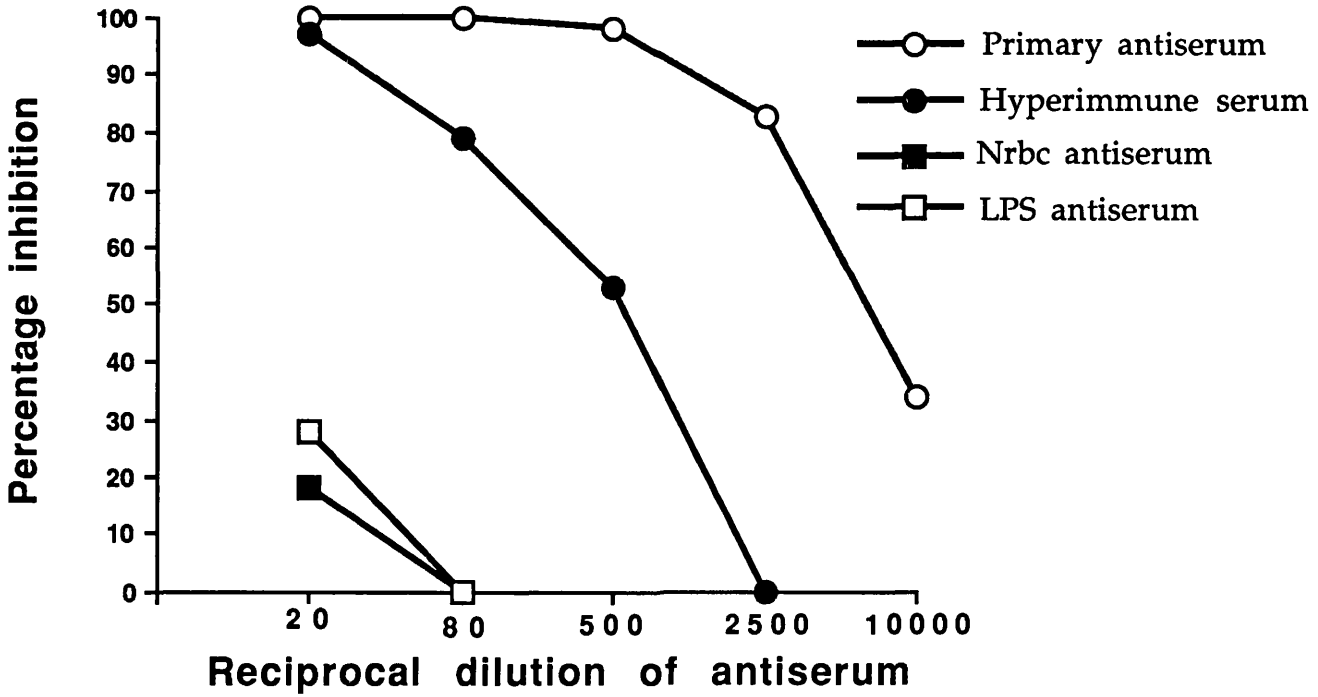


in response either to LPS or to the supernatants (data not shown). The time course and specificity shown by the immunization procedures suggested an antibody-mediated process. This could be tested by collection of the sera from immunized animals 12 days after initial challenge.

7.2.3 Inhibition of TNF induction by antiserum to exoantigens

When mice were injected with 0.5 ml of a parasite supernatant and bled 12 days later, the serum collected inhibited the induction of TNF by parasite exoantigens. We have called such sera from mice bled 12 days after a single injection of parasite supernatants **PRIMARY ANTISERUM**. The results of a titration of the inhibitory effect of a typical primary antiserum raised against exoantigens from lethal *P.yoelii* against a preparation of homologous parasite exoantigens in the presence of 5μg/ml polymyxin B are illustrated (Fig 7.2.3.a.). The antiserum blocked TNF production stimulated by a parasite supernatant specifically in a dose-dependent manner, but had no inhibitory action on TNF production by macrophages stimulated by 200 ng/ml of LPS. Numerous titrations of primary antisera raised against parasite exoantigens showed that they always possessed greater blocking activity (average titre 10,000) than hyperimmune serum (highest titre 800). Sera collected from mice during the course of infection with non-lethal *P.yoelii* also inhibited the triggering of TNF secretion by exoantigens *in vitro* suggesting that similar triggering exoantigens must have been produced during infection. Control sera raised either against LPS or against boiled supernatants from uninfected red blood cells sometimes showed inhibition at a 1/40 dilution, which was not significant. Preincubation of macrophages with antiserum did not affect the subsequent induction of TNF by parasite exoantigens, indicating that the inhibition was not mediated by an effect of the antiserum on the macrophages. Unless otherwise stated antiserum raised against parasite exoantigens is antisera raised against *P.yoelii* supernatants.

Figure 7.2.3.a. Inhibition of exoantigen-induced TNF secretion by various antisera



7.2.4 Activity of primary antiserum *in vivo*

Since primary antiserum neutralizes the induction of TNF by exoantigens *in vitro*, it should also do so *in vivo*, and be able to protect D-galactosamine sensitized mice injected with exoantigens from death. The effect of prior incubation of a 1/50 dilution of primary antiserum with either LPS or with a parasite supernatant on TNF titres they induced from groups of 5 *C.parvum* primed mice is shown (Fig 7.2.4.a.). The titres of TNF stimulated by the parasite supernatant were significantly reduced by the presence of primary antiserum whereas the TNF titres stimulated by LPS were unaffected. Similarly the effect of preincubation of a 1/50 dilution of primary antiserum with either LPS or a parasite supernatant before their injection into D-galactosamine sensitized mice was investigated. Primary antiserum protected mice injected with supernatants but had no effect on the mortality of mice injected with LPS (Fig 7.2.4.b.).

7.2.5 Specificity of inhibitory antiserum

Serum collected 12 days after immunization with 10 μg/mouse of LPS was tested to see if it inhibited TNF production by macrophages stimulated with either 200 ng/ml LPS or a 1/10 dilution of a parasite supernatant (Fig 7.2.5.a.). This antiserum did not inhibit TNF responses stimulated by parasite exoantigens to any greater extent than normal mouse serum. Indeed, it produced little inhibition even against the homologous LPS. Antibody to LPS is known to be directed primarily against the serologically diverse polysaccharide side chains, the active moiety, lipid A, being poorly antigenic. Thus these antibodies generally react with LPS from the homologous strain but are of little use against LPS from different strains of Gram -ve bacteria.

Primary antiserum may contain antibodies that are not directed to the triggering moiety itself but to associated structures, the binding of which results in steric inhibition of the active moiety. If these associated molecules were constituents of nrbcs then absorption of primary antiserum with nrbcs would be expected to reduce the inhibitory titre. To investigate this question, primary antiserum was absorbed with nrbcs at least 3 times and it was then titrated in parallel with untreated primary antiserum against one dilution of an exoantigen preparation. No significant loss of inhibition was detected (Fig

Figure 7.2.4.a. Primary antiserum against the exoantigens specifically inhibit the amount of TNF in released into the serum of mice injected with parasite supernatants

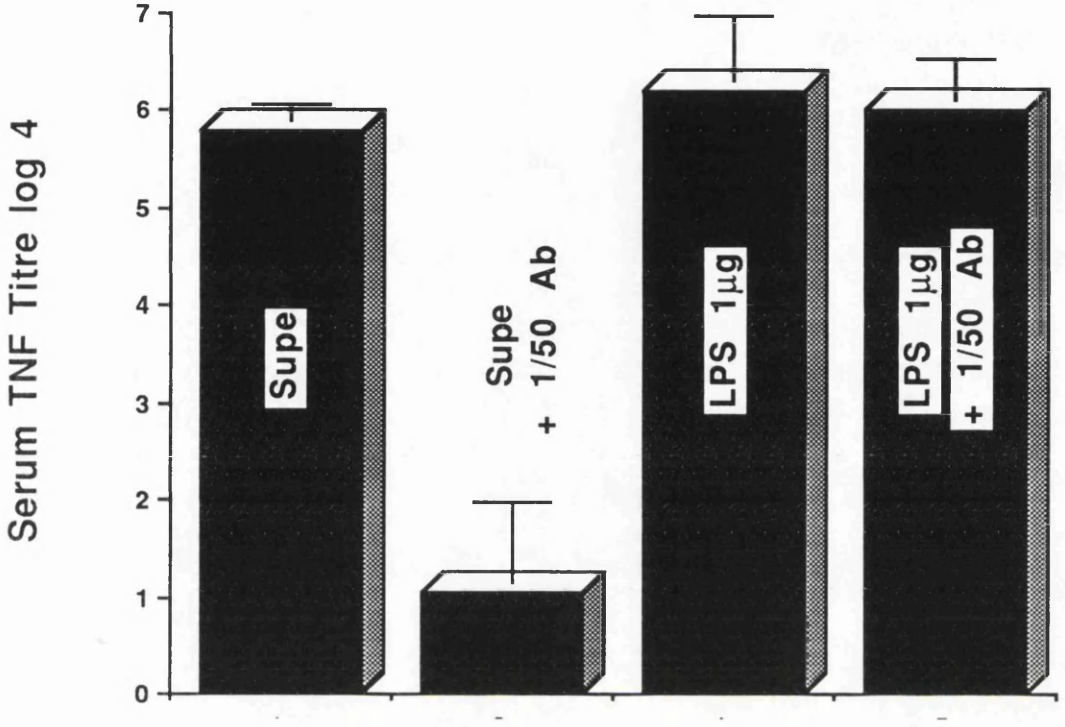


Figure 7.2.4.b. Primary antiserum raised against the exoantigens specifically protects mice injected with parasite supernatants

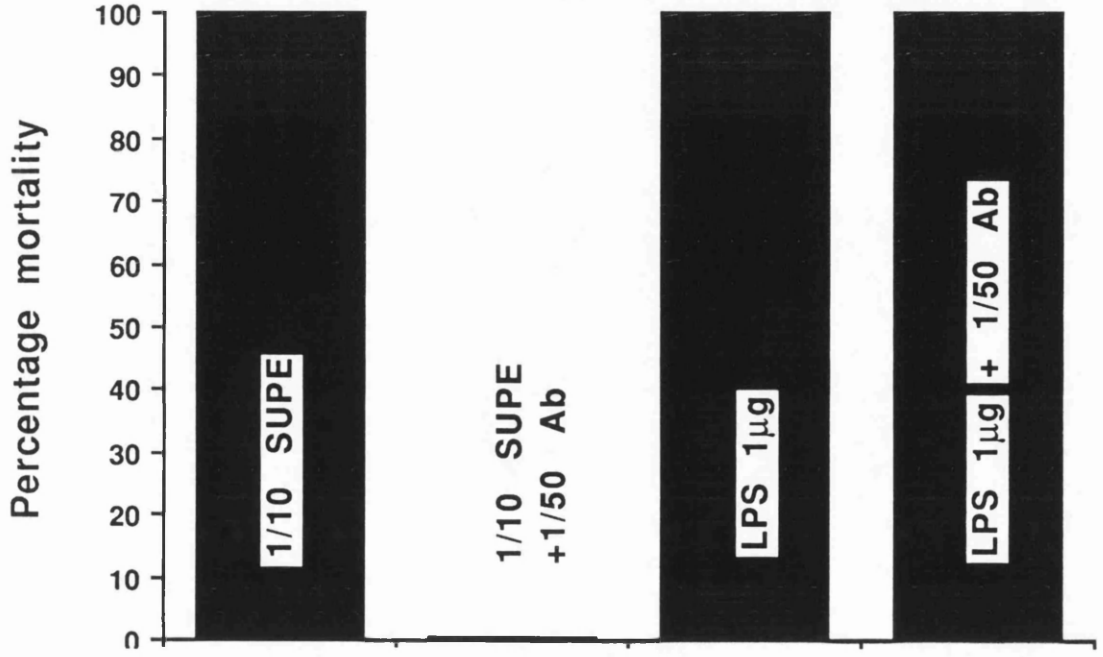


Figure 7.2.5.a. Antiserum raised against LPS did not inhibit induction of TNF by parasite exoantigens

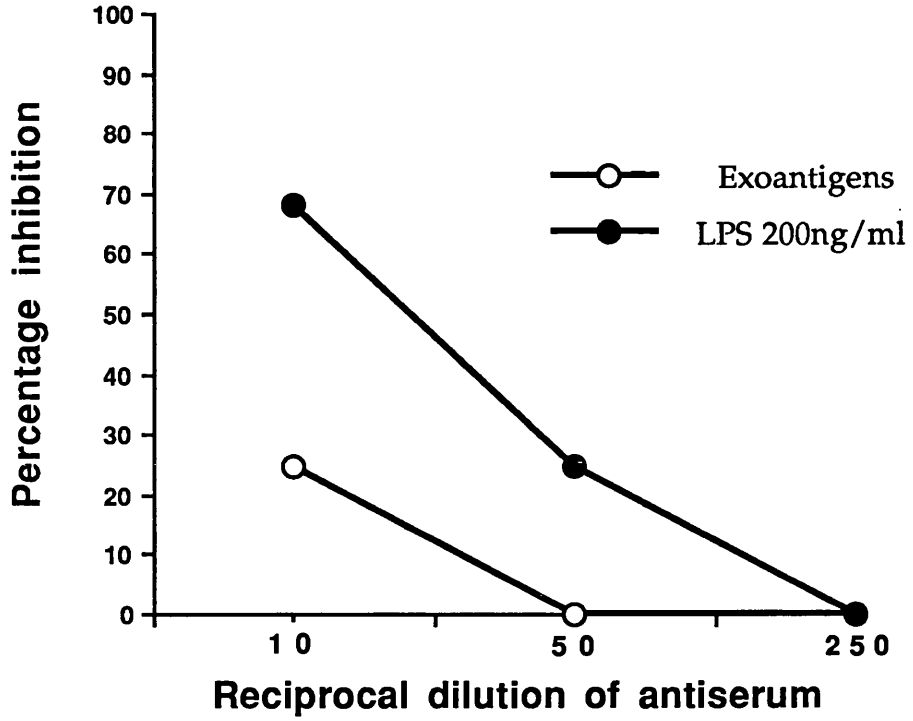
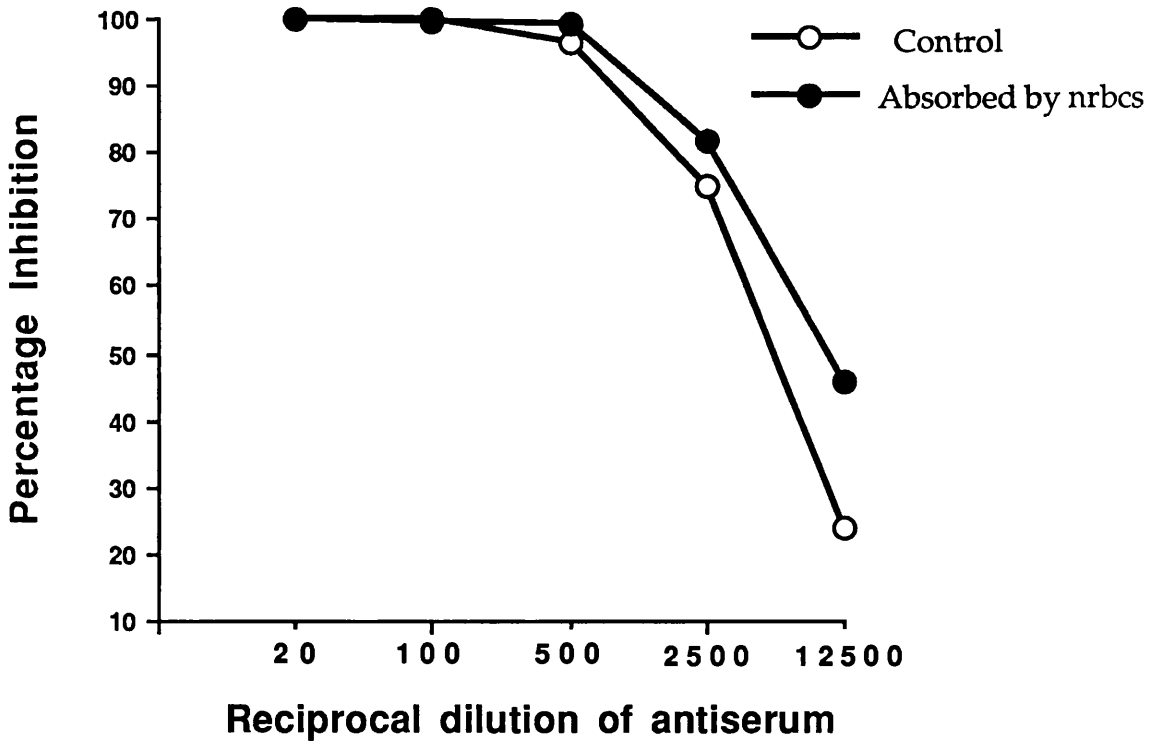


Figure 7.2.5.b. Absorption of primary antiserum with uninfected erythrocytes



7.2.5.b.).

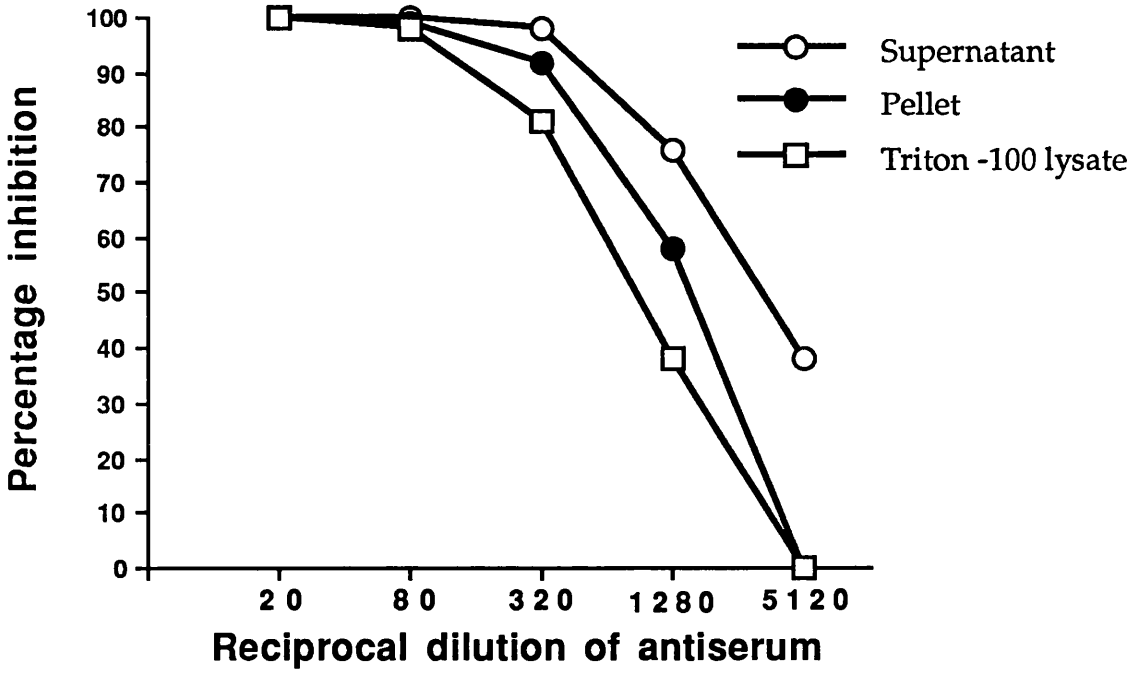
7.2.6 Serological homogeneity of exoantigens in different parasite preparations

Preparations of *Plasmodium* exoantigens, whether released into the supernatant, collected from homogenized pellets or extracted with Triton X-100 into lysates, shared the same sensitivities to chemical and enzymatic degradation. This suggests that the triggering activity from each of these preparations is due to the same molecule or molecules. This hypothesis was therefore tested further. Primary antiserum raised against a pronase-digested supernatant was incubated with a supernatant, a homogenized pellet and a Triton X-100 lysate to see if it inhibited their ability to trigger macrophages (Fig 7.2.6.a.). The primary antiserum inhibited all 3 preparations with equal efficacy, which indicates that the same antigens in the different preparations stimulated the macrophages to produce TNF. Titrations shown are from a typical experiment.

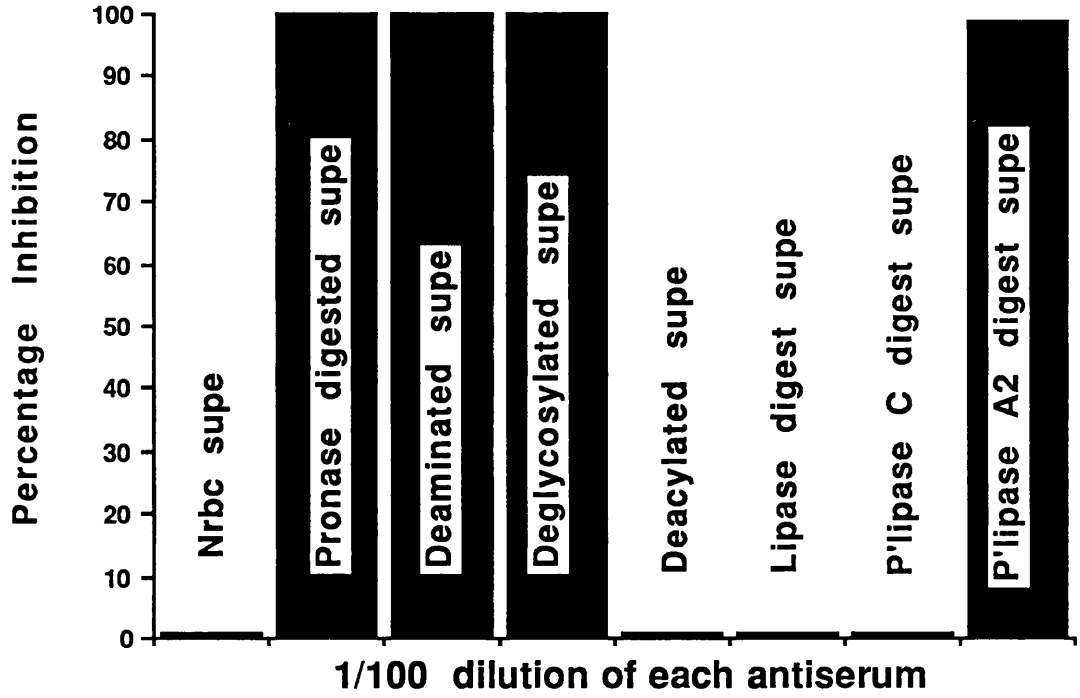
7.2.7 Correlation between the nature of exoantigens stimulating TNF production and the production of inhibitory antiserum

As it seemed likely that the same molecules are responsible for stimulating TNF secretion *in vitro* and for killing sensitized mice, I investigated the nature of the exoantigens that stimulate the production of inhibitory antiserum to see if they had similar properties (Fig 7.2.7.a.). Pooled data from several experiments in which antisera collected 12 days after injection of exoantigens after enzyme digestion or chemical modification were tested for their ability to inhibit TNF production by macrophages stimulated with an exoantigen preparation. Enzymatic digestion or chemical treatment of the exoantigens that caused a loss of their ability to stimulate macrophages to secrete TNF caused a corresponding loss in the production of inhibitory antiserum. These results show therefore that a direct correlation exists between the nature of the exoantigens stimulating TNF secretion and the nature of the exoantigens stimulating the production of inhibitory antisera.

Figure 7.2.6.a. Inhibition of TNF induction by different parasite preparations by primary antiserum against a parasite supernatant



7.2.7.a. Inhibition of P.yoelii exoantigen activity by a 1/100 dilution of antisera raised against variously treated supernatants



The inhibitory titres of antisera raised against two highly purified preparations of *P.yoelii* exoantigens, a nuclease and pronase digested, deaminated supernatant, and a pronase and nuclease digested and TFMS treated supernatant, were compared with the titre of an antiserum raised against an equivalent concentration of a boiled but untreated supernatant (Fig 7.2.7.b.). There was no evidence that epitopes destroyed either by nuclease and protease digestion and then chemical deamination or deglycosylation of the exoantigens stimulate the production of inhibitory antisera.

7.2.8 Immunoglobulin isotypes responsible for inhibitory activity

To determine the Ig isotypes responsible for the blocking activity, a pool of antiserum made against lethal *P.yoelii* exoantigens was depleted of IgM or IgG by chromatography on anti-mouse IgM or IgG coated agarose. Fractions containing the antibody recovered by elution from the columns were titrated against exoantigens in parallel with the original antiserum. To confirm that all the activity was indeed due to immunoglobulin and not to any other inhibitors that might have been present in the serum, a sample of antiserum depleted consecutively of IgM then of IgG was also titrated. Titrations of the samples obtained, which are typical of several experiments, are shown (Fig 7.2.8.a.): serum depleted of both IgG and IgM did not block the stimulation of macrophages by the exoantigens. Inhibitory activity was found in fractions eluted off both isotype-specific columns but greater inhibition was always observed in IgM eluates than in IgG eluates, showing that most of the inhibition was mediated by IgM antibodies.

Figure 7.2.7.b. Inhibition of pronase-digested exoantigens by antisera raised against purified exoantigen preparations

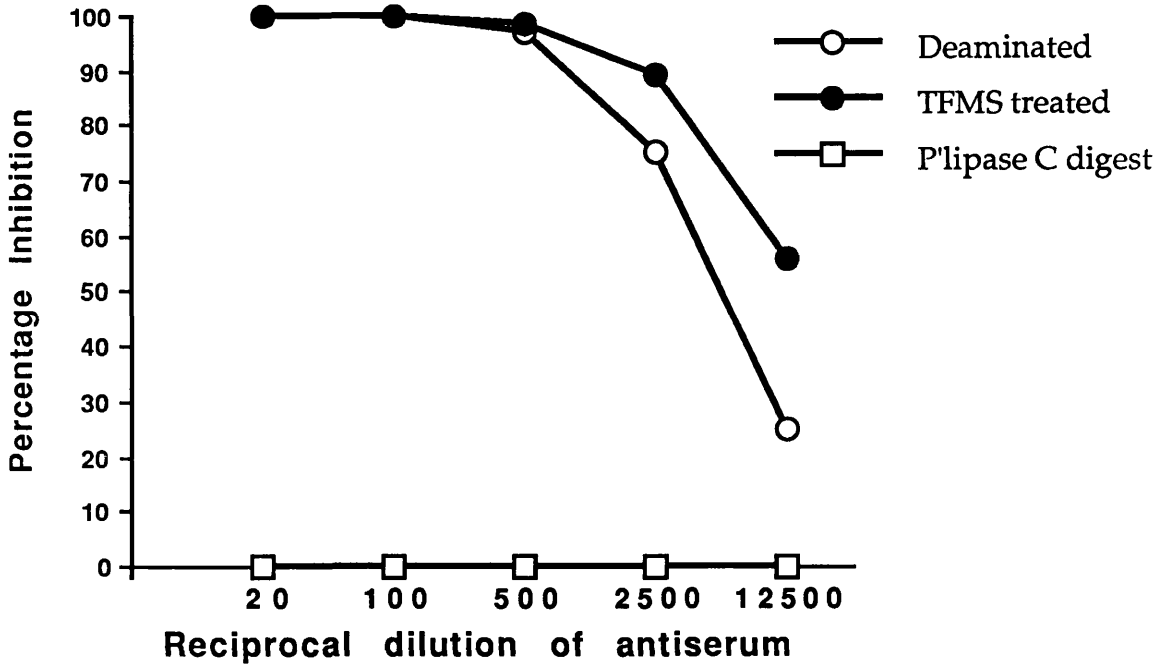
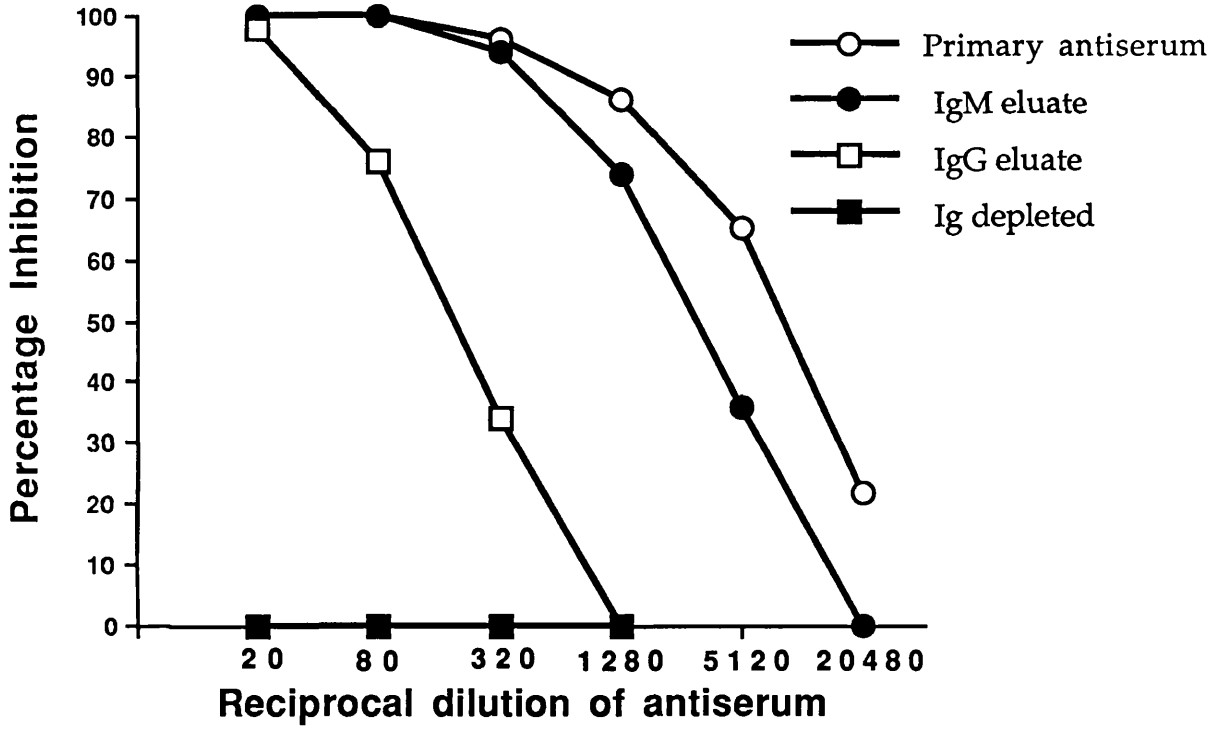


Figure 7.2.8.a. Immunoglobulin isotypes responsible for inhibition by primary antise



7.2.9 Time course of the development of inhibitory antibody

Our observation that the inhibitory antibody in antiserum against exoantigens was predominantly IgM suggested that the exoantigens might be T-independent antigens. The time course of appearance of serum blocking antibody was therefore determined to see if it was characteristic of such antigens. Pooled sera from groups of mice bled at intervals after immunization with exoantigens from lethal *P. yoelii* were tested against one concentration of an exoantigen preparation and their titres were determined and plotted against time after immunization (Fig 7.2.9.a.). Serum taken at day 3 did not inhibit TNF production but some inhibitory effect was detectable by day 4 and day 5. The peak of inhibition occurred at about day 12 and then declined rapidly, so that inhibitory titres were demonstrably reduced by day 14 and by day 21 no significant inhibition was observed.

Comparison of the inhibitory titres of antiserum obtained from mice 12 days after injection of a boiled exoantigen preparation with serum from mice previously injected with exoantigens 6 times at 2 week intervals, and from mice injected simultaneously with the adjuvant saponin at 20 μg/mouse showed that there was no enhancement of the antibody response in either the boosted mice or the mice given exoantigens + saponin (Table 7.2.9.a.). Furthermore, the ratio of the inhibitory isotypes was not altered significantly in either case. The titres of inhibitory antisera were not increased by addition of an adjuvant at other times (data not shown).

Table 7.2.9.a. Fractionation of antisera raised against parasite exoantigens

		Inhibitory Titres	
Immunizing antigen	Original antiserum	IgM	IgG
P.yoelii supernatant	12,800	5,820	260
Same injected 6 times	8,900	6,420	220
Same + saponin (25 μg)	12,200	10,600	400

Groups of mice were injected i.p. and bled 12 days after the last injection. Titres are the reciprocal of the dilution giving 50% inhibition of TNF secretion by mouse macrophages stimulated with pronase-digested supernatants of *P.yoelii YM*.

Figure 7.2.9.a. Time course of the development of inhibitory antiserum

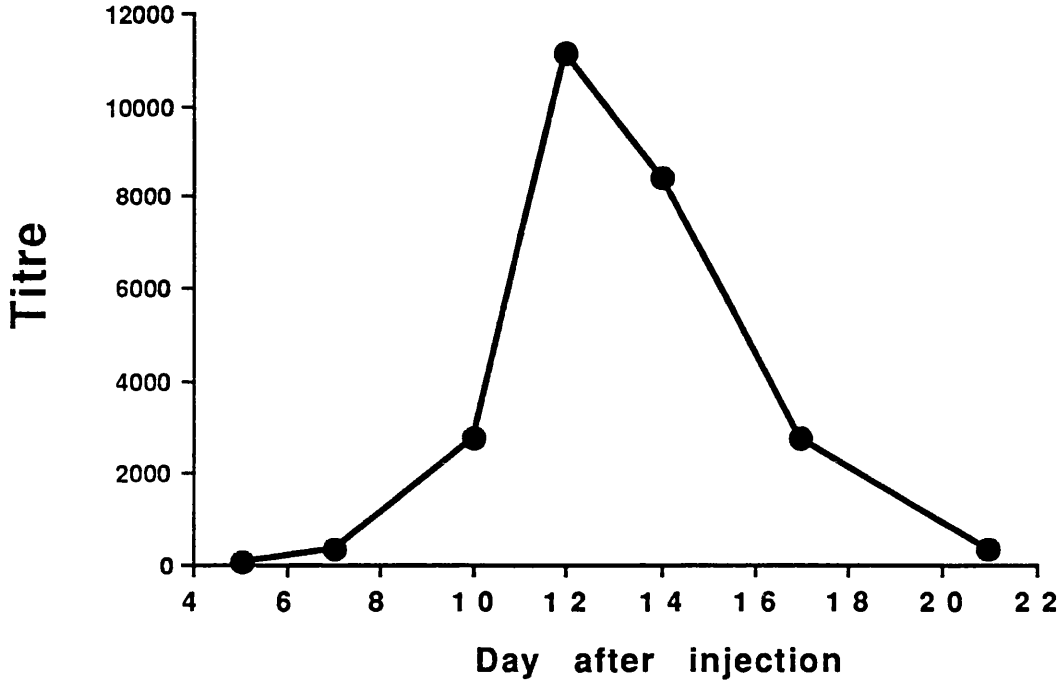
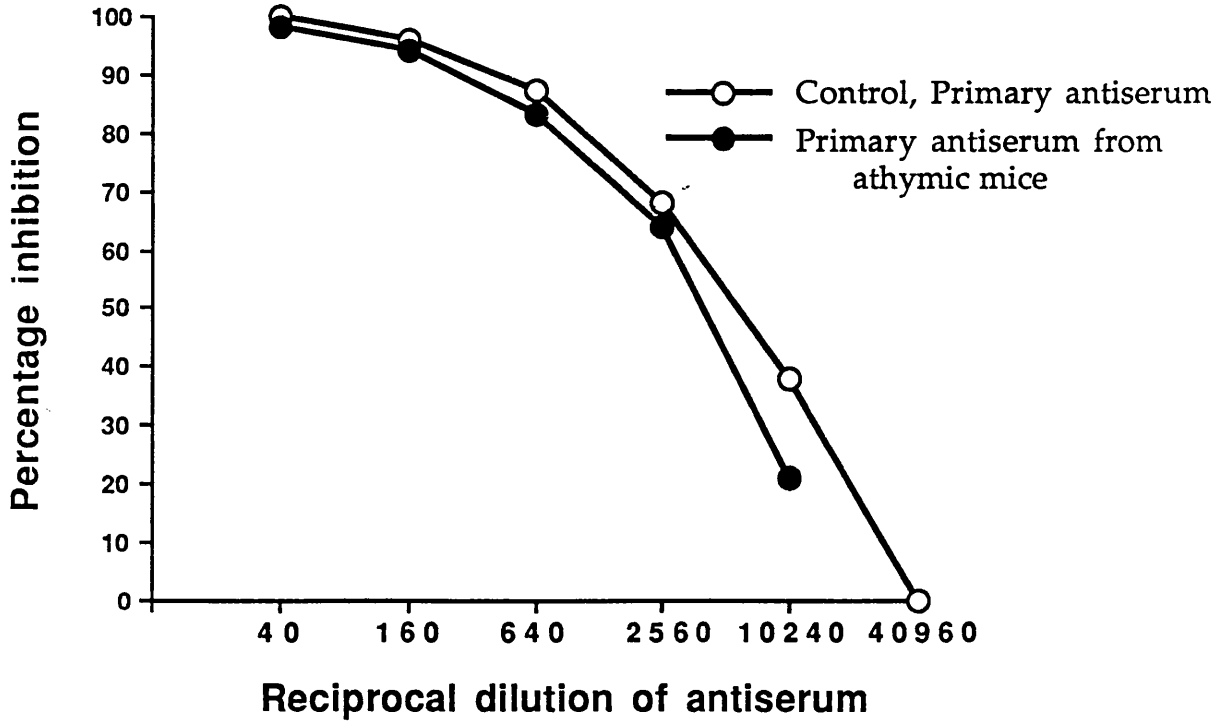


Figure 7.2.10.a. Titrations of primary antisera from normal and athymic mice



7.2.10 Development of inhibitory antiserum in T-cell deficient mice

The lack of increased inhibitory activity in the secondary response suggested that exoantigens do not give rise to immunological memory and thus may not require T cells to induce the production of antibody. Antiserum obtained 10 days after injection of exoantigens into mice that had been depleted of their functional CD 4+ T cells by pretreatment with cytotoxic antibodies had an inhibitory titre equal to that of a control serum raised in normal mice (data not shown). Furthermore, sera from groups of control and athymic nude mice, bled 10 and 12 days after injection of exoantigens of *P.yoelii YM*, had similar blocking activity. A typical experiment is shown (Fig 7.2.10.a.) Antisera collected from athymic mice 12 days after immunization were separated by chromatography on anti-mouse IgM or IgG agarose columns, and the eluted antibodies were titrated against parasite exoantigens in parallel with the original antiserum as a control (Fig 7.2.10.b.). The absence of functional T-cells did not affect the isotype of the inhibitory antibody, the predominant class of inhibitory antibody was still IgM (inhibitory titre = 13200), with some inhibitory IgG (titre = 220).

Two distinct B cell populations have been identified in mice which respond to antigens in the absence of T cells: TI-1 antigens stimulate B cells that are Lyb 3, 5 and 7+. TI-2 responses are mediated by B cells that are Lyb 5 -. The latter fail to respond to a number of antigens whose general characteristics include high molecular weight with repeating epitopes. Immunization of CBA/N mice, a strain of mouse which lacks the Lyb 5 + subset of B cells, led to the production of inhibitory antisera at day 12 with equivalent titres to those of antisera collected from control mice (Fig 7.2.10.c.). Antisera were collected from 3 groups of 3 mice. Thus parasite exoantigens act like a TI-2 antigen; this class of antigens is characterized as inherently mitogenic and they are classically polyclonal B cell activators.

Figure 7.2.10.b. Immunoglobulin isotypes responsible for inhibition by primary antisera from athymic mice

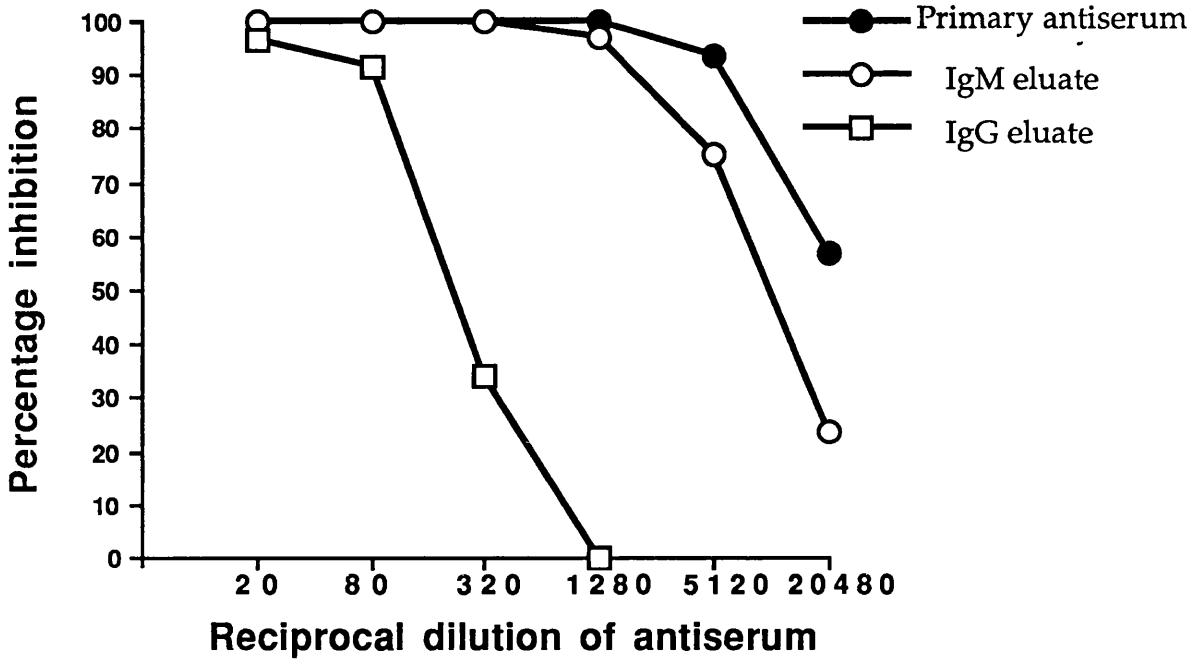
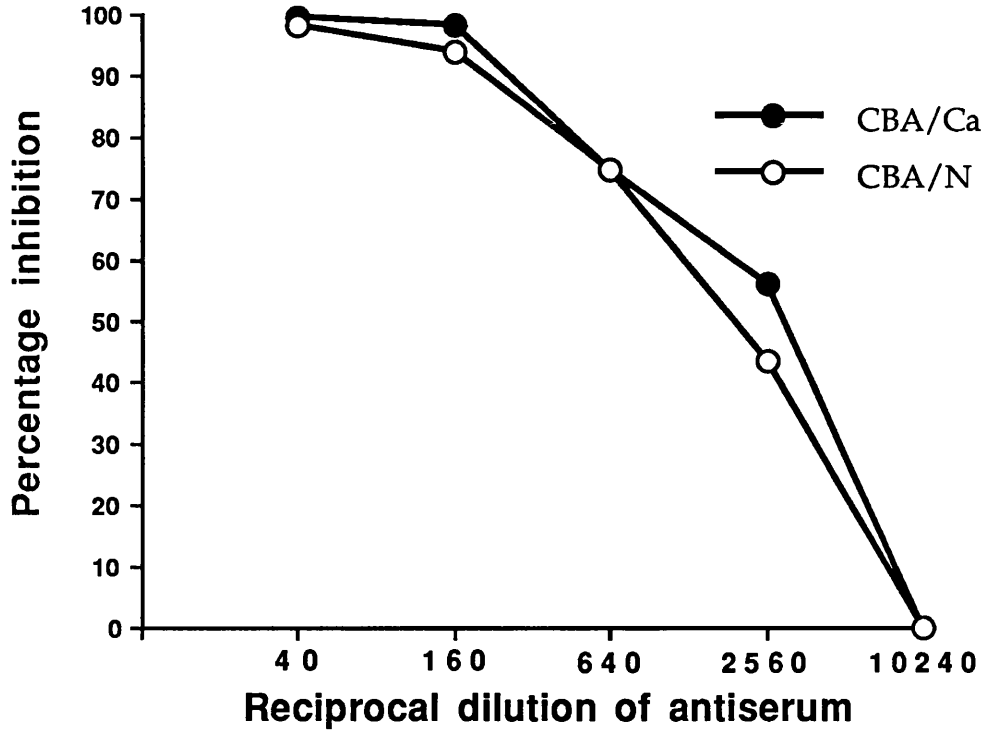


Figure 7.2.10.c. Titration of the inhibitory effect of antiserum from CBA/N compared with control CBA/Ca mice



7.2.11 Cross-reactivity of exoantigens from different *Plasmodium* species

We prepared exoantigens from the different *Plasmodium* species in our possession and investigated their serological relationships. Antisera raised against pronase-digested exoantigens from the different rodent parasites all inhibited TNF induction stimulated by a preparation of pronase-digested *P.yoelii* exoantigens (Fig 7.2.11.a.). Pooled sera raised against one species of parasite successfully block the triggering of macrophages by exoantigens derived from both the same and different species of rodent parasites. This suggests that the triggering exoantigens from *Plasmodium* species infecting rodents do not show marked antigenic variation. It is, of course, possible that by digestion and purification of the antigen we are unearthing species cross-reactions which do not occur in the native state. However, these cross-reactions were observed irrespective of the purity of the preparations used either as the trigger or as the immunizing antigen (data not shown).

7.2.12 Cross-reactivity with exoantigens from *P.falciparum*

I have shown that there are functionally similar exoantigens in *P.falciparum* and that they share the same sensitivities to chemical and enzymatic modification. Serological cross-reactions between *P.falciparum*-derived exoantigens and the rodent parasite exoantigens were therefore investigated. TNF secretion stimulated by *P.falciparum* exoantigens was inhibited by primary antisera raised against exoantigens of *P.berghei, P.yoelii* and *P.falciparum;* typical titrations from one such experiment are shown (Fig 7.2.12.a.). This cross-reaction was reciprocal, in that primary antiserum raised against *P.falciparum* exoantigens also inhibited the exoantigen preparations from *P.berghei, P.yoelii* and *P.falciparum* (data not shown). The cross reactions between antisera and exoantigens isolated from different species are summarized in Table 7.2.12.a.

Figure 7.2.11.a. Inhibition of TNF induction by P.yoelii pronase-digested exoantigens by antisera raised against pronase-digested exoantigens from different parasite species

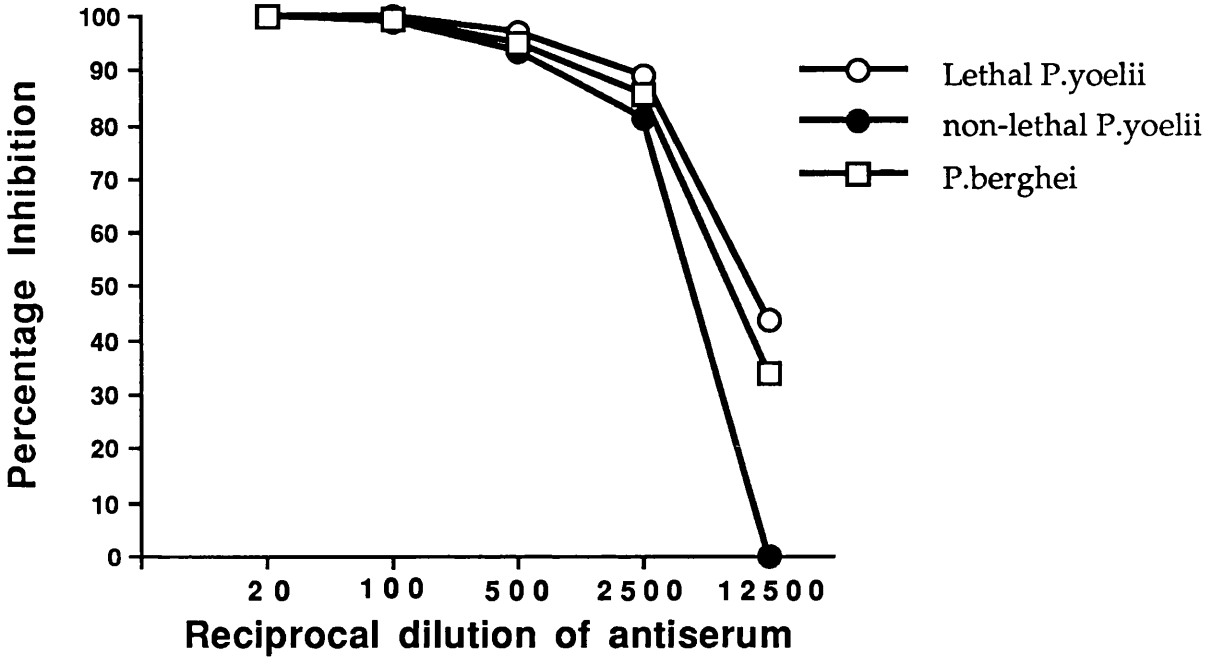


Figure 7.2.12.a. Inhibition of TNF induction by exoantigens of P.falciparum by antisera raised against exoantigens of rodent parasites

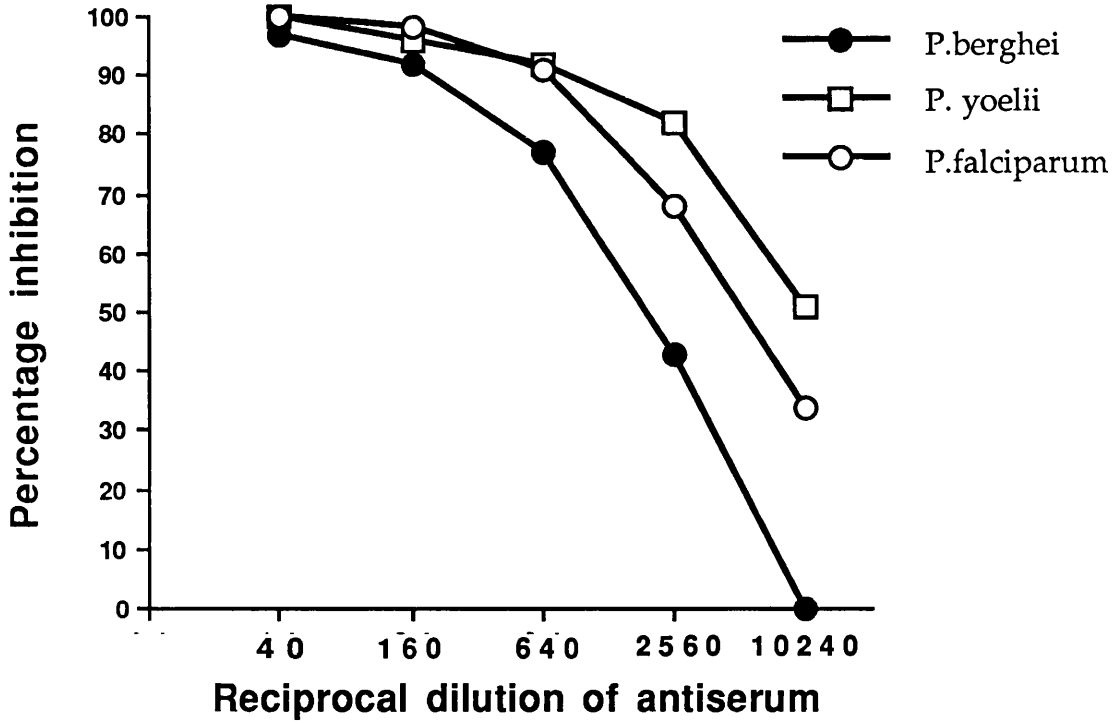


Table 7.2.12.a. Serological cross-reactions between exoantigens of different *Plasmodium* species: inhibition by primary antisera of exoantigen stimulation of TNF secretion *in vitro*.

Antiserum against	Exoantigens stimulating TNF induction		
	P.yoelii YM	*P.berghei*	*P.falciparum*
P. yoelii YM	13,400	12,500	12,000
P. berghei	12,500	7,760	8,560
P. falciparum	15,000	12,400	15,000

Mice were bled 12 days after injection of exoantigens. The figures shown are reciprocals of the dilution of antiserum giving 50% inhibition of TNF release. None of the antisera blocked TNF release induced by LPS.

In these experiments, crude lysates of *P.falciparum* parasitized erythrocytes that had been pronase-digested were used as exoantigens both as macrophage stimulants and for immunization. It is possible that no inhibition would have been seen if an undigested supernatant had been used. However, primary antiserum raised against a pronase-digested, deaminated supernatant of *P.yoelii* prbcs inhibited the activity of the exoantigens in an unboiled crude lysate of *P.falciparum* prbcs(Fig 7.2.12.b.). Conversely, antisera raised against a crude untreated lysate of *P.falciparum* which inhibited stimulation by the unboiled supernatant was also effective against the activity of pronase-digested, deaminated exoantigens of *P.yoelii* (data not shown). This suggests that even antiserum raised against untreated *P.falciparum* exoantigens recognizes a common triggering moiety directly and that there are no associated molecules that either block antibody interactions or induce the production of non-specific antibodies that cause steric inhibition of this active moiety.

Our laboratory, in collaboration with P. Jakobsen, previously described the finding that 2 antigens, named antigen 1 and antigen 7, isolated from continuous culture supernatants of *P.falciparum* by affinity chromatography stimulated TNF secretion by macrophages. We reported that these antigens differed in their pattern of inhibition by primary antisera raised against rodent exoantigens, in that TNF responses to antigen 7 were

Figure 7.2.12.b. Inhibition of TNF induction by a crude P.falciparum lysate by antisera raised to a crude lysate and to purified (deaminated) P.yoelii exoantigens

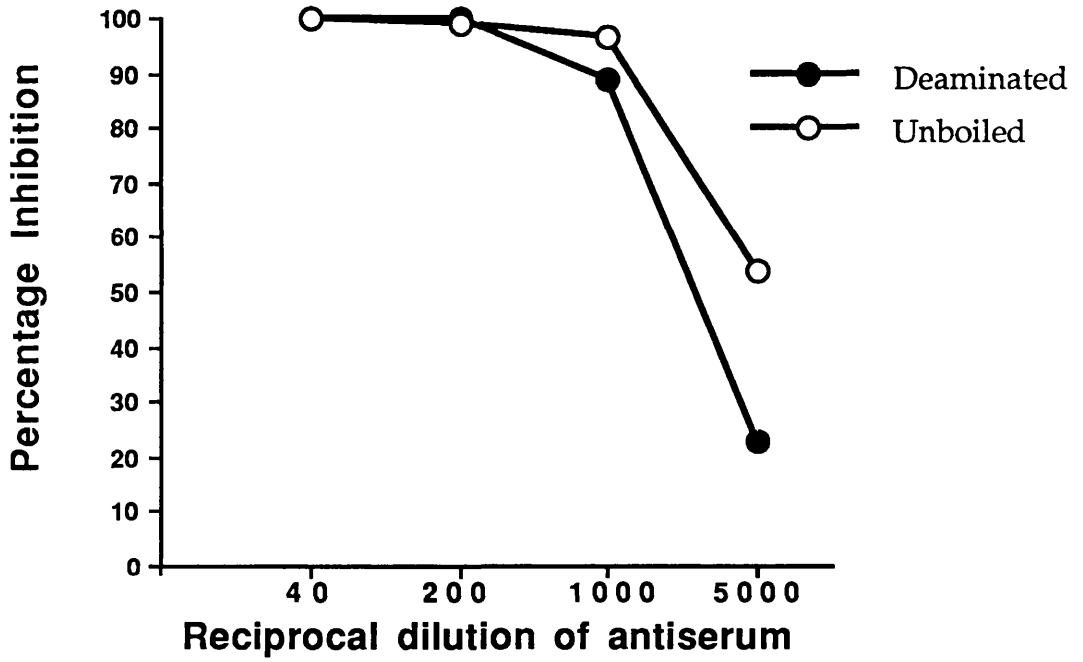
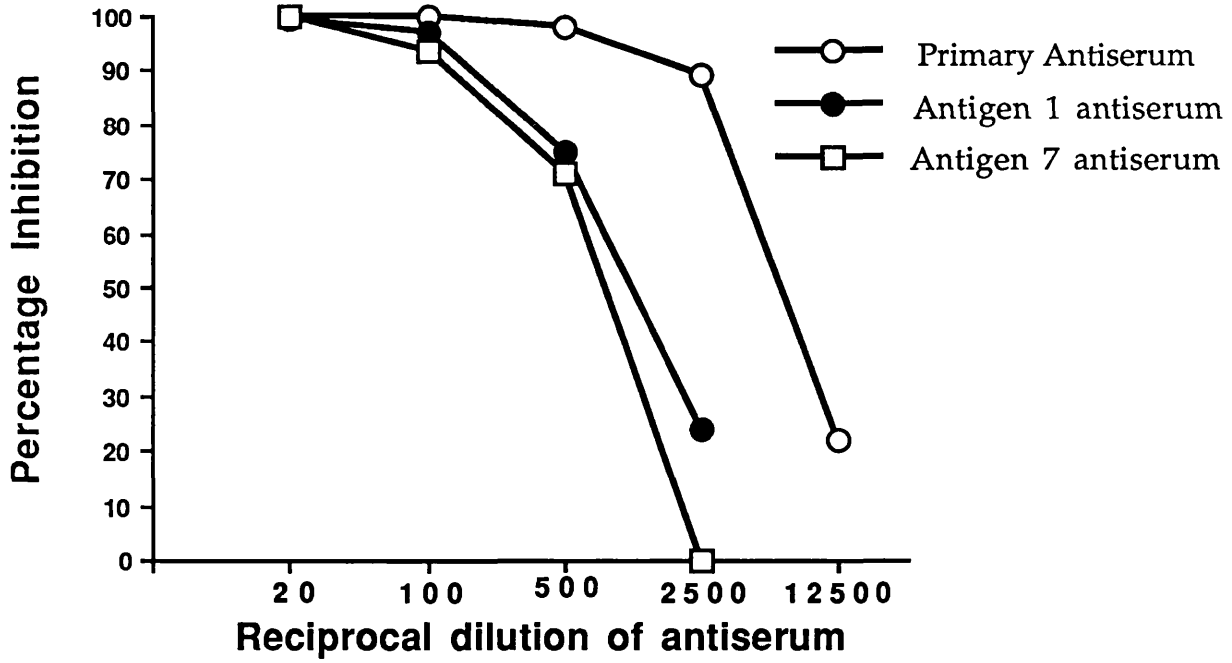


Figure 7.2.12.c. Inhibition of TNF induction by pronase-digested P.yoelii exoantigens by antisera raised to pronase-digested Ag 1 and Ag 7



inhibited while responses to antigen 1 were not (Taverne *et al.*, 1990). We subsequently found that the triggering activity of both antigens resisted digestion by pronase E. Immunization with pronase-digested preparations of antigens 1 and 7 stimulated the production of inhibitory antiserum that blocked the induction of TNF by the exoantigens of *P.yoelii* (Fig 7.2.12.c.). The results of a typical experiment are shown; more than 2 pools of inhibitory antiserum were produced using 2 different samples of each antigen. The results obtained for such antisera are compared to those for an antiserum raised against pronase-digested *P.yoelii* exoantigens.

We had previously reported that primary antisera raised against rodent parasite supernatants did not inhibit the activity of antigen 1 (Taverne *et al.*, 1990). However, antigen 1 preparations that had been digested with pronase E were now found to be inhibited by the above inhibitory antiserum. (Fig 7.2.12.d.). The triggering moiety in antigen 1 preparations therefore seems to be associated with a protein which, while it allows interaction with the macrophage prevents the binding of antibodies. As this type of interaction was not seen with *P.falciparum* exoantigens obtained in serum free conditions, these results could be explained by masking of the trigger moiety resulting from the binding of host molecules. In conclusion, however different the soluble antigen complexes between *Plasmodium* species and strains may be, the TNF triggering moiety appears to constitute a serologically conserved antigen.

7.2.13 Inhibition of the activity of live prbcs by primary antiserum

The exoantigens responsible for triggering macrophages to induce TNF have the same properties regardless of the species from which they were derived or the method of preparation, suggesting that they bear a single invariable active moiety. As macrophages *in vivo* would also be stimulated by live prbcs it was necessary to confirm that there were no other macrophage triggers, perhaps even species specific, that might be active. Freshly obtained *P.yoelii* prbcs were washed and incubated with activated macrophages in the presence and absence of a 1/20 dilution of primary antiserum (which had an inhibitory titre of >10,000 tested against a pronase-digested supernatant). Rather to our surprise, no significant differences were seen between the TNF

Figure 7.2.12.d. Inhibition of TNF induction by antigen 1 before and after pronase digestion by primary antiserum raised against P.yoelii exoantigens

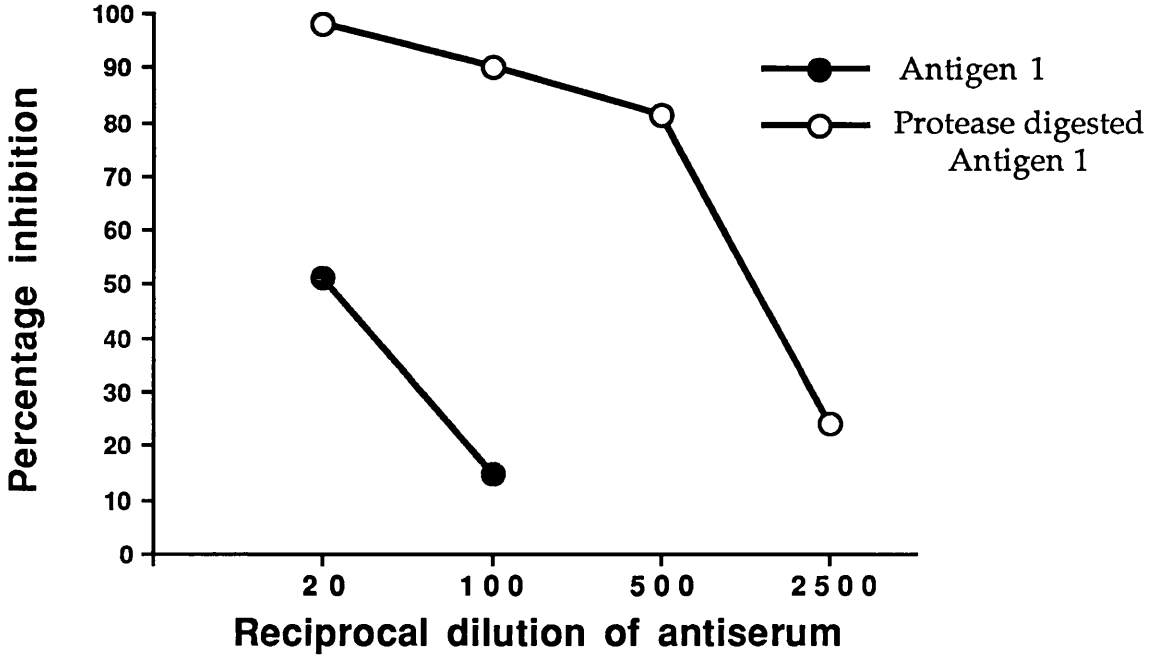
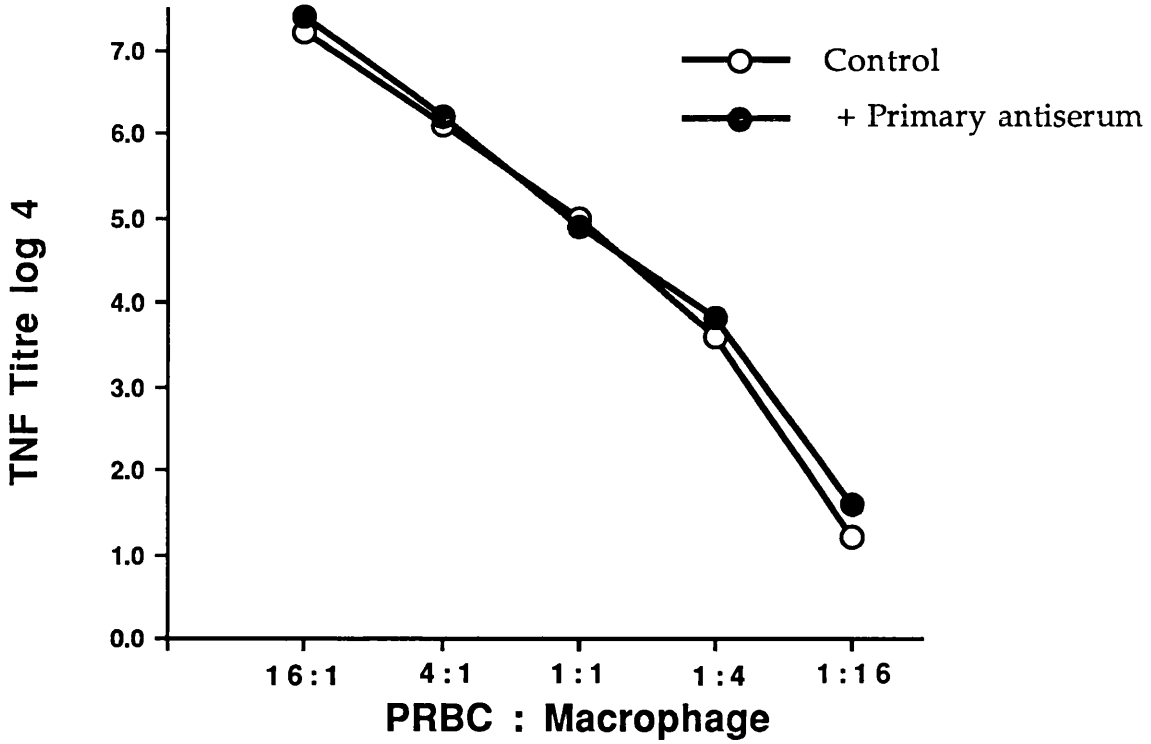


Figure 7.2.13.a. TNF titres stimulated by P.yoelii prbcs in the presence and absence of a 1/10 dilution of primary antiserum



titres stimulated by live *P.yoelii* in the presence and absence of this primary antiserum (Fig 7.2.13.a.). Although these are the results of a single representative experiment, several experiments all showed an absence of inhibition of the activity of live prbcs by primary antiserum. The simplest interpretation of these findings is that the live prbcs synthesizes a molecule that triggers macrophages but is not released into the supernatant. Alternative explanations are that triggering molecules become inserted into the prbc membrane when the cell is in close contact with the macrophage so that there is physical exclusion of antibody, or that the expression of the triggering molecule(s) on the surface of a prbc is different from their expression in solution.

7.2.14 Inhibition of fixed prbcs by primary antiserum

The possibility that live prbcs were inserting exoantigens into the erythrocyte membrane directly adjacent to macrophages and thereby physically excluding antibody was next tested by using formaldehyde-fixed prbcs, which can stimulate TNF secretion but cannot alter their external membrane. It was found that primary antiserum that failed to inhibit the effect of live prbcs did however inhibit the stimulation of macrophages by formaldehyde fixed prbcs (Fig 7.2.14.a.). These results were reproducible and the cross-reactions observed between supernatants from different rodent species were also found using prbcs from different rodent species. Although inhibition was clearly seen, inhibitory titres were lower than when the same antiserum was titrated against parasite supernatants, and inhibition was not total. This implies either that there are other serologically different triggering molecules present on the fixed prbc surface or that the same triggering molecules as those released into the supernatant are associated with other structures on the prbc membrane that prevent the binding of antibody.

When fixed prbcs that were known to stimulate macrophages were deacylated by treatment with 0.05 M NaOH or digested with phospholipase C they then failed to stimulate macrophages (Fig 7.2.14.b.). Furthermore antisera raised against such inactive preparations was not inhibitor , nor were antisera raised against deacylated or phospholipase C-digested Triton X-100 lysates, homogenates or unboiled supernatants. This suggests that all the *Plasmodium* derived molecules that are capable of stimulating TNF secretion are lipids.

Figure 7.2.14.a. TNF titres stimulated by formaldehyde-fixed P.yoelii prbcs in the presence and absence of a 1/10 dilution of primary antiserum

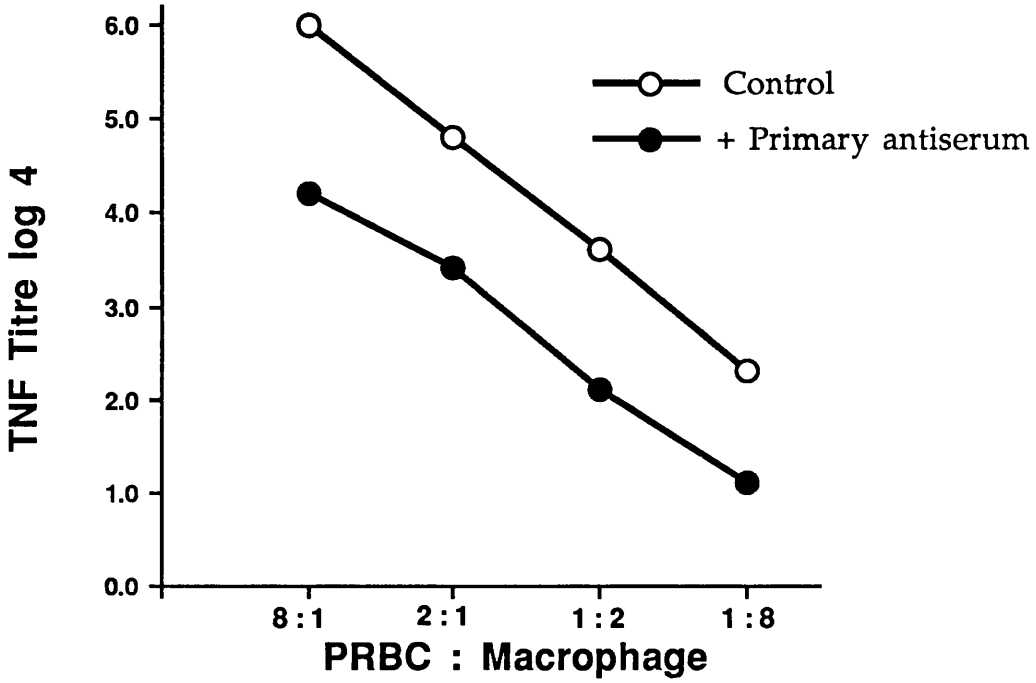
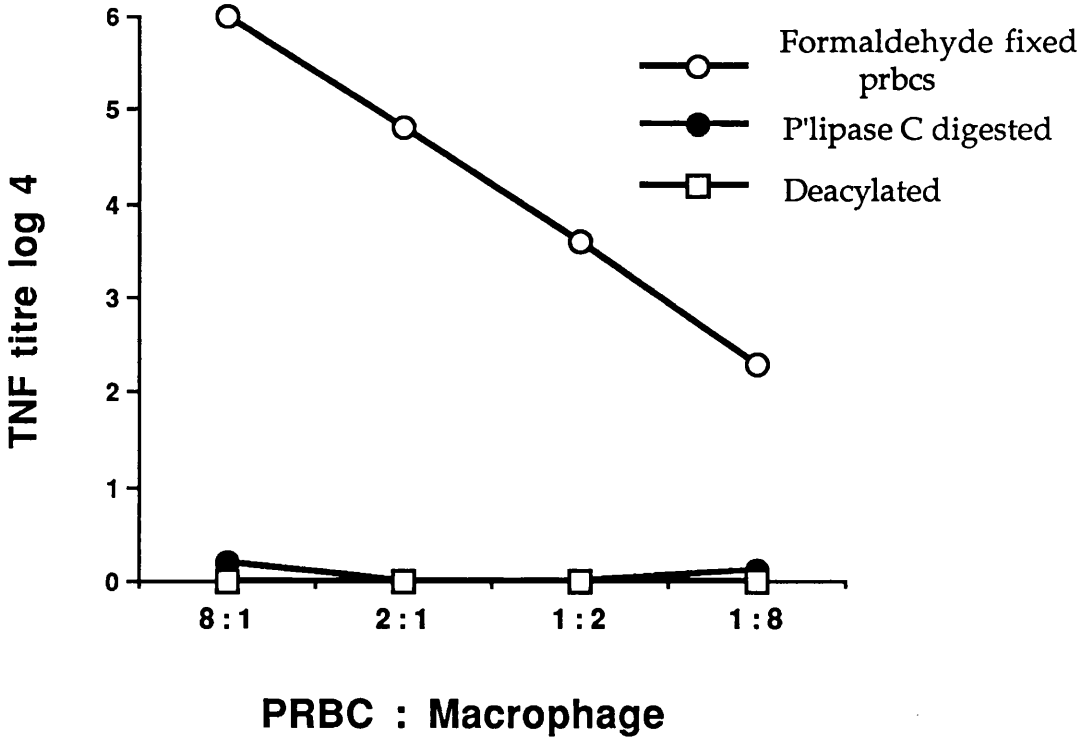


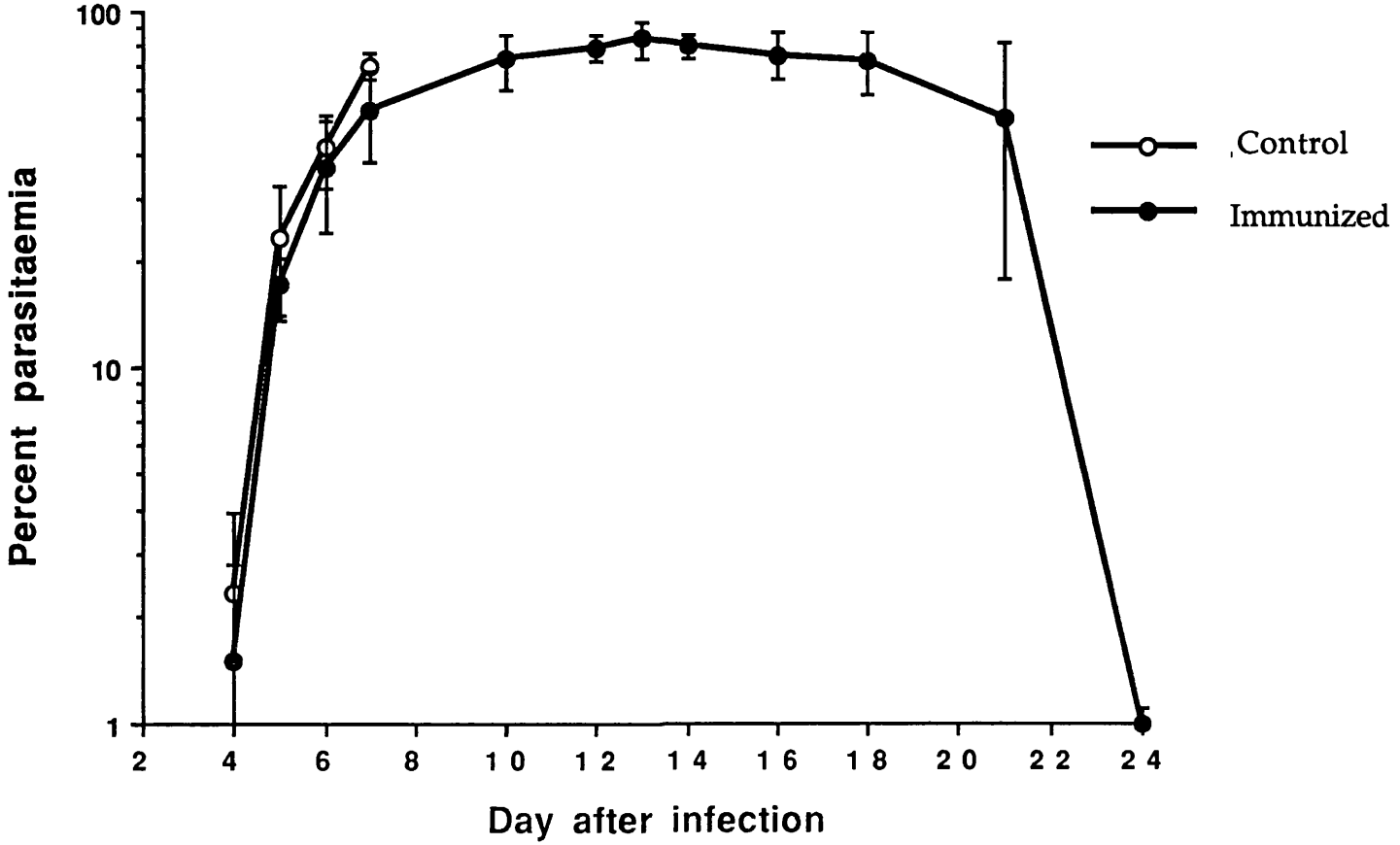
Figure 7.2.14.b. TNF titres stimulated by fixed prbcs before and after deacylation or digestion with P'lipase C



7.2.15 Vaccination with exoantigens

Antigens separated from a membrane detergent lysate of blood-stage *P.yoelii* YM induce protection against the lethal variant of *P.yoelii* in (Balb/c x C57/Bl) F1 mice of two main types: in the first, parasites show signs of damage ("crisis forms") around the 5th-7th day of infection and are completely cleared a day or two later. In the second, mice maintain high parasitaemias, often averaging over 80%, for two or three weeks before recovery, but do not show signs of severe illness (Playfair *et al.*, 1990). This latter type of protection would be expected of a vaccine which induces immunity to the toxic effects of the parasite. Immunization with boiled supernatants obtained from *P.yoelii* prbcs 10 days before challenge with 1×10^4 *P.yoelii* infected prbcs led exactly to this type of protection (Fig 7.2.15.a.). The results are given from an experiment showing mean parasitaemias, +/- standard deviation, for groups of control (5) and immunized (15) mice. This type of protection was also provided by boiled supernatants obtained from *P.berghei* and *P.falciparum*, pronase-digested supernatants, deaminated supernatants and by lipids isolated from a deaminated supernatant by a two-phase extraction procedure. However, not all the mice were protected by the latter preparations; protection varied from 33 - 75 %, so that further investigation is required to determine optimum vaccination regimens for purified materials. Groups of mice immunized with either LPS or with nrbc supernatants 10 days before infection did not protect any mice.

Figure 7.2.15.a. Protection against the lethality of infection with P.yoelii YM by prior immunization with parasite supernatants



7.3 Discussion

During the course of *Plasmodium* infections, exoantigens appear to be released which cause toxicity, probably through the stimulation of macrophages to release TNF. In this chapter I have examined some immunological methods that might reduce this toxicity. The inhibition of monokine release induced by parasite exoantigens provides one possible strategy to block the clinical disease associated with *Plasmodium* infections.

Exoantigen-induced toxicity in the D-galactosamine model can be inhibited by prior injection of immunomodulators. Two distinct types of protection can be achieved. A short term protection lasting up to 48 hours occurs in response to low doses of either LPS or of exoantigens (Bate *et al.*, 1989). This "early" protection can also be mediated by cytokines, since TNF and IL-1 both protect against a subsequent lethal dose of LPS (Wallach *et al.*, 1988; Vogel *et al.*, 1988). Animals rendered tolerant have a reduced capacity to produce CSFs and IFNs and show characteristic haemopoetic changes. It is possible that the endotoxin tolerance of human malaria may be mediated by a similar mechanism.

The protection afforded 10 days after injection is different. Thus immunization with parasite exoantigens specifically inhibits both the release of TNF into the serum and the toxicity of exoantigens in sensitized mice, but TNF responses to, and the toxicity of, LPS are unaffected. Similarly, immunization with LPS inhibited responses to LPS but did not affect responses to parasite exoantigens. These results suggest that the protection afforded to mice challenged 10 days later with exoantigens is mediated by specific antibody.

Both human and rodent derived parasite exoantigens injected into mice stimulate the production of inhibitory antiserum 12 days later which we have called **Primary antiserum**. Primary antiserum specifically blocks exoantigen, but not LPS, stimulated TNF secretion by macrophages *in vitro*, and protects against the toxicity of exoantigens in D-galactosamine-treated mice. There is a direct correlation between the properties of the exoantigens stimulating TNF secretion, exoantigens mediating toxicity and exoantigens stimulating antibody formation. Inhibitory antibodies cannot be raised by

preparations that do not trigger macrophages, regardless of the method used to abolish their activity and the species of parasite from which they were derived. However, only antibodies that react directly with the triggering moiety were measured in this assay. In contrast, the active moiety of LPS, lipid A is poorly antigenic and antibodies are directed primarily against the polysaccharide side chains. There was some evidence that the affinity purified antigen 1 isolated from *P.falciparum* may be associated with protein that prevents its recognition by primary antisera. This may be host rather than parasite derived, since preparations of *P.falciparum* exoantigens in a serum-free system do not have this property.

Primary antiserum that was depleted of IgG and IgM on isotype-specific columns was incapable of inhibiting exoantigen responses, indicating that the inhibition is mediated by antibody. The isotype responsible for the inhibition of macrophage stimulation by parasite exoantigens is predominantly IgM, but there is also measurable inhibitory IgG. Inhibition was first detectable in the serum between 5 and 7 days after immunization. Antibody titres rise quickly, reaching a peak on day 12 and falling rapidly so that by day 21 inhibitory antibody is barely detectable. Prior injections of homologous exoantigen preparations, or the injection of 25 μg/mouse of saponin used as an adjuvant did not alter either peak antibody titres or the time course of antibody production. The pattern of inhibition by antibody isotypes was also unaffected by these regimens. These results suggest that antibody formation is stimulated in a T-cell independent manner.

Exoantigens stimulate the production of inhibitory antiserum in CD 4+ depleted mice and athymic mice, showing that T-cells are not required for the production of inhibitory antibody. In most systems anti-parasite immunity is considered to be T-cell dependent, and it has been claimed that *P.yoelii* lacks T-independent antigens (Taylor *et al.*, 1982). However, it is widely held that persistence of the parasite within the host is necessary for the maintenance of effective immunity: elimination of parasitaemia by chemotherapy rapidly restores susceptibility, and individuals returning to an endemic area after a period of absence as little as 6 months suffer symptoms of disease at low parasite densities (Maegraith., 1974). These observations could be explained by the retention of T-dependent anti-parasite immunity, but the loss of a T-independent anti-disease immunity.

I have shown that inhibitory antibody acts against phospholipase C-sensitive sites and that these sites are necessary for the production of functionally inhibitory antibodies that cross-react with exoantigens from different species. The lack of antigenic variation seen in exoantigen preparations suggests that, in theory, antibodies that block the triggering molecules might protect against the toxicity of infection by a variety of different strains or species of *Plasmodium.* In contrast, antitoxic antibodies to LPS are generally protective against challenge with the homologous strain but not against LPS from different strains.

It is theoretically possible to use passive transfer of neutralizing antibodies as therapy. Passive transfer of primary antiserum inhibited toxicity in the D-galactosamine model and provided some protection against infection with lethal *P.yoelii* (data not shown). In experimental animals antiserum raised against the lipid A of LPS has been shown to increase the dose of LPS required to kill half a group of mice (LD_{50}) and to block the dermal Shwartzman reaction (Kirkland & Ziegler., 1984). Antisera (Greisman *et al.*, 1978) and IgM monoclonal antibodies (Teng *et al.*, 1985) can confer protection against the lethal toxicity of a broad spectrum of Gram-negative bacteria in mice. Antisera raised against a heat-killed mutant of *Escherichia coli* (J5) which lacks oligosaccharide side-chains so that the core lipid A is exposed induces antibodies against lipid A, (J5 antiserum). Prophylaxis with plasma from volunteers taken after immunization with J5, when given intravenously to patients near to the onset of disease did not lower levels of bacteria within the bloodstream, but did reduce mortality in patients with septic shock. In bacteraemic patients, mortality was reduced from 39 % to 22 % and in patients with profound shock, mortality was reduced from 77 % to 44 % (Ziegler *et al.*, 1982). There are inherent risks in the way such antiserum is produced, the injection of a toxin and the scarcity of such serum, has highlighted the advantages of monoclonal antibodies. Several monoclonal antibodies to lipid A have been made and clinical trials using them are in progress. So far, we have been unable to produce a monoclonal antibody that inhibits exoantigen-induced TNF secretion. Such an antibody would be of use to check the similarities of exoantigens from different species or preparations and an affinity column using from such antibodies could help in the identification of associated molecules.

In direct contrast to the conclusions drawn above, primary antiserum did not inhibit the stimulation of macrophages by live prbcs. These results could indicate the existence of other, non-lipid, triggering antigens. If they exist, however, they are not released into lysates or homogenates since the stimulation of macrophages by such preparations is completely blocked by primary antisera raised against purified preparations. Triggering antigens in these preparations were totally destroyed by deacylation and by digestion with phospholipase C. This result does present considerable problems as far as potential for vaccination is concerned, because if there are several antigens that stimulate macrophage TNF secretion then an effective vaccine would have to inhibit all of them.

Vaccination with a crude boiled supernatant protects mice against an otherwise lethal infection of *P.yoelii*. There was no reduction in parasite load, however, but an apparent reduction in the severity of disease and in mortality of infection which are the results we would expect from an "anti-toxin" vaccine. Similar but unexplained results have been described in *P.chabaudi* infection of susceptible NIMR and B.10 mice (Wunderlich *et al.*, 1988). In these mice, vaccination with plasma membranes (erythrocyte ghosts) from prbcs led to protection, but did not alter the time course or peak of parasitaemia of infection. Furthermore, cerebral lesions in mice infected with *P. berghei* can be inhibited by antibody to soluble antigens (Schetters *et al.*, 1989).

The crude supernatants used in the initial vaccination studies contain many molecules which might be protective. In further vaccination studies, mice were protected from lethal *P.yoelii* by both deaminated exoantigens, and exoantigens prepared from *P.falciparum* Furthermore there was no evidence that 3 injections of antigen provided better protection (data not shown), suggesting that this type of immunity was T cell-independent. Thus the properties of the immunogens providing protection resemble the properties of the exoantigens that stimulate TNF production *in vitro* and the production of inhibitory antibody. However further cross-reactivity between species, correlation with antibody production and the time course of protection has not been fully determined in vaccination studies.

If exoantigens are responsible for the toxicity of *Plasmodium* infections and antibodies blocking exoantigen responses are capable of protection; then we are faced with problem of how to generate immunological memory by vaccination. The exoantigens, although highly antigenic, do not seem to stimulate T-cell proliferation (G. Grau, personal communication) suggesting that they are not presented with MHC II antigens. There is little literature on the ability of lipid antigens to induce T-cell responses, although it is claimed that DTH responses to the LPG (lipophosphoglycan) of *Leishmania major* are directed against the lipid moiety (Moll *et al.*, 1989).

A possible strategy for the conversion of a thymus-independent antigen into a thymus-dependent immunogen may be to develop a surrogate protein image of the antigen. This is effectively seen at the combining site of an anti-idiotype antibody. Mice have been protected against the toxicity of LPS by immunization with an anti-idiotype antibody to a monoclonal antibody to lipid A, which produced protective anti-anti-idiotype antibodies (Kato *et al.*, 1990).

Another approach is to study the immunology of carrier molecules associated with the triggering moiety. If these show similar species cross-reactivity, then memory might be generated to these molecules. The antibody responses would not show up in *in vitro* assays as they do not block the active moiety, but *in vivo* they might function by facilitating the removal of the triggering complex. The adjuvant, saponin did not increase antibody titres as determined *in vitro*; however, there is preliminary evidence that saponin may increase the percentage of mice protected by a boiled supernatant in vaccination studies (data not shown). This suggests that there may not be a direct correlation between protection and antibody production. More work is required to evaluate other mechanisms of protection that might be mediated by these exoantigens and other adjuvant systems.

Since these molecules which might form the basis of a potential vaccine are in effect toxins, some methods to reduce their toxicity would be necessary. Currently, experiments are being carried out to assess the use of liposomes as adjuvants. Liposomes offer many advantages: they are themselves non-toxic and they reduce the activity both of lipid A (Dijkstra *et al.*, 1989) and of the exoantigen preparations to stimulate macrophage TNF

secretion (data not shown). Liposomes can be targeted by addition of specific carrier molecules, they can be combined with other adjuvants and they have even been claimed to confer T-dependency upon T-independent antigens (Gregoriadis., 1990).

A surprizing recent finding is that phospholipid liposomes themselves stimulate the production of ~~inhibitory~~ antibody that inhibit parasite exoantigens (unpublished data). Liposomes made from a variety of different phospholipids stimulate the production of antibodies that inhibit exoantigen induced TNF secretion from macrophages. Furthermore soluble forms of phosphatidyl inositol and its derivative inositol monophosphate also stimulate the production of such inhibitory antibodies. Inhibitory antibodies in primary antiserum raised to parasite exoantigens are specifically absorbed by liposomes made with phosphatidyl inositol but were not absorbed by liposomes consisting of phosphatidyl choline, phosphatidic acid, cardiolipin or phosphatidyl serine. Preliminary experiments suggest that the inhibitory antibodies raised against phospholipids share the some of the immunological properties of antiserum raised against parasite exoantigens. The inhibitory antibodies in antiserum raised against inositol monophosphate and phosphatidyl inositol are predominantly IgM, inhibitory titre is not increased by repeated injection and inhibitory antibodies are not absorbed by phosphatidyl choline liposomes. Furthermore vaccination ~~with~~ with either phosphatidyl inositol or inositol monophosphate protected some mice from an otherwise lethal infection with *P.yoelii* (unpublished data). These cross-reactions suggest that the molecular identification of parasite exoantigens may not be necessary as inhibitory antibody can be raised by non-toxic phosphatidyl inositol, inositol monophosphate and various phospholipid liposomes, but the possibility of autoimmune reactions against "normal" phospholipids would need to be explored.

8.0 Modulation of TNF cytotoxicity

8.1 Introduction

One of the interesting differences between human and mouse malaria is that clinical disease in man occurs at very low parasitaemias; this correlates with the relative lower sensitivity of humans to endotoxins and to TNF (Clark, 1982). Mice tolerate high parasitaemias, and are relatively tolerant of LPS and TNF (Galanos & Freudenberg., 1987) when compared to humans (Spriggs *et al.*, 1987). The presence of factors that either enhance the activity of TNF in man or inhibit the activity of TNF in mice might explain these differences in the toxicity of parasite infections. Such factors are already made use of in experimental design, *in vitro* L929 cells are pretreated with inhibitors of RNA and protein synthesis, either actinomycin D or emetine which enhance cell destruction. *In vivo* D-galactosamine pretreatment of mice leads to an increased sensitivity to the toxic effects of TNF (Lehmann *et al.*, 1987).

8.2 Results

8.2.1 Inhibition of the cytotoxicity of TNF by pretreating target cells with indomethacin

Macrophages pretreated with indomethacin secrete more TNF. This suggests that mice pretreated with indomethacin might be more sensitive to an injection of exoantigens. However, instead indomethacin-treated D-galactosamine sensitized mice were protected against an otherwise lethal injection of exoantigens. The inhibition of the cytotoxic activity of TNF *in vitro* by indomethacin could explain such a result. Therefore L929 cells were incubated in different concentrations of indomethacin before titration of r human TNF. Pretreatment of these target cells with indomethacin inhibited the cytotoxicity of TNF in a dose-dependent manner. A typical titration is presented (Fig 8.2.1.a.); indomethacin pretreated target cells were always less sensitive to murine and human r TNF than untreated cells.

Figure 8.2.1.a. Reduction in the cytotoxicity of r TNF by pretreatment of target cells with the cycloxygenase inhibitor Indomethacin

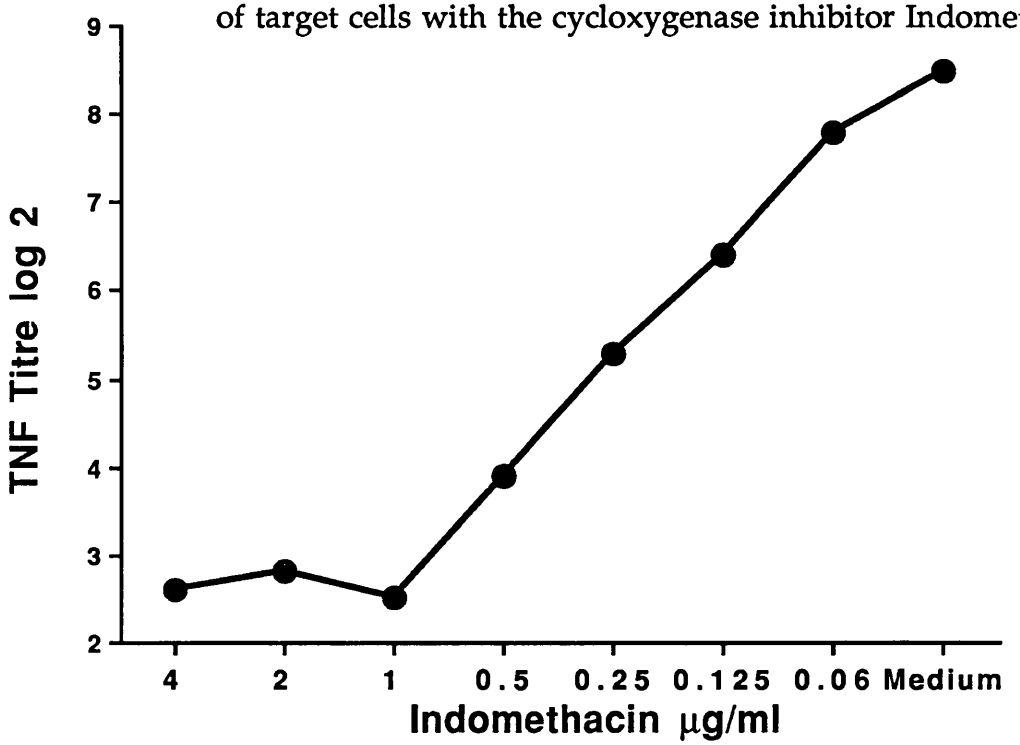
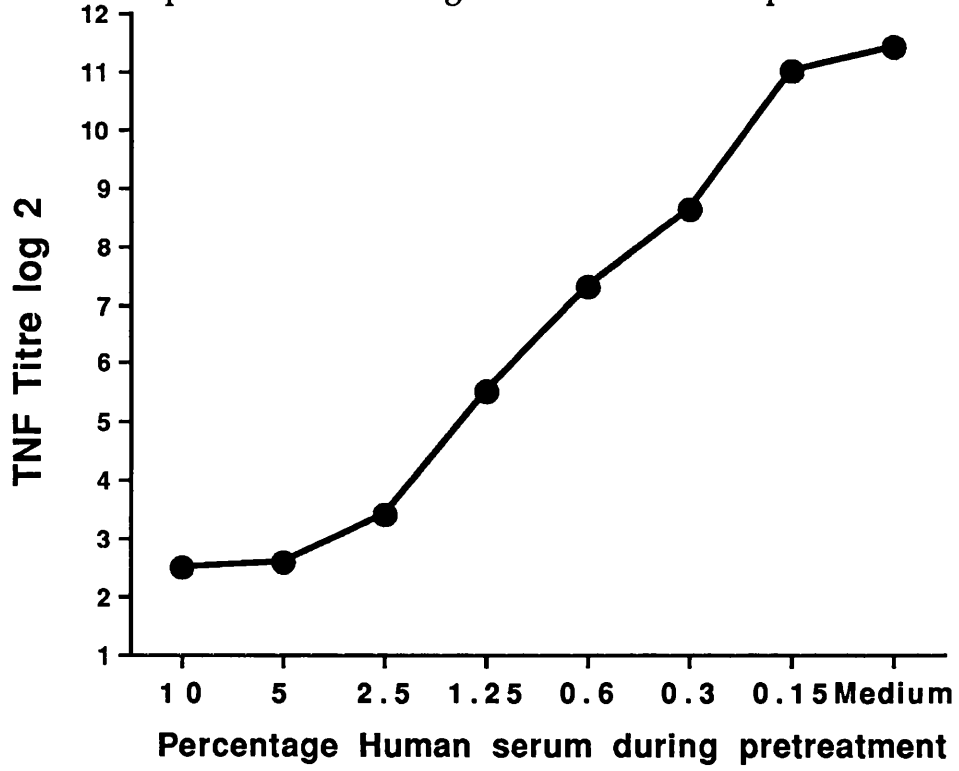


Figure 8.2.2.a. Reduction in the cytotoxicity of r TNF by pretreatment of target cells with human post exercise serum



8.2.2 Inhibition of the cytotoxicity of TNF by pretreating target cells with human sera

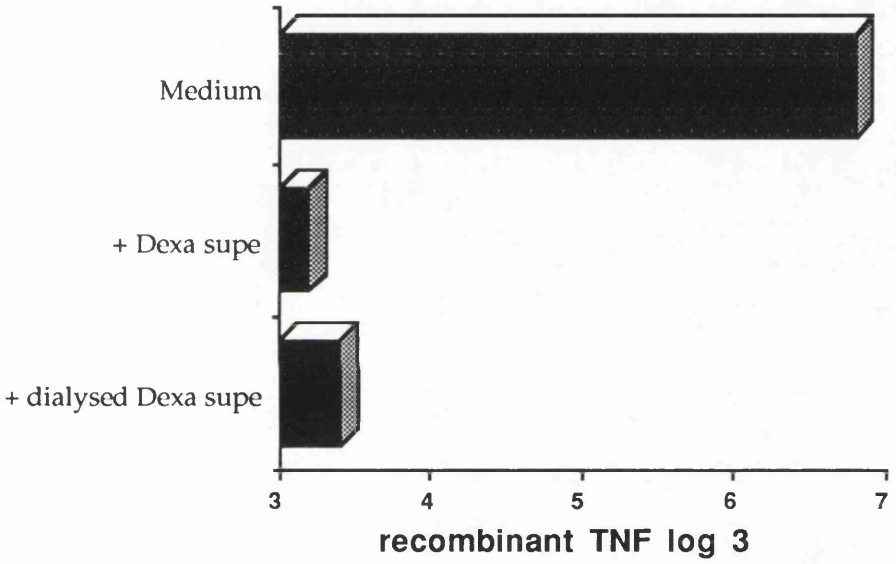
The cytotoxicity of human r TNF for L929 cells when titrated in the presence of 5 % human serum from different individuals was highly variable, some serum samples showing inhibition. Out of 12 samples tested, 2 showed highly significant inhibition of TNF (unpublished data). Dilutions of one of these incubated with L929 cells for 24 hours before removal and titration of recombinant human TNF, reduced the sensitivity of the cells to the toxicity of recombinant human TNF in a dose-dependent manner (Fig 8.2.2.a.). Serum samples with the highest inhibitory titres were those collected from donors within an hour after undertaking vigorous exercise, and preliminary studies suggest that TNF cytotoxicity can be inhibited by factors that are released during or within an hour after exercise (unpublished data). The controls were target cells incubated in 5 % FCS or in 5 % human serum. As the inhibition of TNF occurred when serum samples were incubated with the target cells, the inhibition does not appear to be due to the presence of free TNF receptors but rather, like indomethacin, to an inhibition of the sensitivity of target cells to TNF.

8.2.3 Inhibition of TNF cytotoxicity by factors from dexamethasone-treated macrophage cultures

Cortisone and its analogue dexamethasone inhibit the toxicity of LPS *in vivo*, furthermore dexamethasone-treated macrophages secrete lipomodulin, a naturally occurring anti-inflammatory factor which is functionally similar to indomethacin (Blackwell, 1983). We investigated the possibility that the inhibition of the toxicity of LPS *in vivo* might be caused by the inhibition of TNF cytotoxicity through the production of cortisone sensitive factors such as lipomodulin. Thioglycollate-elicited macrophages were incubated with 100 ng/ml dexamethasone and the supernatant collected after 1 hour. This supernatant, which is not itself toxic, was incubated for 1 hour with L929 cells before the addition of serial dilutions of recombinant human TNF. The results of titrations on control cells and cells which had been pretreated with this supernatant are compared (Fig 8.2.3.a.). To ensure that this effect was due to a macrophage-derived factor and not to dexamethasone in the macrophage supernatant, it was dialysed against PBS

before addition to the L929 cells. The dialysed macrophage supernatant still inhibited the cytotoxicity of recombinant TNF. Protection against the cytotoxicity of recombinant TNF was obtained on 3 different occasions with macrophages incubated with dexamethasone, and equivalent protection was observed with culture supernatants from both thioglycollate elicited and unelicited macrophages.

Figure 8.2.3.a. Protection of target cells by pretreatment with the culture supernatant from dexamethasone-treated macrophages



8.3 Discussion

Protection of mice sensitized by D-galactosamine against the toxic effects of parasite exoantigens was achieved by prior injections of human post-exercise sera, indomethacin and phenoxybenzamine. The protection provided by such reagents might be due to the inhibition of the toxicity of TNF, perhaps by inhibition of TNF interaction with target cells or by the functional inhibition of TNF at a post receptor level. TNF inhibitors have been found in the serum of patients with Sarcoidosis, Tuberculosis and Crohn's disease (Foley *et al.*, 1990) and there is growing evidence for the inhibition of TNF by soluble TNF receptors (Seckinger *et al.*, 1988).

TNF and IL-1 both generate fever by the stimulation of hypothalamic prostaglandins and the induction of this fever can be inhibited by indomethacin and other non-steroidal anti-inflammatory drugs. The signal transduction mechanism mediating TNF cytotoxicity also involves the generation of prostaglandins, as the cytotoxicity of TNF can be inhibited by indomethacin. Indomethacin is not a physiological factor, but there is an endogenous inhibitor of prostaglandin synthesis, lipomodulin, a protein which is secreted by macrophages in response to cortisone. Dexamethasone-treated macrophages give rise to a supernatant that inhibits TNF toxicity; this activity is not due to dexamethasone itself as it is still present after dialysis.

Inhibition of TNF cytotoxicity mediated by pretreatment with human sera suggests that there are compounds that decrease target cell sensitivity rather than directly inhibit TNF. The limited data collected suggests that the basis of this insensitivity to TNF was not genetic, but rather that it was mediated by factors present in the serum after physical exercise. These results apply to the toxicity of TNF upon L929 cells; toxicity *in vivo* is mediated by effects upon different cell types which may use different signal transduction mechanisms. The nature of these experiments has been to examine some methods of inhibiting TNF toxicity.

It has been proposed that sensitivity to TNF *in vivo* is largely a function of the condition of an animal. Animals infected with pathogens are more sensitive to TNF, for example infection with either BCG or *P.vinckei* (Clark *et al.*, 1987) increases sensitivity to the lethal effects of TNF. The detoxified form of LPS, monophosphoryl Lipid A (MPL), is not lethal in mice

at doses that stimulate the production of large amounts of TNF (Kiener *et al.*, 1988). That there is no direct correlation between toxicity and levels of TNF suggests that factors modulating the actions of TNF are also of importance. It is possible that *P.falciparum* causes greater pathology, including death, in some individuals because they are either more sensitive to TNF or because they produce more factors in response to infection that increase sensitivity to the toxicity of TNF. Cytotoxicity *in vitro*, lethality *in vivo* and antitumoricidal activity can be modulated separately by the actions of different drugs (Haranaka *et al.*, 1988).

IFN-γ has been shown to increase the expression of TNF receptors by cells (Ruggerio *et al.*, 1986), and to increase the sensitivity of L929 cells to TNF toxicity (Taverne & Bate, unpublished data). Recombinant human TNF is significantly less effective against murine tumours in nude mice than in normal mice indicating that the activity of TNF, in this model, depends upon the presence of T-cells (Palladino & Figari., 1988). IFN-γ has been reported to enhance the toxicity of TNF *in vitro* (Talamadge *et al.*, 1987).

Interleukin-1 is another possible mediator of pathology associated with LPS-like toxins, as treatments that sensitize mice to LPS also render mice hypersensitive to IL-1 (Bertini *et al.*, 1988). TNF and IL-1 have been shown to synergize over a wide variety of functions, including the induction of lethal shock (Waage & Espevik., 1988). During infection of mice with *P.vinckei*, the toxicity of exogenous TNF is enhanced by even relatively small amounts of IL-1 (Clark, personal communication).

In vivo there are feedback mechanisms that modulate TNF toxicity. Adrenalectomy (Bertini *et al.*, 1988) sensitizes mice to the lethal effects of both TNF and IL-1. Adrenalectomized mice show a 40-60 fold increase in TNF production in response to a constant dose of LPS compared with control mice, but a 300-400 fold increase in sensitivity to its lethal effects (Rangappa *et al.*, 1989). This is thought to be due to the loss of the glucocorticoids which are intrinsic opponents of TNF (Aabe *et al.*, 1987). Endogenous TNF production is always followed by glucocorticoid production some 2-3 hours later, which is believed to mediate negative feedback by reducing TNF secretion. The drug RU-38486, an inhibitor of glucocorticoid receptors, sensitizes mice to the toxic effects of TNF (Flad *et al.*, 1989).

9.0 Conclusions and Discussion

In this study I have shown that in malaria components of a potentially toxic reaction exist, primarily the presence of exoantigens that stimulate TNF production, and that these might explain some of the pathology of *Plasmodium* infections. However not all infections with *P.falciparum* in man, or *Plasmodium* species in other hosts are associated with pathology or mortality. I have considered some of the factors that might modulate this type of reaction *in vivo* and which might possibly explain the spectrum of disease.

9.1 Production of the exoantigens

Prbcs cultured with activated macrophages stimulate the macrophages to secrete TNF. This response is mediated by molecules which have been partially purified and which we have called exoantigens. However there is no simple correlation between the production of exoantigens by a given species and the outcome of an infection with that species of *Plasmodium*. The synchronicity of the developmental cycle, the rate of parasite multiplication, strain differences in the amount of exoantigen produced, the ages of the infected erythrocytes and the method by which exoantigens are liberated may all be important factors which alter the stimulus presented to macrophages.

The cellular location of these exoantigens is unknown. Fixed prbcs stimulate macrophages, suggesting that they are expressed upon prbc surface membranes, but there is also some evidence of increased release during schizont rupture (Kwiatkowski *et al.*, 1989). Although the exoantigens are parasite derived, it is not known whether they are produced by parasite metabolism of host molecules or by parasite-specific anabolic pathways. If the production of exoantigens involves parasite-specific enzymes, then there exists the potential for pharmacological intervention. It is possible that exoantigens are produced only in response to certain stimuli, such as oxidative stress. If this is so, then the nature of these stimuli is important; one rather intriguing possibility is that the synthesis of the exoantigens is controlled by host factors.

The active moiety of the exoantigens seems to be a low molecular weight phospholipid, and as sera taken from infected animals react with the exoantigens, I have concluded that these molecules are a feature of infection rather than just an artifact of *in vitro* incubation. If such exoantigens are to be used as part of a vaccine, the purification and characterization of the active moiety(s) is essential. The ultimate goal is to synthesize parasite exoantigens or structural analogues and to check that they have the same biological activities as the natural molecule. The active moiety of the exoantigens might be linked to other structures, probably parasite derived, which could alter their properties such as solubility in aqueous media, ability to stimulate cytokines or antibody production, serum half-life or interactions with inhibitory antibody.

The identity of the receptor of macrophages which mediates exoantigen induced TNF secretion and the second messengers initiated by receptor occupation are just some of the areas suitable for further investigation. Initial experiments suggest the existence of a " non-toxic analogue", molecules which bind the macrophage receptor for exoantigens but which do not stimulate TNF secretion. TNF induction by exoantigens can be inhibited either by a lipase digested exoantigen preparation or by specific phospholipids; inositol monophosphate and phosphatidyl inositol inhibit this activity of exoantigens but do not themselves stimulate TNF secretion (unpublished data). It might be possible to use such " non-toxic analogues" as antagonists which could inhibit TNF production by exoantigens *in vivo* and hence reduce the toxicity of infection.

9.2 Requirement for activated macrophages

If our hypothesis is correct, then corticosteroids, which inhibit TNF secretion, should block the pathology of infection. However, previous studies have shown that pharmacological intervention with high doses of dexamethasone provide no benefit in the treatment of either cerebral malaria (Warrell *et al.*, 1982) or severe sepsis (Bone *et al.*, 1987). It could be argued that this was due to the timing of treatment, as corticosteroids only affect TNF secretion if macrophages are treated before addition of the the stimulus.

In the D-galactosamine model, TNF seems to tolerize to the toxic effects of subsequent injection of parasite exoantigens, perhaps through inhibition of the release of bone marrow macrophage precursors, which provide new waves of activated macrophages (Vogel *et al.*, 1988). Cytokines and individual genetic influences of both the host and the parasite might possibly affect tolerance and hence the toxicity of infection. The artificial induction of tolerance and its effects upon the toxicity of infection provide further areas of interest.

In general, malaria causes activation of myeloid cells which would lead to their increased capacity to produce TNF. The variable antigenic profile of different *Plasmodium* strains or species might result in the proliferation of different T-cell populations. These T-cells might secrete a different repertoire of cytokines which could result in the production of populations of macrophages with different activation states. For example, *P. falciparum* might preferentially activate TH-1 cells resulting in IFN-γ production and the appearance of a population of macrophages that are highly activated to secrete TNF. Host genetic influences and the nature of previous infections may also affect T-cell responses and hence the activation state of macrophages. Individual genetic variations may predispose certain individuals to producing large amounts of TNF, and MHC class II molecules have been shown to be involved in LPS-induced IL-1 and TNF secretion (Santamaria *et al.*, 1989). It may be possible to identify "high responders" to exoantigens which could identify those at risk of suffering complications.

It may also be possible to experimentally manipulate the T-cell repertoire so as to reduce the overall activation state of macrophage populations. For example, a vaccine that increased the proportion of TH-2 cells, leading to the subsequent secretion of large amounts of IL-4, would theoretically prevent toxicity as IL-4 inhibits TNF secretion in response to LPS (Reynaud *et al.*, 1989). Therapy which lead to either the specific or non-specific induction of factors that inhibit macrophage TNF secretion are an obvious exciting area for future investigation.

9.3 Identification of toxins and their mode of action

This study has been based upon the assumption that TNF has an important role in the production of pathology during infection. TNF seems to be the prime mediator of toxicity in the D-galactosamine model, but this may be a function of the model used. In D-galactosamine sensitized mice antibodies to IFN-γ do not affect LPS induced mortality; in contrast, the pathology of LPS induced Shwartzman reactions can be blocked by antibody to IFN-γ. The identification of the role of other "toxic" cytokines, the contribution these mediators might make to the pathology of infection and the nature of the antigens that stimulate their secretion should be considered if an anti-toxic vaccine is to be effective. If these cytokines are predominantly stimulated as part of an immune cascade, then it is logical to look at methods of inhibiting the initiator of that cascade.

The possibility that there are other toxins which act directly upon cells in a similar manner to pertussis and cholera toxins is another topic for further investigation. The chemical and enzymatic sensitivities of exoantigens are similar to those of phosphotidylinositol-glycans (PI-Gs), molecules that are released after stimulation with insulin and are thought to mediate the intracellular effects of the hormone. This similarity suggests that exoantigens might cause hypoglycemia directly, and it has been shown that the injection of parasite supernatants into mice caused a significant fall in blood glucose levels (K. Taylor, personal communication). This was not secondary to the production of TNF as in these mice hypoglycemia still occurs in the presence of a neutralizing Mab to TNF.

The most severe complication of infection with *P.falciparum* is cerebral malaria. It is possible that altered neurological function may occur as the result of excessive cytokine production, perhaps within the cerebral microenvironment. The amphipathic nature of the exoantigens suggests that they may be able to cross the blood brain barrier and I am interested in the possibility that *Plasmodium* antigens affect neurological function directly, either as neurotransmitters or neuromodulators. Altered membrane phospholipid organization due to the intracellular growth of *P. falciparum* is associated with increased monocyte and endothelial adherence (Maguire, Prudhomme & Sherman., 1991). Another possibility is that our phospholipid exoantigens, which are expressed upon the surface of fixed prbcs, might be

involved in cytoadherence. Furthermore, the loss of membrane phospholipid asymmetry has been shown to reduce clotting times by a factor of 3 (Zwaal *et al.*, 1977), it is not inconceivable that parasite phospholipid exoantigens are also involved in this process. Exoantigens are chemically similar to the lyso phosphatidylcholines which are potent haemolytic agents that can lyse erythrocytes and liberate haemoglobin. In the assays outlined in this thesis these types of toxin would not be recognized and more work needs to be done to assess the relative importance of such toxins.

9.4 Sensitivity to TNF

In mice and monkeys infected with malaria, disease is not seen until parasitaemias are quite high, which contrasts with the small numbers of parasites required to cause disease in man. Infections of *Aotus* monkeys with *P.falciparum* suggest that mice and monkeys are innately tolerant of malaria, rather than that the species that parasitize mice or monkeys are less toxic. As well as tolerating malaria well, mice are also more tolerant of both endotoxin and TNF than are human subjects (Clark *et al.*, 1982). Moreover, during infection this sensitivity to TNF increases (Clark *et al.*, 1987). No identifiable genetic basis has been found that could explain this sensitivity or act as an indicator of patients likely to suffer severe disease. Altered sensitivity to TNF may be the result of either exogenous inhibitors or factors that affect cellular sensitivity to TNF.

The presence of factors that directly inhibit TNF may also be able to reduce the toxicity of infection. A molecule of 40-60 KD has been isolated from the urine of febrile patients, (Seckinger *et al.*, 1988) was shown to inhibit TNF cytotoxicity *in vitro*. Furthermore the immunosuppressive glycoprotein uromodulin, synthesized by the kidney, has been shown to bind TNF. There is potential for the amelioration of TNF induced toxicity by the addition of non-toxic TNF analogues or exogenous TNF receptors.

Cytokines such as TNF stimulate a cascade of mediators each of which in turn stimulates further cascades resulting in a complex, integrated system of feedback regulatory systems. I would speculate that these feedback networks have evolved to balance the toxic effects of cytokines, and that pathology may be the result of an imbalance in the system, possibly through the tolerance or depletion of one feedback system. TNF can induce inhibitory feedback at several different levels, including the induction of acute phase

proteins which can inhibit exoantigen interactions with macrophages. TNF also stimulates the release of factors such as prostaglandins, cortisone, IL-6 and TGF-β which act to inhibit the further secretion of TNF from macrophages. Finally, TNF may induce the release of factors such as cortisone, which inhibit the sensitivity of target cells to TNF. Interactions between the genetic make-up of both host and parasite could alter any one of several feedback mechanisms and hence the toxicity of infection.

Preliminary experiments suggest that sensitivity to TNF can be reduced by factors that are not present in normal human serum, but whose presence is increased after exercise. The synthesis and secretion of such factors might be stimulated during infection as part of a feedback system. The identification of these factors, how and when they are produced, and their effects on the course and toxicity of infection provide an exciting area for future investigation.

In an ideal situation it would be possible to dissociate the useful and deleterious effects of TNF. As two different TNF receptors have been identified (Brockhaus *et al.*, 1990) it is possible that the toxic actions of TNF are mediated either by a different receptor, or through a different second messenger system, from the beneficial actions of TNF. It is perhaps possible that drugs acting as functional rather than direct antagonists could selectively inhibit the "toxicity" (desensitize target cells) of TNF without affecting the beneficial actions of TNF upon the immune system. This requires the identification of a population of cells *in vivo* that are responsible for "toxicity" and also identification of the second messengers stimulated by TNF. Nitric oxide (NO) is a possible candidate for such a toxic second messenger, it is generated by a range of cell types in response to TNF, and if produced within the CSF might interfere with normal neurotransmission (Clark *et al.*, in press).

It has been postulated that much of the toxicity of TNF is mediated through the actions of cytokines upon endothelial cells. Indeed TNF has powerful effects upon vascular endothelium, as extensively reviewed by Pober, (1988). In a murine model of cerebral malaria an antibody that bound to LFA-1 inhibited the development of the cerebral condition (G.Grau personal communication). TNF has been shown to upregulate ICAM-1, a ligand for LFA-1, on endothelium (Pober *et al.*, 1986). It is attractive to hypothesize that there are genetic factors that regulate the production of such

adhesion molecules in different areas of the vasculature in response to cytokines (do cerebral blood vessels express more of such molecules ?) which might explain the variation in individual sensitivities to the toxicity of TNF. It is also possible that different species of parasite induce such molecules to a greater or lesser extent. It may be possible to use immunological manipulation of the expression of such molecules to desensitize patients to the toxic effects of TNF.

The use of drugs to reduce the toxicity of recombinant TNF relative to the anti-tumour activity has been explored (Haranaka *et al.*, 1988). Initial results suggest there to be some dissociation between the the two activities in that the toxicity of TNF can be reduced without affecting its tumoricidal activity. It would be of interest to study the effects of these drugs upon malarial infection.

9.5 Vaccination and potential side effects

The malarial antigens currently on trial as vaccines, from both the sporozoite and the blood stage, are increasingly feared to display considerable antigenic variation in both their T and B cell epitopes (Good & Miller., 1990, Good *et al.*, 1988), as well as to display a degree of MHC-related variability in immunogenicity (Arnot, 1989).

Molecules causing excessive TNF release during malaria behave, in effect, like toxins, and might therefore be modified for use as vaccines; an appropriate vaccine might induce antibody that blocks exoantigen-induced activities, thus conferring "anti-disease" immunity. Immunization with parasite exoantigens inhibits toxicity in the D-galactosamine model, this inhibition being mediated by *Plasmodium* specific antibodies. Preliminary evidence suggests that protective antibodies show cross-reactivity between *Plasmodium* species and are directed at specific, evolutionary conserved sites. We are currently investigating sera from *P.falciparum* infected patients in order to determine the presence of inhibitory antibodies and their correlation with immune status. Our mouse studies suggest that inhibitory antibodies are short lived, predominantly IgM, and are produced in the absence of T-cell help. Crude supernatants provide protection against an otherwise lethal infection, which has been interpreted as an anti-toxic immunity. Further investigation to determine the nature of this protection in vaccination studies, the extent of cross-reactions between species, the use of different

vaccination schedules, the use of adjuvants and the nature of the immunizing antigens is ongoing. If such exoantigens are to be used as a vaccine, it is essential that they stimulate immunological memory. Exoantigens entrapped in liposomes or bound to proteins with T-cell epitopes might be used to induce memory.

Total inhibition of TNF production during infection may not in-fact be possible since its production can be stimulated by live prbcs in the presence of primary antisera, and TNF may be synthesized as part of an immune cascade in response to other cytokines or to antigens present in concurrent infections with other organisms. Indeed total inhibition of TNF production may not be desirable, Clark & Chaudhri (1988) have argued that there is a balance between the useful and harmful effects of TNF during infection. TNF is essential to host resistance against *Listeria monocytogenes* (Nakane *et al.*, 1988) and can provide protection against lethal bacterial infection in C3H/HeJ mice (Cross *et al.*, 1989). TNF has been implicated in host protection against viruses (Vilcek *et al.*, 1988) and it can selectively lyse virus infected cells (Mestan *et al.*, 1986). In murine malaria, recombinant TNF can inhibit parasite multiplication *in vivo*, probably through the activation of polymorphs, but not *in vitro* (Taverne *et al.*, 1987). It is widely believed that a little TNF, particularly in the right local micro-environment is probably useful during infection and that pathology only occurs when there is an excess liberated into the circulation.

LPS has been shown to stimulate the production of serum factors that can kill parasites and our exoantigens may also be able to stimulate the release of such factors. Indeed, TNF in the presence of as yet unidentified complementary factors present in "crisis" or "paroxysm" serum is involved in the destruction of gametocytes (Mendis *et al.*, 1990). *In vitro*, febrile temperatures inhibit the growth of *P.falciparum* and synchronize parasitaemia (Kwiatkowski., 1990). A monoclonal antibody neutralizing TNF reduces fever in human patients (Kwiatkowski, personal communication). Since an "anti-toxin" vaccine that inhibits TNF production would also inhibit the production of factors that reduce parasitaemia, indeed recent experiments in our laboratory have shown that injection of exoantigens into *P.yoelii* infected mice precipitates a fall in parasitaemia (unpublished data), it is envisaged that such exoantigens would not constitute a vaccine on their own but might be included as part of a multicomponent vaccine.

10.0 References

Aabe, S., Yamamotto, T., Inagawa, T., Oshima, H., Soma, G.I., Yamazaki, M., & Mizuno, D. (1987). A possible role of glucocorticoids: a natural and endogenous inhibitor of tumour necrosis factor ? *Immunobiol.* 175. 124-128.

Alemany, S., Mato, J.M., & Stralfors, P. (1987). Phospho-dephospho-control by insulin is mimicked by a phospho-oligosaccharide in adipocytes. *Nature.* 330. 77-79.

Arnot, D. (1989). Malaria and the major histocompatibility complex *Parasitol. Today,* 5, 138-143.

Ash, C. (1991). First Impressions of the Malaria Vaccine. *Parasitol. Today.* 7, 4, 63-64

Bate, C.A.W., Taverne, J., & Playfair, J.H.L. (1988). Malarial parasites induce tumour necrosis factor production by macrophages. *Immunology* 64, 227-231.

Bate, C.A.W., Taverne, J., & Playfair, J.H.L. (1989). Soluble malarial antigens are toxic and induce the production of tumour necrosis factor *in vivo. Immunology* 66: 600-605

Bate, C.A.W., Taverne, J., Davé, A., & Playfair, J.H.L. (1990). Malaria exoantigens induce T-independent antibody that blocks their ability to induce TNF. *Immunology*, 70, 315-320.

Berendt, A.R., Simmons, D.L., Tansey, J., Newbold, C.I., & Marsh, K. (1989). Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for *Plasmodium falciparum. Nature* 341. 57-59

Bertini, R., Bianchi, M., & Ghezzi. (1988). Adrenalectomy sensitizes mice to the lethal effect of IL-1 and TNF. *J.Exp.Med.* 167, 1708-1712.

Bertini, R., Bianchi, M., Mengozzi, M., Erroi, P.G., & Mantovani, A.M. (1988). Chloropromazine as an antagonist of interleukin-1 mediated activities *in vivo. 7th International Congress of Immunology, Abstracts* W-69-12

Bernton, E.W., Beach, J.E., Holaday, J.W., Smallridge, R.C., & Fein, H.G. (1987). Release of multiple hormones by a direct action of interleukin-1 on pituitary cells. *Science*. 238. 519-522.

Beutler, B., Milsark, I.W., & Cerami.A. (1985). Cachectin / Tumour Necrosis Factor: Production, distribution, and metabolic fate *in vivo*. *J. Immunol*. 135. 3972-3977.

Beutler, B., Milsark, I.W., & Cerami.A. (1985). Passive immunization against Cachectin / Tumour Necrosis Factor protects mice from the lethal effect of endotoxin. *Science*. 229. 869-871.

Beutler, B., Krochin, N., Milsark, I.W., Luedke, C., & Cerami, A. (1986). Control of cachectin (Tumour Necrosis Factor) synthesis: Mechanisms of endotoxin resistance. *Science*. 232. 977-979.

Beutler, B., & Cerami, A. (1987). Cachectin: More than a Tumour necrosis factor. *New. Eng. J. Med*. 316. 379-385.

Beutler, B., & Cerami, A. (1990). The biology of Cachectin/TNF a primary mediator of the host response. *Ann. Rev. Immunol*. 7. 625-655.

Blackwell, G.J. (1983). Specificity and inhibition of glucocorticoid-induced macrocortin secretion from rat peritoneal macrophages. *Br.J.Pharmac*. 79. 587-594.

Bloksma, N., Hofhuis, F.M.A., & Willers, J.M.N. (1982). Effects of adrenoreceptor blockade on hemorrhagic necrosis of Meth A sarcomata induced by endotoxin or tumour necrosis serum. *Immunopharmacol*. 4, 49-55.

Bloksma, N., Van de Eiel, P.A., Kuper, C.F., & Hofhuis, F.M.A. (1988). Multiple facets of induction of tumour necrosis. *Ann. Inst. Pasteur/Immunol*. 139, 294-299.

Bomfors, R. (1989) Adjuvants for anti-parasite vaccines. *Parasitol. Today*. 5. 41-46.

Bone, R.C, Fischer, C.J., Clemmer, P.J., Slotman, G.J., Metz, C.A., & Balk, R.A. (1987). A controlled clinical trial of high dose methylprednisolone in the treatment of severe sepsis. *New. Eng. J. Med.* 317, 653-658.

Brade, H., Brade, L., & Reitschel, E.T. (1988). Structure-activity relationships of bacterial lipopolysaccharides (Endotoxins) *Zbl. Bakt. Hyg.* A 268, 151-179.

Brake, D.A., Weidanz, W.P., & Long, C.A. (1986). Antigen-specific interleukin 2 propergated T lymphocytes confer resistance to a murine malarial parasite *Plasmodium chabaudi adami. J.Immunol.* 137. 347-352.

Bray, R.S., & Anderson, M.J. (1979). Falciparum malaria and pregnancy. *Trans. Roy, Soc. Trop. Med & Hyg.* 73. 427-431.

Breder, C.D., Dinarello, C.A., & Saper, C.B. (1988). Interleukin-1 immunoreactive innervation of the human Hypothalamus. *Science.* 240. 321-323.

Brockhaus, M., Schoenfeld, H-J., Schlaeger, E-J., Hunziker, W., Lesslauer, W., & Loetscher, H. (1990). Identification of tumour necrosis factor receptors on human cell lines by monoclonal antibodies. *Proc. Natl. Acad. Sci.* 87. 3127-3131.

Brooks, M.H., Malloy, J.P., & Bartelloni, P.J, Tigertt, W.D., Sheedy, T. W., & Barry, K.G. (1967). Pathophysiology of acute falciparum malaria. Correlation of clinical and biochemical abnormalities. *Am. J. Med.* 43. 735-744.

Brown, S.L., Smith, L.R., & Blalock, J.E. (1987). IL-1 and IL-2 enhance proopiomelanocortin gene expressionin pituitary cells. *J.Immunol.* 139, 3181-3185.

Bruce-Chawatt, L. (1985). Essential malariology. 2nd Edition., W.Heinmann Med. Books. Ltd., London.

Butcher, G.A., & Clark, I.A. (1990). SLE and Malaria: Another look at an old idea. *Parasitol. Today.* 6. 8. 259-261.

Camussi, G., Bussolini, F., Salvidio, G., & Baglioni, C. (1987). Tumour necrosis factor/Cachectin stimulates peritoneal macrophages, polymorponuclear neutrophils and endothelial cells to synthesize and release platelet activating factor. *J. Exp. Med.* 166. 1390.

Carlson, J., Helmby, H., Hill, A.V.S., Brewster, D., Greenwood, B.M., & Wahlgren. M. (1990). Human cerebral malaria: association with erythrocyte rosetting and lack of anti-rosetting antibodies. *Lancet.* 336. 1457-1460.

Carswell, F.A., Old, F.J., Kassel, R.L., Green, S., Fiore, N., & Williamson, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumours. *Proc. Natl. Acad. Sci.* (USA) 72, 3666-3671.

Cavallion, J., Fitting, C., Haeffner-Cavallion, N., Kirsch, S., & Shaw-Warren, H. (1990). Cytokine responses by monocytes and macrophages to free and lipoprotein bound Lipopolysaccharide. *Infect. Immun,* 58.7,2375-2382.

Chantry, D., Turner, M., Abney, E., & Feldman, M. (1989). Modulation of cytokine production by transforming growth factor–β. *J.Immunol.* 142, 4295-4300.

Cianco, S.B, Jones, S.B, Yelich, M.R., & Filkins, J.P. (1987). Glucoregulatory and sympathoadrenal responses to TNF in conciouss rats. *Fed. Proc. Fed. Am. Soc, Exp. Biol.* 46. 561.

Clark, I.A. (1974) *Babesia microti* and *Plasmodium berghei* infections in nude mice. *Nature.* 252. 328-329.

Clark, I.A., Allison, A.C., & Cox, F.E.G. (1976). Protection of mice against *Babesia* and *Plasmodium* with BCG. *Nature,* 259. 309-311.

Clark, I.A., & Coutsen, W.M. (1980). Effects of endotoxin on the histology of intact and athymic mice infected with *Plasmodium vinckei petteri. J. Pathol.* 131. 221.

Clark, I.A., Virelizier, J-L., Carswell, E.A., & Wood, P.R. (1981). Possible importance of macrophage-derived mediators in acute malaria. *Infect. Immun.* 32. 1058-1066.

Clark, I.A. (1982). Correlation between susceptibility to malaria and babesia parasites and to endotoxicity. *Trans. Roy. Soc. Trop. Med. Hyg.*,76, 4-7.

Clark, I.A. (1987). Cell mediated Immunity in protection and pathology of malaria. *Parasitol. Today.* 3. 300-305.

Clark, I.A., & Chaudhri, G. (1988). Tumour necrosis factor may contribute to the anaemia of malaria by causing dyserythropoesis and erythrophagocytosis. *Br. J. Haematol.* 70. 99-104.

Clark, I.A., & Chaudhri, G. (1988). Tumour necrosis factor in malaria induced abortion. *Am. J. trop. Med. Hyg.* 39. 246-249.

Clark, I.A., & Chaudhri, G. (1988). Relationship between Inflammation and immunopathology of malaria. 127-147. In, *Malaria: Host responses to infection. CRC press. Boca Raton. Florida.*

Cobbold, S.P, Jayasuriya, A., Nash, A., Prospero, J.D., & Waldmann, H. (1982). Therapy with monoclonal antibodies by elimination of T-cell subsets *in vivo.* *Nature.* 312. 548-550

Cohen, S., McGregor, I.A., & Carrington, S. (1961). Gamma-globulin and acquired immunity to human malaria. *Nature.* 192. 733-737.

Collart, M.A., Relin, D., Vassali, J.D., DeKassado, S., & Varsalli, P. (1986). γ-interferon enhances macrophage transcription of the tumour necrosis factor/cachectin, interleukin 1, and urokinase genes, which are controlled by short lived suppressors. *J. Exp. Med.* 164. 2113-2118.

Coppel, R.L., Cowman, A.F., & Anders, R.F. (1984). Immune sera recognize on erythrocytes a *Plasmodium falciparum* antigen composed of repeated amino acid sequences. *Nature.* 310. 789-792.

Cottrell, B.J., Playfair, J.H.L., & de Souza, B. (1977). *Plasmodium yoelii* and *Plasmodium vinckei*: The effects of non-specific immunostimulation on murine malaria. *Exp. Parasitol.* 43. 45-53.

Cox, F.E.G., & Voller, A. (1966). Cross-immunity between the malaria parasites of rodents. *Ann. Trop. Med. Parasitol.* 60. 297-303.

Cox, F.E.G. (1970). Protective immunity between malaria parasites and Piroplasms in mice. *Bull. W.H.O.* 43, 325

Cox, F.E.G. (1988). Malaria Vaccines, The shape of things to come. *Nature,* 333, 702

Cross, A.S, Sadoff, J.C, Kelly, N, Bernton, E., & Gemski, P. (1989). Pretreatment with recombinant murine tumour necrosis factor-α /Cachectin and murine Interleukin-1α protects mice from lethal bacterial infection. *J. Exp. Med.* 169. 2021-2027.

David, P.H, Hommel, M., Miller, L.H, Udeinya, I.J., & Oligino, L.D. (1983). Parasite sequestration in *Plasmodium falciparum* malaria: spleen and antibody modulation of cytoadherence of infected erythrocytes. *Proc. Natl. Acad. Sci.* (USA). 80. 5075-5079.

Day, K.P., & Marsh. K. (1991). Naturally acquired immunity to *Plamodium falciparum. Immunoparasitol. Today. (Immunology Today 12, Parasitology Today, 69)* A 68-71.

Deans, J.A., & Cohen, S. (1983). Immunology of Malaria. *Ann. Rev. Microbiol.* 37. 25-49.

Diggs, D., & Osler, A.G. (1975). Humoral immunity in rodent malaria 111 : Studies on the site of antibody action. *J. Immunol.* 114. 1243-1247.

Dijkstra, J., Mellors, J.W., & Ryan, J.L. (1989). Altered *in vivo* activity of Liposome-incorparated Lipopolysaccharide and Lipid A. *Infect. Immun.* 57, 11, 3357-3363.

Dinarello, C.A, Bernheim, H.A., Duff, G.W., Le, V., Nagabhushan, L., Hamilton, N.C., & Coceani, F. (1984) Mechanisms of fever induced by recombinant human interferon. *J. Clin. Invest.* 74. 906-912

Dinarello, C.A., Cannon, J.G., Wolff, S.M., Bernheim, H.A, Beutler, B., Cerami, A., Figari, I.S., Palladino, M.A., & O'Connor, J.V. (1986). Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin-1. *J. Exp. Med.* 163: 1433 - 1435

Dinarello, C.A, Ikejima, T., Warner, J.C, Lonnemann, G., Cannon, J.G., & Libby, P. (1987). Interleukin 1 induces Interleukin 1. *J. Immunol.* 139. 1902-1910.

Djeu, J.Y., Blanchard, K.D., Richards, A.L., & Freidman, H. (1988). Tumour Necrosis Factor induction by *Candida Albicans* from human natural killer cells and monocytes. *J.Immunol.* 141. 4047-4052.

Dockrell, H.M., Alavi, A., de Souza, J.B., Leveton, C., Taverne, J., & Playfair, J.H.L. (1986). Liver macrophage changes in murine malaria. Cells of the Hepatic Sinusoid Vol. 1. 1986, pp 337-338. Ed: A. Kirn, D.L. Knook & E. Wisse, Kuppfer Cell Foundation.

Dockrell, H.M., Alavi, A., & Playfair, J.H.L. (1986). Changes in the oxidative burst capacity during murine malaria and the effect of vaccination. *Clin. Exp. Immunol.* 66. 37-43.

Dukes, D.C., Sealy, B.J., & Forbes, J.I. (1968). Oliguric renal failure in blackwater fever. *Amer. J. Med.* 45. 899-903.

Edge, A.S.B., Faltynek, C.R., Hof, L., Reichert, L.E., & Weber, P. (1981). Deglycosylation of glycoproteins by trifluoromethansulphonic acid. *Anan. Biochem.* 118, 131-137.

Edwards, C.F., Ghiasuddin, S.M., Schepper, J.M., Yunger, L.M., & Kelley, K.W. (1988). A newly defined property of somatropin: priming of macrohages for production of superoxide anion. *Science.* 239. 769-771.

Facer, C.A. (1980). Direct antiglobulin reactions in Gambian children with *P.falciparum* malaria. 111. Expression of IgG subclasses determinants and genetic markers and association with anaemia. *Clin. Exp. Immunol.* 41. 81-90.

Fast, D.J., Schlievert, P.M., & Nelson, D.N. (1989). Toxic shock syndrome associated *staphylococcal* and *streptococcal* pyrogenic toxins are potent inducers of Tumour Necrosis Factor production. *Infect. Immun.* 57, 291-294.

Ferreira, S.H., Lorenzetti, B.B., Bristow, A.F., & Poole, S. (1988). Interleukin-1β as a potent hyperalgesic agent antagonized by a tripeptide analogue. *Nature* 334, 698-701

Ferguson, M.A.J. (1988). Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol structures. *Ann. Rev. Biochem.* 58. 285-370.

Flad, H.D., Kirchner, H., & Resch, K. (1989). Interleukin-1 and related cytokines. *Lymphokine. Res.* 8. 227-233

Foley, N., Lambert, C., McNicol, M., Johnson, N., & Rook, G.A.W. (1990). An inhibitor of Tumour Necrosis Factor in the serum of patients with sarcoidosis, tuberculosis and Crohn's disease. *Clin. exp. Immunol.* 80, 395-399.

Freeman, R.R., Trejdosiewicz, A.L., & Cross, G.A.M. (1980). Protective monoclonal antibodies recognizing stage specific merozoite antigens of a rodent malaria parasite. *Nature.* 284. 366-368.

Freeman, R.R., & Holder, A. (1983). Characteristics of the protective response of BALB/c mice immunized with a purified *Plasmodium yoelii* schizont antigen. *Clin. exp. Immunol.* 54 609-616.

Galanos., C., & Freudenberg, M. (1987) Tumour necrosis factor (TNF), a mediator of endotoxin lethality. *Immunobiology.* 175. 13.

Garnham, P.C.C. (1970). The role of the spleen in protozoal infections with special reference to splenectomy. *Acta. Tropica.* 27. 1-14.

Goldfield, A.E., & Manatis, T. (1989). Coordinate viral induction of Tumour Necrosis Factor α and Interferon β in human B cells and monocytes. *Proc. Natl. Acad. Sci.* 86, 1490-1494.

Good, M.F., Kumar, S., & Miller, L.H. (1988). The real difficulties for malaria sporozoite vaccine development: nonresponsiveness and antigenic variation.

Immunol. Today, 9, 351-355.

Good, M.F., & Miller, L.H. (1990). T-cell antigens and epitopes in malaria vaccine design. *Current Topics in Microbiol. & Immunol.* 155, 63-78.

Gordon, J.R., & Galli, S.J. (1990). Mast cells as a source of both preformed and immunologically inducible TNF-α/cachectin. *Nature,* 346. 274-276.

Girardin, E., Grau, G., Dayer, J., Lombard, P., & Lambert, P. (1988). Tumour Necrosis Factor and Interleukin-1 in the serum of children with severe Infectious Purpura. *New. Eng. J. Med* 319, 7. 397-400.

Gregoriadis, G. (1990). Immunological adjuvants: a role for liposomes. *Immunol.Today,* 11. 67-102.

Greisman, S.E., DuBuy, J.B., & Woodward, C.L. (1978). Experimental Gram-negative sepsis : Reevaluation of the ability of rough mutant antisera to protect mice. *Proc. Soc. Exp. Bio & Med.* 158, 482-490.

Grau, G.E., Fajardo, L.F., Piguet, P-F, Allet, B., Lambert, P.-H., & Vassali, P. (1987). Tumour necrosis factor (Cachectin) as an essential mediator in murine cerebral malaria. *Science.* 237. 1210

Grau. G.E, Piguet, P-F., Vassalli, P., & Lambert, P-H. (1989) Tumour necrosis factor and other cytokines in cerebral malaria: Experimental and clinical data. *Immunol. Revs.* 112. 49-70.

Grau, G.E., Taylor, T.E., Molyneux, M.E., Wirima, J.J., Vassalli, P., Hommel, M., & Lambert, P-H. (1989). Tumor necrosis factor and disease severity in children with falciparum malaria. *New Eng. J. Med.* 320: 1586-1591

Grau, G.E., Bieler, G., Pointaire, P., de Kossodo, S., Tacchini-Cotier, F., Vassalli, P., Piguet, P.F., & Lambert, P-H. (1990). Significance of cytokine production and adhesion molecules in malarial immunopathology. *Immunol Letters,* 25, 189-194

Gravely, S.M., & Krier, J.P. (1976). Adoptive transfer of immunity to *Plasmodium berghei* with T and B lymphocytes. *Infect. Immun.* 14. 184-190.

Grun, J.L., & Weidanz, W.P. (1981). Immunity to *Plasmodium chabaudi adami* in the B cell deficient mouse. *Nature*. 290. 143-145.

Haeffner-Cavallion, N., Weiss, L., Laude, M., & Kazatchkine, M.D. (1989) Interleukin 4 inhibits IL-1 secretion by human monocytes stimulated with LPS. *7th International Congress of Immunology Abstracts* W-69-30, 422.

Haranka, K., Satomi, N., Sakurai, A., & Haranka, R. (1988). Necrotizing activity of tumour necrosis factor and its mechanism. *Ann. Inst. Pasteur/Immunol.* 139, 288-294.

Harbour-McMenamin, D., Smith, E., & Blalock, J.E. (1985). Bacterial lipopolysaccharide induction of leucocyte derived corticotrophin and endorphins. *Infect. Immun.* 48. 813-817.

Harte, P.G., Rogers, N.C., & Targett, G.A.T. (1985). Monoclonal anti-gamete antibodies prevent transmission of murine malaria. *Par. Immunol.* 7. 607-615.

Hesse, D.G., Tracey, K.J., Fong, Y., Manogue, K.R., Palladino, M.A, Cerami, A., Shires, G.T., & Lowry, S.F. (1988). Cytokine appaerance in in human endotoxaemia and primate bacteraemia. *Surg. Gynecol. Obstet.* 166. 147-151.

Hogan, M.M., & Vogel, S.N. (1987). Lipid-A associated proteins provide an alternative "second signal"in the activation of recombinant Interferon-γ primed C3H/HeJ macrophages to a fully tumouricidal state. *J.Immunol.* 139, 3697-3702.

Holder, A.A., & Freeman, R.R. (1981). Immunization against blood stage rodent malaria using purified parasite antigens. *Nature*. 294. 361-364.

Hollingdale, M.R., Nardin, E.H., Tharavanij,S., Schwartz, A.L., & Nussenzweig, R.S. (1984). Inhibition of entry of *Plasmodium falciparum* and *P.vivax* sporozoites into cultured cells; an *in vitro* assay of protective antibodies. *J. Immunol.* 132. 909-913.

Hollingdale, M.R. (1991). Novel protective sporozoite and exoerythrocytic antigens. *British. Soc. Parasitol. Malaria Meeting, Abstracts.*

Holz, G.G. (1977). Lipids in the malarial parasite. *Bulletin. W.H.O.* 55. 237-248.

Hommel, M., David, P.H., & Oligino, L.D. (1983). Surface alterations of erythrocytes in *P.falciparum* malaria. *J . Exp. Med.* 157. 1137-1148.

Hotez, P.J., Le Trang, N, Fairlamb, A.H., & Cerami, A. (1984). Lipoprotein lipase suppression in 313-L1 cells by a haemo protozoan induced mediator from peritoneal exudate cells. *Para. Immunol.* 6. 203-208.

Howard, R.J. (1987). Antigenic variation and antigenic diversity in malaria. *Con. Micro. Immun.* 8, 176-218.

Hughes, M. (1987). Bone marrow in human cerebral malaria: parasite sequestration in sinusoids. *Br.J.Haematol,* 66. 295.

Hutchings, P.R., & Cooke, A. (1990). The transfer of autoimmune diabetes in NOD mice can be inhibited or accelerated by distinct cell populations present in normal splenocytes taken from young males. *J. Autoimmun.* 3. 175-180.

Issekutz, A.C & Szpejda, M. (1986). Evidence that Platelet activating factor may mediate some acute inflammatory responses. *Lab. Invest.* 54. 3. 275-280.

Jakobsen, P.H., Riley, E.M., Allen, S.J., Larsen, S.O., Bennett, Jepsen, S., & Greenwood, B.M. (1991). Differential antibody response of Gambian donors to soluble *Plasmodium falciparum* antigens. *Trans. Roy. Soc. Trop. Med. Hyg.* 85, 26-32.

Jakobsen P.H., Theander, T.G., Jensen, J.B., Mølbak, K., & Jepsen, S. (1987). Soluble *Plasmodium falciparum* antigens contain carbohydrate moieties important for immune reactivity. *J. Clin. Microbiol.* 25: 2075-2079

Jakobsen, P.H., Baek, L., & Jepsen, S. (1988). Demonstration of soluble *Plasmodium falciparum* antigens reactive with Limulus amoebocyte lysate and polymyxin B. *Parasite Immunol.* 10, 593-598.

Jakobsen, P.H., Hviid, L., Theander, T.G., Riley, E.M, Grellier, P., Bruun, L., Dalsgaard, K., Schrevel, J., & Jepsen, S. (1991). Isolation and characterisation of a soluble antigen of *P.falciparum* with pyrogenic properties. *Acta Path.*

Microbiol. Scand. 99. 21-29.

Jayawardena, A.N., Targett, G.A.T., Carter, R.L., Leuchars, E., & Davies, A.J.S. (1977). The immunological responses of CBA mice to *Plasmodium yoelii,* I. General characteristics: the effects of T cell deprivation and reconstitution with thymus graft. *Immunology.* 32. 849-861.

Jayawardena, A.N., Murphy, D.B., Janeway, C.A., & Gershon, R.K. (1982). T-cell mediated immunity in malaria. 1. The Ly phenotype of T cells mediating resistance to *Plasmodium yoelii. J.Immunol.* 129. 377-381.

Jensen, J.B., Boland, M.T., Allan, J.S., Carlin, J.M., Vande Waa, J.A., Divo, A.A., & Akood, M.A.S. (1983). Association between human serum induced crisis forms in cultured *P.falciparum* and clinical immunity to malaria in the Sudan. *Infect. Immun.,* 41, 1302-1311

Jepsen, S., & Axelsen, N.H. (1980). Antigens and antibodies in *Plasmodium falciparum* malaria studied by the immunoelectrophoretic method. *Acta Path. Microbiol. Scand. [C].* **88:** 263-270

Jepsen, S., & Andersen, B.J. (1981). Immunoadsorbent isolation of antigens from the culture medium of *in vitro* cultivated *Plasmodium falciparum, Acta. Path. Microbiol. Immunol. Scand.* **89:** 99-103.

Jones, E.Y., Stuart, D.I., & Walker., N.P.C. (1989). Structure of tumour necrosis factor. *Nature.* 338. 225-228.

Jones, K.R., Cottrell, B.J., Targett, G.A., & Playfair, J.H.L. (1989). Killing of *Plasmodium falciparum* by human monocyte-derived macrophages. *Par. Immunol.* 11. 585-592.

Jupin, C., Anderson, S.M., Damais, C., Alouf, J.E., & Parant, M. (1988). Toxic shock syndrome toxin 1 as an inducer of human tumor necrosis factors and γ–interferon. *J.Exp.Med.* **167:** 752-761.

Kato, T, Takazoe, I., & Okuda. (1990). Protection of mice against the lethal toxicity of a Lipopolysaccharide (LPS) by immunization with anti-idiotype antibody to a monoclonal antibody to lipid A from *Eikenella corrodens* LPS.

Infect. Immun. 58. 416-420.

Keiner, P.A, Marek, F, Rodgers, G, Lin, P-F, Warr, G., & Desiderio, J. (1988). Induction of tumour necrosis factor, IFN-γ, and acute lethality in mice by toxic and non-toxic forms of lipid A. *J.Immunol*. 141, 870-874.

Kelker, H.C., Oppenheim, J.D., Stone-Wolff, D., Henrikson-deStefano, D., Aggarwal, B.B., Stevenson, H.C., & Vileck, J. (1985). Characterization of human tumour necrosis factor produced by peripheral blood monocytes and its separation from lymphotoxin. *Int. J. Cancer.* 36. 69-73.

Kern. P., Hemmer, C.J., van Damme, J., Gruss, H-J., & Dietrich. M. (1989). Elevated tumor necrosis factor α and interleukin-6 serum levels as markers for complicated *Plasmodium falciparum* malaria. *Amer. J. Med.* **57**: 139-143.

Kettlehut, I.C., Fiers, W., & Goldberg, A.L. (1987). The toxic effects of Tumour Necrosis Factor *in vivo* and their prevention by cyclooxygenase inhibitors. *Proc. Natl, Acad. Sci. USA.* 84. 4273-4277.

Kettlehut, I.C., & Goldberg, A.L. (1988). Tumor Necrosis Factor can induce fever in rats without activating protein breakdown in muscle or lipolysis in adipose tissue. *J. Clin. Invest.* 81, 1384-1389

Kirkland, T.N & Ziegler, E.J. (1984). An immunoprotective monoclonal antibody to lipopolysaccharide. *J.Immunol*. 132. 2590-2593.

Klayman, D.L (1989). Weeding out malaria. *Natural History*. 10. 18-26.

Krishna, K., Cowan, G., Meade, C., Wells, R., Weatherall, D., & Robson. K. (1991). A family of cation ATPase-like molecules in *Plasmodium falciparum*. *British. Soc. Parasitol. Malaria Meeting. Abstracts.*

Kunkel, S.L, Larrick, J.W & Remick, D.G. (1986). Prostaglandin E_2 (PGE_2) and Prostacyclin (PGI_2) regulate tumour necrosis factor-α (TNF) production at the cellular and molecular level; an analysis of autocrine, paracrine and endocrine effects. *Immunobiology*. 175. 80-83.

Kwiatkowski, D., & Greenwood, B.M. (1989). Why is malaria fever periodic ? A hypothesis. *Parasitol. Today*, 5, 8, 264-266.

Kwiatkowski, D., Cannon, J.G., Manogue, K.R., Cerami, A., Dinarello, C.A., & Greenwood. B.M. (1989). Tumour necrosis factor production in *falciparum* malaria and its association with schizont rupture. *Clin. Exp. Immunol.* **77**: 361-366

Kwiatkowski, D., Hill, A.V.S., Sambou, I., Twumasi, P., Castracane, J., Manogue, K.R., Cerami, A., Brewster, D.R., & Greenwood, B.M. (1990). TNF levels in fatal and non-fatal cerebral malaria compared to uncomplicated *Plasmodium falciparum* infection *Lancet* 336. 1201-1204.

Kwiatkowski, D. (1990). Tumour Necrosis Factor, fever and fatality in *falciparum* malaria. *Immunol Letts*, 25, 213-216

Lambert, C. (1961) Notes sur le traitement de la malaria cerebrale algid de l'adulte. *Acta. Tropica.* 18. 71-77.

Landau, A., & Boulard, Y. (1978). Life cycles and morphology. In Rodent Malaria. Eds. Killick-Kendrick and Peters. Academic Press, London, 53-84.

Le, J., & Vileck, J. (1987). Tumour necrosis Factor and Interleukin 1 : Cytokines with multiple overlaping biological activities. *Lab. Invest.* 561. 234-248.

Le, J., Lin, J.X., Henrickson-DeStefano., & Viléck, J. (1986). Bacterial lipopolysaccharide-induced interferon-γ production: roles of interleukin 1 and interleukin 2. *J.Immunol.* 136. 4525-4530.

Lee, S.H., Crocker, P., & Gordon, S. (1986). Macrophage plasma membrane and secretory properties in murine malaria. Effects of *Plasmodium yoelii* blood-stage infection on macrophages in liver, spleen and blood. *J. Exp. Med.* 163. 54-74.

Lehmann, V., Freudenberg, M.A., & Galanos, C. (1987) Lethal toxicity of lipopolysaccharide and tumour necrosis factor in normal and D-galactosamine treated mice. *J. Exp. Med.* 165; 657-663.

Liew, F.Y., & Cox, F.E.G. (1991). Nonspecific defense mechanism: the role of nitric oxide. *Immunoparasitol. Today. (Immunology Today 129, Parasitology Today, 69)* A17-21.

Lelchuk, R., Dockrell, H.M., & Playfair, J.H.L. (1983). T-independant macrophage changes in murine malaria. *Clin. Exp. Immunol.* 51. 487-493.

MacPherson, G., Warrell, M.J., White, N.J., Looareesuwan, S., & Warrell, D.A. (1985). Human cerebral malaria: a quantatative ultrastructural analysis of parasitized erythrocyte sequestration. *Am. J. Pathol.* 119. 385-401.

Maegraith, B.G. (1946). Pathological processes in malaria and blackwater fever. Blackwell Scientific. Oxford.

Maegraith, B.G., & Fletcher, A. (1972). The pathogenesis of mammalian malaria. *Advances in Parasitology.* 10. 49-75.

Maegraith, B.G. (1974). Malaria. In: Medicine in the tropics.Ed. Woodruff, Churchil Livingston. Edinburgh. 27-43.

Maguire, P.A., Prudhomme, J., & Sherman, I.W. (1991). Alterations in erythrocyte membrane phospholipid organization due to the intracellular growth of the human malaria parasite *Plasmodium falciparum*. *Parasitology*. 102. 1769-186.

Maehara, N., & Ho, M. (1977). Cellular origin of interferon induced by bacterial lipopolysaccharide. *Infect. Immun.* 15. 78-85.

Mannel, D.N., Rosenstreich, D.I., & Mergenhagen, B.E. (1979). The mechanism of lipopolysaccharride-induced tumour necrosis: requirements for LPS-sensitive lymphoreticular cells. *Infect. Immun.* 24. 573-576.

Marsh, K & Howard, R.J. (1986). Antigens induced on erythrocytes by *P.falciparum*: Expression of diverse and conserved determinants. *Science.* 231. 150-153.

Marsh, K, Otoo, L & Greenwood, B.M. (1987). Absence of crisis form factor in subjects immune to *Plasmodium falciparum* in the Gambia, West Africa.

Trans. Roy. Soc. Trop. Med. Hyg. 81 514-515.

Mathison, J.C., Wolfson, E., & Ulevitch, R.J. (1988). Participation of Tumour Necrosis Factor in the mediation of Gram negative Bacterial Lipopolysaccharide- induced injury in Rabbits. *J. Clin. Invest.* 81. 1925-1937

Matthews, N., Neale, M.L., Jackson, S.K., & Stark, J.M. (1987). Tumour cell killing by tumour necrosis factor; inhibition by anaerobic conditions, free-radical scavengers and inhibitors of arachidonate metabolism. *Immunology* 62, 153-155.

Matsumura, H., & Nakano, M. (1988). Endotoxin induced interferon-γ production in culture cells derived from BCG-infected C3H/HeJ mice. *J. Immunol.* 140, 494-500.

Mendis, K.N., Naotunne, T.deS., Karunaweera, N.D., Giudice, G.D., Grau, G.E., & Carter, R. (1990). Anti-parasite effects of cytokines in malaria. *Immunol. Letts.* 25, 217-220.

Mendis, K.N., David, P.H., & Carter. R.C. (1991). Antigenic polymorphism in malaria: is it an important mechanism for immune evasion ? *Immunoparasitol. Today. (Immunology Today 129, Parasitology Today, 69)* A34-37

Mestan, J, Digel, W, Mithnacht, S, Hullen, H, Blohm, H, Moller, D, Jacobsen, H., & Kirchner, M. (1986). Antiviral effects of recombinant tumour necrosis factor *in vivo. Nature.* 323, 816-818.

Michie, H.R., Manogue, K.R., Spriggs, D.R., O'Dwyer, S., Dinarello, C.A., Cerami, A., Wolff, S.M., & Wilmore, D.W. (1988). Detection of tumour necrosis factor after endotoxin administration. *New. Eng. J. Med.* 318. 1481.

Miller, L.H, (1969). Distribution of mature trophozoites and schizonts of *Plasmodium falciparum* in the organs of *Aotus trivirgatus,* the night monkey. *American. J. Trop. Med & Hyg.* 18. 860-865.

Miller, K.L., Silverman, P.H., Kullgren, B., & Mahlmann, L.J. (1989). Tumour necrosis factor alpha and the anaemia associated with murine malaria.

Infect. Immun. 57, 1542-1546.

Mizuno, D., & Soma, G-I. (1988). Endogenous and exogenous TNF therapy (EET therapy): Conceptual and experimental grounds. *Ann. Inst. Pasteur / Immunol.* 139. 282-285.

Moll, H., Mitchell, G.F., McConville, M.J., & Handman, E. (1989). Evidence for T-cell recognition in mice of a purified Lipophosphoglycan from *Leishmania major. Infect. Immun.* 57 , 3349-3356.

Moreno, C., Taverne, J., Mehlert, A., Bate, C.A.W., Breally, R.J., Maeger, A., Rook, G.A.W., & Playfair, J.H.L. (1989). Lipoarabinomannan from Mycobacterium tuberculosis induces the production of tumour necrosis factor from human and murine macrophages. *Clin. exp. Immunol.* 76. 240-245.

Mori, L, Righi, M, de Libero, G, Donini, S.D, Sironi, M, Mantovani, A & Riccardi-castagnoli, P. (1989). Production of TNFα, IL-1 and IL-6 in microglial cell clones. *7th International Congress of Immunology Abstracts* W-92-65, 608

Morrison, D.C. & Jacobs, D.M. (1976). Binding of polymyxin B to the lipid A portion of bacterial polysaccharides. *Immunochem.* **13:** 813-818.

Murphy, M.T, Koeing, J.I., & Lipton, J.M. (1983). Changes in central concentrations of β-endorphin in fever *(Abstract no,1010) Fed. Proc.* 42.464.

Nakane, A, Minagawa, T & Kato, K. (1988). Endogenous Tumour necrosis factor (Cachectin) is essential to host resistance against *Listeria monocytogenes* infection. *Infect. Immun.* 56, 10, 2563-2569.

Nawroth, P.P., Bank, I., Handley, D., Cassimeris, J., Chess, L., & Stern, D. (1986). Tumour necrosis factor/Cachectin interacts with endothelial cell receptors to induce release if interleukin 1. *J. Exp. Med.* 163. 1363

Nedwin, G.E., Naylor, S.L., Sakaguchi, A.Y., Smith, D., Jarret-Nedwin, J., Pennica,D., Goeddel, D.V., & Gray, P. W. (1985). Human lymphotoxin and tumour necrosis factor genes: structure homology and chromosomal localization. *Nuc. Acid. Res.* 13. 17. 6361-6373.

Nedwin, G.E., Svedersky, L.P., Bringmann, L.P., Palladino, M.A., & Goeddel, D.V. (1985). Effect of interleukin-2, interferon-γ and mitogens upon the production of tumour necrosis factors α and β . *J. Immunol,* 135. 2492-2495.

Nishikawa, K., Arai, H., & Inoue, K. (1990). Scavenger receptor-mediated uptake and metabolism of lipid vesicles containing acidic phospholipids by mouse peritoneal macrophages. *J. Biol. Chem.* 265. 5226-5231.

Nussenzweig, R.S., Vanderberg, J., & Most, H. (1969). Protective Immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei.* IV. Dose response, specificity and humoral immunity. *Mil. Med.* 134 (10). 1176-1182.

Nussenzweig, R.S., Vanderberg, J.P., Most, H., & Sanabria, Y. (1972). *Plasmodium berghei*: accelerated clearance of sporozoites from blood as part of immune-mechanism in mice. *Exp. Parasitol.* 31. 81-97.

Nussenzweig, R.S., & Nussenzweig, V. (1985). Development of a sporozoite vaccine. *Parasitol. Today.* 1. 150-152.

Ohlsson, K., Bjork, P., Bergenfeldt, M., Hageman, R., & Thompson, R.C. (1990). Interleukin-1 receptor antagonist reduces mortality from endotoxin shock. *Nature.* 348. 550-552.

Okusawa, S, Yancey, K.B, Van Der Meer, W.M, Endres, S, Lonneman, G, Hefter, K, Burke, J.F, Dinarello, C.A & Gelfand, J.A. (1988). C5a stimulates secretion of Tumour Necrosis Factor from human mononuclear cells *in vitro. J.Exp.Med.* 168. 443-448.

Okusawa, S., Gelfand, J.A., Ikejima, T., Connolly, R.J., & Dinarello, C.A. (1988). Interleukin-1 induces a shock-like state in rabbits. *J. Clin. Invest.* 81, 1162-1172.

Ovadia, H., Abramsky, O., & Weidenfeld, J. (1989). Involvement of the adrenergic system in the febrile and adrenocortical responses induced by IL-1. *7th International Congress of Immunology. Abstracts.* W-92-72

Palladino, M.A., & Figari, I.S. (1988). *In vivo* anti-tumour activity of recombinant tumour necrosis factor-alpha against Meth A sarcoma requires L3T4-positive T cells. *Ann. Inst. Pasteur/Immunol.* 139. 299-301.

Patarroyo, M.E., Amador, R., Clavijo, P., Moreo, A., Guzman, F., Romero, P., Tascon, R., Franco, A., Murillo, L.A., Ponton, G., & Trujillo, G. (1988). A synthetic vaccine protects against challenge with asexual blood stages of *Plasmodium falciparum* malaria. *Nature,* 332. 158-161.

Philips, R.E., & Warrell, D.A. (1986). The pathophysiology of severe *falciparum* malaria. *Parasitol. Today.* 2. 10. 271-281.

Picot, S., Peyron, F., Vuillez, V.P., Barbe, G., Marsh, K., & Ambroise-Thomas, P. (1990). Tumor necrosis factor production by human macrophages stimulated *in vitro* by *Plasmodium falciparum. Infect. Immun.* 58: 214-216

Playfair, J.H.L., de Souza, J.B, & Cottrell, B.J. (1979). Antibody responses in mice protected against malaria by a killed vaccine: differences in effectiveness against *P.yoelii* and *P.berghei. Immunol.* 33. 507-515.

Playfair, J.H.L., Taverne, J., & Matthews, N. (1984). "What is tumour necrosis factor really for ?" *Immunol. Today,* 5, 165-166.

Playfair, J.H.L., de Souza, J.B., Freeman, R.R., & Holder, A.A. (1985). Vaccination with a purified blood stage malaria antigen in mice: correlation of protection with T cell mediated immunity. *Clin. Exp. Immunol.* 62. 19-23

Playfair, J.H.L., Dockrell, H.M., & Taverne, J. (1985). Macrophages as effector cells in immunity to malaria. *Immunology Letters,* 11, 233-237

Playfair, J.H.L., & de Souza, J.B. (1986). Vaccination of mice against malaria with soluble antigens. I The effect of detergent, route of injection and adjuvant. *Parasite Immunol.* 8: 409-414

Playfair, J.H.L., & Taverne, J. (1987). Anti-parasite effects of tumour necrosis factor *in vivo* and *in vitro.* in "Tumour Necrosis Factor and Related Cytotoxins" Ciba Foundation Symposium. vol. 131, p 192-198. Published by John Wiley & Sons.

Playfair, J.H.L., Taverne, J., Bate, C.A.W., & de Souza, J.B. (1990). The malaria vaccine: anti-parasite or anti-disease? *Immunol. Today*. 11, 25-28.

Pober, J.S., Gimbrone, M.A., Lapierre, L.A., Mendrick, D.L., Fiers, W., Rothlein, R., & Springer, T.A. (1986). Overlapping patterns of activation of human endothelial cells by interleukin-1, tumour necrosis factor and immune interferon. *J. Immunol.*, 137, 1893-1896.

Pober, J.S. (1988). TNF as an activator of vascular endothelium. *Ann. Inst. Pasteur/Immunol.* 139. 317-323.

Rangappa, R.N., Berczi, I., & Sehon, A.H. (1989). Neuro-immune host defence in endotoxic shock. *7th International Congress of Immunology Abstracts* W-92-79, 611.

Ramasamy, R., & Reese, R.T. (1986). Terminal galactose residues and the antigenicity of *Plasmodium falciparum* glycoproteins. *Mol. Biochem. Parasitol,* 19. 91-101

Reynaud, C., Dayer, J., Rochemonteix, B.G., Leung, D.J.M., & Polla, B.S. (1989). Interleukin-4 decreases superoxide, IL-1α and β, and tumour necrosis factor production by human monocytes-macrophages. *7th International Congress of Immunology Abstracts* W-69-70, 429.

Rhodes-Feuillette, A., Bellosguado, M., Fruihle, M., Ballet, J.J., Choustermann, S., Canuvet, M., & Penes, P. (1985). The interferon compartment of the immune response in human malaria. 11. Presence of serum interferon-gamma following the acute attack. *J. Interferon. Res.* 5. 169. 2113.

Riley, E.M., Andersson, G., Otoo, L.N, Jepsen, S., & Greenwood, B.M. (1988). Cellular immune responses to *Plasmodium falciparum* antigens in Gambian children during and after an acute attack of *falciparum* malaria. *Clin. Exp. Immunol.* **73:** 17-22

Riley, E.M., Andersson, G., Otoo, L.N., Jepsen, S., & Greenwood, B.M. (1988). Cell-mediated immune response to *Plasmodium falciparum* antigens in adult Gambians. *Clin. Exp. Immunol.* **71:** 377-382

Robertson, B., Dostal, D., & Daynes, R.A. (1988). Neuropeptide regulation of inflammatory and immunologic responses. *J.Immunol.* 140, 4300-4307.

Romero, P., Maryanski, J.L., Corradin, G., Nussenzweig, R.S., Nussenzweig, G., & Zavala, F. (1989). Cloned cytolytic T cells recognize an epitope in the circumsporozoite protein and protect against malaria. *Nature.* 341. 323-325.

Ross, R. (1905). Researches on malaria. *J. Roy. Arm. Corps.* IV, 450-474, 541-579, 705-740.

Ruggerio, V., Tavernier, J., Fiers, W., & Baglioni, C. (1986). Induction of the synthesis of of tumour necrosis factor receptors by interferon-γ. *J. Immunol.* 136. 2445-2450.

Santamaria, P., Gehrz, R., Bryan, M.K., & Barbosa, J.J. (1989). Involvement of class II MHC molecules in the LPS induction of IL-1/TNF secretion by human monocytes. *J.Immunol.* 143. 913-922.

Schetters, T., Curfs, J., van Zon, A., Hermsen, C., & Eling, W. (1989). Cerebral lesions in mice infected with *Plasmodium berghei* are the result of an immunopathological reaction. *Trans. Roy. Soc. Troop. Med & Hyg.* 83. 103-104.

Schmidt, L.H. (1978). *Plasmodium falciparum* and *Plasmodium vivax* infections in the owl monkey (*Aotus trivirgatus*).1. The course of untreated infections. *Am.J Trop. Med & Hyg.* 27. 671-702.

Schofield, L., Villaquiran, J., Ferriera, A., Schellekens, H., Nussenzweig, R & Nussenzweig, V. (1987). γ-Interferon, CD8+ T cells and antibodies required for immunity to malarial sporozoites. *Nature.* 330. 664-666.

Schumann, R.R., Leong, S.R., Flaggs, G.W., Gray, P. W., Wright.S.D, Mathison, J.C., Tobias, P.S. & Ulevitch, R.J. (1990). Structure and Function of Lipopolysaccharide Binding protein. *Science,* 249, 1429-1431.

Seckinger, P., Isaaz, I., & Dayer, J-M. (1988). A human inhibitor of tumour necrosis factor. *J.Exp.Med.* 167, 1511-1516.

Sedegah, M., Tosta, C.E., Henderson, D.C., & Wedderburn, N. (1982). Cross-reactivity and cross-protection in murine malaria. *Anns. Trop. Med. & Parasitol.* 76. 219-221

Shatney, C.H & Benner, C. (1985). Sequential serum complement (C3) and immunoglobulin levels in shock/trauma patients developing acute fulminant systemic sepsis. *Circ. Shock.* 16. 9-17.

Sherman, M.L., Spriggs, D.R., Arthur, K.A., Imamura, K., Frei, E.F., & Kufe, D.W. (1988). Recombinant human tumour necrosis factor administered as a five-day continuous infusion in cancer patients. *J. Clin. Oncol.* 6. 344-352.

Sherwood, J.A., Spitalnik, S.J., Aley, S.B., Quayki, I.A., & Howard, R.J. (1986). *Plasmodium falciparum* and *P.knowlesei*: initial identification and characterization of malaria synthesized glycolipids. *Exp. Parasitol.* 62. 127-141.

Shparber, M., & Nathan, C. (1986). Autocrine activation of macrophages by recombinant tumour necrosis factor but not recombinant IL-1. *Blood.* 68.86a

Sinton, J.A. (1939). A summary of our present knowledge of the mechanism of immunity in malaria. *J. Mal. Inst. India,* 2, 71-83.

Sitprija, V. (1971). Urinary excretion patterns in renal failure due to malaria. The effects of phenoxybenzamine in two cases. *Aus & NZ .J. Med.* 1. 44-48.

Skirrow, M.B., Chongsuphajaisiddhi, T., & Magraeith, B.G. (1964). The circulation in malaria. 11. Portal angiography in monkeys (*Macaca mulatta*) infected with *Plasmodium knowlesi* and in shock following manipulation of the gut. *Anns. Trop. Med & Parasitol.* 58. 502-510.

Spriggs, D.R., Sherman, M.L., Frei, E., & Kufe, D.W. (1987). Clinical studies with tumour necrosis factor, Tumour Necrosis Factor and related Cytotoxins, *Ciba Foundation Symposium No 131* John Wiley & sons, Chichester, England, 206.

Stocker, R., Hunt, N.H., Clark, I.A., & Weidemann, M.J. (1984) Production of lumonol-reactive oxygen radicals during *Plasmodium vinckei* infection. *Infect. Immunol.* 45. 708-712.

Sun, H-M., & Huseh, W. (1988). Bowel necrosis induced by tumour necrosis factor in rats is mediated by platelet activating factor. *J. Clin. Invest.* 81. 1328-1331.

Sung, S.J., Bjorndahl, J.M., Wang, C.Y., Kao, H.T., & Fu, S.M. (1988). Production of tumour necrosis factor/cachectin by human T cell lines and peripheral blood T lymphocytes stimulated by phorbol myristate acetate and anti-CD3 antibody. *J. Exp. Med.* 167. 937-953.

Suss, G., Eichmann, K., Kury, E., Linke, A., & Langhorne, J. (1988). Roles of CD4+ and CD8+ T lymphocytes in the immune response to the erythrocytic stages of *Plasmodium chabaudi*. *Infect. Immun.* 56. 3081-3088.

Talamadge, J.E., Bowersox, O., Tribble, H., Lee, S.H., Shepard, H.M., & Liggitt, D. (1987). Toxicity of tumour necrosis factor is synergistic with gamma-interferon and can be reduced with cyclooxygenase inhibitors. *Am.J. Pathol.* 128. 410-415.

Targett, G.A.T., & Sinden, R.E. (1985). Transmission blocking Vaccines. *Parasitol. Today*. 1, 6, 155 - 157.

Targett, G.A.T. (1989). Status of malaria vaccine research. *J. Roy. Soc. Med.* Supplement 17, 82, 52-56

Taverne, J., Dockrell, H.M., & Playfair, J.H.L. (1981). Endotoxin-induced serum factor kills malarial parasites *in vitro*. *Infect. Immun.*, 33, 83-89

Taverne., J, Depledge, P., & Playfair, J.H.L. (1982). Differential sensitivity *in vivo* of lethal and non-lethal malarial parasites to endotoxin-induced serum factor. *Infect. Immun.* 37, 927-934.

Taverne, J., Treagust, J.D., & Playfair, J.H.L. (1986). Macrophage cytotoxicity in lethal and non-lethal murine malaria and the effect of vaccination. *Clin. Exp. Immunol.* 66. 44-51.

Taverne, J., Rahman, D., Dockrell, H.M., Alavi, A., Leveton, C., & Playfair, J.H.L. (1987). Activation of liver macrophages in murine malaria is enhanced by vaccination. *Clin. exp. Immunol.* 70, 508-514.

Taverne, J., Tavernier, J., Friers, W., & Playfair, J.H.L. (1987). Recombinant tumour necrosis factor inhibits malarial parasites *in vivo* but not *in vitro*. *Clin. Exp. Immunol.* 67. 1-8.

Taverne. J., Bate. C.A.W., & Playfair, J.H.L. (1989). Induction of TNF *in vitro* as a model for the identification of toxic malaria antigens. *Lymphokine Res.* 8: 317-322

Taverne, J., Bate, C.A.W., Kwiatkowski, D., Jakobsen, P.H., & Playfair, J.H.L. (1990). Two soluble antigens of *Plasmodium falciparum* induce tumor necrosis factor release from macrophages. *Infect. Immun.* 58, 2923-28.

Taverne, J., Bate, C.A.W., Sarkar, D.A, Meager, A., Rook, G.A.W., & Playfair. J.H.L. (1990). Human and murine macrophages produce TNF in response to soluble antigens of *Plasmodium falciparum*. *Parasite Immunol.* 12, 33-43.

Taverne, J., Bate, C.A.W., & Playfair. J.H.L. (1990). Malaria exoantigens induce TNF, are toxic and are blocked by T-independent antibody. *Immunol. Letters* 25, 207-212.

Taylor, D.W., Bever, C.T., Rollwagen, F.M., Evans, C.B., & Asofsky, R. (1982). The rodent malaria parasite *Plasmodium yoelii* lacks both types 1 and 2 T-independant antigens. *J. Immunol.* 128. 1854-1858

Teng, N.N.H., Kaplan, H.S., Hebert, J.M., Moore, C., Douglass, H., Wunderlich, A., & Braude, A.I. (1985). Protection against Gram-negative bacteraemia and endotoxaemia with human monoclonal IgM antibodies. *Proc. Nat. Acad. Sci.* 82. 1790-1794.

Toro, G., & Roman, G. (1978). Cerebral malaria. a disseminated vasculomyelinopathy. *Archives of Neurology*. 35. 271-275.

Tracey, K.J., Beutler, B., Lowry, S.F., Merryweather, J., Wolpe, S., Milsark, I.W., Hariri, R.J., Zentella, A., Albert, J.D., Shires, G.T., & Cerami, A. (1986). Shock and tissue injury induced by recombinant human cachectin. *Science.* 234. 470

Tracey, K.J., Fong, Y., Hesse, D.G., Manogue, D.R., Lee, A.T., Kuo, G.C., Lowry, S.F., & Cerami, A. (1987). Anti-cachectin/TNF monoclonal antibodies prevent

septic shock during lethal bacteraemia. *Nature.* 330. 662-665.

Tubbs, H. (1980). Endotoxin in human and murine malaria. *Trans. Roy. Soc. Med. Hyg.*, 74, 538-545.

Turchik, J.B., & Bornstein, D.L. (1980). Role of the central nervous system in acute phase responses to endogenous pyrogen. *Infect. Immun.* 30, 439-444.

Usawattanakul, W., Tharavanji, S., Warrell, D.A., Looareesuwan, S., White, N.J., Supavej, S., & Soikratoke, S. (1985). Factors contributing to the development of cerebral malaria 11. Endotoxin. *Clin. Exp. Immunol.* 61. 562-568.

Valone, F.H., & Epstein, L.B. (1988). Biphasic platelet activating factor synthesis by human monocytes stimulated with Il-1β, tumour necrosis factor or IFN-γ. *J. Immunol.* 141. 3945-3950.

Vial, H.J., Thuet, M.J., & Philippot, J.R. (1982). Phospholipid biosynthesis in synchronous *Plasmodium falciparum* cultures. *J. Protozoology* 29, 258-263.

Vial, H.J., Thuet, M.J., Ancelin, M.J., Philippot, J.R., & Chavis, C. (1984). Phospholipid metabolism as a new target for malaria chemotherapy : mechanism of action of D-amino-1-butanol. *Biochem. Pharmacol.* 33. 2761-2770.

Vial, H.J., Thuet, M.J., & Philippot, J.R. (1984). Cholinephosphotransferase and ethanolaminephosphotransferase activities in *P.knowlesi* infected erythrocytes. Their use as parasite specific markers. *Biochem. Biophys. Acta.* 795. 372-383

Vial, H.J., Ancelin, M.L., Thuet, M.J., & Philippot, J.R. (1989). Phospholipid metabolism in *Plasmodium* infected erythrocytes: guidelines for further studies using radioactive precursor incorporation. *Parasitology*, 98,351-357.

Vileck, J., Palombella, V.J., Zhang, Y., Lin, J.X., Feinman, R., Reis, L.F.L., & Le, J. (1988). Mechanisms and significance of the mitogenic and antiviral actions of TNF. *Ann. Inst. Pasteur / Immunol.* 139 307-311.

Vogel, S.N., Kaufman, E,N., Tate, M.D., & Neta, R. (1988). Recombinant Il-1α and recombinant tumour necrosis factor α synergize *in vivo* to induce early endotoxin tolerance and associated haematopoetic changes. *Infect. Immun.* 56. 2650-2657

Vogel, S.N., & Havell, E.A. (1990). Differential inhibition of lipopolysacharride induced phenomena by anti-tumour necrosis factor alpha antibody. *Infect. Immun.* 58. 2397-2400

Waage, A., & Espevik, T. (1988). Interleukin-1 potentiates the lethal effect of tumour necrosis factor/cachectin in mice. *J.Exp.Med.* 167. 1987-1992.

Waage, A., Brandtzaig, P., Kierulf, P., & Espevik, T. (1989). The complex pattern of cytokines in serum from patients with meninogococcal septic shock. Association between interleukin 6, interleukin 1 and fatal outcome. *J. Exp. Med.* 169. 333-338.

Wahlin, B., Wahlberg, M., Perlmann, H., Berzins, K., Bjorkman, A., Patarroyo, M.E., & Perlmann, P. (1984). Human antibodies to a M_r 155,000 *Plasmodium falciparum* antigen efficiently inhibit merozoite invasion. *Proc. Natl. Acad. Sci.* 81. 7912-7916.

Wallach, D., Holtmann, H., Engelmann, H., & Nophar, Y. (1988). Sensitization and desensitization to the lethal effects of tumour necrosis factor and IL-1. *J.Immunol.* 140. 2994-2999.

Wallach, D., Holtmann, H., Aderka, D., Hahn, T., Engelmann, H., & Nophar, Y. (1988). Sensitizing effects and mechanisms of desensitization in regulation of the *in vivo* response to TNF. *Ann. Inst. Pasteur/Immunol.* 139, 323-327.

Wang, A.M., Creasy, A.A., Ladner, M.B., Lin, L.S., Strickler, J., Van Arsdell, J.N., Yamamoto, R., & Mark, D.F. (1985). Molecular cloning of the complementary DNA for human tumour necrosis factor. *Science.* 228. 149-154.

Warrell, D.A., Looareesuwan, S., & Warrell, M.J., Kasemsarn, P., Intaraprasert, R., Bunnag, D., & Harinasuta, T. (1982). Dexamethasone proves deleterious in cerebral malaria: a double blind trial in 100 comatose patients. *N. Eng. J. Med.*

306. 313-319.

Warrell, D.A., Molyneux, M.E., & Beales, P.F. (1990). Severe and complicated Malaria. *Trans. Roy. Soc. Trop. Med. 84. Supplement 2.*

Weinbaum, F.I, Evans, C.B., & Tigelaar, R.E. (1976). An *in vitro* assay for T cell immunty to malaria in mice. *J.Immunol.* 116. 1280-1283.

Welsh, N., Nilsson, T., Halberg, A., Arkhammar, P., Bergren, P-O., & Sandler, S. (1989). Human interleukin 1β stimulates islet insulin release by a mechanism not dependant on changes in phospholipase C and protein kinase C activities or Ca^{2+} handling. *Acta. Endocrinologica.* 121, 698-704.

White, N.J., Warrell, D.A., & Chantavich, P., Looareesuwan,S., Warrell, M.J.,Krishna, S., Williamson, D.H., & Turner, R.C. (1983). Severe hypoglcaemia and hyperinsulinaemia in *falciparum* malaria. *New. Eng. J. Med* 309. 61-66

White, N.J., & Warrell, D.A., & Looareesuwan, S. (1984). Cerebral blood flow and metabolism in cerebral malaria. *XI Int. Cong. Trop. Med & Malaria,* Calgray. 116 (abstract).

Wickramasinghe, S.N., Philips, R.E., Louareesuwan, S., Warrell, D.A,. & Hughes, M. (1987). The bone marrow in human cerebral malaria: parasite sequestration in sinusoids. *Br.J.Haematol,* 66. 295.

Woloski, B.M.R.N.J., Smith, E.M., Meyer, W.J., Fuller, G.M., & Blalock, J.E. (1985). Corticotrophin releasing activity of cytokines. *Science.* 230. 1035-1038.

W .H.O., (1986). Principles of malaria vaccine trials : memorandum from a W.H.O meeting. *Bull W.H.O.,* 64 , 185-204.

W .H.O., (1987). World malaria situation 1985. *World Health Stat. Q.* 40.142-170

World Health Organization-Malaria Action Programme. (1986). Severe and complicated malaria. *Trans. Roy. Soc. Trop. Med. Hyg.* 80. S1-S50.

Wozencraft, A.O., Dockrell, H.M., Taverne, J., Targett, G.A.T., & Playfair, J.H.L. (1984). Killing of human malaria parasites by macrophage secretory products. *Infect. Immun.* 43. 664-669.

Wunderlich, F., Brenner, H.H., & Helwig, M. (1988). *Plasmodium chabaudi* malaria : Immunization with surface membranes of infected erythrocytes. *Infect & Immun.* 56. 3326-3328.

Wyler, D.J., Oster, C.N., & Quinn, T.C. (1979). The role of the spleen in malarial infections. In The role of the Spleen in the Immunology of Parasite diseases. *Tropical disease Research Series 1.* UNDP/World bank/W.H.O. 183-204.

Ziegler, E.J., McCutchan, J.A., Fierer, J., Glauser, M.P., Sadoff, J.C., Douglass, H., & Braude, A.I. (1982). Treatment of Gram-negative bacteraemia and shock with human antiserum to a mutant *Esherichia coli. New. Eng. J. Med.* 307,1225-1230.

Zwaal, R.F.A., Comfurius, P., & van Deenen, L.L.M. (1977). Membrane asymmetry and blood coagulation. *Nature.* 268. 358-360.