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A thesis submitted to the Open University for the degree of Doctor of Philosophy

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Title: Analysis of DNA and recombinant viral vaccines against *P. falciparum* in malaria-naïve and malaria-exposed humans

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Abstract

The hypotheses under test were as follows. Firstly that sequential immunisation of humans with two candidate vaccines recombinant for the same malarial DNA sequence would be safe, would produce higher frequencies of antigen-specific T cells in peripheral blood and greater efficacy than repeated immunisation with one vaccine. Secondly that recombinant viral malaria immunization would be more immunogenic in malaria-exposed than malaria-naïve individuals. The third hypothesis was contingent on the conduct of a field efficacy trial; liver-stage specific T cells induced by the regimen with greatest immunogenicity would provide protection against natural infection. Three delivery systems were evaluated - a circular plasmid DNA molecule (DNA), modified vaccinia virus Ankara (MVA) and fowlpox strain 9 (FP9); each recombinant for the multiple epitope – thrombospondin related anonymous (or adhesion) protein (ME-TRAP) *P. falciparum* DNA sequence. DNA ME-TRAP and MVA ME-TRAP were safe but poorly immunogenic when given alone in both UK and Gambian adults. Two doses of 1mg DNA ME-TRAP administered intramuscularly followed by one dose of 3×10^7 plaque forming units (pfu) MVA ME-TRAP administered intradermally induced higher effector T cell frequencies as measured by *ex vivo* γ-interferon ELISPOT (enzyme-linked immunospot) assay than three doses of either alone. A second MVA ME-TRAP immunisation did not increase immunogenicity above that after a single immunisation. At these doses the DNA/MVA regimen was more immunogenic in malaria-experienced Gambian adults than in malaria-naïve British adults. Increasing the dose of DNA to 2mg and MVA to 1.5×10^8 pfu increased immunogenicity further. Two doses of FP9 ME-TRAP showed a trend towards to being less immunogenic than two doses of DNA ME-TRAP prior to a single MVA immunisation. The heterologous DNA/MVA regimen afforded protection manifested by delay in time to parasitaemia in a clinical challenge model in the UK. Therefore a randomised double-blind controlled trial was conducted in men aged 15-45 in The Gambia. This trial confirmed safety and

high immunogenicity but there was 10.3% (95%CI –22% to +34%) efficacy for the time to infection primary endpoint. Potential reasons for failure of the intervention include an unfavourable CD4+/CD8+ T cell ratio, inadequate TRAP expression in infected hepatocytes, inadequate cross-reactivity and the duration or magnitude of the peak T cell immunogenicity. (363 words)

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A CD containing clinical trial protocols and raw data is included. The raw data is provided in the form of excel spreadsheets with an explanatory text sheet as part of each file entitled "Notes".

Abbreviations

ADL – Activities of Daily Living

ALT – Alanine Transaminase

AMA1 – Apical Membrane Antigen 1

AP – Alkaline Phosphatase

APC – Antigen Presenting Cell

BC – Boiled Casein

BCG – Bacillus Calmette-Guerin

BGH- Bovine Growth Hormone

CEF – Chicken Embryo Fibroblast

cGMP – current Good Manufacturing Practice

CISM – Centro de Investigacao em Saude de Manhica

CMV – Cytomegalovirus

CO2 – Carbon Dioxide

CS(P) –Circumsporozoite (protein)

CTL – Cytotoxic T lymphocytes

D – i.m. DNA ME-TRAP immunisation

DC – Dendritic cell

DDT - Dichlorodiphenyltrichloroethane

DGP – Daniel Gordon plasma (positive control for NANP ELISA)

DMSO - Dimethylsulfoxide

DNA – Deoxyribonucleic acid

EIR – Entomological Inoculation Rate

ELISA – Enzyme-Linked Immunoadsorbent Assay

ELISPOT – Enzyme-Linked Immunospot

EPI – Expanded Programme of Immunisation

EXP-1 – Exported Protein 1

F – FP9 ME-TRAP immunization

FACS – Fluorescence Activated Cell Sorting

FBS – Foetal Bovine Serum

FMP1 – Falciparum Malaria Protein 1

FP9 – Fowlpox Strain 9

G – needleless delivery of DNA ME-TRAP (G for gene gun)

GAVI – Global Alliance for Vaccines and Immunization

GIS – Geographical Information Systems

GLURP – Glutamate Rich Protein

GDP – Gross Domestic Product

GSK – GlaxoSmithKline

HCT – Haematocrit (also known as PCV)

HIV – Human Immunodeficiency Virus

HLA – Human Leucocyte Antigen

HP-B – Heterologous Prime-Boost Immunisation

HRP – Horseradish Peroxidase

ICH-GCP – International Committee on Harmonisation Good Clinical Practice

IDT – Impfstoffwerke Dessau-Tornan

IE1 – Immediate Early 1

i.d. – intradermal

i.m. – intramuscular

KanR- Kanamycin Resistance Gene

KEMRI – Kenya Medical Research Institute

LSA-1 – Liver Stage Antigen 1

LSA-3 – Liver Stage Antigen 3

LSHTM – London School of Hygiene and Tropical Medicine

M – MVA ME-TRAP immunization

MACS – Magnetic Activated Cell Sorting

MCA – Medicines Control Agency (name since changed to MHRA, Medicines and Healthcare products Regulatory Agency)

MCV – Mean Cell Volume

ME – Multiple Epitope

MRC – Medical Research Council

MSP1,2,3 – Merozoite Surface Protein 1,2 and 3

MVA – Modified Vaccinia virus Ankara

MVDB – Malaria Vaccine Development Branch

MVI – Malaria Vaccine Initiative

NDM – Nuffield Department of Medicine

NIAID – National Institute for Allergy and Infectious Disease

NIH – National Institutes of Health

NYU – New York University

OXREC – Oxfordshire Research Ethics Committee

OXTREC – Oxfordshire Tropical Research Ethics Committee

Pb – *Plasmodium berghei*

PBMCs- Peripheral Blood Mononuclear Cells

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PCV – Packed Cell Volume (also known as haematocrit)

Pf – *Plasmodium falciparum*

pfu – plaque forming units

PLG – Poly Lactide co-Glycolide

polyA – polyAdenylation

R0 – Sigma RPMI-1640 with l-glutamine and penicillin/streptomycin

RBC – Red Blood cell Count

RN10 – R0 with 10% human AB serum.

RESA – Ring Infected Erythrocyte Surface Antigen

SCC – Scientific Coordinating Committee

SFCs – Spot Forming Cells

SSP2 – sporozoite surface protein 2 (synonym of TRAP)

TRAP – thrombospondin related adhesion (or anonymous) protein

UNDP – United Nations Development Programme

VAC – code for clinical trial conducted as part of Oxford malaria vaccine programme

VLP – Virus Like Particle

WBC – White Blood cell Count

WRAIR – Walter Reed Army Institute of Research

Chapter 1
Preface

1.1 Background to the thesis

In 1996 workers in Professor Hill's Molecular Immunology group at the John Radcliffe Hospital, Oxford had discovered that DNA immunisation followed by MVA immunisation with vaccines encoding either *P. berghei* circumsporozoite protein (CSP) or TRAP greatly increased T cell immunogenicity and efficacy in a mouse model of malaria.

In August 1999 I started working as a clinical research fellow for Professor Adrian Hill. My task was to design and conduct a series of phase I trials of DNA and recombinant viral vaccines against liver-stage *P.falciparum* malaria to evaluate the hypothesis that the animal model finding would transfer to enhanced immunogenicity and protection against *P. falciparum* in humans.

In early 2000 I wrote the application for and was awarded a Wellcome Trust Training Fellowship in Clinical Tropical Medicine for the Gambian studies in this thesis.

Through the year 2000 I conducted several small phase I studies with DNA ME-TRAP and MVA ME-TRAP in Oxford. In addition I conducted the first malaria vaccine study with experimental challenge to occur in the UK in collaboration with Imperial College, London.

By September 2000 we had found that three immunisations with 1mg i.m. DNA ME-TRAP or MVA ME-TRAP 3 x 10^7 i.d. were safe but poorly immunogenic. We were then faced with the difficult question of how to discover the most

protectively immunogenic regimen with three available routes of administration, an unknown optimum immunisation interval and dose-escalation necessary. Professor Hill and I designed studies comparing i.m. DNA and DNA administered by needleless delivery device as priming immunisations for subsequent i.d. MVA ME-TRAP boosting with varying numbers of priming and boosting immunisations and increasing dose.

From September 2000 onwards I spent some of my time at the MRC Laboratories, The Gambia. Once it became clear that we were unable to disprove the heterologous prime-boost hypothesis in terms of immunogenicity my aim was to optimise immunogenicity of a regimen with some evidence of protection in the IIa model. Between September 2000 and April 2002 I fulfilled this aim with data gathered in two phase I trials in adults and one phase I trial in children aged 1-5. The first Gambian adult phase I trial generated data consistent with the finding that immunogenicity of DNA ME-TRAP followed by MVA ME-TRAP or MVA ME-TRAP alone was higher in Gambian than British volunteers. Between June 2001 and January 2002 we designed and conducted a study to explore immunogenicity of MVA ME-TRAP alone in children. If such increased immunogenicity was seen in children then it may have been appropriate to proceed to an efficacy trial in children. Data from this phase I trial however indicated that immunogenicity in Gambian children was similar to that in British adults.

During 2001 data from phase I trials in Oxford indicated that FP9 ME-TRAP could replace DNA ME-TRAP for priming prior to MVA ME-TRAP boosting with at least equivalent IIa efficacy and that increasing the dose of DNA and MVA greatly increased immunogenicity. The UK FP9 ME-TRAP data are not part of this thesis.

Between November 2001 and June 2002 we designed and conducted a phase I trial in Gambian adults of either higher dose DNA ME-TRAP, or for the first time in The Gambia, FP9 ME-TRAP, followed by higher dose MVA ME-TRAP.

At this point I designed a field efficacy trial with a DNA ME-TRAP/ MVA ME-TRAP regimen with advice from Professor Hill, Professor Brian Greenwood of the London School of Hygiene and Tropical Medicine and several other scientists. Professor Hill wrote the grant application to the Gates' Malaria Partnership. I wrote the trial protocol and standard operating procedures and designed the case report form for the field efficacy study. I conducted the field efficacy study between June 2002 and April 2003 as a Principal Investigator with help from many scientists and staff of the MRC unit particularly Tunde Imoukhuede. Pauline Kaye was the trial data manager. Paul Milligan analysed the data from the trial. Margaret Pinder gave advice on laboratory work throughout the Gambian trials.

I completed the writing of this thesis in Farafenni, The Gambia and Bethesda, Maryland, USA.

1.2 Oxford Malaria Vaccine Trials Code Designations

Trial	Vaccine Regime	No. of Vaccinees	No. Challenged
VAC 01	DDD, GGG	12	-
VAC 02	MMM	6	-
VAC 03	DDDM, DMM, DDD, MMM	16	12
VAC 05	DDMM, GGMM	9	9
VAC 06	DDMM, MMM in The Gambia	20	-
VAC 10	DDDMM – higher dose DNA (2mg) + higher dose MVA,	17	9
VAC 11	MVA in children 1 to 5 in The Gambia	20 (+20 controls)	-
VAC 14	FFM, DDM, DDDM (all at higher doses) in The Gambia	29	-
VAC 20	Double-Blind Randomised Efficacy Trial of DDM with Rabies Controls	372 (1:1)	-

D – i.m. DNA ME-TRAP immunisation

G – needleless delivery of DNA ME-TRAP

M – MVA ME-TRAP immunization

F – FP9 ME-TRAP immunisation

1.3 Acknowledgements

I thank my parents for their belief in me and their sacrifices for me. This thesis is dedicated to them. I greatly appreciate the kind support of my wife Caroline Bradley during the writing of this thesis. I also wish to thank my brother Anand and my aunt Sothy for valuable support.

I would like to thank Adrian Hill for his supervision throughout these studies. He has provided scientific clarity and guidance and has nurtured my belief that it is possible to achieve progress towards important strategic goals by focussing on each small step. He provided a training in both the formulation of scientific method and leadership.

NAME	AFFILIATION	ROLE
Bojang, Kalifa	MRC Gambia	Advisor on VAC006, 020
Dunachie, Susie	NDM	Oxford Investigator
Everaere, Simone	NDM	VAC011 ELISPOTS
Greenwood, Brian	LSHTM	Advisor on VAC020
Imoukhuede, Tunde	MRC Gambia	Local Investigator
Keating, Sheila	NDM	VAC020 ELISPOTS
Keita, Saikou	MRC Gambia	VAC014 ELISPOTS
McAdam, Keith	MRC Gambia	Head of MRC Unit
McConkey, Sam	NDM	Oxford Investigator
Milligan, Paul	MRC Gambia	Statistician on VAC020
Peto, Tim	NDM	Second Supervisor
Pinder, Margaret	MRC Gambia	Supervisor in The Gambia
Reece, William	NDM	Oxford Immunologist
Walraven, Gijs	MRC Gambia	Head of Farafenni field station
Watkins, Kate	NDM	VAC006 ELISPOTS
Webster, Daniel	NDM	Oxford Investigator
Whittle, Hilton	MRC Gambia	Advisor on VAC011, 020

1.4 Detail of the role of each contributor to each project

I had the lead role in all projects detailed below from which data are quoted for this thesis other than 1.4.6 (VAC 10) which was co-led by Sam McConkey and Daniel Webster. Sam McConkey was co-lead for VAC 05. For all UK studies Adrian Hill performed the process of Medicines Control Agency submission (MCA). 1.4.1 to 1.4.4 were funded by a Wellcome Trust project grant to Adrian Hill. Sarah Gilbert made the initial DNA, MVA and FP9 recombinants. Joerg Schneider, Carolyn Hannan and Pilar Degano performed the pre-clinical work necessary for MCA submission. Except where stated the data presented are my analyses.

1.4.1 VAC 01 Phase I trial of DNA ME-TRAP immunisations with MVA ME-TRAP boosting of some volunteers

I conducted the trial. Adrian Hill wrote the protocol, designed the studies and obtained OXREC approval. Mark Roberts recruited the volunteers. William Reece and Philip Gothard performed the ELISPOTS.

1.4.2. VAC 02 Phase I trial of MVA ME-TRAP immunisations

I wrote the protocol, obtained OXREC approval, recruited the volunteers and conducted the study. William Reece and Philip Gothard performed the ELISPOTS.

1.4.3 VAC 03 First Phase IIa trial of DNA ME-TRAP and MVA ME-TRAP

I wrote the protocol, obtained OXREC approval and conducted the study. Kate Watkins performed the ELISPOTS. Geoff Butcher of Imperial College and Jackie Williams of Walter Reed Army Institute of Research performed the falciparum sporozoite culture in *Anopheles stephensi.*

1.4.4. VAC 05 Second Phase IIa trial of DNA ME-TRAP and MVA ME-TRAP

I wrote the protocol, obtained OXREC approval, recruited the volunteers and conducted the first part of the study. Sam McConkey co-ordinated the latter part of the study. Kate Watkins, William Reece and I performed the ELISPOTS. Both Sam McConkey and I analysed the data. Geoff Butcher of Imperial College performed the falciparum sporozoite culture.

1.4.5 VAC 06 Phase I trial of DNA ME-TRAP and MVA ME-TRAP in adults living in Bakau, The Gambia

I wrote the grant application to the Wellcome Trust which funded 1.4.5, 1.4.7, 1.4.8 and co-funded 1.4.9. I wrote the protocol, Gambian scientific committee submission and standard operating procedures, obtained OXREC and Gambian Government/ MRC Ethics Committee approval and conducted the study. I performed the ELISPOTS after cell separation. Kate Watkins performed the ELISPOTS on fresh cells. Sowsan Atabani performed the cell separations and co-staining by FACS. Carolyn Hannan performed anti-TRAP antibody ELISAs.

1.4.6 VAC 10 Phase I trial of high dose DNA ME-TRAP and MVA ME-TRAP in Uk adults

Sam McConkey wrote the protocol, obtained OXREC approval and recruited the volunteers. Sam McConkey and Dan Webster conducted the trial. Kate Watkins performed the ELISPOTS. I analysed the data

1.4.7. VAC 11 Phase I trial of MVA ME-TRAP in children aged 1-5 living in Sibanor, The Gambia

I wrote the protocol, Gambian scientific committee submission and standard operating procedures, obtained OXREC and Gambian Government/ MRC Ethics Committee approval and conducted the study. I supervised the ELISPOTS and analysed the data. Simone Everaere performed the ELISPOTS. Tunde Imoukhuede supervised the follow-ups during the study.

1.4.8 VAC 14 A Phase I trial of DNA ME-TRAP, FP9 ME-TRAP and MVA ME-TRAP in adults living in Farafenni, The Gambia

I wrote the protocol, Gambian scientific committee submission and standard operating procedures, obtained OXREC and Gambian Government/ MRC Ethics Committee approval and conducted the study. Tunde Imoukhuede was the medical officer. I supervised the ELISPOTS and analysed the data. Saikou Keita performed the ELISPOTS.

1.4.9 VAC 20 A Randomised Double-blind Efficacy Trial of DNA ME-TRAP followed by MVA ME-TRAP with Chiron Rabipur rabies vaccine as controls.

Adrian Hill wrote the grant application to Gates Malaria Partnership at LSHTM which co-funded this study. I wrote the protocol, Gambian scientific committee submission and standard operating procedures, obtained Gambian Government/ MRC Ethics Committee approval, OXTREC and LSHTM ethics committee approval, supervised Tunde Imoukhuede, Simon Correa and Fanta Njie and co-ordinated the study. Tunde Imoukhuede recruited the volunteers and supervised immunisations, post-vaccination follow-up and the surveillance period follow-ups. Pauline Kaye was the data manager and supervised data entry by 4 data entry clerks. Paul Milligan contributed to study design and analysed the efficacy data. Adrian Hill, Brian Greenwood, Gijs Walraven, Margaret Pinder and Kalifa Bojang contributed to study design. Samba Baldeh was the supervisor of the 10 field assistants and 4 study nurses. Simon Correa supervised the slide reading by 4 lab assistants. Fanta Njie supervised the haematology and biochemistry assays and performed the HIV antibody assays.

Chapter 2

Background information on the epidemiology of malaria, the development of malaria vaccines and the Gambian study settings

2.0 Mortality and morbidity of *P. falciparum* malaria

Any visitor to a pediatric ward in a malaria-endemic sub-Saharan African country will be confronted with a vivid sense of the importance of malaria as a public health problem. If the visitor attended a primary health clinic in a village they would witness the scale of the problem readily. Is it therefore necessary to attempt to accurately estimate the mortality and morbidity of malaria in sub-Saharan Africa? I believe this is important for two reasons. Firstly in a competitive climate for provision of funding for research into interventions against diseases with an impact primarily on poor countries, it is a necessary pre-condition for advocacy. Secondly, unfortunately, there are other diseases which cause more deaths than malaria in some parts of some sub-Saharan African countries. Pneumonia and gastroenteritis are examples in low malaria transmission areas(Greenwood, 1999). Therefore for public health decisions to be made about deployment of interventions where funding can only allow prioritization, estimates are needed not just at a country level but also by region. Early estimates used data from studies in a few settings and then interpolated these with population densities to give figures for all Africa. These estimates were 0.5 – 1 million per year(Sturchler, 1989).

More recently Snow et al. used a variety of methods to estimate disease burden (Snow et al., 1999). They identified 76 sources of data for annual malaria mortality in children in Africa between 1931 and 1997. In parallel they developed a fuzzy logic model of malaria transmission based on temperature and rainfall data. This model makes certain assumptions about parasite and vector biology in relation to temperature which will lead to some errors. The rainfall data as variables for calculation of vector abundance are underestimates as they do not include river/flood plain complexes as a source of breeding sites. Snow et al. constructed a GIS population density database from the most recent censuses of 4000 African administrative units. They adjusted these for a common base year to give an interpolated 1995 population density map of Africa. By combining the malaria risk map and population density map they arrived at a figure of 987,466 deaths in Africa, of whom 765, 442 would have been children under the age of 5.

These figures do not include indirect deaths due to *P. falciparum* particularly in children. Many children who are infected with, for example, a respiratory virus are also parasitaemic. It is very difficult to estimate the number of additional deaths caused by contribution of parasitaemia to other disease conditions and to undernutrition. Another example of an indirect effect is the number of new HIV infections thought to be due to malarial anaemia related blood transfusions. One estimate of this figure is 20,000 per year in Africa. Furthermore the above model does not include provision for epidemics in areas with moderate malaria transmission or for deaths of pregnant women. In addition malaria mortality in Africa is thought to be increasing for several reasons. Resistance to chloroquine is widespread and is increasing to the only other widely used affordable antimalarial, sulphadoxine/pryimethamin (Mutabingwa et al., 2001) . Pyrethroid resistance in west and southern Africa is decreasing the effectiveness of insecticide-treated bednet and residual spraying programmes(Hemingway et al., 2002). Migration is increasing in Africa, in part due to civic instability leading to movement of refugee populations, which have increased malaria disease burdens, and also because migrant labour is increasing(Martens and Hall, 2000).

Construction of small dams(Ghebreyesus et al., 1999) and other environmental changes, possibly including global warming, (Lindblade et al., 1999) have increased malaria disease burden in parts of Africa. For example the el nino rains were followed by increased malaria mortality in Africa(Brown et al., 1998). About 7,000 cases of imported malaria are reported in Europe each year and this figure is likely to increase(Muentener et al., 1999). Finally the population of endemic countries is rising, increasing the numbers of individuals at risk.

The morbidity and mortality of malaria is thought to be increasing in many areas of Asia and South America. Multiple resistance to antimalarials in *P. falciparum* is common in parts of south-east asia(White et al., 1999) and chloroquine-resistant *P. vivax* has emerged(Fryauff et al., 1998). Some areas such as parts of the former Soviet Union have seen sharp economic declines leading to collapse of public health infrastructures and the resurgence of malaria(Pitt et al., 1998).

When figures are obtained of decrease in mortality after the introduction of a successful malaria control programme, they tend to be higher than predicted by conventional models or malaria epidemiology(Snow et al., 1999). To conclude on mortality due to malaria, the consensus is that 1 million deaths a year is the minimum but that the true figure is up to 3 million or more. Thus an effective malaria vaccine might reduce overall mortality in endemic countries much more substantially than previously thought.

In much of sub-Saharan Africa young children experience several attacks of clinical malaria a year. Estimates vary from 200 – 450 million clinical attacks a year in Africa(Breman, 2001). About 1-2% go on to develop severe malaria(Greenwood and Mutabingwa, 2002). Socioeconomic, parasite and host genetic factors are all likely to be involved in susceptibility(Miller et al., 2002). Mortality from cerebral malaria remains at about 20% with 10% suffering long-term neurological deficits likely to lead to long-term education and employment

problems(Holding and Snow, 2001). Mortality from severe anaemia is increasing due to drug resistance(Breman, 2001).

Malaria in pregnant women in low transmission areas may lead to death of the mother or still-birth of the child. In stable transmission areas malaria in pregnancy is associated with heavily parasitised placentas with low birth weight babies and almost certainly, increased infant mortality(Miller et al., 2002). The direct economic costs of malaria due to loss of education and absence from work are very high, but the true cost is likely to be far higher than those estimated by direct costs alone(Sachs and Malaney, 2002). Recent research in this area has suggested that there is a very strong argument for large, sustained increases in malaria research funding on economic grounds alone(Sachs, 2002).

2.1 The development of malaria vaccines

An edited version of this review was published(Moorthy et al., 2004a).

Summary

After large reductions in malaria in the early twentieth century, its mortality has increased substantially over recent decades. Mortality now stands at 2-3 million annually. Advances in vaccine technology and immunology have transformed malaria subunit vaccination. Novel approaches, which might yield effective vaccines also for other diseases, are first being evaluated for malaria. I describe major progress in malaria vaccine development in the last 5 years and reasons for cautious optimism. I discuss what sort of vaccine might realistically be expected, and how this might be developed more rapidly. Timescales remain uncertain, but if more funding can be mobilized, a deployable effective malaria vaccine is a realistic medium to long term goal.

Introduction

In 1955 a book entitled “Man’s Mastery of Malaria”(Russell, 1955) reflected the generally held views of that time. Half a century later 2-3 million people are dying

from malaria annually(World Health Organisation, 1996), mostly children under the age of 5 in sub-Saharan Africa. In the 21st century, an era of accelerating scientific progress, an African child dies from malaria every 30 seconds(World Health Organisation, 2000). In few other diseases has scientific optimism been so misplaced. With the advent of both chloroquine resistance and DDT resistance, malaria reemerged in many parts of the world. In recent years, the burden of disease and death has increased substantially in endemic countries(Shanks et al., 2000) and transmission has spread to new areas(le Sueur et al., 1996). This resurgence (described in section 2.0) in malaria contrasts with a background of decline in all-cause mortality in children in many developing countries(Snow et al., 2001). The economic prospects for endemic countries are closely linked to the malaria disease burden, making a strong economic and political case for large increases in malaria intervention funding(Sachs, 2002).

Three key intermediate goals for malaria vaccinology are induction of strong, strain-transcending and durable immune responses; identification of protective antigens for stage-specific immunity; and successful combination of candidate immunogens. Until recently the level of malaria vaccine funding was pegged at the level of less than 50 million dollars worldwide. Current funding, mainly for human trials, has increased somewhat to 60-70 million dollars, but this is still an order of magnitude lower than that for HIV vaccine development, a disease which affects a comparable number of individuals.

In this section I start by summarising the complex life cycle of malaria and then outline the differences between immunity acquired in nature and the immunity likely to be induced by a vaccine(Beverley, 2002), discuss the relevance of the recently published *P. falciparum* genome sequence(Gardner et al., 2002) to vaccine development and highlight some new vaccine candidates that have reached the clinical evaluation stage.

The history of malaria vaccines

The history of malaria vaccine research and development has been full of salutary tales, good and bad science and disappointments. 1973 saw the first publication of human protection from malaria by vaccination(Clyde et al., 1973). This protection was produced by the bite of about a thousand mosquitoes infected with malaria parasites, which had been X-ray irradiated(Hoffman et al., 2002). This was a demonstration of the principle that it is feasible to protect by a vaccine, but is obviously unlikely to be a practical means of mass vaccination. For about 20 years, progress occurred mainly in experimental models, not in human vaccine trials(Cochrane et al., 1980, Kwiatkowski and Marsh, 1997). Much speculation and excitement was generated by the Spf66 candidate vaccine championed by Pattoroyo, despite real uncertainty about how such a construct could work. In the end, multiple phase III efficacy trials were required to demonstrate that this candidate lacked efficacy(Patarroyo et al., 1992, Alonso et al., 1994, D'Alessandro et al., 1995, Nosten et al., 1996, Acosta et al., 1999). Over the last 5 years several new vaccine approaches have reached clinical trials (Moorthy and Hill, 2002). Numerous potential future candidate vaccines now warrant pre-clinical evaluation.

The life-cycle of the P. falciparum *parasite*

An infected female anopheline mosquito requires a blood meal for oviposition. During such a meal, she will inject perhaps 5-20 sporozoites(Rosenberg et al., 1990, Ponnudurai et al., 1991), which invade hepatocytes within minutes. Sporozoites migrate through several hepatocytes before entering one to begin the liver-stage(Mota et al., 2001, Mota et al., 2002). The sporozoite and liver-stages together constitute the pre-erythrocytic stages of the life cycle. Over on average 6.5 days parasites develop within the liver into schizonts which rupture releasing 20-30,000 merozoites per original sporozoite into the hepatic venous circulation, from where they disseminate systemically. Each merozoite that is not picked up by phagocytic cells invades an erythrocyte, starting the 48 hour cycle of replication. This is followed by schizont rupture and invasion of new red blood

cells, and this is designated the blood-stage of malaria. The blood-stage culminates either in death of the human or control by the immune system. Some merozoites differentiate into male or female gametocytes which can be ingested by an anopheline mosquito. Fertilisation occurs within the mosquito midgut, leading ultimately to completion of the life cycle, with sporozoites migrating to the salivary glands, and becoming infective.

Introduction to subunit vaccination

It became clear that producing live, attenuated or killed, inactivated vaccines was not practical for many diseases. The concept of subunit vaccination is that partial or complete antigens are identified from a pathogen's proteomic complement which can induce protective immunity to the whole pathogen following immunisation. An example of an effective subunit vaccine is the hepatitis B vaccine(Crosnier et al., 1981). This vaccine, as with most other vaccines in widespread use (BCG being the notable exception), was designed to maximise antibody (humoral) immune responses. Unfortunately, different proteins vary greatly in their immunogenicity for antibody induction. Many of the vaccines described below embody attempts to induce antibodies having the correct avidity (ability to bind), specificity, biological activity and at high enough titre to result in blocking of infection. New understanding of antigen processing, adjuvants and their effects on innate immunity, genetic engineering techniques and novel delivery systems are gradually increasing such antibody immunogenicity. Duration of the induced immune responses remains a very considerable problem though. Moreover, such recombinant protein subunit vaccines are generally poor at induction of the effector T cell responses, such as CD8+ cytotoxic T lymphocytes (CTL), that are necessary for elimination of intracellular pathogens such as liver-stage malaria parasites. The newest generation of subunit vaccines are DNA(Ulmer et al., 1993) and improved recombinant viral vaccines(Li et al., 1993). In the case of malaria, DNA sequences from *falciparum* parasites have been inserted into plasmid DNA molecules (DNA vaccines(Wang et al., 1998) or various recombinant attenuated DNA viruses (recombinant viral

vaccines(Schneider et al., 1998) to generate candidate vaccines. The DNA vaccines are taken up by host cells, protein is expressed and T cell epitopes bound to HLA molecules prime naïve T cells to form memory T cell populations (Gurunathan et al., 2000). Recombinant viral vaccines work similarly but actively infect cells and express the recombinant malaria proteins before aborting infection(Miyahira et al., 1998). These vaccines provide new methods of inducing high levels of effector T cell immune responses(Paoletti, 1996). Methods for assessing T cell responses have been revolutionised by two assays – the enzyme linked immunospot (ELISPOT) assay and the tetramer assay. ELISPOT(Kabilan et al., 1990, Lalvani et al., 1997) allows highly sensitive quantitative detection of functional antigen-specific T cells. Tetramers allow detailed characterisation of antigen-specific T cells(Altman et al., 1996). These advances together with those of subunit vaccination in malaria raise the possibility of identifying antibody and T cell immune correlates of protection or, in other words, an understanding of how partially effective vaccines provide their level of protection. Such an understanding should allow tailoring of vaccine design around immune correlates of protection to systematically improve vaccine efficacy – a process dubbed iterative vaccine development.

Naturally acquired immunity & types of vaccine-induced immunity

Natural exposure to *P. falciparum* gradually elicits, in humans, relatively short-lived strain-specific malaria immunity first to severe disease and death and then to mild disease(McGregor, 1974). Repeated infections are required to maintain immunity. This is both antibody and T cell-based, although the clearest evidence for protective immunity acquired in nature is antibody-mediated immunity to blood stage malaria(Cohen et al., 1961, Marsh and Howard, 1986, Bouharoun-Tayoun et al., 1990). It is not known on which of the 5,300 antigens encoded by the *P. falciparum* parasite the key protective immune responses are focused, although some evidence exists for about 20 antigens. Immunity acquired in endemic areas is likely to be mediated by an integration of low to moderate level responses to many antigens. Immunity to one stage of the parasite is restricted only to that

stage (although sporozoite and liver-stage immunity overlap to some extent) increasing the complexity of vaccine design. However recent data from the malaria genome project call into question some of the previously held convictions about stage-specific expression of antigens(Florens et al., 2002); many antigens believed to be specific to one stage have been found at other stages of the life-cycle using proteomics techniques. Most vaccines aim to induce antibody and T cell responses to one or a few antigens but for effective vaccination these will need to be of greater magnitude, duration and strain-transcendence than naturally acquired immunity. T cell responses have been neglected to date particularly for blood stage vaccines; little is known or understood of the required T cell responses apart from the need to produce T cell help for an antibody response. An alternative ambitious approach for the longer term is to attempt to develop a vaccine using a "cocktail" of very many antigens to attempt to mimic natural immunity(Doolan and Hoffman, 2001) but this could lead to a product of considerable complexity and cost.

A note on malaria vaccine trial terminology

Clinical trials of new drug, vaccine or medical device products are divided into phase 1,2 and 3 trials. Phase 1 trials enrol small numbers of volunteers (10-60) and are conducted primarily to assess the safety and reactogenicity of malaria vaccines. Immunogenicity assessment is another important objective of phase 1 trials. Phase 2 trials in the malaria vaccine field are divided into two quite different types of trial: phase 2a and phase 2b trials. Phase 2a trials are no larger than phase 1 trials and, in addition to safety and immunogenicity objectives, a preliminary efficacy measurement is obtained. In Phase 2a trials mosquitoes infected with *P. falciparum* sporozoites are allowed to bite volunteers after completion of the immunisation course. This deliberate infection is known as "challenge"; phase 2a trials are also known as challenge trials. Clones of highly characterised *P. falciparum* (either 3D7, NF54 or 7G8) are used to infect *A. stephensi* mosquitoes for challenge. Unvaccinated volunteers serve as controls for the infectiousness of the challenge. Phase 2b trials are medium-sized

randomised controlled field efficacy trials. The objectives of phase 2b trials are safety, immunogenicity and efficacy measurement. The primary endpoint is either a natural infection or a clinical disease endpoint. Between 300 – 2000 volunteers are required depending on the primary endpoint, the transmission setting, the age group of volunteers and the precision required. Infection can be measured in either adult or paediatric populations. Clinical disease requires paediatric populations. A phase 3 trial in the malaria vaccine field is a critical pre-licensure safety and efficacy trial. Efficacy endpoints chosen for phase 3 trials will dictate the indications for which the vaccine could be marketed. If efficacy is known after such a phase 3 trial to be correlated with an immunoassay, then further work (for example on dose, schedule, age groups or combination vaccines) could be conducted with immunogenicity as the primary endpoint.

Pre-erythrocytic vaccines

The ideal vaccine for this stage would induce high levels of functional antibodies against sporozoites to prevent all parasites entering the liver-stage and potent T cell immunogenicity against the liver-stage to kill infected hepatocytes (harmless to the human host). Much of the recent expansion in clinical trials has been using this type of vaccine. The lead candidate is RTS,S, a recombinant protein vaccine developed by GlaxoSmithKline Biologicals(Stoute et al., 1997) . At the DNA level hepatitis B surface antigen was fused to a large part of the best characterised pre-erythrocytic malaria antigen, the circumsporozoite (CS) protein(Potocnjak et al., 1980, Nardin et al., 1982). When expressed in yeast this fusion product (RTS) binds hepatitis B surface antigen (S) to form RTS,S particles. When formulated with a proprietary adjuvant, AS02 – a mixture of de-acylated monophosphoryl lipid A, QS21 and an emulsion - and given intramuscularly on 2-3 occasions RTS,S vaccination induces high titre antibodies to both CS and hepatitis B and gives 41% protection of volunteers against challenge with parasites of the same strain used in the vaccine in an established sporozoite challenge model(Kester et al., 2001). In this challenge model vaccinees from industrialised countries (in America and Europe) are bitten by five mosquitoes infected with the 3D7 strain of

falciparum, known to be fully sensitive to chloroquine. Volunteers are closely monitored by malaria blood smears (or increasingly also by PCR techniques) and promptly treated once blood stages are detected by microscopy(Church et al., 1997). The landmark result on the efficacy of RTS,S/AS02 in 1996-97 at the Walter Reed Army Institute of Research (WRAIR) , followed several years of iterative development of CS-based vaccines - trials either with no challenge, or with only partial protection(Ballou et al., 1987, Herrington et al., 1987, Sherwood et al., 1991, Vreden et al., 1991, Herrington et al., 1992, Brown et al., 1994, Gonzalez et al., 1994, Hoffman et al., 1994a). Notably several different adjuvants used with the RTS,S construct were far less protective than the AS02 adjuvant. In a subsequent randomised controlled field trial of the efficacy of 3 dose RTS,S in Gambian adults vaccine efficacy was 34% (p=0.014) over the 15 week surveillance period, but with 71% efficacy over the first 9 weeks and 0% over the next 6 weeks(Bojang et al., 2001). Although the duration of protective efficacy induced was short-lived, RTS,S is the first pre-erythrocytic vaccine to show clear protection against natural *P. falciparum* infection. Further development of RTS,S has been accelerated by the provision of funding by the Malaria Vaccine Initiative, sponsoring an efficacy trial of RTS,S in children aged 1-5 in Mozambique, due to start in July 2003.

Several other pre-erythrocytic candidates have reached clinical evaluation over the last 5 years. A pre-erythrocytic vaccine candidate known as ICC-1132, developed by New York University School of Medicine investigators together with Apovia (a biotechnology company), is currently being tested in different formulations in the U.S., Germany and the U.K. ICC-1132, is a hepatitis B core particle genetically engineered to include a region of CS for high level antibody induction. Very high titres of biologically active CS antibody have been seen in pre-clinical studies(Birkett et al., 2002) and clinical trials have recently started.

The University of Oxford has focused in humans on the concept of heterologous prime-boost vaccination. Two different vaccine vectors encoding the same

antigen are given sequentially. Viral vectors can be given first (priming) or second (boosting); DNA vaccines are efficient priming vaccines but do not boost efficiently(Schneider et al., 1999). Three carriers have been clinically evaluated; DNA, MVA (modified vaccinia virus Ankara) and another attenuated poxvirus FP9 (an attenuated fowlpox virus strain once used to vaccinate chickens against fowlpox). The insert in these three vaccine components includes TRAP, a well characterised pre-erythrocytic antigen, and a string of T cell epitopes (ME for multiple epitope); these ME-TRAP vaccines are given in prime-boost sequence(Moorthy and Hill, 2002) (DNA then MVA or FP9 then MVA). This has induced T cell frequencies unprecedented in the vaccination field and partial protection in terms of delay in time to parasitaemia in artificial challenge studies. A randomised controlled efficacy trial of DNA ME-TRAP followed by MVA ME-TRAP has been completed in The Gambia with 372 adult volunteers. MVA encoding the CS protein and given before or after RTS,S is also currently in phase I and IIa studies in Oxford.

The US Navy's Naval Medical Research Centre has led an intensive effort to provide effective vaccination with DNA-based vaccines to both the liver-stage and blood stages. Various DNA vaccines each encoding a pre-erythrocytic antigen have been evaluated in Phase I studies(Doolan and Hoffman, 2001). Most recently a mixture of five pre-erythrocytic DNA vaccines were administered in phase I studies but no evidence of protection was found using sporozoite challenge. It has become clear that DNA vaccines require viral boosting for strong T cell immunogenicity in macaques(Rogers et al., 2002) as well as humans and that antibody induction in humans is generally very low after DNA vaccination(Wang et al., 1998, Wang et al., 2001), in contrast to some animal models. The findings on the immunogenicity and efficacy of protein adjuvant vaccines containing the AS02 adjuvant and the principles of prime-boost vaccination using non-replicating vectors identified in these malaria clinical studies are likely to impact on the design of vaccines for other diseases.

Both New York University (NYC) and the University of Lausanne have conducted Phase I studies, to date without challenge, of different CS-based vaccines. One NYU candidate vaccine is a multiple antigen peptide(Nardin et al., 2001, Nardin et al., 2000), a type of synthetic delivery system. It induced strong antibody responses but also generalised urticaria in two volunteers (Edelman et al., 2002). NYU also used a polyoxime construct, containing a universal T cell epitope. The University of Lausanne candidate, a long synthetic peptide in an oil-based adjuvant, induced detectable antibody, CD4+ and CD8+ T cell responses with a good safety profile(Lopez et al., 2001).

Blood stage vaccines: anti-invasion and anti-complication

There are two possible classes of blood stage vaccine: anti-invasion and anti-complication. A vaccine which could prevent invasion of red blood cells by the merozoite would prevent malaria disease. Blood-stage vaccines have been hampered by the lack of an established human challenge model, by the limitations of available animal models and by unclear immunological correlates of protection. A major impediment to vaccine development has been antigenic polymorphism of candidate vaccine antigens. Merozoite surface protein-1, MSP-1, is the best characterised antigen involved in invasion and the basis of several candidate vaccines. The existence of parallel pathways for invasion and the elegant demonstration that some antibodies to MSP-1 can block the activity of protective antibodies has alerted researchers to the difficulty of the task(Holder et al., 1999). A blood stage vaccine incorporating the antigen MSP-2 with two other blood-stage antigens reduced parasite density in vaccinees in a small efficacy study in Papua New Guinea(Genton et al., 2002). Vaccinees were protected most from infection with the vaccine strain of malaria, implying that for polymorphic antigens such as MSP2, a vaccine including just one allelic form of the antigen is not likely to be sufficient.

WRAIR, which was primarily responsible for establishing the laboratory challenge model for pre-erythrocytic vaccines, developed a recombinant viral vaccine,

NYVAC Pf-7, encoding 7 antigens from various life-cycle stages(Ockenhouse et al., 1998). This showed encouraging delays in time to parasitaemia in a sporozoite challenge study and some antibody and CTL immunogenicity, but was withdrawn by the pharmaceutical company involved. WRAIR is clinically evaluating a blood stage candidate, FMP-1, an anti-invasion vaccine based on MSP-1, and has progressed quickly to an adult phase I study in western Kenya.

Two blood stage candidates GLURP(Oeuvray et al., 2000) and MSP3(Oeuvray et al., 1994) have been evaluated clinically in Europe. A key issue for all such protein candidates is the identification of a safe, immunogenic adjuvant as the traditional adjuvant, alum, appears to be insufficiently immunogenic for many malaria proteins. In addition alum-adjuvanted vaccines induce T cell responses of a Th2 rather than Th1 type considered likely to be more protective for many antigens. Induction of biologically relevant antibodies is also a challenge and it is unclear how often this will require a native conformation of the recombinant protein.

An alternative approach to blood-stage vaccine design has been suggested by the demonstration that vaccine induced T cell responses against blood-stage antigens can be protective in animal models(Makobongo et al., 2003) and the finding that human volunteers can be protected against infection by immunization with low doses of blood-stage parasites that do not induce detectable antibodies(Pombo et al., 2002). With the development of blood stage challenge models(Cheng et al., 1997) and numerous new antigens becoming available a significant expansion in clinical blood stage candidate evaluation looks set to occur in the next few years.

Sequestration of *P. falciparum* by adherence to vascular endothelial cells in the brain, the kidneys and the placenta is an important cause of severe malaria. The PfEMP-1 antigen, known to be the main ligand for such adherence is the subject of intense research. However, its high degree of variability and high copy number

within each parasite isolate make a PfEMP-1 anti-complication vaccine highly problematic, although some scientists believe this possible based on a conserved part of the antigen. At schizont rupture inflammatory mediators are released which lead to many of the severe manifestations of disease. The *falciparum* GPI (glycosyl phosphatidyl inositol) molecule is a lead candidate for this mediator, which has been called the "malaria toxin". Immunisation with *falciparum* GPI protected mice from severe disease manifestations on malaria challenge(Schofield et al., 2002), although this finding was not reproducible by NYU investigators(Molano et al., 2000) and the pathway from this work to an effective clinical vaccine is unclear. .

Sexual stage vaccines: the altruistic vaccine

Induction of antibodies to gametocyte antigens can prevent fertilisation in the mosquito; the mosquito ingests antibodies with its blood meal which block fertilisation. As a result, efficacy evaluation of gametocyte vaccines is possible with a simple *ex vivo* assay. Mosquitoes are fed on gametocytes with or without the addition of human sera from vaccinated volunteers. The National Institute for Allergy and Infectious Disease Malaria Vaccine Development Unit plans clinical evaluation of a *falciparum* gametocyte candidate vaccine Pfs25, a recombinant protein. There is little commercial funding for sexual stage vaccine candidates as they have no market in industrialised countries. They could however contribute to malaria control particularly if linked with other interventions. A sexual stage vaccine utilizing an antigen not expressed in humans during natural infection would not select for escape mutants. Therefore combination of such a vaccine with a blood-stage or pre-erythrocytic vaccine could prevent immune selection that might otherwise occur. Sexual stage vaccination has been called "altruistic" because it would not protect vaccinees from disease but would protect communities from infection.

Vaccine development in the post-genomic era

Whole genome sequencing indicates a likely 5,300 P. falciparum antigens(Gardner et al., 2002). The genome databases can be used for identifying hundreds of candidate antigens for vaccination. Currently the number of possible antigens is not rate-limiting for malaria vaccine development. Increasing greatly the number of available antigens does not help solve some key problems in malaria vaccine development i.e. how to induce strong, durable immune responses and how to combine multiple antigens without interference or competition between them. Post-genomic antigen identification should generate a wealth of information of long-term potential value to vaccine development but solving other vaccinology problems could yield an effective vaccine sooner. Clearly, diversion of funding from clinical development of the well-characterised antigens already available would be counter-productive. A distinction can be made here with drug development where there are likely to be shorter term promising applications of the genome sequence information.

Discussion

The clinical evaluation of malaria vaccines is accelerating. In the last 5 years the number of groups evaluating candidate vaccines in the clinic has increased from 3 to 11. The number and quality of field sites with the capability to conduct field efficacy studies is increasing. Funding has increased and looks set to increase further. Progress has been facilitated by several agencies that are making important contributions. For example the Malaria Vaccine Initiative (MVI), at the Program for Appropriate Technology in Health, funded primarily by the Bill and Melinda Gates Foundation, has applied positive aspects of the corporate ethos to vaccine development undertaken by academic research groups. About nine MVI-funded candidate vaccines are in development (www.malariavaccine.org) and may reach clinical evaluation in the near future. Several other funding agencies, such as the NIH, the US Department of Defense, the Wellcome Trust, the European Commission and the European Malaria Vaccine Initiative have maintained and increased their commitment to this area. However funding

remains very inadequate for the task in hand and must increase substantially if an effective vaccine is to be developed in a time frame appropriate to the scale of global mortality and morbidity. A strong economic argument for very large increases in malaria intervention funding has recently been made(Sachs, 2002). It is vital that funding increases are allocated appropriately between vaccine and non-vaccine malaria researchers. The funding crisis could be addressed more effectively if a co-ordinated malaria advocacy community existed. Such a community is an important factor in the much more substantial funds available for HIV vaccine research. The lack of advocacy is largely due to the small number of "western" educated individuals who suffer severe malaria as a proportion of the total disease burden, although the number will probably continue to increase modestly. The challenge therefore is to form such advocacy effectively from a disparate group of researchers, non-governmental organisations and government officials. As malaria is a poor country disease, rich country markets offer only a modest incentive for development of a malaria vaccine. Therefore a significant scaling-up of the financing of malaria vaccine research can only occur through mobilisation of non-private sector funding. Company involvement in public-private partnerships is vital to success but on very different terms to those in private-sector funded initiatives.

The urgent need for an effective vaccine should be stressed strongly. Figure 2.1 shows a reasonable timeline for vaccine development in which a large efficacy trial in infants is still 8 years away. Efficacy studies will often have to progress through adults and children aged 1-5 before reaching their target age group of 4-6 months. There is a likely need for combination vaccines and therefore most likely a requirement for fusion of vaccine development efforts from two or more groups. More cooperation between vaccine groups is therefore necessary. We believe there is an ethical cost of pursuing a slow, linear approach to vaccine trials progression and propose staggered, rapid evaluation of vaccines in field settings, after data are available from the country of vaccine origin. At all stages care must be taken to ensure high quality data and the safety of study

participants is paramount: these are facilitated by clinical trial monitors and data and safety monitoring boards for each field efficacy trial. An essential component of field studies must be detailed immunology so that partial efficacy can be characterised immunologically. Although one candidate vaccine has recently moved from first use in humans to a phase I trial in developing countries in months, an additional greater challenge is speeding the progression from demonstrated efficacy to licensure. With current development paradigms a new vaccine that provided near complete efficacy against malaria infection in African adults might require another decade of field testing and development before licensure for use in young children (figure 2.1).

Cost of vaccines should be considered from the outset, before large scale efficacy trials are planned. This is complicated by the unpredictable but anticipated decrease in price of a candidate vaccine over time. With the increase in field trials there will be increasing populations of study participants who should be followed up in the long term. Funding however rarely exists for more than 1-2 years per trial: the best way to maintain long term follow up is to conduct sequential trials in the same field setting, where possible, and to include demographic surveillance infrastructures. A plan should be made in conjunction with local governments for provision of vaccine to the country or region participating in key pre-licensing field trials. Increasingly, ceasing vaccinations once such a trial is over is seen as unacceptable if the intervention has been shown conclusively to work.

Informed consent is a complex issue for the conduct of field efficacy studies. In many rural African settings, community consent is as important as individual consent. The American-European-Japanese ICH-GCP (International Committee on Harmonisation-Good Clinical Practice) guidelines are moving towards the status of law in much of the industrialised world. These were drawn up by regulatory authorities and pharmaceutical companies with little non-industrialised country contribution. The issue of informed consent is often particularly complex

for GCP application in non-industrialised countries. GCP consent forms must be very detailed in part for the legal protection of sponsors. In the rural Gambian setting, for example, the local consensus (of lay Gambian and Gambian Government ethical review board members) is that ICH-GCP compliant consent forms are not always appropriate. It is important that complex trials are clearly explained to participants and local experience is that by repeated delivery of complex messages with reinforcement throughout the study adequate understanding is possible, but this undertaking is far from trivial. In particular the concept that the vaccine under evaluation is not known to protect against malaria must be stressed throughout the consent procedure and the study.

If funding continues to increase in line with recent increases I believe that in time a malaria vaccine will be shown to demonstrate useful efficacy in infants. The next step would be the conduct of several larger trials in different epidemiological settings, perhaps including multiple interventions such as long term insecticide-treated bednets. These should be designed, with severe disease or death as an endpoint and sufficient sample size, so as to convince local policy makers and international funding organisations of the need to implement such a multiple intervention (figure 2.2). If vaccine development continues to be funded and to accelerate at current rates we can hope for a time when malaria mortality will be substantially reduced through multiple interventions including a vaccine. When an effective vaccine is developed, public sector funding needs to be provided to deliver the product to African infants. Organisations such as GAVI (Global Alliance for Vaccines and Immunisation) and the Global Fund for AIDS, Tuberculosis and Malaria could have sufficient funds for widespread vaccination in the medium term providing the cost is low (of the order of a few dollars a dose). The expanded programme of immunisation (EPI) infrastructure would need to be improved in many countries to enable adequate population coverage however.

The technology and required infrastructure now exists for development of an effective malaria vaccine; success or failure will depend on the mobilisation of financial resources and political commitment.

2.2 The Gambia

The Gambia was the site of studies which highlighted the T-cell based approach to malaria vaccine design (Hill et al., 1991, Hill et al., 1992, Aidoo et al., 1995). The MRC unit in The Gambia has long been a leader in the development of vaccines for African populations. Notable examples of vaccines which have been developed in The Gambia are the hepatitis B and haemophilus influenzae type b vaccines. Both are now part of the Gambian routine infant immunization programme largely as a by-product of their field efficacy evaluation by MRC Gambia. The MRC Laboratories possess exceptionally good laboratory and field trials infrastructure for sub-Saharan Africa. For these reasons we chose The Gambia as the site for the first African clinical trials of DNA-based malaria vaccines.

The Gambia gained independence from the UK in 1965. It is a small country surrounded on three sides by Senegal and bordering the Atlantic ocean. The GDP per capita was $430 in 1994 (World Bank 1996). The Gambia was 163 out of 174 countries in the UNDP's human development index in 1997. The economy is based on subsistence farming with maize, millet and rice as the main food crops. Groundnuts are the most important cash crop although cotton, seafood, fruits and vegetables are also exported. The tourism sector is expanding with over 100,000 tourists visiting annually (accounting for 11% of GDP).

The population of The Gambia was 1,411,205 in 2001 and is growing by approximately 3.2% each year. The population consists of 14 different ethnic groups including the mandinka, wollof, fula, jola, serahuli, serrer, tukulor, balanta, bambara, manjago and aku. These have widely differing cultures and many unrelated languages. The lingua franca is English although Arabic and French

are also spoken by some Gambian residents. In urban areas English language education predominates. In rural areas Arabic language education by Muslim scholars is more common.

Life expectancy remains at 54 (in 2001) amongst the lowest in the world. The infant mortality rate was 78 per 1000 live births in 2001. Child survival is one of the lowest in West Africa. Maternal mortality is also high and shows a clear increase in rural vs. urban populations (12 vs. 6 per 1000 live births). Annual population growth is one of the highest in the world at 3.2% in 2001 with a very high fertility rate.

The Gambia can be divided into urban and rural areas with one atypical relatively large conurbation known as the Kombos. The Kombos, of which Bakau is one part, is coastal and the population consists mostly of Gambians and West Africans who have moved in search of work. There is increasing contact with and reliance on Western tourists for revenue in this area.

Malaria is highly seasonal in The Gambia with the bulk of infections occurring during the rainy season between July and November. Malaria transmission has been estimated in coastal Gambia, including the site of the study described in Chapter 8, at <1 infectious bite per year. However most adults travel regularly to inland areas where the transmission is up to >100 infectious bites per year. In The Gambia the age-incidence of malaria mortality peaks at 2-4 (van Hensbroek et al., 1996) and is very low after the age of 10.

Farafenni was the site of studies described in Chapters 10 and 11 and is 170km inland, to the east of the Atlantic coast and part of the North Bank Division of The Gambia. The population of an area with a 5km radius of the center of Farafenni town is about 26,000. There is a district general hospital in Farafenni town. The regional government primary health care administrative unit, the divisional health team, is also based in Farafenni town. The 13 efficacy study villages comprising

the setting for the efficacy study are in 2 groups. 10 villages are east of Farafenni and three are west of Farafenni town at distances of up to 41km. The vegetation in the area surrounding Farafenni is Guinea Savannah and is similar to many areas in West Africa for example parts of Senegal, southern Mali and northern Nigeria. The rural population lives in discrete village units segregated by ethnic group. Isolated compounds outside villages are not found in the study area. The extended family returns to its ancestral village for the farming season during and after each wet season. At the beginning of the dry season men migrate in search of labour to urban areas. Rainfall is restricted to between June and November with wide year-to-year variations, for example there was a threefold variation between 1998 and 1999 from 402mm to 1320mm. No data are available for entomological inoculation rates (EIR) for Farafenni town. However a relationship for village dwellers has been documented between EIR and distance from the flood plain. EIR varies from <10 to >50 over as little as 3km from the river in this area of The Gambia (Clarke, 2001). As residents of urban Farafenni, which is over 3km north of the river, visit villages near the river often, the volunteers in VAC 14 (Chapter 10) will have been intermittently exposed to high EIR. The volunteers enrolled in the efficacy trial were residents of villages chosen for their proximity to the flood plain and so will have been subject to higher still cumulative exposure (Chapter 11).

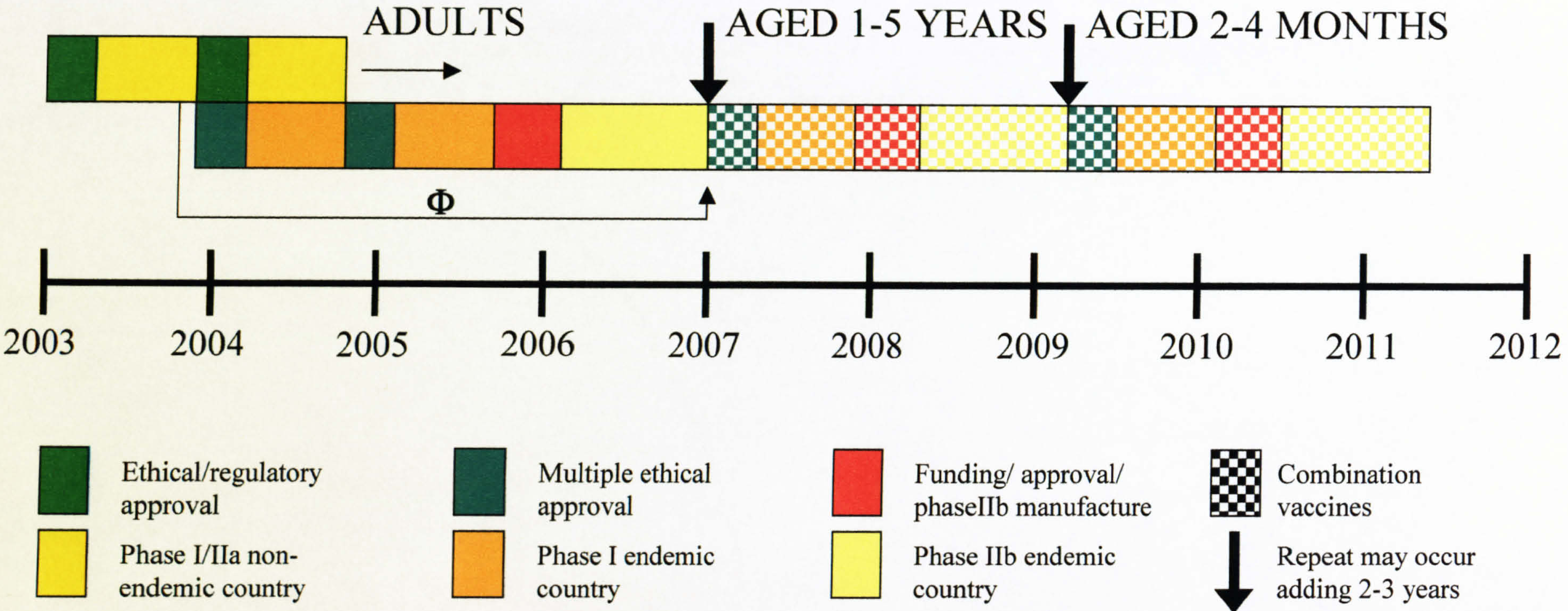
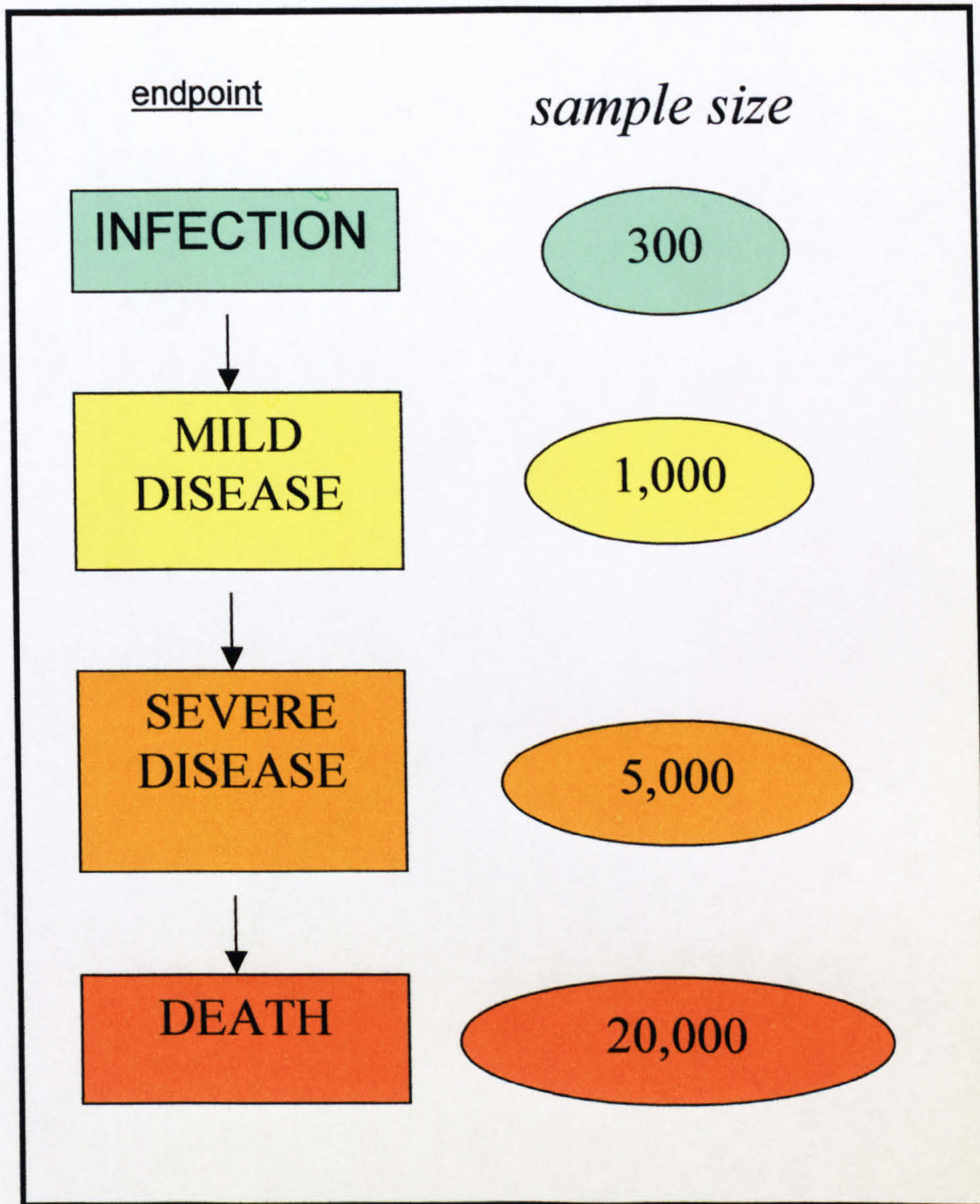


Figure 2.1:This is a simplified timeline for evaluation of a hypothetical candidate malaria vaccine. Regulatory and ethics approval must be obtained before the first clinical trial. The first trial has always occurred to date in a non-endemic country. With safety data and ethics approval from both country of origin and locally, the vaccine can start evaluation in an endemic country. Once safety, immunogenicity and perhaps artificial challenge efficacy data warrants field efficacy testing, the candidate moves to a phase IIb trial. This is a malaria-specific term indicating a small scale safety, immunogenicity and efficacy study usually involving a few hundred subjects (see figure 2.2). At some point the candidate will probably require combination with other antigens and the earlier this occurs, the earlier a final combined product can be licensed. Φ - if efficacy in the laboratory challenge model is sufficient and safety requirements allow, it may be possible to bypass adults entirely in endemic countries. The vaccine will usually be required to show safety and efficacy in children aged 1-5 before progressing to infants. Prior to efficacy studies in infants, studies would be performed ensuring that co-administration with vaccines routinely administered to infants does not cause interference of immunogenicity. After the final study in the above timeline a safe and efficacious vaccine would move to critical pre-licensing safety and efficacy evaluation involving several thousand infants. Note that this timeline assumes that the novel vaccine candidate enters the clinic in 2003; many current candidate vaccines entered trials prior to this date and could reach licensure sooner.

Figure 2.2: Endpoints in malaria vaccine field trials



An extremely simplified cartoon of sample size and endpoints in malaria vaccine trials. A trial in adults can detect 40% efficacy against infection but not disease with only 300 subjects even in moderate transmission settings. The higher the transmission intensity the smaller the necessary sample size. In children aged 1-5 about 1,000 subjects are needed to measure efficacy against mild malaria, whereas 5,000 such children would be required to measure efficacy against severe malaria and about 20,000 against death. The more clinically relevant the endpoint the larger and more complex the trial but the more likely the trial would be to change public health policy locally.

Table 2.1: Some candidate malaria vaccines in clinical trials

GROUP (FIELD COLLABORATION)	VACCINES (TYPE)	TARGET
APOVIA INC., GERMANY AND USA/ NEW YORK UNIVERSITY, USA	ICC-1132 (PROTEIN)	PRE-ERYTHROCYTIC
GLAXOSMITHKLINE BIOLOGICALS, BELGIUM WITH WRAIR, USA (MRC LABORATORIES, THE GAMBIA & CISM, MOZAMBIQUE)	RTS,S (PROTEIN)	PRE-ERYTHROCYTIC
MALARIA VACCINE DEVELOPMENT BRANCH, NATIONAL INSTITUTES OF HEALTH, USA	Pvs25 (PROTEIN)	TRANSMISSION-BLOCKING
NAVAL MEDICAL RESEARCH CENTER, USA/ VICAL INC., USA	Pf-CS, Pf-SSP2/TRAP, Pf-LSA-1, Pf-EXP-1, Pf-LSA-3 (DNA VACCINES)	PRE-ERYTHROCYTIC
NEW YORK UNIVERSITY, USA	CS (SYNTHETIC PEPTIDES, POLYOXIMES)	PRE-ERYTHROCYTIC
OXFORD UNIVERSITY, UK (MRC LABORATORIES, THE GAMBIA AND WELLCOME-KEMRI UNIT, KILIFI, KENYA)	DNA ME-TRAP, MVA ME-TRAP, FP9 ME-TRAP, MVA-CS (DNA & RECOMBINANT VIRAL)	PRE-ERYTHROCYTIC
SSI, COPENHAGEN/ INSTITUT PASTEUR, PARIS/ UNIVERSITY OF LAUSANNE, SWITZERLAND	GLURP, MSP-3, CS (SYNTHETIC PEPTIDE)	PRE-ERYTHROCYTIC, BLOOD-STAGE
WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH, MELBOURNE/ QIMR, BRISBANE/ SWISS TROPICAL INSTITUTE/ BIOTECH AUSTRALIA PTY LTD (PAPUA NEW GUINEA INSTITUTE OF MEDICAL RESEARCH)	MSP-1, MSP-2, RESA (PROTEIN)	BLOOD-STAGE
WALTER REED ARMY INSTITUTE OF RESEARCH, USA (KEMRI, KISUMU, KENYA)	FMP-1 (PROTEIN)	BLOOD-STAGE

Only candidates in clinical trials as of January 2003 are listed. Similarly field collaborations are only listed if field trials of the candidate have begun as of January 2003. (see next page for abbreviations specific to this table)

Abbreviations for table 2.1

SSI – Statens Serum Institut, GLURP – Glutamate Rich Protein, MSP – Merozoite Surface Protein, Pvs - Plasmodium vivax surface protein, CISM – Centro de Investigacao em Saude de Manhica, RESA – Ring Infected Erythrocyte Surface Antigen, CS – Circumsporozoite Protein, Pf- Plasmodium falciparum, SSP2 & TRAP are synonyms – sporozoite surface protein 2 & thrombospondin-related adhesion protein, LSA - liver stage antigen, EXP – exported protein, KEMRI – Kenya Medical Research Institute, ME – multiple epitope, FMP – falciparum malaria protein.

Chapter 3

Background information on the pre-clinical and clinical development of DNA and recombinant viral vaccines

3.0 Rationale for T-cell induction based vaccine design in malaria.

Most licensed vaccines in widespread use are thought to mediate protection by induction of long-lived antibody responses. In contrast development of vaccines that induce long-lived protective cellular immune responses has proved difficult. Three of the most important diseases for developing countries from a public health perspective, HIV, malaria and tuberculosis, require induction of cellular immune responses for protection against intracellular pathogens. Induction of CD8+ T cells has been thought to be particularly desirable for elimination of intracellular pathogens and cancer immunotherapy(Gurunathan et al., 2000). A large proportion of host cell types express HLA class I molecules and are thus amenable to surveillance by CD8+ T cells. Antigen-specific CD8+ T cells are able to secrete a variety of effector molecules which directly destroy the pesenting cell. Some effector cells release perforin which destroys the infected cell by lysis. Others induce apoptosis through the action of granzymes, fas ligand or γ-interferon, the last of which induces nitric oxide mediated apoptosis.

HLA class II molecules, which are necessary for interaction with antigen-specific CD4+ T cells, are expressed only by a small subset of cell types. In the case of malaria vaccination, infected hepatocytes are likely to have higher HLA class I expression than class II expression. Even with good expression of the relevant HLA molecules there are other prerequisites for translation of T-cell

immunogenicity to efficacy. The malaria epitopes to which vaccine-induced memory T cells are specific must bind to HLA molecules intracellularly and then be expressed on the cell surface in association with the HLA molecule. If any one of expression, proteolytic processing or HLA presentation is inadequate, inhibition of liver-stage parasites will not occur. Experiments assessing processing and presentation of malaria epitopes during liver-stage malaria in human hepatocytes *in vivo* are not possible currently. There is evidence that human T cells specific for liver-stage malaria epitopes are primed *in vivo*. Such T cells with a γ-interferon effector function have been detected in the peripheral blood of humans living in malaria-endemic countries, but at very low frequency(Plebanski et al., 1997, Aidoo et al., 1995). However this may occur by antigen transfer to professional antigen-presenting cells and there is no direct evidence that such T cells have a protective effect against infected hepatocytes. There are conflicting data from longitudinal studies in endemic countries on the protective role of antibody responses to pre-erythrocytic antigens(Hoffman et al., 1987). Limited data are available to support a protective role for T cell responses to pre-erythrocytic stages in naturally acquired immunity (Reece et al., submitted) (Kurtis et al., 1999), although this could be due to the use of inadequate T cell assays or a current lack of knowledge of the critical antigens for T-cell mediated pre-erythrocytic immunity. T cell migration may play a key role in protective immunity(Rodrigues et al., 1992, Renggli et al., 1995).

There are however several persuasive types of indirect evidence for a protective role for T-cells specific for pre-erythrocytic *P. falciparum* antigens in humans. The first and, in my opinion most compelling evidence, stems from the irradiated sporozoite immunization model. Exposure of humans to the bites of *P. falciparum* infected X-ray irradiated mosquitoes allows inoculation of attenuated sporozoites. In the early 1970s it was discovered that exposure to >1000 such bites, in batches over several weeks to months, could afford sterile immunity to challenge with infectious sporozoites(Clyde et al., 1973). The dose of radiation must be such that attenuated sporozoites are able to invade hepatocytes but not complete

the liver-stage. They persist within hepatocytes for months in mouse models(Scheller and Azad, 1995). Increasing the dose of attenuation to a level at which sporozoites are rendered unable to invade hepatocytes abrogates protection. In addition chemotherapy that clears intra-hepatic parasites also abrogates protection. There is thus a strong implication that protection is cell-mediated and against infected hepatocytes in this model. Protection is species-specific but not strain-specific and is of up to 10 months duration(Hoffman et al., 2002). Protected volunteers possess circulating CD8+ and CD4+ T cells specific for liver-stage epitopes(Malik et al., 1991, Moreno et al., 1991, Wizel et al., 1995). There is no immunodominance in protected humans and the consensus is that immunity is conferred by an integration of low to moderate level T-cell responses to many antigens expressed in the liver-stage, although data on characterization of immune responses in protected individuals are limited.

A second line of evidence is the finding that humans who possess the B53 allele of the HLA-B gene are protected from severe malaria in The Gambia(Hill et al., 1991). Further work showed that such individuals have a specific cell-mediated immune response to liver-stage malaria. CD8+ T cells in their peripheral blood are able to recognise a short peptide sequence from LSA-1, a pre-erythrocytic antigen(Hill et al., 1992). This finding would be strengthened by confirmation by other studies.

Thirdly, some *P. falciparum* CD8+ epitopes are in highly polymorphic regions in which all nucleotide substitutions encode amino acid changes. The observation that no synonymous changes occur argues strongly that these regions are subject to selection pressure from T cells(Good et al., 1988) and the identification of altered peptide ligand antagonism of CTL as an immune escape mechanism supports this view(Gilbert et al., 1998). The evidence for T cell selection pressure opens up an uncertainty about whether candidates for T cell based vaccination should be from conserved regions of antigens or from those regions with demonstrated T cell selection pressure. DNA-based vaccines including

conserved regions would be less likely to be susceptible to T-cell escape but may be less likely to induce protective responses. These regions may be conserved because they are not processed or presented during natural infection. However T-cell escape is likely to occur if regions subject toT-cell selection pressure are used.

The direct evidence for a protective role of T cells is from animal models. Early evidence that CTL induction might be relevent to protection was provided by analysis of the mechanisms of protection in a rodent model of irradiated sporozoite immunisation(Schofield et al., 1987). CTL clones induced by this means were found to transfer protective immunity to *Plasmodium berghei* sporozoite challenge in mice(Romero et al., 1989). CTL recognition of circumsporozoite protein-derived peptide on infected hepatocytes led to lysis of the infected cell and parasite death(Weiss et al., 1990).

3.1 DNA vaccines.

A major challenge in vaccine design has been the induction of CD8+ T cells. In the case of malaria cellular immune responses mediated by γ-interferon are critical for protection against the liver-stage. In recent years the class of vaccines known as DNA vaccines has been highlighted as being of particular promise for induction of strong, durable cellular immune responses.

A standard DNA vaccine consists of a plasmid DNA backbone into which gene(s) of interest are cloned. A promoter suitable for good mammalian expression such as cytomegalovirus IE1 (immediate early 1) is necessary. Polyadenylation termination sequences are important for mRNA transcript stability. A prokaryotic origin of replication allows propagation in for example *E. Coli.* It has been the norm to include an antibiotic resistance marker gene. A study in 1990 showed that direct intramuscular injection of plasmid DNA could induce protein expression in myocytes(Wolff et al., 1990). In 1993 protective CTL mediated immunity by DNA vaccination in mouse models was demonstrated(Ulmer et al.,

1993). In 1994 this was demonstrated in a mouse model of malaria, confirming that in mice, the principle also worked for a complex parasitic pathogen(Hoffman et al., 1994b).

CpG motifs have an important role in the immunogenicity of DNA vaccines(Klinman et al., 1999). These motifs are made up of cytosine-phosphate-guanosine repeats with flanking regions. These sequences are optimally unmethylated and 20 fold more common in prokaryotic than mammalian DNA(Sato et al., 1996). They induce γ-interferon and IL-12 *in vitro* and thus may be important in the apparent Th1 bias of most DNA vaccines(Klinman et al., 1996). DNA vaccination facilitates *de novo* synthesis of recombinant protein within cells for optimal antigen-presentation and priming of naïve CD8+ T cells. Intradermal DNA vaccination by gene gun is known to transfect a proportion of the class of highly efficient antigen-presenting cells known as dendritic cells (DCs) (Corr et al., 1996). Transfected DCs prime naïve CD8+ T cells directly. However after intramuscular DNA vaccination antigen transfer from transfected myocytes to DCs may be necessary for CD8+ T cell priming because significant DC transfection may not occur (Ulmer et al., 1996) (Fu et al., 1997). This is known as cross-priming.

A study published in 1998 demonstrated for the first time induction of CTL responses in humans after DNA vaccination(Wang et al., 1998). The DNA vaccine was recombinant for *Plasmodium falciparum* CS and was given intramuscularly three times at doses up to 2.5 mg with CTL responses measured after the third dose. Subsequently there have been clinical trials of malaria DNA vaccines for CS and 4 other pre-erythrocytic vaccines with CD8+ T cell responses obtainable after *in vitro* expansion but low magnitude responses by γ-interferon ELISPOT, no antibody induction and no protection in the IIa model(Wang et al., 2001) (Richie et al., 2001). Development of detectable antibody titres has occurred in some individuals after challenge although this finding is of questionable significance. Similar poor immunogenicity has been

seen in HIV clinical DNA vaccine trials(MacGregor et al., 1998) (Boyer et al., 2000) (MacGregor et al., 2002). Antibody induction through clinical DNA vaccination has been achieved with a Hepatitis B DNA vaccine administered by a needleless delivery device (also known as a gene gun) (Roy et al., 2000). It appears that Hepatitis B is an unusual immunogen. This result is therefore probably an exception. Most data indicate that antibody induction after DNA vaccination in man is very poor in contrast to some non-human primate models.

3.2. Recombinant viral vaccines.

The successful worldwide eradication of smallpox via vaccination with live vaccinia virus highlighted vaccinia as a candidate for recombinant use(Moss, 1996). The prospect of recombinant viral vaccines was heralded by studies in 1982 which described the genetic engineering of vaccinia virus to express foreign genes(Mackett et al., 1982) (Panicali and Paoletti, 1982). An early example of a successful veterinary recombinant viral vaccine is a recombinant vaccinia expressing a rabies virus antigen. This vaccine is licensed and has been effective by the oral route for control of rabies in foxes in Europe and raccoons in the USA(Kieny et al., 1984). Double stranded DNA viruses such as vaccinia are the most commonly used class of virus as candidate vectors, although there are methods for insertion of cDNA-derived RNA into some RNA viruses(Li et al., 1993). However there are many viruses for which stable expression of inserted foreign genes has not yet proved possible. The greater part of the clinical experience with recombinant viral vaccines has been with recombinant poxviruses(Paoletti, 1996).

Although millions of humans have been vaccinated with conventional live vaccinia virus with no ill effect, its small but definite risk to both researchers and future patients led to the development of several highly attenuated replication-deficient strains of vaccinia during smallpox eradication(Fenner et al.) and more recently(Tartaglia et al., 1992, Paoletti et al., 1994, Paoletti, 1996, Kaplan, 1989).

One such strain, NYVAC, was developed as the basis for the first malaria recombinant viral vaccine NYVAC-Pf7 by Tine et al. (see section 2.1). As the immunogenicity of NYVAC and modified vaccinia virus Ankara appeared comparable in pre-clinical studies, this suggested that MVA recombinants could also be of some value as malaria vaccines, as well as being potentially more effective in heterologous prime-boost immunisation regimes(Schneider et al., 1998).

3.3 Modified vaccinia virus Ankara.

Attenuated vaccinia virus strains were developed during the smallpox vaccination era to reduce possible side effects associated with using live vaccinia virus vaccines. In particular the host-range restricted modified vaccinia virus Ankara (MVA) proved to be extremely attenuated compared to wild-type(Mayr, 1976, Mayr et al., 1978, Werner et al., 1980). MVA was originally derived from the vaccinia strain Ankara by over 570 serial passages in primary chicken embryo fibroblasts. MVA has six major fully characterized genomic deletions compared to the parental genome severely compromising its ability to replicate in mammalian cells(Meyer et al., 1991). Deletions include host range genes and genes encoding cytokine receptors. Viral replication is blocked late during infection of cells but importantly viral and recombinant protein synthesis is unimpaired even during this abortive infection(Sutter and Moss, 1992), making MVA an efficient single round expression vector incapable of causing disseminated infection in mammals.The entire DNA sequence of MVA has been published(Antoine et al., 1998). In the final stages of the smallpox eradication programme MVA was administered to over 120,000 individuals. No significant side-effects were recorded despite the deliberate vaccination of high risk groups(Stickl et al., 1974, Mahnel and Mayr, 1994). This safety in man is consistent with the avirulence of MVA in animal models(Mayr et al., 1978). Replication-deficient recombinant MVA has been seen as an exceptionally safe viral vector(Sutter and Moss, 1992). When tested in animal model studies recombinant MVAs have been shown to be avirulent, yet protectively immunogenic as vaccines against viral diseases and

cancer(Sutter et al., 1994, Hirsch et al., 1996, Wyatt et al., 1996, Carroll et al., 1997, Hanke et al., 1999).

3.4. Fowlpox strain 9.

Some avipoxviruses have been characterised as candidate vectors, in particular the ALVAC strain of canarypox(Fries et al., 1996). Extensive studies of ALVAC have been undertaken for HIV(Clements-Mann et al., 1998). Fowlpox is an avipoxvirus which causes disease in chickens but not mammals. Recombinant attenuated fowlpox has been used as a rabies vaccine in mammals, in which it shows good recombinant protein expression(Taylor et al., 1988). Fowlpox strain 9 (FP9) was derived from wild type fowlpox virus by 400 passages in tissue culture, leading to marked attenuation and loss of pathogenicity in chickens by all routes of administration to the extent of avirulence in one-day old chicks(Mayr and Malicki, 1966). Recombinant FP9 has been used as a vaccine in chickens (Mayr et al., 1971). This attenuated virus, then called PIND-AVI, was used in clinical studies in a variety of formulations and with various routes of administration in several hundred people. These studies are reported in the German literature and it appears that no serious adverse events occurred. In the UK a clone of this vector has been developed and characterised in detail as a candidate vector by researchers at the Institute of Animal Health in Compton where it was named FP9(Mockett et al., 1992). Like all avipoxviruses, FP9 does not replicate in mammalian cell lines in vitro. Attenuated strains of fowlpox virus, such as TROVAC have been used safely as recombinant viral vaccines in many human clinical trials and have been demonstrated to be non-virulent in a variety of immunosuppressed animals and human volunteers. The extensive clinical experience with ALVAC as a vector is relevant as this is a closely related avipoxvirus. FP9 ME-TRAP is the first recombinant FP9 to have been used in clinical trials to our knowledge.

3.5. Enhanced immunogenicity and efficacy by immunisation with a combination of delivery systems

In order to induce immune responses against an antigen a candidate vaccine must present the immune system with foreign antigen, usually in the form of protein sequences or alternatively as DNA-based vaccines, two classes of which have been described above – DNA vaccines and recombinant viral vaccines. (Lipids and carbohydrates have also been components of some non-malaria vaccines, but will not be discussed here.) Protection obtained with DNA vaccines and recombinant viral vaccines in animal models of malaria tended to be unsatisfactory. A minority of animals were protected against low dose sporozoite challenge. Immunisation with an influenza virus recombinant for a single *P. yoelii* CS CD8+ T cell epitope followed 3 weeks later by a vaccinia recombinant for whole *P. yoelii* CS induced complete protection of 9/15 mice against high dose sporozoite challenge(Li et al., 1993). The vaccination approach of using two delivery systems encoding the same or related constructs has been called heterologous prime-boost immunisation.

The first delivery system in the combination is known as the prime and the second is known as the boost. DNA vaccines can prime efficiently but cannot boost. Poxviruses can boost efficiently. The greater the attenuation of vaccinia virus from replication-competent to NYVAC or MVA, the greater the immunogenicity of boosting(Schneider et al., 1998). FP9 and ALVAC boost efficiently. Both FP9 and MVA have also been shown to prime for heterologous boosting(Anderson et al., 2004). Recombinant protein subunit vaccines have also been used as part of prime-boost strategies, clinically in combination with ALVAC-HIV recombinants(Clements-Mann et al., 1998) and malaria DNA vaccines (Wang et al., 2004) or malaria MVA vaccines (S Dunachie et al., unpublished data).

The experiments in our laboratory which provided the impetus for the clinical trials which form the basis of this thesis were as follows. In a series of

immunisation studies in mice various delivery systems (recombinant particles, peptides, plasmid DNA, numerous adjuvants and recombinant vectors such as recombinant BCG, Salmonella, adenovirus, MVA and FP9), encoding malaria epitopes and antigens, were compared. Most of these approaches induced only modest levels of T lymphocyte response. A priming immunisation with plasmid DNA encoding an entire murine malaria pre-erythrocytic antigen followed by a booster immunisation with MVA vector carrying the same antigen (either CS or TRAP) induced complete protection in strains of mice highly susceptible to sporozoite challenge in some experiments(Schneider et al., 1998). By 1998 the DNA/MVA heterologous prime boost combination had repeatedly shown very high immunogenicity and efficacy in mouse models(Plebanski et al., 1998) (Gilbert et al., 1999). Similar results for a different mouse malaria model were reported by an American group(Sedegah et al., 1998). Induction of CD8+ T cells was assessed by both conventional Cr^{51} lysis assays after *in vitro* restimulation and fresh γ-interferon ELISPOT assays. The latter assay showed the best association with efficacy.

In several further studies DNA/MVA and other prime-boost regimes have been highly immunogenic for CD4+ and CD8+ T cell induction against Hepatitis B (McConkey et al. unpublished data), tuberculosis(McShane et al., 2001), HIV(Hanke et al., 1999), ebola(DNA/adenovirus) (Sullivan et al., 2000) and melanoma in both murine and non-human primate studies. DNA/MVA polyprotein vaccinations controlled a mucosal challenge of a highly pathogenic SIV/HIV chimaera and prevented AIDS in a macaque model. In this study challenge occurred 7 months after final vaccination(Amara et al., 2001). Interestingly two vaccinations with a DNA-HIV construct followed by a single adenovirus-HIV boost gave much higher immunogencity (about 20% vs 5% in a class I tetramer assay) but slightly lower efficacy than three vaccinations with the recombinant adenovirus without DNA priming in a macaque model (Shiver et al. 2002).

3.6 Mechanism of CD8+ T cell induction by heterologous prime-boost immunisation

Many viral vectors, mostly poxviruses, amplify CD8+ T cell frequencies in heterologous prime-boost regimes. DNA vaccines are limited in their immunogenicity by the nanogram to picogram quantities of recombinant protein which can be expressed. An advantage of such immunogenicity is that it is highly focussed. Viral vectors produce greater quantities of recombinant protein but also induce polyclonal responses to many vector immunogens. If a viral vector immunisation is administered after a DNA immunisation, DNA vaccine-induced memory T cells are amplified rapidly with the kinetics of a secondary immune response. Although a primary immune response also occurs to vector antigens the tendency of the immune system to produce a few immunodominant responses probably leads to the high amplitude responses being directed against the recombinant insert. Thus the priming immunisation generates insert-specific T cells which are amplified strongly by the viral vector boost(Schneider et al., 1999).

If a viral vector boost is administered after a heterologous viral priming immunisation the efficiency of the boosting effect is presumably determined by the proportion of immunogens with cross-reactivity between vectors. The more cross-reactive epitopes, the smaller the likelihood that epitopes from the insert will be "selected" for amplification. In murine malaria experiments by several groups, recombinant vaccinia viruses have been particularly good for CD8+ T cell induction after boosting, whether after DNA(Schneider et al., 1998), recombinant influenza(Li et al., 1993). adenovirus(Gilbert et al., 2002), Ty-VLPs(Plebanski et al., 1998), fowlpox(R Anderson, unpublished data) or a recombinant protein particle in a murine hepatitis B model(P Gothard, unpublished data). A time interval is necessary before the prime-boost phenomenon can be observed. In the BALB/c mouse model 9 days are required after DNA immunisation before induced T cells are fully boostable. Not all delivery systems are able to boost T

cell induction as part of a combination. DNA vaccines and Ty-VLPs were unable to boost after MVA immunisation(Plebanski et al., 1998). So delivery systems are either boosting or non-boosting systems. Possible reasons for this difference are recombinant protein expression levels, the need for additional as yet unconfirmed signals for either APC maturation or T cell modulation and the relatively low rate of APC infection with these carriers compared to viral vectors.

Chapter 4

The Candidate Malaria Vaccines DNA ME-TRAP, MVA ME-TRAP and FP9 ME-TRAP

4.0 The Multiple Epitope String

Antigens or epitopes for inclusion in a malaria vaccine should be chosen for induction of protective immunity. This choice has been complicated by the lack of both relevant animal models and immune correlates of protection for *P. falciparum*. The malarial insert, ME-TRAP, combines two complementary approaches. Firstly the identification of CD8+ T cell epitopes and inclusion as an epitope string. Secondly the inclusion of a whole antigen.

Possession of the HLA-B53 allele confers protection against severe malaria in Gambian children(Hill et al., 1991). The molecular basis of this protection is thought to be the ability of HLA-B53 to bind ls6, a nonamer epitope from LSA-1, and thus induce protective CTL to infected hepatocytes(Hill et al., 1992). The specificity of this HLA restriction was discovered by a "reverse immunogenetics" approach, which was extended in later work. The DNA sequences of 6 pre-erythrocytic *P. falciparum* antigens (CS, TRAP, LSA-1, EXP-1, LSA-3 and STARP) were screened using allele-specific motifs for HLA-B53, HLA-B35, HLA-B7, HLA-B8, HLA-A2 and HLA-B17. These 6 HLA types are found in 75% of Gambians and 70% of caucasians. Assembly assays were used to assess the binding of potential epitopes thus identified to HLA molecules. CTL assays on Gambian adults and children were then used to confirm functional epitopes. Blood was obtained from children either during acute malaria or convalescence. 14 CD8+ T cell epitopes were thus identified. More recently another HLA-B53-

restricted epitope was identified in another antigen expressed by liver-stage parasites, LSA3 (Aidoo et al., 2000)

The ME string consists of the DNA sequences for all 14 epitopes fused to the following additional sequences: three CD4+ T cell epitopes from *P. falciparum* CS, BCG and tetanus toxoid; a *P. falciparum* CS B cell epitope; a *P. falciparum* TRAP B cell epitope and pb9, a *P. berghei* CD8+ mouse T cell epitope to enable potency testing of each clinical batch in mice. Six Ty-VLP constructs including various combinations of the ME string constituent epitopes were used to evaluate processing and presentation of single epitopes from a string(Gilbert et al., 1997). All 5 *P. falciparum* epitopes tested were processed and presented *in vitro* for both restimulation of CTL responses from malaria-exposed donors and induction of primary CTL responses. pb9 was processed and presented *in vivo* from various positions in a string for priming of CTL in murine experiments. A Ty-VLP construct including pb9 as the only murine malaria sequence was shown as part of a Ty-VLP/MVA immunisation regime to not only induce high level γ-interferon effector T cell immunogenicity but also to be 100% protective in a murine experiment. More recently T cell responses to these epitopes have been found to correlate with protection from malarial anaemia in Kenyan children (Ong'echa et al., 2003)

An Advanced Blast P2.0.8 search was conducted using the National Center for Biotechnology Information protein database in May 1999 to search for homologies between the multiple epitope string and all reported human protein sequences. This revealed the expected homologies between the TRAP AM peptide and members of the human thrombospondin/properdin family. As this peptide is not a novel sequence created by the fusion of epitopes, but is contained in *P falciparum* parasites, and has been a constituent of other malaria vaccines this is not believed to be a safety concern. Nucleotide homology searches were also undertaken. No new homologies were found which are not

present in the individual epitopes and to which humans are therefore exposed due to natural infection.

4.1 The TRAP antigen as a vaccine candidate

The ME string is fused in frame to the entire T9/96 strain coding sequence of *P.falciparum* TRAP to generate the 2389 base pair ME-TRAP hybrid malarial DNA sequence, encoding a single 789 amino acid polypeptide. The CS antigen is the best characterised pre-erythrocytic *P. falciparum* antigen. Most previous published work on malaria candidate vaccines has been on CS-based candidates. TRAP was chosen as a well-characterised pre-erythrocytic antigen with a homologue that is protective in rodent models and with less polymorphism than CS. Most T cell responses to CS are focussed on a few highly variable regions(Good et al., 1988). T cell responses to TRAP are broadly distributed with no immunodominant loci(Flanagan et al., 1999). TRAP is an abundant pre-erythrocytic stage antigen(Rogers et al., 1992). Human volunteers immunised with irradiated sporozoites and protected against malaria develop T cell responses against TRAP(Wizel et al., 1995), as do residents of malaria-endemic countries(Flanagan et al., 1999).

4.2 DNA ME-TRAP

The DNA vaccine DNA ME-TRAP consists of the 6773 base pair malarial expression vector pSG2. This includes the ME-TRAP hybrid malaria insert. Expression of this cloned malarial DNA sequence is regulated by a standard set of control elements, consisting of the human cytomegalovirus (CMV) IE1 (immediate early 1) enhancer/promoter and intron A and the bovine growth hormone–derived polyadenylation signal (bGH.polyA). The polyadenylation serves to improve mRNA transcript stability. A kanamycin antibiotic resistance gene is included as a marker gene suitable for clinical use as kanamycin is no longer used in the clinic. A prokaryotic origin E1 is included for propagation within *E. coli* but not mammalian tissue.

4.3 MVA ME-TRAP

MVA ME-TRAP vaccine consists of MVA, and the identical ME-TRAP hybrid encoded by DNA ME-TRAP. The ME-TRAP sequence was ligated into the vaccinia shuttle vector pSC11 such that it will be expressed by the vaccinia P7.5 early/late promoter.

This vector includes the *E. coli* β-galactosidase gene expressed by the vaccinia P11 late promoter. The region including ME-TRAP and the β-galactosidase gene is flanked by a sequence from the vaccinia thymidine kinase locus to allow insertion into the vaccinia genome at this locus. CEF cells infected with wild type MVA virus were transfected with pSC11 ME-TRAP and *in vitro* recombination allowed to take place. Recombinant virus was identified using β-galactosidase substrate X-gal overlay of infected chicken embryo fibroblast (CEF) monolayers and isolated by multiple rounds of plaque picking(Chakrabarti et al., 1985). A stock of MVA ME-TRAP was supplied to contract manufacturer IDT (Rosslau, Germany) for production of the master seed virus and clinical lot, which was produced under cGMP manufacturing conditions.

4.4 FP9 ME-TRAP

The vaccine consists of the attenuated fowlpox virus, FP9 and the identical ME-TRAP insert encoded by DNA ME-TRAP and MVA ME-TRAP.

The ME.TRAP fusion protein sequence was ligated into the unique cloning site after the P7.5 promoter in the FP9 shuttle vector pEFL29 (Qingzhong et al., 1994). This vector includes the *E. coli* β-galactosidase gene expressed by the FPV p4b promoter, late fowlpox promoter, to allow detection of the recombinant virus. The region including the gene of interest driven by the Vaccinia P7.5 promoter, and the β-galactosidase gene is flanked by sequence from the FP9 ORF1 locus to allow insertion into the FP9 genome at this locus.

Specific pathogen free CEF cells were infected with wild type FP9 supplied by Michael Skinner, Institute of Animal Health, Compton, UK. The infected cells were then transfected with the pEFL29 ME.TRAP shuttle vector and *in vitro* recombination was allowed to take place. Virus harvested from the *in vitro* recombination mixture was diluted and plated on fresh unpassaged CEF monolayers which were overlaid with medium containing agarose after infection and incubated for four days to allow viral plaques to develop. A second overlay containing the β-galactosidase substrate X-gal was then added and the monolayers were incubated for a further 24 hours, after which time plaques containing recombinant virus had turned blue.

Well isolated blue plaques were picked from the monolayer using a sterile pipette tip and placed in a small volume of sterile phosphate buffered saline (PBS). This was then frozen and thawed three times to lyse the CEF cells and release the virus. The resulting virus was again diluted and plated on CEF monolayers and the recombinant plaque picking was repeated five times in total to ensure the purification of the recombinant FP9 ME.TRAP away from the wild type virus.

Unpassaged CEF monolayers were then inoculated with the purified virus to obtain a stock of FP9 ME.TRAP. This was then titred using CEF cells. The infected monolayer was incubated until white plaques were visible (five days). A second agarose overlay containing X-gal was added. The monolayers were incubated for a further 24 hours and then examined to see if all plaques were blue. Any remaining wild-type virus would form a white plaque, but all plaques were found to be blue.

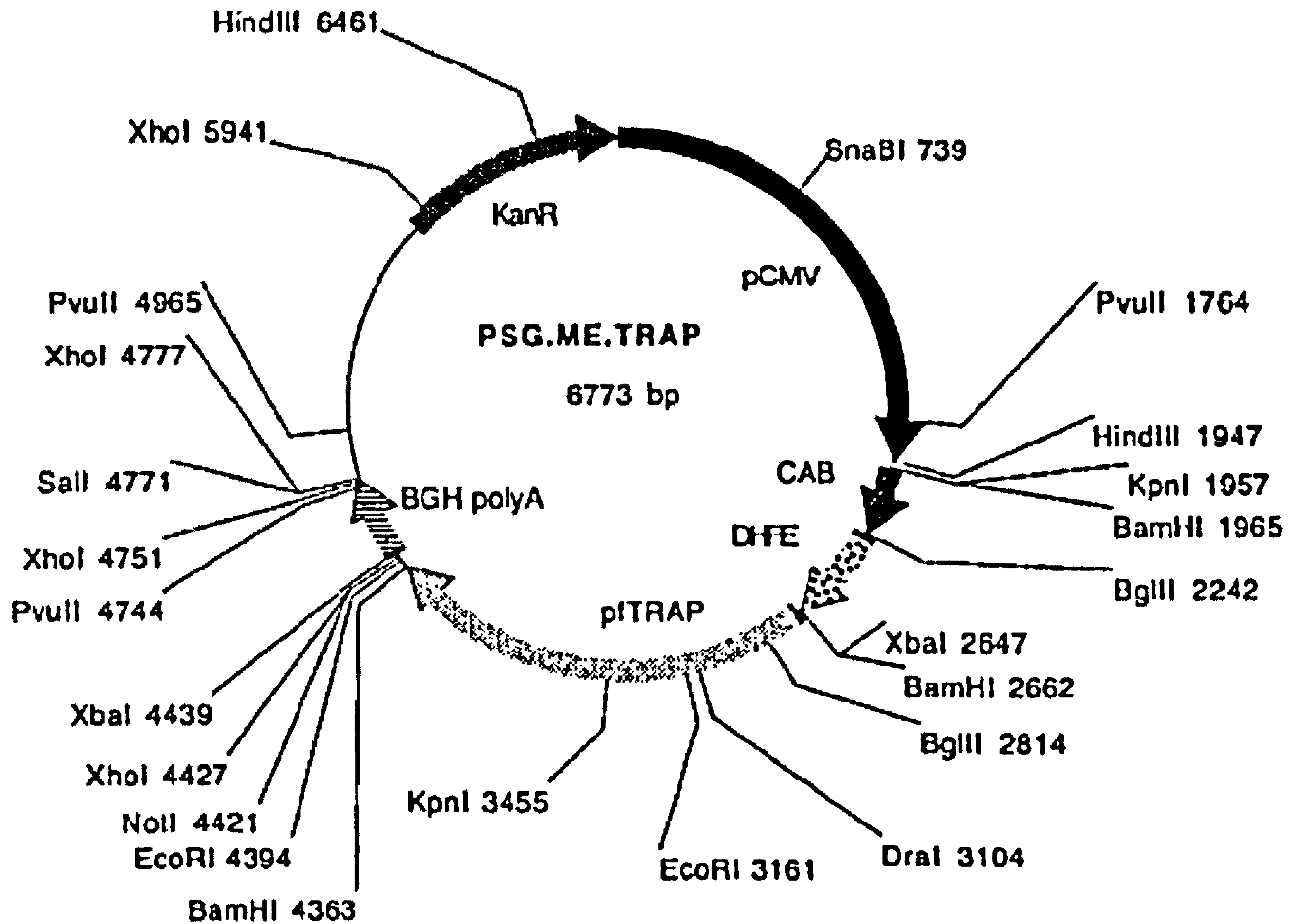
The initial stock of recombinant FP9 was then inoculated into fresh CEF monolayers to produce a manufacturers stock which was passed through a 0.2 micron filter to ensure absence of bacterial or fungal contamination, then supplied to IDT for production of the master seed virus and the clinical lot.

Table 4.1: Epitopes within the ME string

Epitope	Antigen	Amino acid Sequence	Type	HLA restriction
st8	STARP	MINAYLDKL	CTL	A2.2
ls50	LSA-1	ISKYEDEI	CTL	B17
pb9	*Pb* CS	SYIPSAEKI	CTL	$H2\text{-}K^d$
ls8	LSA-1	KPNDKSLY	CTL	B35
cp26	CS	KPKDELDY	CTL	B35
ls6	LSA-1	KPIVQYDNF	CTL	B53
tr42/43	TRAP	ASKNKEKALII	CTL	B8
tr39	TRAP	GIAGGLALL	CTL	A2.1
cp6	CS	MNPNDPNRNV	CTL	B7
tr26	TRAP	HLGNVKYLV	CTL	A2.1
ls53	LSA-1	KSLYDEHI	CTL	B58
tr29	TRAP	LLMDCSGSI	CTL	A2.2
CS	CS	DPNANPNVDPNANPNV	T helper	Universal epitopes
38H	BCG	QVHFQPLPPAVVKL	T helper	
FTTp	TT	QFIKANSKFIGITE	T helper	
cp39	CS	YLNKIQNSL	CTL	A2.1
la72	LSA-3	MEKLKELEK	CTL	B8
ex23	EXP-1	ATSVLAGL	CTL	B58
NANP	CS	NANPNANPNANPNANP	B cell	
AM	TRAP	DEWSPCSVTCGKGTRSRKRE	Putative B cell	

The epitopes were encoded end to end within the ME string in the order shown above. The final epitope is encoded end to end with the PfTRAP sequence.

Figure 4.1: DNA ME-TRAP



Schematic representation of the plasmid DNA vaccine encoding ME-TRAP. The MVA and FP9 vaccines expressed the same insert. KanR represents the kanamycin resistance gene. pCMV represents the human CMV immediate early 1 promoter. CAB and DHFE together represent the ME string. CAB represents the first 9 epitopes of the string. PfTRAP represents the TRAP gene. BGHpolyA represents the bovine growth hormone polyadenylation site.

Chapter 5

General Methods

5.0 *Ex vivo* γ-interferon ELISPOT assay

The vaccines were designed for maximal induction of cellular immune responses. Pre-clinically DNA/MVA leads to efficient induction of antigen-specific T cells with rapid effector function for γ-interferon secretion as assayed by a γ-interferon ELISPOT assay. Previous published antigen-specific T cell induction through clinical DNA vaccination was assayed in a CTL assay performed on *in vitro* restimulated cell lines(Wang et al., 1998). These assays provide high sensitivity for detection of antigen-specific T cells either present at very low frequency or without rapid effector function. However T cells able to secrete relevant effector molecules such as γ-interferon without a requirement for cell division or prolonged stimulation are more likely to eliminate infected hepatocytes *in vivo* than those with such a requirement. T cells specific for influenza epitopes and with rapid γ-interferon effector function have been shown to be present in the peripheral blood of humans many months to years after the most recent exposure to pathogen(Lalvani et al., 1997). These cells were enumerated by an *ex vivo* γ-interferon ELISPOT which was modified for use in these clinical trials.

ELISPOTs were performed on Millipore MAIP S45 96-well plates with MabTech human γ-interferon monoclonal antibodies. Plates were coated overnight at 4°C with 50µl per well of 10µl/ml 1-D1K capture antibody diluted in Sigma pH9.6 carbonate/bicarbonate coating buffer or sterile PBS. The plates were washed 6 times with sterile PBS and blocked for at least 1 hour at room temperature with Sigma RPMI-1640 containing 10% foetal bovine serum (FBS) and with l-glutamine and penicillin/streptomycin supplementation (R10). Medium was Sigma RPMI-1640 with 10% human AB serum and l-glutamine and

penicillin/streptomycin supplementation (RN10). 4 x 10^5 PBMCs in a total volume of 100μl were assayed for 18-20 hours at 37°C in a humidified 5% CO_2 incubator on the ELISPOT plates in the presence of 25 μg ml^{-1} peptides. Blood volume limitations precluded assaying peptides individually. 20-mer peptides overlapping by 10 were used in pools to span the entire T9/96 and 3D7 strain of the TRAP antigen. 8-mer to 17-mer epitopes from the ME string were also assayed in pools. Although 20-mer peptides are suboptimal for formation of the T cell-APC synapse, it is known that both CD8+ and CD4+ T cell responses can be detected with 20-mer peptides when used at high concentrations such as 25 μg ml^{-1}.

After the 18-20 hour incubation period, plates are flicked and washed 6 times with PBST (PBS with 0.05% Sigma Tween20 added). 50μl of 1μl/ml of 7-B6-1 detector antibody diluted in PBS is added per well and incubated at room temperature for 2-4 hours or overnight at 4°C. Plates are then flicked, washed 6 times with PBST before addition of 50μl per well of 1μl/ml of streptavidin-alkaline phosphatase antibody diluted in PBS. This is incubated for 1 hour at room temperature before flicking and washing 6 times with PBST. Development is with BioRad alkaline phosphatase reagents which lead to production of a blue/purple spot. Plates must be washed several times with tap water and left to soak in water preferably overnight before thorough drying. The colour of the spots fades gradually with time but this fading can be slowed by storage in the dark. In this way plates can be read without loss of data several months after the day of development. Ideally however plates should be read as soon as they are dry.

5.0.1 Analysis of *ex vivo* ELISPOT immunogenicity

Means of duplicates were calculated for negative controls and test wells. A > 50 SFC per million PBMC (spot forming cells per million peripheral blood mononuclear cells) level in the negative control was used to exclude plates with high background from the analysis. Spots were summed across relevant pools with the negative control subtracted the requisite number of times and values were adjusted to SFCs per million PBMCs. As 20-mer peptides overlapping by

ten were used it is possible that some responses were counted twice by this method. This procedure derives summed responses for T9/96 TRAP, 3D7 TRAP, ME peptides and all peptides in the construct (the sum of T9/96 TRAP and ME responses). The pre-vaccination summed responses for each volunteer were subtracted from the post-vaccination responses and negative induced responses were set to zero. By setting negative responses to zero, the sensitivity of the analysis was maximized and the absolute number of reactive cells is likely to have been slightly inflated. Biologically plausible hypotheses were made, thus specifying comparisons to be performed between vaccination groups or between T9/96 and 3D7 TRAP responses in the same group. All comparisons in the text are either of two groups of induced responses or an post-vaccination response with a pre-vaccination response. Groups of responses are positively skewed (i.e. to the right on a conventional graphical representation). The median is therefore usually smaller than the arithmetic mean. The variance of datasets is related to the size of the response and therefore when comparing two groups for magnitude of immunogenicity the variances are often markedly dissimilar. These datasets are often not normally distributed with or without log-transformation. Arithmetic means are used for visual representation of data as this has intuitive meaning to the reader whereas geometric means or medians with interquartile ranges are used in the text as these have more meaning and validity particularly for the purposes of significance tests. A table (table 7.2) giving comparisons within the UK data of geometric means and arithmetic means is provided to allow interpretation of the distibution of the data. Individual test responses in the *ex vivo* ELISPOT are not defined as positive or negative, rather they are summed as described above and then summed responses are compared between timepoints or between groups by the Mann-Whitney test. This statistical method was chosen to allow comparison of the magnitude of responses between timepoints and between groups. The view of our group was that negative responses do not have any biological meaning leading to our decision to set negative responses to zero.

5.0.2 Characterisation Of T cell subsets by CD4 and CD8 cell depletion.

Cell separations were performed on PBMCs frozen in 90% Foetal Bovine Serum/10% Dimethylsulfoxide. Once thawed, washed and counted, the cells were incubated for 15 minutes at 4ºC with CD4 or CD8 Microbeads (Mitenyi Biotec), washed once in separation buffer (PBS + 0.5% human AB serum + 2mM EDTA) and then passed through a magnetic separation column. Undepleted, CD4 depleted and CD8 depleted cell populations were then assayed as above in ex vivo ELISPOT assays. Cell separations were checked by co-staining aliquots of purified populations and whole PBMC in cold PBS containing 0.5% BSA and 0.05% sodium azide with Peridinin-chlorophyll-protein complex (PerCP)-conjugated mouse anti-human CD3, fluorescein isothiocyanate (FITC)-conjugated mouse anti-CD4, and phycoerythrin (PE)-conjugated anti-CD8 antibodies. Cells were acquired on a FACSCalibur (Becton Dickinson, San Jose, CA) and analysed using CellQuest software (Becton Dickinson). For analysis, lymphocytes were gated by forward and side scatter properties and CD3+CD4+ & CD3+CD8+ populations were identified. 10,000 live events were collected. All populations shown were separated to >90% purity.

5.1 Cultured γ-interferon ELISPOT assay

Cells are aliquotted at 2 x 10^5 PBMCs per well into standard 96-well U-bottom plates with peptides at 25 μg ml^{-1} in RN10 to make a total volume of 200μl. Stimulant wells are surrounded by wells containing 200μl water or PBS to reduce evaporative losses. On day 3 100μl supernatant is removed and replaced with Lymphocult-T (BioTest) to aid cell proliferation. This is also performed on day 7. On day 9, the day prior to the ELISPOT, 150μl supernatant is replaced with RN10 to reduce background γ-interferon in the assay. On the day of the assay, day 10, the plates are spun at 1400 rpm for 3 minutes, 150μl supernatant removed and replaced with RN10. This washing procedure is repeated for 6 total washes. Cells are then resuspended carefully in 240μl and transferred at 33,000 original input cells (40μl) per well to pre-coated and blocked ELISPOT plates.

Further incubations and development are as described above in the *ex vivo* assay.

5.1.1 Analysis of cultured ELISPOT immunogenicity

Triplicate responses were obtained. These triplicates can vary substantially and there is a degree of variability between experiments in both test and background responses. In order to obtain meaningful outcomes I analysed the data with emphasis on high specificity rather than sensitivity. Each well containing cells stimulated for 10 days with ME or TRAP peptides was split after culture into 6 wells for the overnight ELISPOT assay. One of these assay wells was a negative control with medium but no peptides. Therefore each test response out of the triplicates had a corresponding individual negative control response. An individual well was considered positive if the number of spots appearing in the test well was significantly ($p <= 0.05$) greater than the number of spots appearing in the background well, assuming if the response was not positive that both responses were part of the same poisson distribution. Furthermore I defined as positive only those test responses where all three triplicates were positive in this way. Although this approach will tend to discard some true positives, the positive responses defined by this method are very likely to be true positives.

We used definitions of positive for cultured ELISPOT assays in contrast to our approach for the *ex vivo* assay. There are two main reasons why we chose to use definitions of positive responses for cultured ELISPOTs but not *ex vivo* ELISPOTs. Firstly in our hands variability in the cultured ELISPOT is greater than the variability in the *ex vivo* ELISPOT. Greater variability in an assay requiring 10 days of *in vitro* stimulation compared to an overnight assay is biologically plausible and perhaps expected. Secondly negative control responses tend to be slightly higher in the cultured ELISPOT. Negative control wells were almost always very low in the *ex vivo* ELISPOT assays conducted as part of this thesis. For these reasons in the cultured ELISPOT I was most interested in a method with high specificity but low sensitivity of defining positive responses. The

drawback of such a method is that interpretation of the size of the responses is more problematic when only defined positive responses are used, as most responses are defined as negative for the cultured ELISPOT. This cultured ELISPOT statistical method for defining positives therefore provides a stronger method, in my opinion, for defining the frequency of positive responses than for the magnitude of induced responses.

5.2 Preparation and cryopreservation of PBMCs

Venous blood is collected into BD 10ml lithium heparinised Vacutainers. The blood may be stored for up to 24 h at room temperature. The blood is transferred into a 50ml Falcon tube. Initially this falcon tube was centrifuged, and the plasma removed for storage before ficoll-hypaque layering. However in order to increase efficiency during the course of the studies this was modified as follows. The blood is transferred directly to a Leucosep 50ml Falcon tube. These tubes contain a filter paper horizontally fixed within the tube. 15mls of Lymphoprep, also known as ficoll (Nycomed technologies), is pre-added to the bottom of the tube. Blood could then be transferred onto the filter paper much more rapidly than when layering directly layered onto Lymphoprep when maintenance of good separation requires great care. The blood and Lymphoprep is then centrifuged at 2000 rpm for 20 minutes with the brake off after which aliquots of plasma are taken from the top plasma layer. Plasma is then either used fresh for antibody ELISA or clinical chemistry assays or stored at -20°C. PBMCs are then collected from the interface with a pasteur pipette and transferred to another 50ml Falcon tube. The PBMCs are diluted to 50ml with RPMI-1640 supplemented with l-glutamine and penicillin/streptomycin (R0). This solution is centrifuged at 1600 rpm for 10 minutes, the supernatant poured off, the pellet flicked and one further wash performed in R0 with the centrifuging at 1400 rpm for 7 minutes. Again the supernatant is poured off and the pellet flicked and resuspended in 5-10mls R0.

If there are many red blood cells still in the pellet, these are lysed by resuspending in 5 ml ACK lysis buffer. After 2 minutes, the cell suspension is

underlaid with 5 ml heat inactivated FBS, and centrifuged at 1400 rpm for 7 minutes without brake. Two stains are used for counting cells, Trypan Blue and Crystal Violet both from Sigma. The Crystal Violet is diluted 12 to 1 in water and filtered before use. 10μl of cells in R0 are mixed 1:1 with stain and then added to the counting chamber of an Improved Neubauer Haemocytometer. Whole numbers of chamber squares are counted until the cell count is above 200. Two different Crystal Violet stained counts (CV1 and CV2) are performed to count PBMCs which appear as nucleated purple cells. A single Trypan Blue count is performed to count dead cells only (TB), which stain blue throughout under this stain. Viable cells do not take up stain intracellularly and so appear as outlines only. The following calculation can then be made to calculate the number of live cells per litre of cell suspension.

$$((CV1+CV2)/2 - TB)*(25/\text{No Squares Counted})*(\text{Dilution Factor})*10^7 \text{ cells/litre}$$

This number is then divided by 1000 and mutiplied by the number of mls of cell suspension to give the number of cells in the cell suspension. At this point the number of cells needed for the assay(s) are taken out into a separate container and the cells remaining in the 50ml Falcon are prepared for freezing. The assay cells are centrifuged at 1400 rpm for 7 minutes and made up to $8x10^6$ cells/ml in RN10. The cells to be frozen are centrifuged at 1400 rpm for 7 minutes and resuspended in 0.5ml FBS for each vial to be frozen. One vial is frozen for 5-10 million cells. The cells in FBS are kept on ice for at least 30 minutes. However ice supplies are not straightforward in laboratories in The Gambia and so 4 °C was employed when necessary. After this period the cells are mixed 1:1 with 80% FBS and 20% Dimethylsulfoxide (DMSO, Sigma) in 1ml cryovials and immediately placed in specialised cell freezing containers (Mr. Frosty cryocontainers) which are placed in a –80 °C freezer overnight. The Mr. Frosty cryocontainers must have been filled appropriately with propanol and left at 4 °C at least 4 hours before this time. The vials are transferred the next day to liquid nitrogen.

5.3 Thawing of PBMCs

Traditionally PBMCs are transferred from their liquid nitrogen cryogenic storage container on solid CO2 during thawing. However solid CO2 was not available in The Gambia. Therefore various modifications to the usual technique were employed. What was found best to work was as follows. Two scientists were present in the liquid nitrogen storage room. Once the correct vials were found, one scientist remained in this room and closed the container. The distance from this room to the laboratory was considerable. Therefore the vials were placed in a 50ml Falcon tube whose lid was closed and the second scientist returned to the door to the laboratory as quickly as possible. Once in the laboratory the vials were stirred vigorously in a waterbath containing water at 37 °C watching carefully for thawing of the cell suspension. As soon as the 1ml cell suspension had thawed they were transferred to a 15ml Falcon containing 10ml R10. Cells were centrifuged at 1400 rpm for 7 minutes and washed once further with 10mls R10 before an interval of 1-2 hours in a 37 °C 5%CO2 humidified incubator. Cells were then centrifuged at 1400 rpm for 7 minutes and made up to 0.5ml RN10 for counting as above. Recovery of about 60% of the frozen cell numbers were obtained by this method in The Gambia.

5.4 Haematology

The clinical haematology service of the John Radcliffe Hospital was used for UK studies. A Medonic CA620 automatic cell counter was used for all Gambian studies. High, normal and low controls (Mallinckrodt Baker UK) were run at least once a week on the Medonic. Test samples were mixed well on a roller and then a sample was aspirated into the Medonic. After completion of the analysis, results are automatically displayed on the screen. Platelets, haemoglobin, haematocrit (HCT) and white blood cell count (WBC) were used for analysis. WBC differentials were performed manually when total WBC were outside the normal range.

5.5 Biochemistry

The clinical biochemistry service of the John Radcliffe Hospital was used for UK studies. A Visual (Bio-Merieux) analyser was used in The Gambia. Creatinine standards were used as controls for creatinine assays; zymotrol was used as controls for the ALT assays. Water was the negative control. ALT or creatinine reagents were incubated with plasma samples at 37 °C for 5 minutes before assaying.

5.6 Blood Smears

In The Gambia clinical slides were stained with Field's stain and read immediately. Duplicate smears were made and all slides were also stained with Giemsa. Slides were read to 200 fields in the Phase I studies and 100 fields in the Phase IIb study (see chapter 11). Parasitaemia was calculated as /μl assuming 1 parasite in 1 high power field = 500 parasites/μl.

5.7 Anti-TRAP Antibody ELISA

Recombinant T9/96 TRAP (PfTRAP 1.1) was obtained from Andrea Crisanti. Recombinant 3D7 TRAP and positive and negative control sera were obtained from GSK Biologicals. Antigens were coated at 4°C overnight onto Maxisorp immunoplates (Nunc) at a concentration of 4ug/ml in 0.05M carbonate-bicarbonate buffer, pH 9.6 (Sigma). Plates were washed with PBS/0.05% Tween 20. Plasma samples from volunteers were diluted 1/100 in PBS/0.05% Tween containing 1% BSA and incubated in duplicate wells for 2 hours at 37°C. Plasma samples were also added to wells in which no TRAP had been added to the coating buffer. Plates were then washed and bound antibodies detected with goat anti-human IgG antisera conjugated to alkaline phosphatase (Promega) diluted 1/5000 in PBS/Tween/1%BSA. After 1 hour plates were washed and developed using p-nitrophenyl phosphate tablets (Sigma) and the OD_{405} measured.

5.8 Anti-R32LR ELISA

R32LR is a recombinant *P. falciparum* CS fragment containing 32 NANP repeats. This is the immunodominant B cell epitope from the CS antigen. This ELISA assay was developed by WRAIR and is the most widely used ELISA for antibody to *P. falciparum* CS. The antigen R32LR, which was provided by WRAIR, is diluted in PBS to a concentration of 0.1 μg/50 μl. A 0.5% boiled casein (BC) preparation is added at 4 μl of BC per 5 ml of diluted antigen. Triplicate wells are coated for each dilution of serum with 50 μl of diluted antigen. Incubation is overnight at room temperature in a humidified box. The well contents are aspirated and wells are blocked with 250 μl of 99% BC/1% Tween 20 for one hour at room temperature. The blocking buffer is then exchanged for 50 μl of appropriate serum dilution per well including blanks, negative and positive controls. Samples are run in triplicate starting with a 1 :50 dilution and making two-fold dilutions down the plate. Incubation is for two hours at room temperature. After wells are washed four times with PBS-Tween 20, 50 μl/well of Goat Anti-Human IgG HRP in BC/0.025% Tween 20 diluent is added and incubated for one hour at room temperature. Wells are washed four times with PBS-Tween 20 before development with HRP reagents for one hour at room temperature. 10 μl of 20% SDS is used to stop the reaction. Adsorbance is read at 414 nm. Because the μg/ml antibody concentration of the positive control (DGP, Daniel Gordon Plasma) has been previously calculated, and DGP is available as positive control sera, antibody concentrations of test samples can be calculated from mean sample adsorbances. Daniel Gordon was a US Army researcher protected by RTS,S vaccination with high titers of NANP-specific IgG present in his plasma samples.

5.9 Exclusion criteria

Fo phase 1 trials the exclusion criteria were:

a. Clinically significant history of skin disorder (eczema, psoriasis, etc.), allergy, immunodeficiency, cardiovascular disease, respiratory disease,

endocrine disorder, liver disease, renal disease, gastrointestinal disease, neurological illness, psychiatric disorder, drug or alcohol abuse.

b. History of splenectomy

c. Haematocrit of less than 30%

d. Serum creatinine concentration >130mmol/L

e. Serum ALT concentration >42 IU/L

f. Blood transfusion within one month of the beginning of the study

g. History of vaccination with previous experimental malaria vaccines

h. Administration of any other vaccine or immunoglobulin within two weeks before or two weeks after vaccination.

i. Positive HIV antibody test

j. Current participation in another clinical trial, or within 12 weeks of this study

k. Any other finding which in the opinion of the investigators would increase the risk of an adverse outcome from participation in the trial.

l. Likelihood of travel away from the study area for a period of 6 months from screening.

m. Positive pregnancy tests for females (UK studies)

n. Allergy to gold or previous gold therapy (UK study with needleless delivery device of vaccine coated to gold particles)

o. Allergy to kanamycin for DNA vaccine studies

p. Allergy to Fansidar for phase 2b study only.

q. Previous rabies vaccination for phase 2b study only.

5.10 Reactogenicity assessments

Most adverse events whether local, systemic or unsolicited were assessed according to the following system.

0 = No adverse experience

1 = An adverse experience which is easily tolerated by the subject, causing minimal discomfort and not interfering with everyday activities.

2 = An adverse experience which is sufficiently discomforting to interfere with normal everyday activities.

3 = An adverse experience which prevents normal, everyday activities. (In adults/ adolescents, such an adverse experience would, for example, prevent attendance at work/ school and would necessitate the administration of corrective therapy).

Where the terms mild, moderate and severe are used for adverse events these generally correspond to 1,2 and 3 on this scale. Follow-up was on day 7 and day 21 in the UK and day 1, day 2, day 7 and day 21 or day 28 in The Gambia. Local adverse events were discolouration, induration, blistering, pain and limited arm motion. Systemic adverse events were headache, nausea, malaise and objective fever (axillary temperature >= 37.5°C). Unsolicited adverse events were collected for 28 days after each vaccination and assessed for relation to vaccination. In The Gambia field assistants collected some reactogenicity data after appropriate training.

5.11 Serious adverse events

The following adverse events are considered "serious adverse events":

- Death of a subject or a life threatening event.
- Hospitalization.
- Incapacitating disability.
- Overdose.
- Cancer or congenital anomaly.

A Safety Monitor was designated for each study and clear rules were set out for serious adverse event reporting. Rules were also in place for reporting to ethics committees and the scientific co-ordinating committee of the MRC unit in The Gambia.

5.12 Prior Approvals Sought

All studies required ethics approval from one or more UK institutions and the Gambian Government/MRC Ethics Committee for the Gambian studies. Gambian studies all also received approval or modification by the MRC Gambia Scientific Coordinating Committee. Before the first Gambian study a group of UK MRC Ethicists convened by MRC Head Office UK considered the vaccine programme prior to Gambian SCC approval. UK Medicines Control Agency approval was obtained prior to each UK phase I study. No Gambian regulatory authority exists but prior UK regulatory authority approval was recognised. An independent Data Safety Monitoring Board gave prior approval to both the study protocol and reporting and analysis plan for the field efficacy study.

5.13 Informed Consent in The Gambia

1) Joint Gambian Government/ MRC Ethics Committee (EC) approval and other relevant EC approval must be obtained before any part of the study. In some cases general aspects of a new study design may be discussed with communities as a possibility for future work before formal approval is obtained, although none of the meetings below should be held without formal approval.

2) Important community representatives and government representatives should be informed before volunteer recruitment commences. This may involve representatives from the Ministry of Health, Divisional health teams, Hospital Management and Chiefs of major towns (for example meetings were held with the Chief of Farafenni before VAC014 and VAC020).

3) Initial meetings should be held with the Alkalo and elders of a village during which the study should be discussed in detail. If the elders do not feel the study is appropriate for the village, further general meetings should not be

held and volunteers from the village should not be recruited. Often it is possible for some women and some younger people to be present at this initial meeting and this should be encouraged.

4) General village meetings should then be held at which the study should be described as in the information sheet and consent form. This should include a description of how much blood will be taken and how frequently and explicitly, what risks and benefits the volunteers and for benefits, the community, can expect from the study.

5) Field assistants should then visit potential volunteers and conduct detailed individual discussions in the local languages giving out both English and where appropriate local language/Arabic script versions of the information sheet and consent forms.

6) Further discussion should then take place at a clinic visit between a study physician and volunteers individually, during which consent should be sought. Consent forms may be signed by the field assistant on behalf of MRC and by the volunteer. Although not required by the MRC unit, the consenting study physician should sign the consent form for ICH-GCP compliance. Because of the very detailed nature of the consent procedure and the lack of literate village residents, it has not been thought appropriate to include a requirement for an independent witness who can witness the correct consenting procedure, translation and read the English consent form. This would usually not be possible.

7) Investigators must understand that in a rural setting the culture of most Gambians is to pay a great deal of attention to the opinion of their village elders and their family elders. It is not the culture for all individuals to expect detailed individual explanation of research procedures from research staff and they may find the need for such detailed explanations difficult to understand,

given that their community and their elders have already consented. However it is the investigators duty to ensure that consent is as informed as possible on an individual basis. It is therefore incumbent on investigators to reinforce important messages throughout the trial in a culturally acceptable manner. In particular the facts that the intervention is not proven and that there is a control group (where applicable) should not be assumed to be understood at the outset.

8) Beyond a certain length of information sheet/consent form, comprehension decreases for many of the village residents. Very detailed and in some senses, legally oriented, consent forms are increasingly being asked for by many sponsors. These consent forms which are sometimes stated to be necessary for ICH-GCP compliance and often 4 or more pages in length are not thought to be ideal for village settings. The important points should be condensed into a two page maximum information sheet and a one page consent form. In this way consent should focus on the critical points e.g. risks and benefits to individuals, randomisation, uncertain efficacy. Where ICH-GCP compliance and being appropriate for the target population are both possible they should both occur, but being appropriate for the local population should be the priority.

9) It must be specifically stated that volunteers are free to leave of their own accord during the study as this may be not be assumed.

10) All villages and volunteers involved in a study must be informed of the results of each study in a timely manner. Culturally there is an expectation for villages to be informed through the mechanism of village meetings. In addition any promises made by researchers or field staff such as results of blood tests etc. must be noted and kept meticulously.

5.14 Quality Control, Toxicology, Potency and Stability

Extensive pre-clinical identity, purity, biodistribution, gross pathology and histopathology testing was performed on samples from lots of all three vaccines before all clinical trials as part of MCA submissions. In addition a potency experiment was performed on doses of vaccines from the clinical lots prior to start of each clinical trial. Three groups of 4 female BALB/c mice were immunised on day 0 with either 50μg of DNA ME-TRAP intramuscularly, 2μg of DNA ME-TRAP by gene gun or $1x10^6$ pfu of MVA ME-TRAP or FP9 ME-TRAP intradermally. Those that received DNA ME-TRAP receive a further 50μg or 2μg respectively on day 14. The number of γ-interferon secreting pb9-specific CD8+ T cells in freshly prepared mouse splenocytes was determined by the *ex vivo* ELISPOT(Schneider et al., 1998). These assays were repeated 3 monthly for each clinical lot to ensure vaccines did not degrade during storage.

Chapter 6

Safety of DNA ME-TRAP and MVA ME-TRAP in Malaria-naïve Adults

This phase 1 trial has been reported (Moorthy et al., 2003a).

6.0 Introduction

This series of UK Phase I trials provides, to my knowledge, the first data on immunisation with a recombinant MVA malaria vaccine, the first data on DNA prime-recombinant viral boost malaria immunisation in humans and the first data on administration of a polyepitope infectious disease vaccine in humans.

In this section, I evaluate the safety and tolerability of intramuscular DNA priming followed by intradermal MVA boosting. In addition I evaluate the safety and tolerability of priming by DNA delivered intradermally by a needleless delivery system followed by intradermal MVA boosting.

6.1. Methods

6.1.1 Volunteers

Forty-three healthy, male or female volunteers aged 18-55 were recruited from the communities surrounding the clinical trial site in Oxford, UK. Recruitment was by placement of posters in designated sites and advertisements in the local press. Each volunteer received a detailed information sheet by post concerning the study. The potential risks were discussed with the volunteers. The research protocol and appended submissions were reviewed and approved by OXREC and MCA. All volunteers gave written informed consent.

Volunteers were screened by clinical evaluation for good physical and mental health. Screening consisted of a medical history, physical examination, urinalysis, complete blood count, anti-nuclear antibody, anti-double stranded DNA antibody, HIV-1 antibody, hepatitis B serology and a biochemistry screen including measurement of liver enzymes and creatinine. For females a urine beta-HCG pregnancy test was performed on the day of the first vaccination and prior to each vaccination.

Exclusion criteria included positive pregnancy testing, serological evidence of HIV or hepatitis B infection, clinically significant haematological, hepatic, renal, cardiac, respiratory, dermatological, gastrointestinal, autoimmune, neurological or psychiatric disorder and drug or alcohol abuse. Allergy to gold or previous gold therapy were exclusion criteria for vaccination by needless delivery.

6.1.2 Vaccines

The study vaccines were DNA ME-TRAP and MVA ME-TRAP as described (see chapter 4). The clinical lot of DNA ME-TRAP for intramuscular administration consisted of vials containing 1mg DNA ME-TRAP in 1ml of phosphate-buffered saline. DNA ME-TRAP was stored at –20 ºC and thawed for 30 minutes at room temperature prior to administration. The DNA vaccine was administered through a 23-gauge needle intramuscularly.

MVA ME-TRAP was stored at –80 ºC and vials were thawed for 30 minutes at room temperature prior to vaccination. The clinical lot consisted of 0.2ml vials containing $1x10^8$ pfu. The MVA vaccine was administered intradermally through a 27 gauge needle in a volume of 0.1ml.

6.1.3 Administration of DNA vaccine by gene gun

The Powderject Pharmaceuticals XR1 needleless delivery device has previously been used to administer a hepatitis B DNA vaccine to 19 volunteers in the USA(Tacket et al., 1999). The Powderject Pharmaceuticals device is the version of the device (sometimes called a "gene gun") for which there has been the greatest experience in clinical trial use. Gene gun technology uses a gas-driven ballistic bombardment device that propels gold particles coated with plasmid DNA into the skin. These gold particles are propelled directly into the cytosol of epidermal dendritic cells, improving delivery of plasmid DNA to the nucleus for transcription. This results in transgene expression levels higher than those obtained by comparable doses of "naked DNA". This mode of immunisation produces protective immunity in several animal models(Fynan et al., 1993, Han et al., 2000). DNA ME-TRAP was precipitated onto microscopic gold beads under GMP conditions. The gold beads carrying DNA were helically coated onto the internal surface of a 74 cm length of tefzel tubing, which was dried and cut into one-half inch segments. A coated segment, or 'cartridge', is inserted into a polycarbonate nozzle, approximately the size of a 12 ml syringe, and the cartridge/nozzle unit is sealed with vacuum in a pouch. Each cartridge carried 1 μg of DNA on 500 μg of gold particles. Prior to vaccination the skin of the proximal, posterior upper arm was prepared with alcohol. Each dose of vaccine consisted of a total of 4 μg of DNA ME-TRAP on 2mg of gold particles, given as 4 administrations of one cartridge each to 4 adjacent sites. The helium delivery pressure was 500 psi.

6.1.4 Study design

This is an open-label, dose-ranging, route-finding series of phase I clinical trials in healthy adult volunteers. Intramuscular DNA ME-TRAP and intradermal MVA ME-TRAP were administered to the non-dominant deltoid region and then to alternate arms for booster doses. Scheduled vaccination intervals were 3 weeks, although exceptionally an interval of up to 4 weeks occurred. Table 6.1 details vaccination regimes by groups. Note that three individuals in group 1 and two in

group 2 went on to received 2-3 intradermal MVA immunisations 2-6 months after their final DNA immunisation. Ascending group number corresponds to chronology such that assessment of safety and tolerability was made before increasing the dose of intramuscular DNA ME-TRAP.

6.1.5 Assessment of safety and tolerability

Volunteers in groups 1,2 and 3 were observed for 3 hours after each immunisation with vital signs monitored. Volunteers in groups 4 to 9 were observed for 1 hour after immunisation. They were then seen for assessment at day 7 and 28 and again 6 months (or 4 months for groups 4 to 9) after final immunisation. At each of the follow-up visits detailed history and physical examination, including vital signs were done. All symptoms and signs were considered adverse events and graded as follows: mild if no limitation of activities of daily living (ADL); moderate if limitation but not prevention of ADL; severe if prevention of ADL. On the day of each immunisation and seven days after each immunisation, blood was obtained for full blood count, anti-nuclear antibodies and anti-double stranded DNA antibodies and the following plasma biochemistry: sodium, potassium, urea, creatinine, alanine aminotransferase, aspartate aminotransferase, albumin, total bilirubin, γ-glutamyl transferase, calcium, magnesium, phosphate, urate and amylase. These investigations were also performed on venous blood obtained 4 weeks and 6 (or 4) months after final immunisation.

6.2 Results

6.2.1 Study population

Forty-three volunteers were enrolled and assigned to groups 1-9 sequentially. Thirty-three men and ten women participated in the study. Median age was 27 (range 21-54). All volunteers were Caucasian. One of the volunteers had spent some time in a falciparum-endemic region. This volunteer was in group 2 and had spent 1 month in Uganda in 1998. He had not had clinical malaria. Six volunteers had vaccinia scars. The participants were either students, biomedical

researchers, clinicians, nurses or other hospital staff or employees of other non-biomedical University of Oxford Departments. All of the volunteers in groups 1 and 2 received their 3 vaccinations, but 3 of those in group 1 were not traceable for the final 6 month follow-up. One volunteer in group 3 was withdrawn after first vaccination because he travelled to a falciparum-endemic country during the study. All volunteers in groups 4 to 9 received all planned vaccinations and completed the study.

6.2.2 Safety Overview

All doses of both vaccines were well tolerated when given by all three routes in all volunteers. No changes occurred in vital signs during the observation period and during the follow-up period. Most adverse events were mild (table 6.2). There were 10 moderate adverse events as follows. 4 episodes of mild flu-like episodes consisting of headache, myalgia and malaise. 3 of these episodes occurred the day after the volunteer's first MVA immunisation. During 2 of these 3 episodes, pyrexias were documented of 37.5 ºC and 37.8 ºC. We have therefore documented 3 mild flu-like reactions of duration less than 24 hours to MVA immunisations out of 40 total MVA immunisations in this series of trials. The fourth episode occurred two days after the volunteer's first DNA immunisation. In all 4 cases, the volunteers experienced only mild limitation of activity (still being able to attend work) and were entirely well the day after the episode began.

There were 3 episodes of moderate headache the day after each MVA immunisation in the same volunteer, always of duration of less than 1 day. There was 1 episode of painful lymphadenopathy 2 days after first MVA immunisation in a volunteer who had received three previous DNA immunisations. On examination during the episode two tender 1.5cm right supraclavicular lymph nodes were palpable. There was no restriction of arm movement and no other symptoms or signs. The lymph nodes were no longer palpable on the seventh day after immunisation. There was 1 episode of lethargy from the first to the fifth day after first DNA immunisation. On the fourth day this volunteer was marching

as part of a military training exercise and suffered a brief vaso-vagal episode with loss of consciousness for a few seconds. On clinical assessment including laboratory testing there were no abnormalities on the seventh day. This episode was classified as unrelated to vaccination. There was 1 episode of intermittent nausea, weakness and leg myalgia with groin pain in a male volunteer lasting for 6-8 hours on the third, sixth and ninth day after his first MVA immunisation following one previous DNA immunisation. On clinical assessment on the ninth day the volunteer was well, pyrexial at 38.0 ºC with no other clinical signs and normal genital and inguinal examination. By the tenth day he was entirely well and remained so. This episode was classified as unrelated to vaccination.

There were no significant laboratory abnormalities attributable to vaccination including anti-nuclear antibodies and anti-double stranded DNA antibodies. Swabs taken from MVA sites 3 hours after injection and fluid withdrawn from a blister on the third day after a MVA immunisation were assayed for presence of infectious MVA ME-TRAP by culture in chicken embryo fibroblasts which are permissive for MVA replication. No MVA ME-TRAP was detected. This suggests that there is no or minimal risk of persistence of the recombinant organism in the environment.

6.2.3. Local reactions to MVA ME-TRAP administered intradermally

A total of 52 doses were given to 22 volunteers in groups 1-7. All sites showed absorption of the intradermal fluid within 30 minutes and onset of redness at the site by 1 hour. Redness, sometimes with induration, developed to maximal size at 48 to 72 hours from vaccination. These were either mildly tender on firm pressure or, in most cases, non-tender. There was little or no warmth. There was no limitation of arm movement. The maximum diameter of the redness on the seventh day after vaccination varied from 5 mm to 21 mm. The redness faded to a faint erythema, which rarely was visible four weeks post vaccination. 33% (17/52) of vaccinations were associated with mild pain at some time during the first seven days after vaccination. 32% (16/52) were associated with mild pruritus

during this period. A 1 to 2 mm blister occurred at 15% of sites (8/52) by the seventh day. Blisters healed without complication over three weeks in all cases:

6.2.4 Local reactions to DNA ME-TRAP administered by Powderject needleless delivery device.

A total of 30 doses of 4 μg DNA ME-TRAP were given to 12 volunteers. Therefore a total of 120 cartridges were administered. Erythema sometimes with a sensation of mild tingling or burning developed within minutes at all sites. The erythema was of maximal diameter between 30 minutes and 3 hours. The range for maximal diameter was 17-28 mm. The erythema resolved over 4-7 days leaving hyperpigmentation. This hyperpigmentation was rarely still visible at day 70, in one case co-existing with a faint erythema. In this one case there was hyperpigmentation and faint erythema at up to 10mm diameter at all 12 sites, which has persisted to day 224. Mild dermal oedema is seen within 30 minutes and resolved by day 7. Mild pruritus was experienced after 27% (8/30) of doses.

6.3. Discussion of safety and reactogenicity data

Two specific safety concerns have been raised with reference to DNA immunisation: integration oncogenesis and potential induction of autoimmunity(Mor et al., 1997). Several pre-clinical studies have measured the number of copies of plasmid DNA covalently linked to genomic DNA after intramuscular plasmid DNA injection in mice. It is unknown whether this linked DNA represents integration but if it is assumed that all bound DNA integrates, one arrives at a figure of integration 1000 times less than the spontaneous mutation rate(Ledwith et al., 2000). Whilst this issue now seems extremely unlikely to be a risk to volunteers, it will be necessary to encourage reporting of any possible long-term adverse events once DNA vaccines have been given in larger scale trials and post-licensing. The possibility of autoimmunity now looks similarly remote. Anti-double stranded DNA antibodies are often associated in clinical practice with the connective tissue disorder systemic lupus erythematosis, a multi-system autoimmune condition with a variable clinical course which can

result in death. In mouse studies it has been very difficult to raise anti-DNA antibodies through administration of plasmid DNA preparations(Parker et al., 1999). After injection of crude *E. coli* denatured DNA preparations in a very strong adjuvant, Freund's adjuvant, antibodies can be raised but these are to single-stranded DNA. Such antibodies are not associated with clinical disease in humans. In clinical trials to date no double-stranded anti-DNA antibodies have been induced after DNA immunisations with up to 2.5mg of plasmid DNA. This chapter adds to the negative body of data in this area.

We show that intradermal administration of a malaria DNA vaccine by needleless delivery device has an acceptable safety profile. Currently, however, there are greater limits to the quantity of DNA which can be delivered intradermally than intramuscularly.

We report the safety profile of recombinant MVA in humans either with or without prior DNA immunisations. The small number of acceptable adverse events is comparable with viral vaccines in general use. Indeed there is scope for increasing the dose of MVA.

Table 6.1. Composition of vaccination groups

Group	Trial Code	Regime	DNA			Interval to boost	MVA			No.
			Dose µg	Dose µg	Dose µg		$\times10^7$ pfu	$\times10^7$ pfu	$\times10^7$ pfu	
1	VAC001	DDD(0.5)	500	500	500					6
2	VAC001	GGG	4	4	4					6
3	VAC002	MMM(3)					3	3	3	6
4	VAC003	MMM(3)					3	3	3	5
5	VAC003	DDD(1)M(3)	1000	1000	1000	3	3			3
6	VAC003	D(1)MM(3)	1000			3	3	3		3
7	VAC003	DDD(1)	1000	1000	1000					5
8	VAC005	DD(1)MM(3)	1000	1000		3	3	3		3
9	VAC005	GGMM(3)	4	4		3	3	3		6
10*	VAC010	MM(15)					15	15		8
11*	VAC010	DDD_MM(15)	2000	2000	2000	8	15	15		5
12*	VAC010	DDDMM(15)	2000	2000	2000	3	15	15		4

D - intramuscular DNA ME-TRAP vaccination
G - gene gun DNA ME-TRAP vaccination
M - intradermal MVA ME-TRAP vaccination
Interval between prime and boose shown above; all other intervals were 3 weeks.
Numbers in brackers refer to the dose administered at each vaccination; in mg for DNA and in $\times10^7$ pfu for MVA. As examples:
DDD(0.5) indicates three intramuscular DNA ME-TRAP vaccinations given at weeks 0, 3 and 6; each at a dose of 500µg.
DD(1)MM(3) indicates the group who received two 1mg DNA ME-TRAP vaccinations at weeks 0 and 3 followed by two 3×10^7 pfu MVA ME-TRAP vaccinations at weeks 6 and 9.
The _ symbol in DDD_MM(15) indicates an interval of 8 weeks between third immunization and fourth immunization, rather than the 3 week intervals between all other immunizations. Hence DDD_MM(15) indicates a group who received three 2mg DNA ME-TRAP immunizations at weeks 0,3 and 6 followed by two 15×10^7 pfu (or 1.5×10^8 pfu) MVA ME-TRAP immunizations at weeks 14 and 17.

*safety data not presented

Table 6.2. Solicited local adverse events over 7 days after immunisation.

	Local Pain	Pruritus	Redness	Induration	Blister	Other
Group 1	DDD(0.5) - 6 individuals					
Inj 1 (D)	0	0	0	0	0	1
Inj 2 (D)	0	0	0	0	0	0
Inj 3 (D)	0	0	0	0	0	0
Group 2	GGG - 6 individuals					
Inj 1 (G)	3	4	6	0	1	0
Inj 2 (G)	2	4	6	0	0	0
Inj 3 (G)	3	2	6	0	0	0
Group 3	MMM(3) - 6 individuals					
Inj 1 (M)	3	2	6	4	3	1
Inj 2 (M)	1	1	6	0	0	0
Inj 3 (M)	0	0	6	0	0	0
Group 4	MMM(3) - 5 individuals					
Inj 1 (M)	4	3	5	5	1	1
Inj 2 (M)	0	1	5	1	0	1
Inj 3 (M)	0	1	5	0	0	1
Group 5	DDD(1)M(3) - 3 individuals					
Inj 1 (D)	0	0	0	0	0	0
Inj 2 (D)	0	0	0	0	0	0
Inj 3 (D)	0	0	0	0	0	0
Inj 4 (M)	3	1	3	1	0	2
Group 6	D(1)MM(3) - 3 individuals					
Inj 1 (D)	0	0	0	0	0	2
Inj 2 (M)	3	1	3	1	1	1
Inj 3 (M)	1	1	3	1	0	0
Group 7	DDD(1) - 5 individuals					
Inj 1 (D)	0	0	0	0	0	0
Inj 2 (D)	0	0	0	0	0	0
Inj 3 (D)	0	0	0	0	0	0

Numbers refer to the number of volunteers positive for the symptom or sign.
D - intramuscular DNA ME-TRAP vaccination
G - gene gun DNA ME-TRAP vaccination
M - intradermal MVA ME-TRAP vaccination

Chapter 7

Immunogenicity and Efficacy of DNA ME-TRAP and MVA ME-TRAP in Malaria-naive Adults

The data for this chapter published in Nature Medicine (McConkey et al., 2003: 3 joint first authors; McConkey, Reece, Moorthy).

7.1 Methods

7.1.1 Study Population and Procedures

All volunteers whose safety profiles were outlined in Chapter 6 are included in the immunogenicity and efficacy measurements. In addition three further groups of volunteers are included. The dose, route and order of immunizations in these groups is described in table 1, chapter 6. All immunization intervals were 3 weeks other than the interval between final DNA and first MVA immunization in group 11 which was 8 weeks (this timepoint is abbreviated as DDD_M(15), whereas DDDM(15) represents the same immunization regimen with a 3 week interval in group 12). Blood was drawn pre-vaccination (sometimes referred to as day 0 or baseline), 7 days after each immunization and 28 and 150-300 days after final vaccination. *Ex vivo* γ-interferon ELISPOT assays, anti-TRAP and anti-R32LR antibody ELISAs were performed as described in Chapter 5. Cell separations were performed on frozen/thawed cells using Miltenyi Biotech MACS beads and columns according to the manufacturer's instructions. Separated and unseparated populations were co-stained with BD anti-CD3, anti-CD4 and anti-CD8 antibodies to allow checking of the purity of the separations.

7.1.2 Analysis of Immunogenicity

See section 5.0.1

7.1.3 Challenge with P. falciparum *Sporozoites*

Asexual blood-stage *P. falciparum* parasites of the well characterized 3D7 clone of the NF54 strain were provided to Geoff Butcher by David Walliker of Edinburgh University. NF54 was derived from a sample taken from a Caucasian female who was infected near to an airport in the Netherlands by a mosquito thought to have originated in West Africa. The 3D7 parasites were expanded *in vitro* and enriched for the gametocyte fraction. Laboratory reared *A. stephensi* mosquitoes were membrane fed 3D7 gametocytes and seven days later a sample of the mosquitoes' mid guts were dissected and oocyst counts performed. 14-21 (typically 19-21) days after the membrane feeding groups of mosquitoes thought to be infected were allowed to feed for 5 minutes on unvaccinated control volunteers and vaccinees. Mosquitoes were dissected to check for feeding and salivary gland infection. This process was repeated until 5 mosquitoes each with 10^2-10^4 sporozoites per salivary gland were known to have fed on each volunteer. These challenges took place 14-37 days after final vaccination (see table 7.1). From the sixth day after challenge volunteers were monitored twice daily with microscopy of thick smears for blood-stage infection. Smears were read to 200 fields (or more if volunteers were symptomatic) before being declared negative. Subjects were given a standard adult treatment course of chloroquine after the first positive blood smear. There was no significant difference in time to infection between the controls in the three challenges using the log-rank test.

7.2 Results

7.2.1 Homologous vaccination induces low but detectable effector T cell responses

Repeated vaccination with either DNA ME-TRAP (p value = 0.07 for all peptides in construct) induces responses by *ex vivo* ELISPOT above pre-vaccination values. These are comparable to those seen with DNA vaccine recombinant for Pf CS previously(Wang et al., 2001). The amplitude of these responses is given in table 7.2. Some of these responses are barely detectable and tend to be towards the minimum threshold for detection by *ex vivo* ELISPOT which is

approximately 10 SFCs per million PBMCs (K Flanaghan DPhil Thesis 2000). Repeated MVA ME-TRAP immunizations induce comparable low levels of effector T cells (p=0.04 for all peptides comparison with pre-vaccination values).

7.2.2. Heterologous prime-boost vaccination induces higher effector T cell frequencies than homologous vaccination

A single MVA ME-TRAP immunization at 3 x 10^7 pfu induces increases in T cell response, most markedly after gene gun DNA immunization (see figure 7.1). A single 1.5 x 10^8 pfu MVA ME-TRAP immunization after three 2mg i.m. DNA ME-TRAP immunizations induces very large increases in effector T cell responses (p value = 0.0003, all peptides). The responses are strongest to T9/96 peptides but strong 3D7 responses and weaker ME string responses (p value = 0.01) are also seen.

7.2.3 Dose, route and interval affect Immunogenicity

Delivery of DNA ME-TRAP by gene gun at 4µg is more immunogenic as a priming immunization for subsequent 3x10^7 pfu MVA ME-TRAP boosting than i.m. DNA at 1mg (p value = 0.01). The peak induced responses in the 9 volunteers who received three 2mg i.m. DNA immunizations followed by MVA at 1.5 x 10^8 pfu were higher than those in the 9 individuals who received either one, two or three DNA immunizations at 1mg followed by MVA at 3 x 10^7 pfu (p value = 0.02). No statistically significant difference in response magnitude was observed in comparison of intervals between DNA and MVA (8 weeks vs 3 weeks).

7.2.4 Breadth of the induced effector T cell response

Responses were induced to all peptide pools (see figure 7.4 and 7.5). Strongest responses for one donor at one timepoint after boosting were generally seen in one or two T9/96 peptide pools with subdominant responses in other T9/96 pools, generally weaker responses in 3D7 pools and to CD4+ T cell epitopes in the ME string and weakest to CD8+ T cell epitopes in the ME string. Tissue

typing was performed and on average each volunteer possessed HLA alleles allowing recognition of 1 CD8+ T cell epitope from the ME string. The results are consistent with the ability of volunteers to recognize several epitopes from within the whole TRAP antigen leading to immunodominance of this region over the CD8+ T cell epitope(s) recognized from the ME string.

7.2.5 The T cell responses persist for several months

In the GGMM group in whom the longest follow-up has been documented the T cell response was 38% of the peak at 5-11 months and 61% of the plateau level (day 21-28) at the 5-11 month timepoint (figure 7.2). In the high dose DDDMM groups the response was 86% of the peak response at 6 months (156 geometric mean SFCs per million PBMCs)

7.2.6 Both CD4+ and CD8+ T cells were induced by prime-boost vaccination

Depletion assays on cells cryopreserved 7 days after first MVA immunization in the GGMM group showed that both CD4+ and CD8+ T cell dependent responses were induced by this vaccination regimen (figure 7.3). 4 of the group had CD4+ T cell responses and 2 had CD8+ T cell responses. In the DDDMM groups depletions showed that the responses were mainly CD4+ T cell dependent.

7.2.7 Delay in time to parasitaemia in the highest immunogenicity prime-boost groups

Volunteers in the GGMM and the two high dose DDDMM groups, but not those receiving lower dose or homologous regimens had a significant delay in time to parasitaemia (p=0.013, log rank test).

7.2.8 Limited antibody induction by DNA/MVA vaccination in humans

Many vaccinees had no detectable anti-CS or anti-TRAP antibodies after vaccination. One vaccinated subject had a four-fold rise in antibody titre, and two others had a two-fold rise in titre (see table 7.3). Four subjects developed low-

titre antibody responses to the NANP repeat epitope in the vaccine. These low titre antibody responses did not correlate with protection.

7.3 Discussion

When the studies detailed in this chapter were designed, the aim was to identify a candidate vaccination regimen for efficacy evaluation including DNA ME-TRAP and MVA ME-TRAP within a time frame appropriate to the scale of the disease burden. The following parameters all required evaluation: dose of DNA, route of DNA, number of DNA vaccinations, dose of MVA, number of MVA vaccinations, intervals between homologous vaccination and the interval between heterologous vaccination. The tensions in the choices for study design were: duration to completion of each study; the need to choose from many possibly protective regimens; group size as a statistical consideration. We decided to use a study design with many small groups (n=3-6). This allowed us to rapidly screen potential regimens and enabled identification of large differences in immunogenicity or protection at the expense of inadequate sample size to show statistical significance for smaller differences. With this scheme we arrived at the very high immunogenicity DDDMM(15) regimen within 18 months of the start of the first phase I trial. Even with the small group size the results provide very strong support for the hypothesis that heterologous DNA prime MVA boost (HP-B) immunization is more immunogenic for effector T cell induction than homologous immunization (the HP-B hypothesis).

Gene gun DNA immunization at 4μg is more immunogenic than 1mg DNA i.m. for subsequent MVA boosting (p=0.01 in small group comparison). If it were possible to increase the dose of gene gun delivery it may be that T cell frequencies even greater than those seen in the DDDMM(15) group could arise. Unfortunately this is not possible with current technology and the pharmaceutical firm have withdrawn their interest in the malaria DNA vaccine. If however conventional liquid DNA vaccine preparations could be concentrated, then evaluation of high

dose needle-administered i.d. DNA vaccination would be desirable. Currently it is not feasible to administer mg DNA quantities i.d.

Effector T cell frequencies are lower after second than first MVA immunizations in the DDDMM(15) (p=0.06 in group n=4). The same finding is seen in all other groups with two or more MVA immunizations. This drop is most likely to be due to the normal decline in T cell frequency over 28 days and indicates no boosting of second MVA immunization with a 3 week interval. I believe that this is due to a human immune response to MVA vector antigens which prevents infection of cells and recombinant protein expression for second MVA immunizations with this interval. The timecourse of this vector immunity is unknown.

DDDM(15) and DDD_M(15) were not statistically significantly different in their immunogenicity. Data in two primate models, DNA/adenovirus in a macaque model of Ebola virus (Sullivan et al., 2000) and DNA/MVA (Amara et al., 2000) in a macaque model of HIV infection, had suggested that a longer interval between DNA and MVA may be more immunogenic. Further studies with larger sample size are necessary to resolve the question of the optimum prime-boost interval in humans.

Our data also confirm the fact that dose titrations in humans are empirical and where reactogenicity and clinical considerations allow dose increases they are desirable for maximal Immunogenicity. 2mg of DNA followed by 1.5×10^8 pfu of MVA are more immunogenic than 1mg and 3×10^7 respectively. We have now reached the highest dose feasible with current formulations. Higher doses of MVA as well as DNA may be practical, safe and desirable if newer formulations can increase the concentration. 1×10^9 pfu MVA has been administered to macaques immunocompromised by total body irradiation, anti-thymocyte globulin administration or measles virus infection. No clinical, haematological or pathological abnormalities were detected during a 13-day follow up(Stittelaar et al., 2001). No replication-competent MVA was isolated from the macaques after

inoculation. Therefore higher doses are likely to be safe even in immunocompromised individuals.

However I should state which questions remain unanswered by our approach. The data do not allow us to state with certainty how many DNA priming immunizations (one, two or three) are most immunogenic for subsequent MVA boosting. Although there is an indication that a longer interval is less immunogenic than a shorter interval between DNA prime and MVA boost this has not been shown beyond doubt.

Some interpretation of the delay in time to parasitaemia with HP-B immunisation is necessary. Calculation of the likely reduction in parasite load required to cause a 2 day delay in parasitaemia suggests a >70% reduction in merozoites emerging from the liver in the HP-B vaccinees. If blood-stage multiplication is 8-fold over 48 hours (Simpson et al. 2002) then a 48 hour delay corresponds to a 8-fold reduction (or 87.5%) in parasites leaving the exo-erythrocytic stage. A 75% reduction in sporozoite inoculation leads to a 2 day prolongation of the pre-patent period in the *P. berghei* BALB/c model (RJ Anderson and AV Hill, unpublished data). These data suggest that the three highest immunogenicity groups developed partially effective immune responses against liver-stage Pf parasites. However this theoretical interpretation requires validation against natural infection in a field efficacy study (see chapter 11).

Could the delay in time to parasitaemia be a non-antigen specific effect? No delay is seen in homologous DNA or MVA vaccination groups. A non-specific effect of DNA followed by MVA immunization cannot be excluded although the biological basis for this is difficult to conceive. Ideally empty vectors would be available for mock immunization of controls, as commonly occurs in animal models. The cost of manufacture of empty vectors to cGMP precludes this at the moment. The final unlikely possibility for a spurious result is that the lack of formal blinding confounded the results. The physician investigators are aware of

vaccine allocation and interact with slide readers often. It is therefore conceivable that there could be some effect of unblinding on the results for delay in time to parasitaemia. Unblinding could not affect complete protection. It would be preferable if a formal single-blinding system were introduced for slide readers.

The *ex vivo* γ-interferon assay was strongly associated with protection in the BALB/c *P. berghei* model. Irradiated sporozoite-immunised and malaria-exposed adults possess T cells specific for liver-stage parasites with a γ-interferon phenotype. Such T cells are known to be able to bring about the apoptosis of infected hepatocytes in murine models by induction of reactive nitrogen intermediates(Mellouk et al., 1994) (Tsuji et al., 1995). However it is also possible that other effector T cell phenotypes such as perforin-mediated lysis and fas or granzyme A or B mediated apoptosis may be important. To date we have not ascertained whether or not *P. falciparum* liver-stage specific T cells from HP-B immunized volunteers are able to exhibit these phenotypes. In future HP-B studies in humans these other effector mechanisms could be assessed to exclude the possibility that a clear protective response of one of these alternative phentotypes is missed. However pragmatically there is a tension between how detailed the immunological studies are and how rapidly alternative regimens or constructs can be screened for efficacy in the IIa model. I believe the highest utility derives from identification of an optimally protective regime followed by characterization of the protective responses exhaustively.

The antibody induction presented is very weak and highly unlikely to account for the delay seen. Anti-CS titres (to the NANP repeat epitope) have been associated with protection after RTS,S/AS02 vaccination(Bojang et al., 2001) at several logs higher than titres seen in the few volunteers who seroconverted to TRAP or CS in the studies presented here. There are also several studies which show no association between antibody titres to CS and protection against infection or disease in field settings(Hoffman et al., 1987, Riley et al., 1990).

The interval between DNA and between MVA immunizations has been 3 weeks in all but one group, but with an acceptable window of up to 3 extra days. Ultimately a regimen with no more than 3 doses and intervals of 4 weeks would be desirable for incorporation into the WHO/UNICEF EPI infrastructure through which routine immunizations are administered to infants in many sub-Saharan countries. Although this infrastructure would need to be expanded in many countries, it would likely form the basis of delivery of an effective malaria vaccine to infants in Africa. 4 week intervals for HP-B regimens are likely to be as effective as 3 week intervals but this needs to be evaluated in future studies.

There are various methods under exploration for increasing immunogenicity of DNA vaccines through adjuvants. Co-administration of plasmids containing IL-2 or GM-CSF; delivery as PLG particles and various other techniques are under trial. MVA and other viral vectors will be improved as vectors through recombinant DNA manipulation. The intellectual property issues involved with developing a regimen involving several different patents is complex. For example some very desirable regimens could involve patents over individual optimized vectors, for vector combinations, for epitopes within constructs, for antigens, and for novel adjuvant, formulation and medical device technologies. In addition to intellectual property, development of these combinations may be slowed by complex regulatory pathways. Once a potential product is highlighted through what has been called "the proof of principle" it should be possible to perform a series of studies aimed at licensure even with complex combination regimens. Avoiding these complex regimens would speed licensure, but sufficient efficacy may not be possible with more simple regimens.

The data presented above represent an early body of data of heterologous DNA-based prime-boost vaccination in humans, which can form a basis for further work. The first hypothesis of this thesis (the HP-B hypothesis) is validated by the data presented or more accurately the repeated failure to falsify the hypothesis

provides strong grounds to believe it is true. The work presented in Chapters 8 and 9 address the second hypothesis.

Regimen	Interval to challenge (weeks)	Number challenged
MMM(3)	2-3	4
D(1)MM(3)	2-3	3
DDD(1)	2-3	5
DD(1)MM(3)	2-3	3
GGMM(3)	5-6	6
DDD_MM(15)	2-3	4
DDDMM(15)	3	4

Table 7.1: Interval between final vaccination and sporozoite challenge. See table 6.1 for clarification of nomenclature for vaccination regimen.

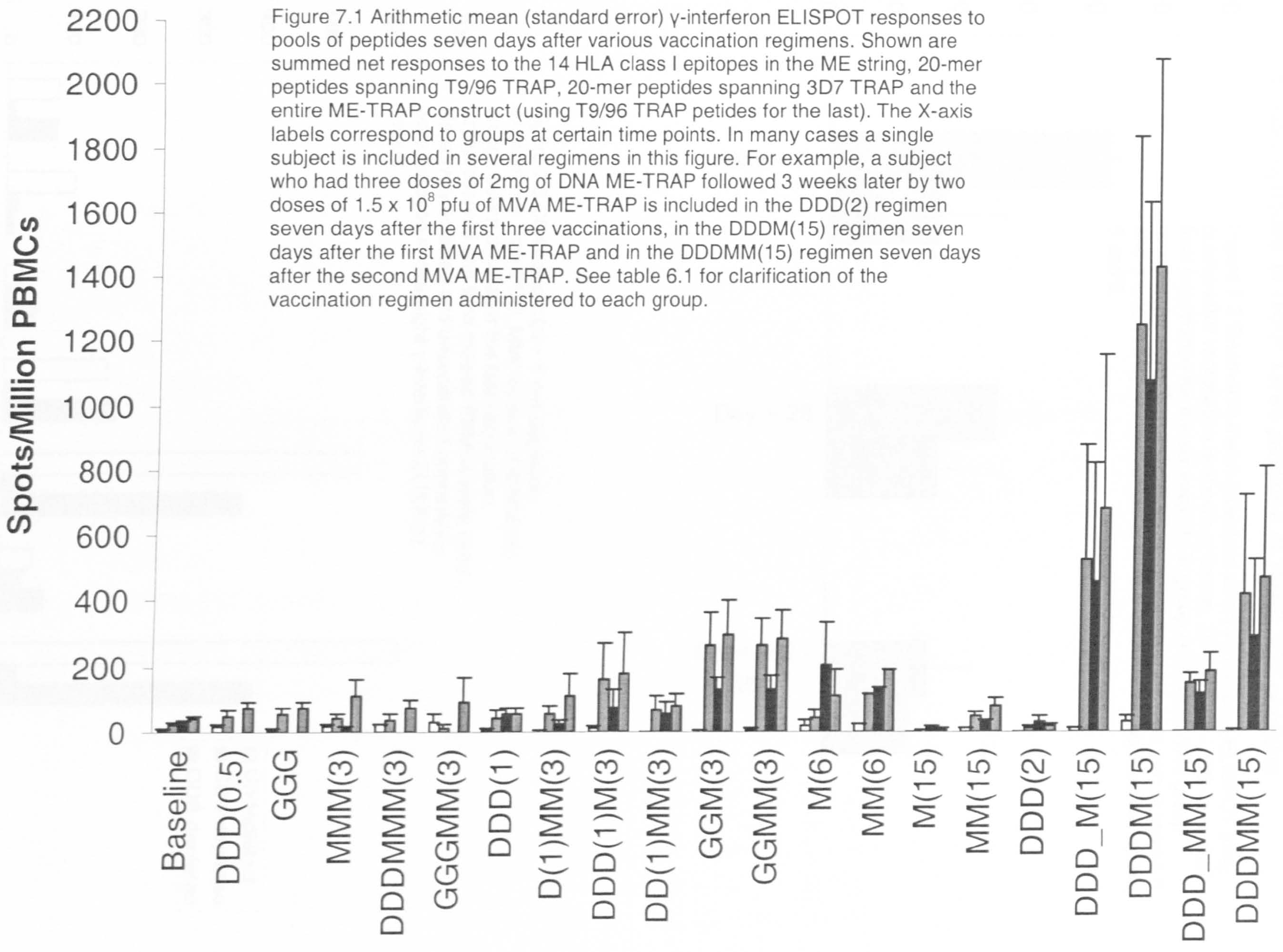


Figure 7.1 Arithmetic mean (standard error) γ-interferon ELISPOT responses to pools of peptides seven days after various vaccination regimens. Shown are summed net responses to the 14 HLA class I epitopes in the ME string, 20-mer peptides spanning T9/96 TRAP, 20-mer peptides spanning 3D7 TRAP and the entire ME-TRAP construct (using T9/96 TRAP petides for the last). The X-axis labels correspond to groups at certain time points. In many cases a single subject is included in several regimens in this figure. For example, a subject who had three doses of 2mg of DNA ME-TRAP followed 3 weeks later by two doses of 1.5×10^8 pfu of MVA ME-TRAP is included in the DDD(2) regimen seven days after the first three vaccinations, in the DDDM(15) regimen seven days after the first MVA ME-TRAP and in the DDDMM(15) regimen seven days after the second MVA ME-TRAP. See table 6.1 for clarification of the vaccination regimen administered to each group.

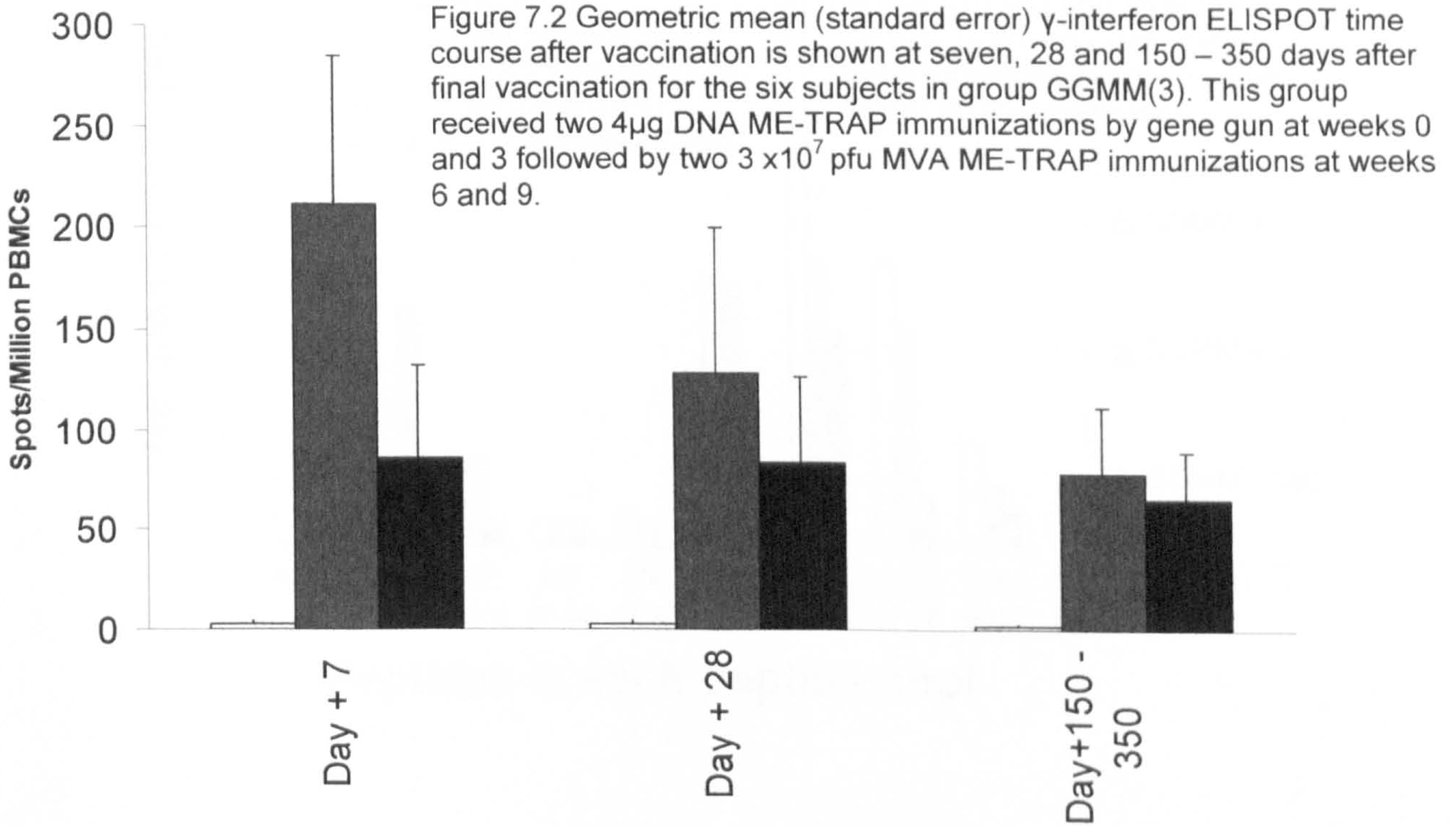


Figure 7.2 Geometric mean (standard error) γ-interferon ELISPOT time course after vaccination is shown at seven, 28 and 150 – 350 days after final vaccination for the six subjects in group GGMM(3). This group received two 4μg DNA ME-TRAP immunizations by gene gun at weeks 0 and 3 followed by two 3 x10^7 pfu MVA ME-TRAP immunizations at weeks 6 and 9.

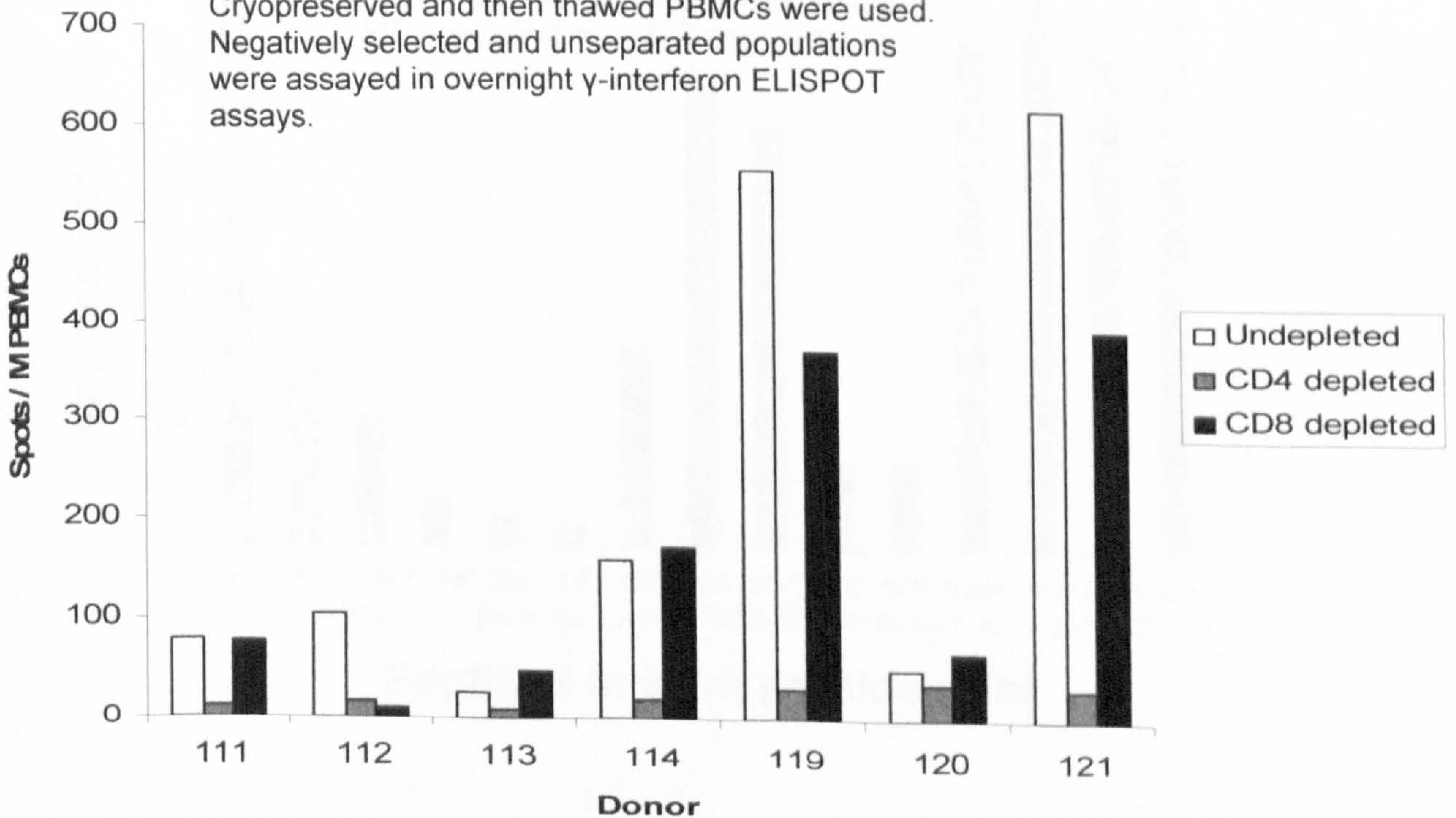


Figure 7.3 CD4+ and CD8+ T cell depletion experiments from DDD_MM(15) and DDDMM(15) groups seven days after the last vaccination. Cryopreserved and then thawed PBMCs were used. Negatively selected and unseparated populations were assayed in overnight γ-interferon ELISPOT assays.

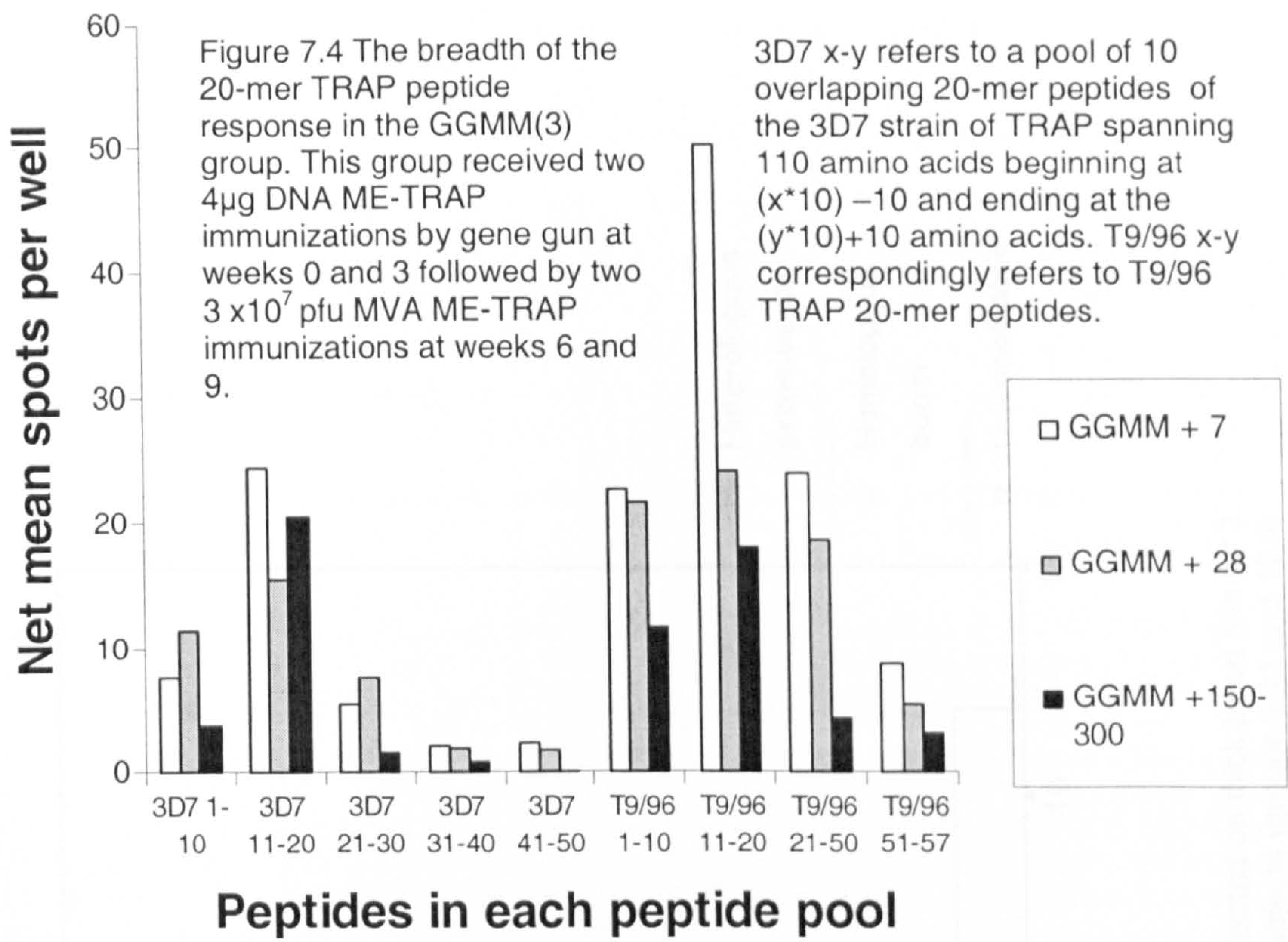


Figure 7.4 The breadth of the 20-mer TRAP peptide response in the GGMM(3) group. This group received two 4μg DNA ME-TRAP immunizations by gene gun at weeks 0 and 3 followed by two 3 x10^7 pfu MVA ME-TRAP immunizations at weeks 6 and 9.

3D7 x-y refers to a pool of 10 overlapping 20-mer peptides of the 3D7 strain of TRAP spanning 110 amino acids beginning at (x*10) –10 and ending at the (y*10)+10 amino acids. T9/96 x-y correspondingly refers to T9/96 TRAP 20-mer peptides.

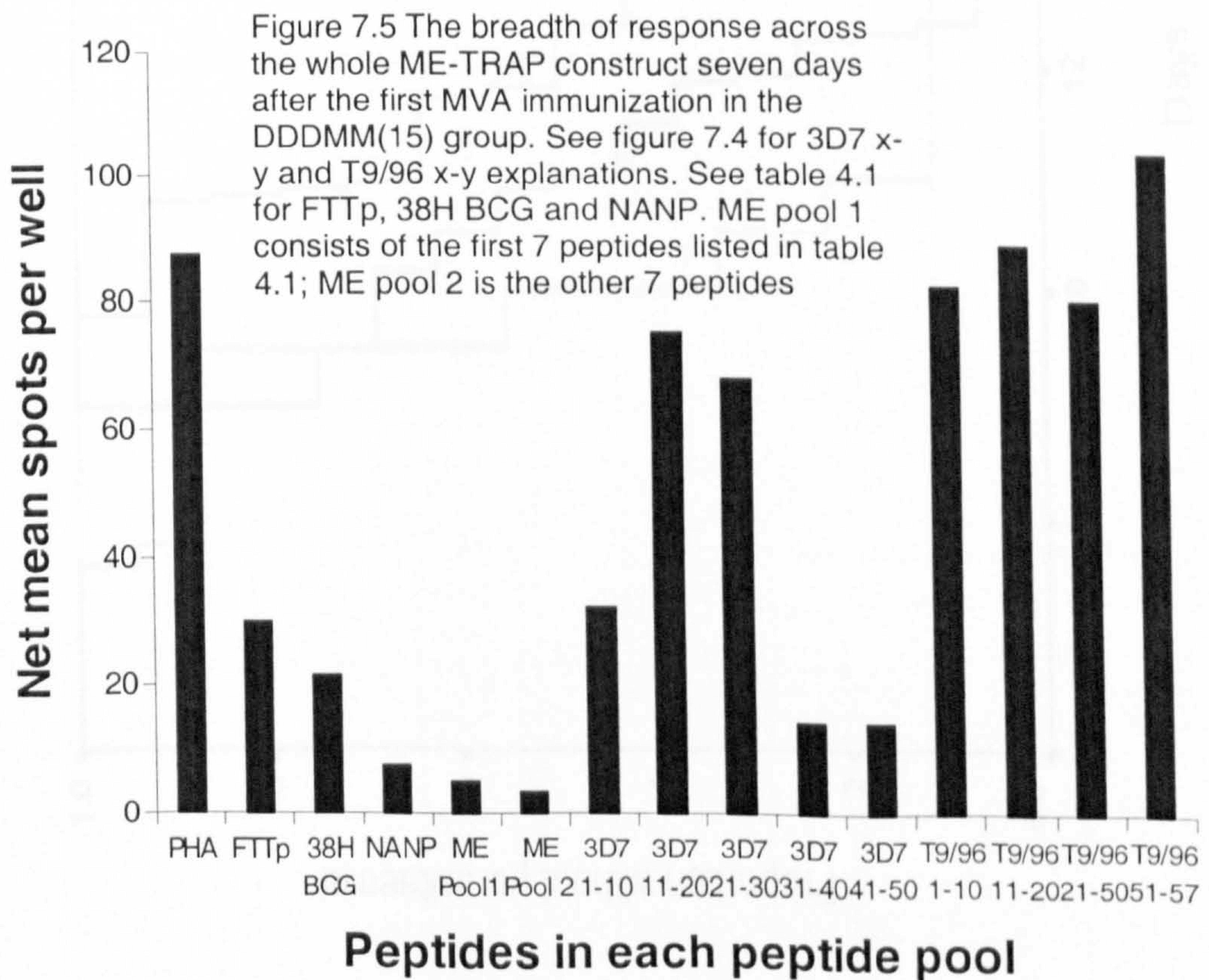


Figure 7.5 The breadth of response across the whole ME-TRAP construct seven days after the first MVA immunization in the DDDMM(15) group. See figure 7.4 for 3D7 x-y and T9/96 x-y explanations. See table 4.1 for FTTp, 38H BCG and NANP. ME pool 1 consists of the first 7 peptides listed in table 4.1; ME pool 2 is the other 7 peptides

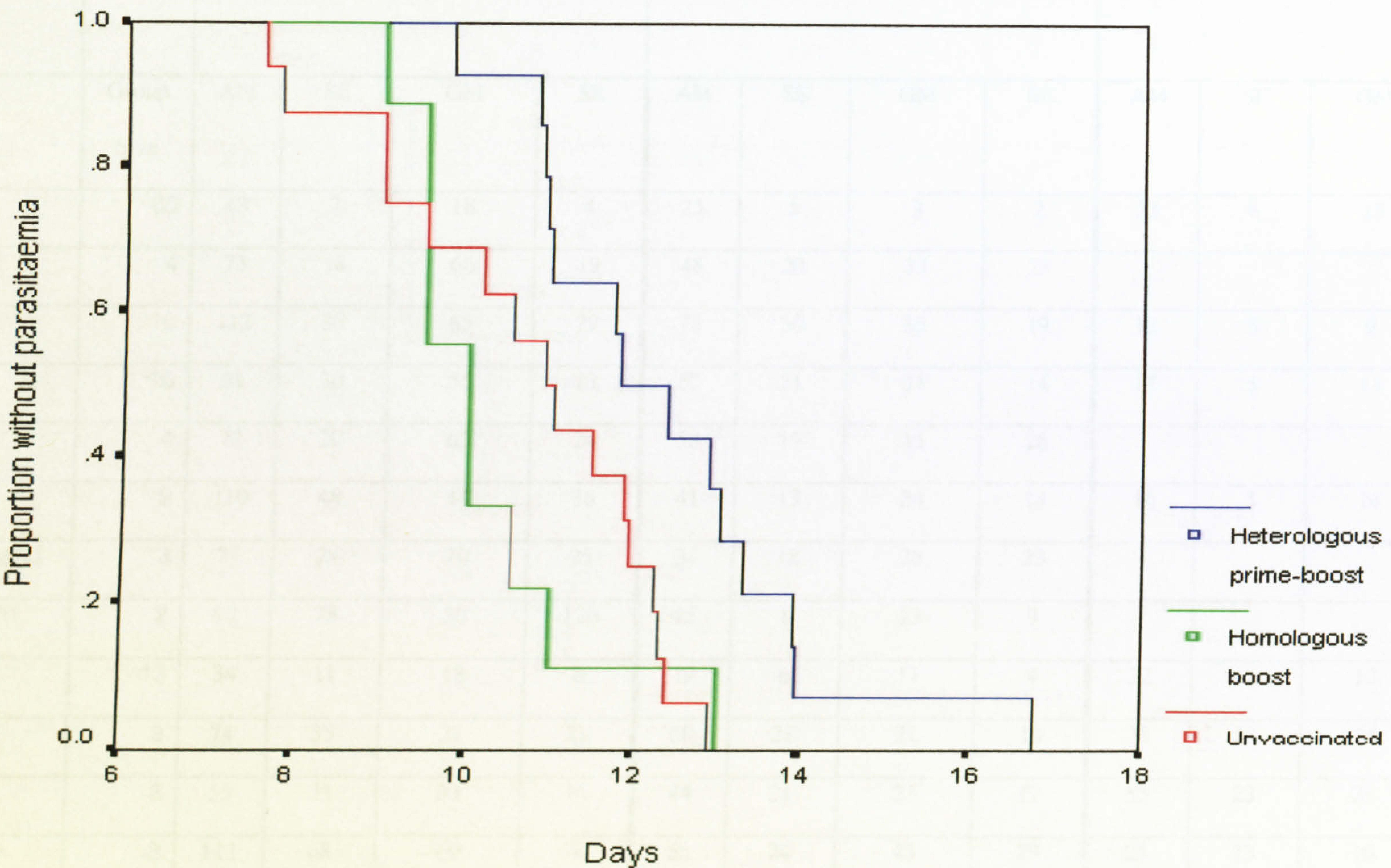


Figure 7.6 Kaplan-Meier curves of time from sporozoite challenge to parasitaemia detected on thick blood film for 3 groups; 16 unvaccinated control subjects, 14 heterologous prime-boost vaccinated subjects (groups 9,11 and 12 in table 7.1), these being the groups with the strongest immune responses, and 9 vaccinated subjects who received just one of the vaccines (groups 4 and 7 in table 7.1). Development of parasitaemia is delayed significantly in the heterologous prime-boost immunisation group (log rank test, $P = 0.013$) compared to the unvaccinated controls but there is no significant difference in time to parasitemia comparing the volunteers who received DNA only or MVA only and the unvaccinated group.

Table 7.2 (see page 107 for legend to this table)

		All peptides in vaccines				T9/96 TRAP				3D7 TRAP			
Vaccine Regimen	Group Size	AM	SE	GM	SE	AM	SE	GM	SE	AM	SE	GM	SE
Baseline	65	43	7	18	4	25	5	9	2	33	6	15	3
DDD(0.5)	4	73	18	66	19	48	20	33	23				
G	10	112	36	65	29	78	30	35	19	13	5	9	7
GG	10	91	30	50	23	57	21	31	14	17	5	14	6
GGG	4	72	20	63	24	58	19	45	26				
MMM(3)	9	110	48	44	36	41	13	24	14	16	3	14	4
DDDMMM(3)	3	77	24	70	25	38	18	28	23				
GGGMM(3)	2	92	78	50	120	15	8	13	9				
D(1)	13	34	11	18	8	19	6	11	4	22	6	13	5
DD(1)	9	74	35	27	21	60	28	21	16	38	17	14	10
DDD(1)	8	55	23	33	16	44	21	25	12	55	23	28	17
D(1)MM(3)	3	112	68	69	78	55	24	43	29	25	13	16	18
DDD(1)M(3)	3	180	122	104	118	162	112	90	109	75	52	46	45

Table 7.2 continued

Vaccine Regimen	Group Size	AM	SE	GM	SE	AM	SE	GM	SE	AM	SE	GM	SE
DD(1)MM(3)	3	79	41	51	56	69	41	21	76	56	36	18	60
GGM(3)	6	297	108	170	124	266	100	148	110	127	41	87	46
GGMM(3)	6	288	83	234	77	265	80	212	73	128	44	85	47
M(6)	2	109	85	68	126	44	27	35	36	208	129	163	174
MM(6)	1	195		195		119		119		139		139	
M(15)	2	5	5	3	7	3	3	2	4	12	5	11	6
MM(15)	4	83	24	74	24	50	10	47	11	32	7	29	9
D(2)	7	22	2	21	2	13	3	10	5	25	5	21	7
DDD(2)	8	19	5.7	14	5	12	4	8	3	33	19	14	9
DDD_M(15)	4	684	474	372	289	528	350	302	226	461	363	195	194
DDDM(15)	4	1430	654	708	1030	1249	593	617	880	1078	555	363	881
DDD_MM(15)	5	188	53	158	53	150	29	137	34	118	35	98	36
DDDMM(15)	2	470	340	316	471	422	304	295	435	294	231	182	340

Table 7.2 (see pages 105-6)

γ-Interferon ELISPOT responses, shown as spot forming cells per million PBMCs, in peripheral blood seven days after the vaccination regimens shown in the left hand column (see table 6.1 for description of nomenclature). For some time points the data are missing due to subjects unavailability, errors in performing the assay or background responses more than 50 spots/million PBMC which were not included in the analysis. The number of subjects in each arm and their vaccination schedule is shown in Table 6.1. Some subjects are included more than once as the results indicate their time course through the trials. For example, a subject who had three doses of 2mg of DNA ME-TRAP followed 3 weeks later by two doses of 1.5×10^8 pfu of MVA ME-TRAP is included in the DDD(2) regimen seven days after the first three vaccinations, in the DDDM(15) regimen seven days after the first MVA ME-TRAP and in the DDDMM(15) regimen seven days after the second MVA ME-TRAP. Arithmetic and geometric means and standard error (SE) are shown for three sets of peptide pools: the summed net responses to all the epitopes in the vaccines, the summed net responses to all peptide pools from T9/96 strain of TRAP and the summed net responses to all peptide pools from 3D7 strain of TRAP.

Table 7.3 Responders by antibody ELISA. Titres shown are greatest dilution which produced an OD of >0.20 in the relevant assay

Volunteer number	Vaccination regimen	Pre-vaccination	Final vaccination +7 days
TRAP IgG ELISA (see section 5.7)			
111	DDD_MM(15)	1/800	1/3200
112	DDD_MM(15)	1/1000	1/2000
121	DDDMM(15)	1/1400	1/3000
R32LR CS IgG ELISA (see section 5.8)			
111	DDD_MM(15)	1/400	1/600
112	DDD_MM(15)	1/1400	1/3000
118	DDD_MM(15)	1/1000	1/3200
121	DDDMM(15)	1/1200	1/1500

Chapter 8

Safety and Immunogenicity of DNA ME-TRAP and MVA ME-TRAP in Gambian adults

8.0 Introduction

There has been a publication reporting this phase 1 trial (Moorthy et al., 2003b)

In my view one of the most interesting observations made in murine malaria studies of DNA and recombinant viral vaccines related to the effects of prior exposure to sporozoite infection on vaccine immunogenicity. Inoculation of a few hundred sporozoites led to only a minimal CD8 T cell response, but this could be boosted very substantially to protective levels (>1000 peptide-specific CD8+ T cells) by a single subsequent immunisation with either the MVA vaccine or, surprisingly, the plasmid DNA vaccine each encoding the *P. berghei* circumsporozoite antigen (Schneider et al. unpublished data). Similar observations were reported by a New York group using a different replicating strain of recombinant vaccinia virus encoding a *P. falciparum* antigen(Miyahira et al., 1998). These results suggest that in individuals primed by natural exposure to sporozoites only one of the vaccines might be needed to induce a substantial immune response. Once promising safety and immunogenicity data from Oxford of DNA ME-TRAP and MVA ME-TRAP at 1mg and 3×10^7 pfu doses were obtained, we decided to plan a phase I trial evaluating whether MVA alone and DNA/MVA would be more immunogenic in malaria-exposed compared to malaria-naïve adults, as the animal model data suggested. This trial was also designed to provide data which would accelerate conduct of a field efficacy trial if subsequent UK IIa efficacy data warranted one.

8.1 Methods

8.1.1 Study setting and volunteers

Volunteers were recruited from the peri-urban community of Bakau, on the coast of The Gambia. Approval was obtained from the Joint Gambian Government/ Medical Research Council Ethics Committee and the Central Oxford Research Ethics Committee. Written informed consent was obtained from all volunteers after initial community consent, discussion with volunteers in the local languages and dissemination of information sheets and consent forms translated into local languages in arabic script. The trial was conducted according to the principles of the Declaration of Helsinki.

Potential volunteers underwent thorough clinical evaluation and were screened for haematological (full blood count), renal (plasma creatinine, urinalysis) and hepatic (plasma alanine aminotransferase (ALT)) dysfunction. 20 semi-immune healthy adults aged 18-45 were enrolled. An independent safety monitor based in The Gambia monitored the study.

For comparison between malaria-exposed and malaria-naïve individuals, ELISPOT data from assays performed on UK volunteers are included in the analysis. The UK DNA/MVA group consists of 9 volunteers (3 volunteers in each group) who received either one, two or three 1mg DNA ME-TRAP immunisations followed by one 3×10^7 plaque forming units (pfu) MVA ME-TRAP immunization (groups 5,6 and 8 in table 6.1). The UK MVA alone group consists of 5 volunteers who received three 3×10^7 pfu MVA ME-TRAP immunizations (group 4 in table 6.1). See chapter 6 for details of recruitment and vaccination of these volunteers.

8.1.2 Vaccines

The two study vaccines were DNA ME-TRAP and MVA ME-TRAP. Regulatory approval for prior U.K. phase I studies with these vaccines was obtained from the U.K. Medicines Control Agency (MCA). The Gambian Government accepted MCA approval in the absence of a Gambian regulatory authority.

8.1.3 Study design

This was a phase I open-label trial designated as trial VAC006 in the Oxford malaria vaccine programme. All Gambian volunteers received a standard treatment with sulphadoxine/pyrimethamine two weeks prior to first vaccination. All vaccinations in both UK and Gambian groups were administered at 3 week intervals. Twelve Gambian volunteers received two 1mg doses of DNA ME-TRAP intramuscularly followed by two 3 x 10^7 pfu doses of MVA ME-TRAP intradermally. Eight Gambian volunteers received three 3 x 10^7 pfu doses of MVA ME-TRAP intradermally. Eligible volunteers were allocated to each of these two groups alternately in order of enrolment. Each volunteer was observed for at least one hour after vaccination. Study visits were scheduled for day 1, 2, 3, 7 and 28 days after each vaccination and 8-10 weeks after final vaccination. Six screened, healthy unvaccinated volunteers were bled three times at day 0, 56-84 and 140-156 to assess background variation in effector T cell responses over the study period in the Gambian population.

8.1.4. Laboratory analysis

Each Gambian volunteer had 30mls venous blood drawn from an ante-cubital vein on five occasions as follows: screening (day -28 to day -7); one week after first vaccination in MVA group only (day 7); one week after second vaccination in both groups (day 28); one week after third vaccination in both groups (day 49); one week after fourth vaccination in the DNA/MVA group only (day 70) and 8-10 weeks (day 142-156) after final vaccination. Full blood counts and ALT and creatinine assays were performed according to the standard operating procedures of the unit (see section 5.4 and 5.5). Normal ranges were as follows: Haemoglobin = 13-16 gm/dl(men). WBC Total = 4-11 x 10^9/L. Diff count = Neutrophils – 40-55%, Lymphoctyes – 25-45%, Monocytes – 3-7%, Eosinophils – 1-5%, Basophils – 0-1%. RBC = 4-6.5 x 10^{12}/L. PCV = 36-54%. MCV= 76-96. Platelets = 150-400 x 10^9/L. ALT<=42 iu/L. Creatinine <=130 mmol/L. Clinically non-significant neutropaenia is

common in Gambians – neutrophil counts of >= 1.0 x 10^9/L were considered normal in asymptomatic subjects.

Ex vivo Enzyme-Linked Immunospot (ELISPOT) Assay: Detection of Antigen-Specific Effector T cells. (see 5.0) ELISPOTs were performed on Millipore MAIP S45 plates with MabTech antibodies according to the manufacturer's instructions. 4 x 10^5 freshly isolated peripheral blood mononuclear cells (PBMCs) were incubated for 18-20 hours on the ELISPOT plates in the presence of 25 μg ml^{-1} peptides, before being developed. The number of spot forming cells (SFCs) were counted by the AutoImmun Diagnostika system. Individual 8-mer to 17-mer epitopes were used for epitopes from the ME string, whereas 20-mers overlapping by 10 were used to span TRAP with both T9/96 and 3D7 strains of TRAP spanned in their entirety. Peptides were assayed in pools due to cell number limitations and cells were assayed in duplicate for each pool. Cell separations were performed on frozen cells with Miltenyi Biotech MACS beads and checked by co-staining and FACS analysis.

Anti-TRAP Antibody ELISA. (see 5.7)

Recombinant T9/96 TRAP and 3D7 TRAP were coated at 4°C overnight onto Nunc immunoplates at a concentration of 4ug/ml. Standard ELISA assays were performed with incubations of 2 hours at 37°C.

8.1.5 Statistical Analysis

The plate layout was changed slightly from the UK studies. Peptide pools 5-9 spanned 3D7 TRAP and 10-13 spanned T9/96 TRAP (figure 8.1). Spots were summed across relevant pools and the "no peptide" negative control spot counts subtracted the requisite number of times (figure 8.3a-d). Arithmetic means, geometric means, medians and interquartile ranges were derived. All four are provided for initial immunogenicity values presented in the text to allow evaluation of the distribution of these summed response data. Two-tailed Mann-Whitney tests were performed on groups of individual summed responses.

8.2 Results

8.2.1 Safety and reactogenicity

Two volunteers had asymptomatic *P. falciparum* parasitaemia (55 and 32 parasites in 200 high power fields respectively) on screening and had subsequent negative blood smears prior to vaccination. Eighteen of 20 vaccinees completed the study protocol. Two withdrew their consent after second DNA immunisation; neither had experienced any adverse events. Two volunteers experienced an episode of clinical malaria (13 and 9 asexual parasites per high power field) between screening and first dose in one case and between first and second doses in another case. One volunteer was in the DNA/MVA group and the other was in the MVA group. In both instances, parasitaemia and symptoms resolved entirely after standard treatment courses of chloroquine and sulphadoxine/pyrimethamine. No other episodes of clinical malaria occurred during the study.

There were no severe or serious adverse events. There were no local or systemic adverse events after a total of 24 DNA ME-TRAP immunisations. The MVA vaccine was well-tolerated (table 8.1). All the tabulated adverse events were mild (no interference with activities of daily living). The one moderate adverse event was transient limitation of arm abduction after MVA vaccination. MVA causes a characteristic local reaction after intradermal administration with redness and induration peaking at 48-72 hours. In 5/18 MVA ME-TRAP first doses, there was a blister < 2mm at the centre of the indurated lesion, which healed without complications over 1-3 weeks in all cases. Analysis of the haematology and biochemistry safety assays performed reveal no adverse events.

8.2.2 High-frequency induction of effector T cells specific for T9/96 TRAP

The six unvaccinated volunteers had geometric mean effector T cell frequencies of 5.3, 7.2 and 6.6 SFCs per million PBMCs at the beginning, middle and end of the study. Effector T cells were induced in vaccinees with specificity for all peptide pools; TRAP-specific frequencies were higher than

ME-specific frequencies (figure 8.1). Following DNA ME-TRAP immunisation there was no statistically significant increase in effector T cell frequency. MVA ME-TRAP immunisation induced much greater effector T cell frequencies. Immunogenicity of MVA ME-TRAP following preceding DNA ME-TRAP (in terms of vaccine-induced T9/96 TRAP-specific effectors) was over three-fold higher in Gambian than in UK volunteers (figure 8.3a vs 8.3c, arithmetic mean 175.4 vs 51.4, geometric mean 69.8 vs 19.8, median 172.0 vs. 31.2, interquartile range 23.4 – 239.4 vs 15.6 – 131.0 spot forming cells (SFCs) per million PBMCs, p value = 0.03). Enhanced immunogenicity was also observed in Gambians who received MVA ME-TRAP immunisation without prior DNA ME-TRAP compared to UK adults who received the same regime (figure 8.3b vs 8.3d, arithmetic mean 55.4 vs 17.2, geometric mean 23.0 vs 11.0, median 28.8 vs 15.2, interquartile range 10.6-105.6 vs 3.4 – 40.7, SFCs per million PBMCs, p value = 0.10). Prior DNA ME-TRAP increased T9/96 TRAP effector T cell frequency after MVA boosting in Gambians compared to Gambians who received MVA without prior DNA (figure 8.3a vs 8.3b, geometric mean 69.8 vs 23.0 SFCs per million PBMCs, p value = 0.07). After second MVA immunisation in the Gambian DNA/MVA group effector T cell frequency was geometric mean 63.3 SFCs per million PBMCs compared with 69.8 after first MVA immunisation. In both UK DNA/MVA (see table 7.2) and Gambian MVA alone (figure 8.3b) groups effector T cell frequency kinetics showed a decay from 7 days after first MVA to 7 days after second MVA. Geometric mean effector frequencies at the 8-10 week follow-up in the group with the strongest responses (DNA/MVA vaccinated Gambian adults) were 70.6% of the peak frequencies.

8.2.3 Enhanced cross-recognition by effector cells for 3D7 TRAP

To assess the ability of a malaria vaccine to induce a cross-reactive T cell response we evaluated the effector response to the 3D7 strain of TRAP, a heterologous strain with 6% sequence variance at the amino acid level compared to T9/96. Cross-recognition of responses induced in Gambians was far in excess of that seen in UK volunteers (vaccine-induced response to 3D7

after DNA then MVA immunisation in Gambian vs UK adults – geometric mean 65.1 vs 5.5 SFCs per million PBMCs, p value 0.01, figure 8.3a vs 8.3c). Prior to vaccination T cell responses in the group of Gambians who received MVA without prior DNA were higher to 3D7 than T9/96. In this group immunogenicity was correspondingly greater for 3D7 than T9/96 (figure 8.3b, vaccine-induced responses of geometric mean 25.9 vs 15.0 SFCs per million PBMCs, p value = 0.16).

8.2.4 Characterisation of vaccine-induced effectors

The induced effectors are of both CD4+ and CD8+ T cell subsets (figure 8.2). Most responses were CD4+ or mixed CD4+ and CD8+ but pure CD8+ responses were also seen.

8.2.5 Anti-TRAP antibodies

12 out of 20 Gambian volunteers had titres of anti-TRAP antibodies (to either or both of T9/96 and 3D7 strains) that are statistically significantly above titres in malaria-naives. There was no statistically significant rise or fall of antibody titres after vaccination (figure 8.4).

8.3 Discussion

Most vaccines in widespread use have been developed and formulated for optimal antibody induction. The prime-boost approach outlined here is an example of a new approach targeted at maximisation of T cell immunogenicity. Potent T cell induction is likely to be necessary to vaccinate effectively against intracellular organisms such as HIV, *M. tuberculosis* and liver-stage *P. falciparum*, and for cancer immunotherapy.

The ability of MVA vaccines to amplify pre-existing T cell responses induced by priming with DNA vaccines in animal models suggested that they may be more immunogenic in African volunteers who have been previously primed by natural exposure to malaria. Previously in a mouse model, it has been shown that immunogenicity of a single dose of a recombinant vaccinia vaccine is not

protective but prior exposure to malaria sporozoites boosts this immunogenicity to protective levels (Miyahira et al., 1998), protection which is T-cell mediated. Our findings confirm this prediction. The immunogenicity of a MVA malaria vaccine (with or without DNA priming) was of greater magnitude in previously exposed Gambian individuals than in malaria-naïve British individuals. There are genetic and environmental differences between the malaria-naïve and malaria-exposed individuals in this study other than their malaria exposure, but we feel these are unlikely to account for the reported altered immunogenicity. Interestingly even with sporozoite priming, further priming by DNA immunisation is still necessary for maximal T cell induction by MVA immunisation. It is unclear whether infants aged less than 6 months will display enhanced immunogenicity as in the adults in this study. Although they are protected from severe disease to some extent by maternal IgG antibodies, some exposure to liver-stage parasites still occurs in high transmission settings in the first few months of life. It may be that such exposure with covering antibody-mediated blood stage immunity would prime for recombinant viral boosting with a liver-stage malaria vaccine. Another unknown is the necessity of immune maturation for immunogenicity of T-cell based vaccines. The little available evidence suggests that B-cell function may be more impaired than T-cell function in early life. BCG affords protection, which is thought to be T-cell mediated, against TB meningitis even when administered at birth. But measles vaccine is known to provide less efficacy below 9 months of age. This effect is only partially due to interference with maternal measles antibody.

It is unclear why MVA ME-TRAP but not DNA ME-TRAP shows enhanced immunogenicity in malaria-experienced adults compared to malaria-naives. This is presumably the same immunological mechanism which causes the failure of DNA (or Ty-virus like particles) to boost in pre-clinical heterologous prime-boost regimens. Perhaps there is insufficient recombinant protein expression or APC transfection for boosting.

The greatly increased cross-recognition demonstrated in Africans in this study provides encouragement for further work with T-cell inducing DNA and recombinant viral vaccines. Lack of strain-transcendence has long been viewed a potential obstacle to malaria vaccination (Genton et al., 2002). The volunteers who received MVA without prior DNA vaccination in The Gambia (figure 8.3b) had higher pre-vaccination effector T cell frequencies to 3D7 strain of TRAP than the vaccine strain, T9/96. After immunisation frequencies of T cells specific for 3D7 TRAP rose more than those to T9/96, consistent with the well-recognised immunological phenomenon termed original antigenic sin (Klenerman and Zinkernagel, 1998).

Not only CD8+ T cells, but also high frequencies of CD4+ T cells, were induced by DNA/MVA and MVA immunisation. Whilst CD4+ T cells have traditionally been considered helper T cells for either antibody production or CD8+ T cell cytotoxicity, it is now clear that many CD4+ T cells have effector activity. Directly cytotoxic CD4+ T cell clones confer protection in murine adoptive transfer experiments(Tsuji et al., 1990), and such CD4+ cytotoxic T cell clones are present in attenuated-sporozoite immunised protected humans(Moreno et al., 1993).

In mouse models, contraction of approximately 90-95% of the effector T-cell pool by apoptosis occurs after infectious challenge over 2 weeks (for CD8+ T cells) or 7 weeks (for CD4+ T cells) in one model(Homann et al., 2001). We examined the kinetics of such contraction in humans. We demonstrated the persistence of a residual memory pool with rapid effector function 8-10 weeks after final vaccination with frequencies at this time greater than 50% of the peak frequencies.

In all groups studies second MVA immunisations 3 weeks after first MVA immunisations show no increase in immunogenicity. In fact, effector T cell frequencies show a reduction consistent with natural decay kinetics from the peak frequency 7 days after first MVA immunisation. This may be because a

host immune response to the highly immunogenic MVA vector prevents infection of host cells and recombinant protein expression after second MVA immunisation at this interval. A study is underway to evaluate immunogenicity of second MVA immunisation after a 12 month interval.

It has been difficult to induce strong effector T cell immune responses through vaccination. We present a safe strategy which is immunogenic for effector T cell induction in malaria and is likely to be applicable to other fields. Prime-boost approaches with viral vector boosts have been seen as an excellent option for improving the efficacy of prophylactic DNA vaccines in diseases where T cell responses are protective. The confirmation of the ability of MVA to boost pre-existing T cell responses in humans implies that MVA and the DNA/MVA combination may also be effective for immunotherapy of chronic infections and tumours. Examples which may merit evaluation are tuberculosis infection prior to onset of disease, hepatitis B-virus infected individuals at risk of disease progression and immunotherapy of HIV-positive and melanoma patients.

Local Adverse Events	Dose 1 (n=18)		Dose 2 (n=18)		Dose 3 (n=7)	
Discolouration	9 (6-18)		7 (4-11)		7 (5-9)	
Itching	7		3		1	
Pain	5		0		0	
Blisters	5		0		0	
Systemic Adverse Events	Total	PB	Total	PB	Total	PB
Temperature ≥ 37.5°C	1	1	0	0	0	0
Headache	2	1	1	1	2	1
Malaise	1	1	0	0	1	0
Myalgia	0	0	1	0	0	0
Arthalgia	1	1	0	0	0	0
Nausea	0	0	0	0	0	0

Table 8.1: **Reactogenicity after each dose of MVA ME-TRAP in VAC006** Data for discolouration are median (range) in mm measured on day 2. PB = probably related. Data for other fields are numbers of volunteers experiencing adverse event during 3-day follow-up. n is the number of volunteers who received each dose for whom diary cards were completed.

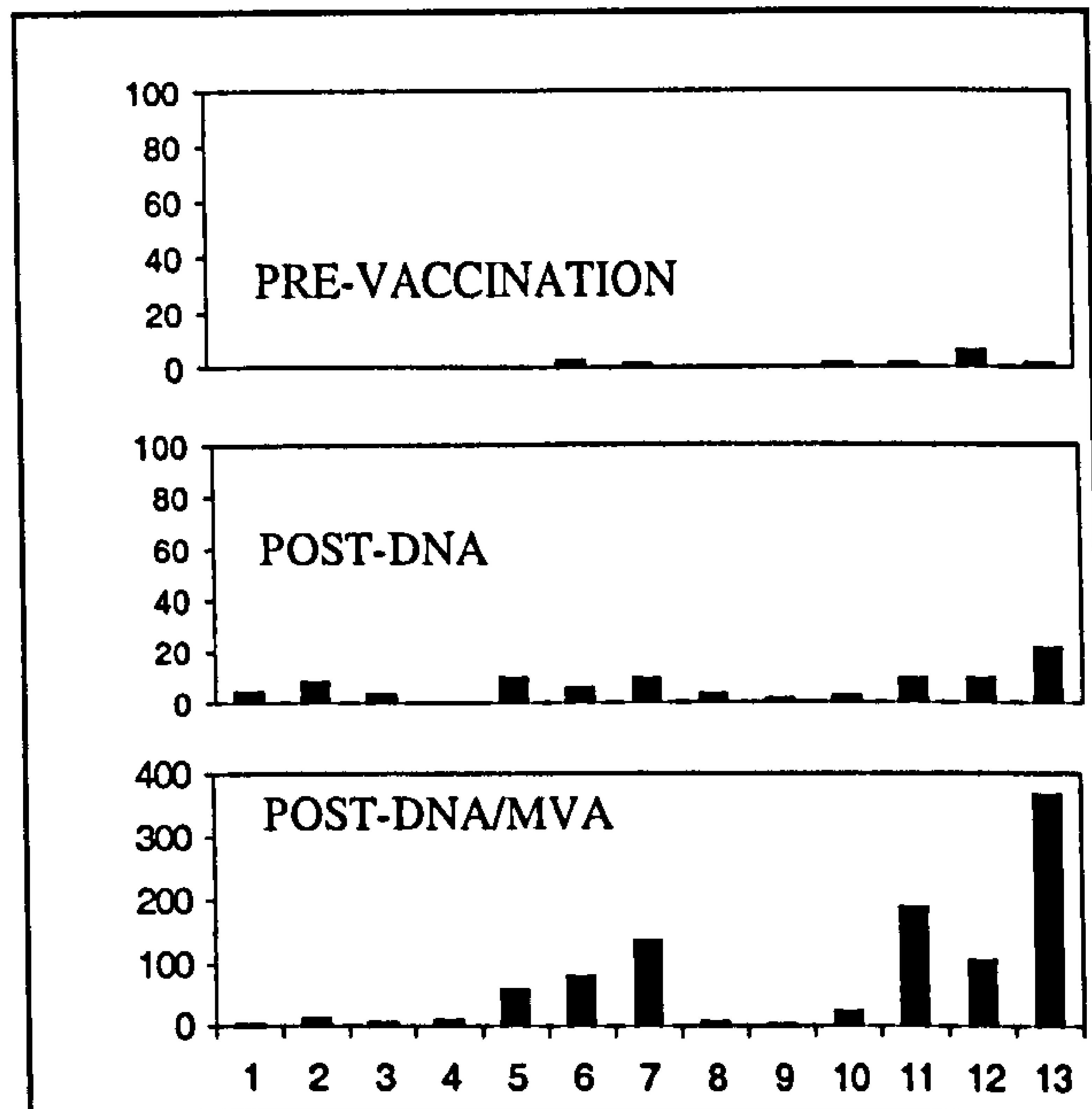


Figure 8.1: **Breadth of effector T cell responses by *ex vivo* ELISPOT**
A timecourse for one Gambian volunteer in VAC006. 13 peptide pools are shown along the x-axis. Pool 1 is the negative control (cells, no peptide). Pools 2-4 span the ME string. Pools 5-9 span 3D7 TRAP. Pools 10-13 span T9/96 TRAP. Pools 5-9 each consisted of ten 20-mer 3D7 TRAP peptides; pools 10 and 12, ten 20-mer T9/96 TRAP peptides; pool 11, thirty 20-mer T9/96 TRAP peptides and pool 13, seven 20-mer peptides which cover the N-terminal region completely conserved between T9/96 and 3D7 TRAP strains. Y-axis units are SFCs per million PBMCs. Post-DNA indicates the timepoint seven days after second DNA immunisation. Post-DNA/MVA indicates the timepoint seven days after first MVA immunisation.

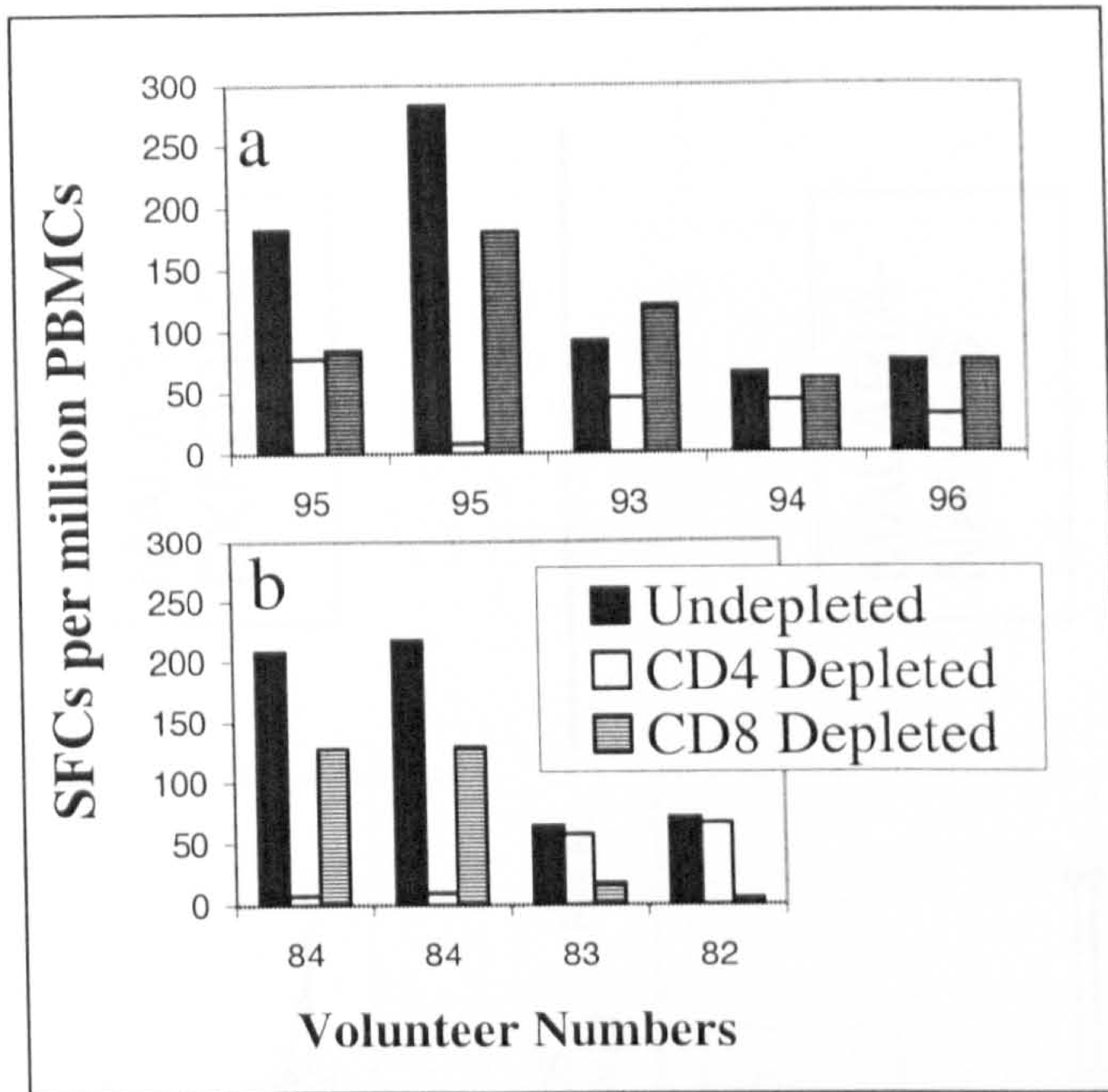


Figure 8.2: **Subset distribution of effector T cell responses.**
T cell subsets for DNA/MVA vaccinated volunteers from VAC006(a) and MVA vaccinated volunteers also from VAC006(b); each set of three bars represents ELISPOT response to a single pool of peptides (various pools from 5-13 in figure 1a) at the maximal timepoint (usually 7 days after first MVA immunisation). Assays were performed on frozen/thawed cells on unseparated, CD4 depleted and CD8 depleted cell populations. The x axis numbers are volunteer numbers. Two different pooled responses were assayed from both volunteers 95 and 84.

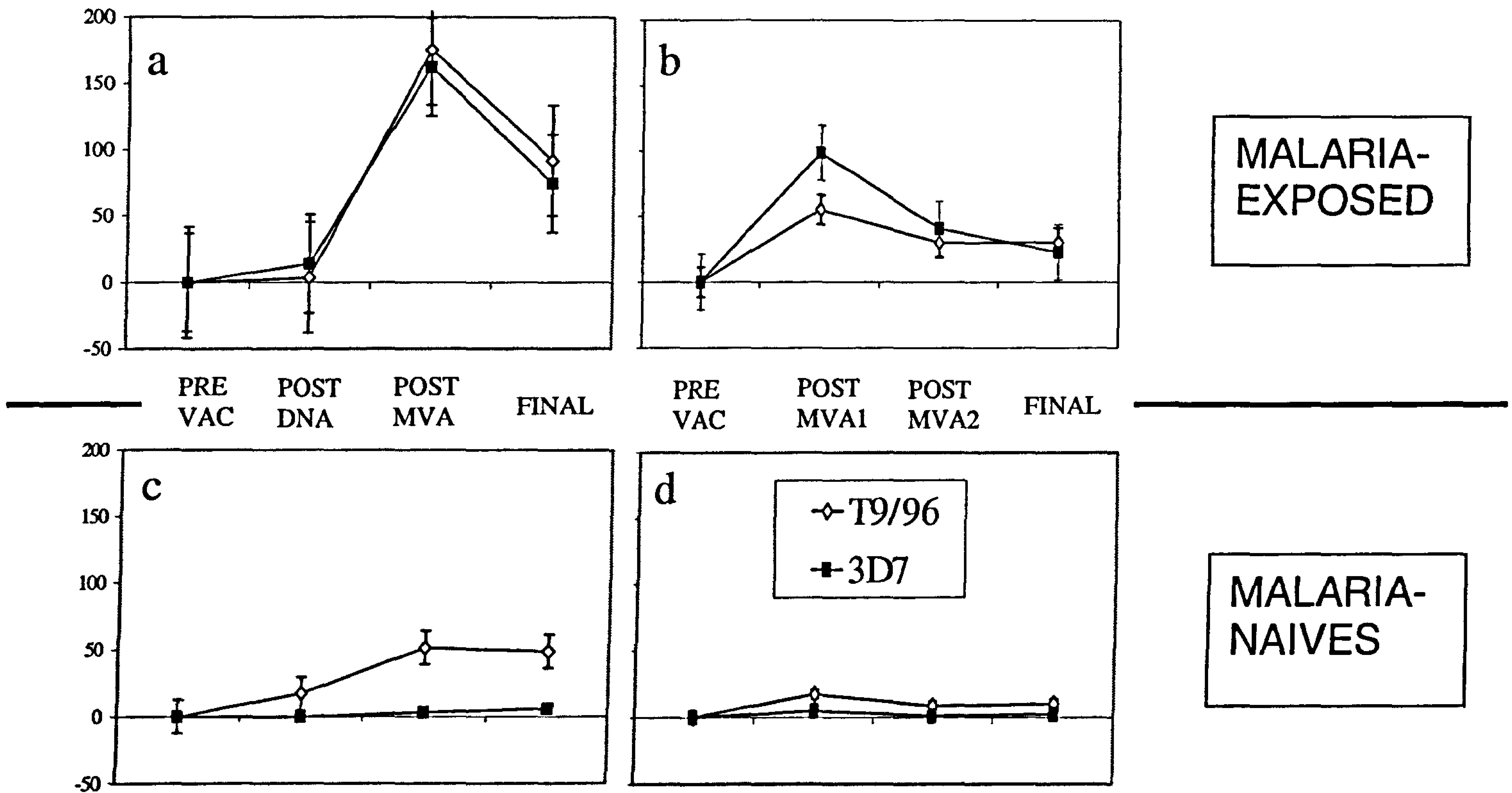
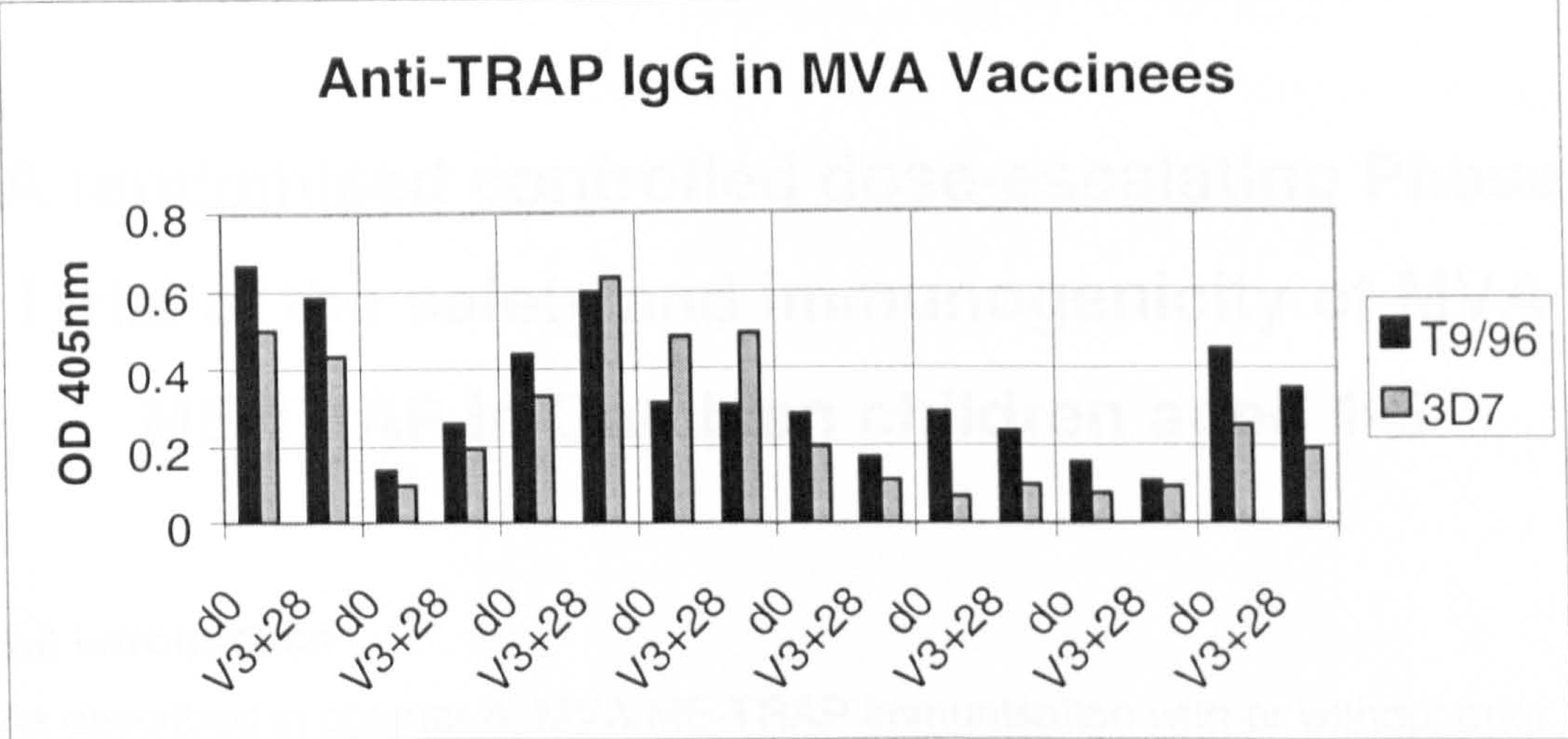


Figure 8.3: **Magnitude, duration and cross-reactivity of effector T cell responses by *ex vivo* ELISPOT.**
Pre-vaccination responses are set to zero for this figure to allow comparison of induction of immune responses. Arithmetic mean (standard error) timecourses for Gambian DNA/MVA group (VAC006) (a), Gambian MVA group (VAC006) (b), UK DNA/MVA group (VAC003) (c), UK MVA group (VAC003) (d). Pre-vac refers to the pre-vaccination time point. Post DNA refers to 7 days after the final DNA immunization. Post MVA in a) and c) refers to 7 days after the first MVA immunization. Post MVA1 and Post MVA2 in b) and d) refers to 7 days after the first and second MVA immunizations respectively. Final refers to the 8-10 weeks after final vaccination timepoint.



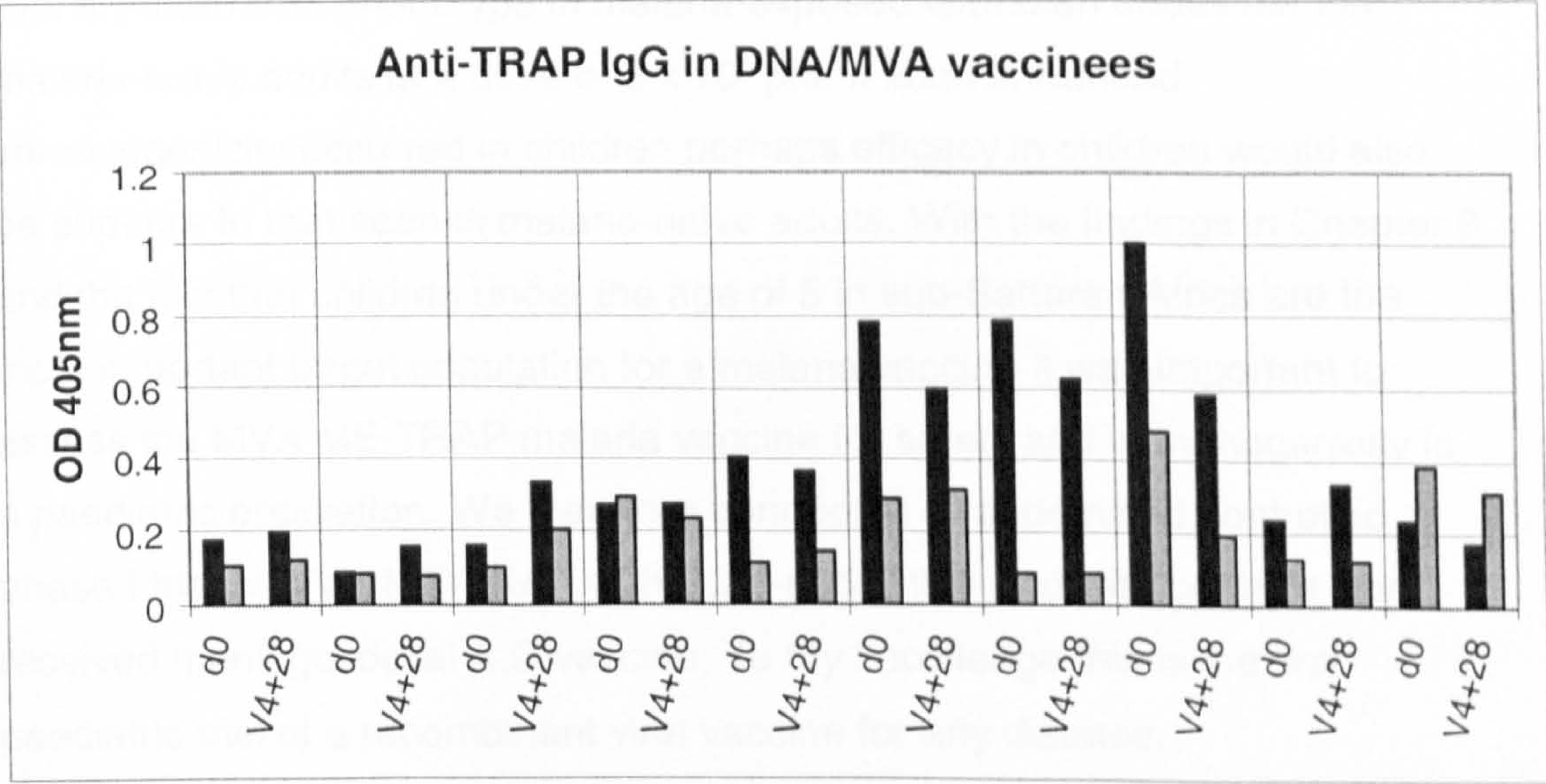


Figure 8.4: Anti-TRAP IgG in MVA and DNA/MVA vaccinated Gambians in VAC006 pre-vaccination and four weeks after final vaccination

Chapter 9

A randomised controlled dose-escalating Phase I trial of the safety and immunogenicity of MVA ME-TRAP in Gambian children aged 1-5

9.0 Introduction

As described in chapter 8, MVA ME-TRAP immunisation with or without prior DNA ME-TRAP immunisation induces higher frequencies of effector T cells with a γ-interferon phenotype in malaria-exposed Gambian adults than in malaria-naïve adults at a dose of 3×10^7 pfu. If such enhanced immunogenicity occurred in children perhaps efficacy in children would also be superior to that seen in malaria-naïve adults. With the findings in Chapter 8 and the fact that children under the age of 5 in sub-Saharan Africa are the most important target population for a malaria vaccine it was important to assess the MVA ME-TRAP malaria vaccine for safety and immunogenicity in a paediatric population. We therefore conducted a randomised controlled phase I trial of MVA ME-TRAP with dose-escalation and with controls who received meningococcal A,C vaccine. To my knowledge this is the first paediatric trial of a recombinant viral vaccine for any disease.

9.1 Methods

9.1.1 Study setting and volunteers

Mothers and children were recruited from the rural Mandinka community of Sibanor, about 100 km inland in The Gambia. EIRs are substantially higher than at the coast and have been estimated as >10 and <30 per person per year. Approval was obtained from the Joint Gambian Government/ Medical Research Council Ethics Committee and the Central Oxford Research Ethics Committee. Written informed consent was obtained from all mothers after

initial community consent, discussion with mothers and fathers in the local languages and dissemination of information sheets and consent forms. Wherever possible fathers were consulted prior to seeking of consent from the mother. An indication by either parent that they did not wish their child to participate was sufficient to exclude the child. The trial was conducted according to the principles of the Declaration of Helsinki.

Potential study participants underwent thorough clinical evaluation and were screened for haematological (full blood count), renal (plasma creatinine, urinalysis) and hepatic (plasma alanine aminotransferase (ALT)) dysfunction. After pre-test counseling of mothers HIV-1 and HIV-2 antibody rapid tests were performed with confirmation of positives by ELISA. 40 healthy children aged 1-5 were enrolled. An independent safety monitor based in The Gambia monitored the study.

9.1.2 Vaccines

The two study vaccines were MVA ME-TRAP and Mencevax (a licensed meningococcal polysaccharide A,C vaccine manufactured by GlaxoSmithKline).

9.1.3 Study design

This was a controlled phase I trial with partial blinding and random allocation to vaccine group. All children received single dose immunisation only. Ten children received a 6×10^6 pfu dose of MVA ME-TRAP i.d. and ten children received 0.5ml Mencevax i.m. during the same week. Twenty further children were immunized after review of safety and reactogenicity data; ten received a 3×10^7 pfu dose of MVA ME-TRAP i.d. and ten received 0.5ml Mencevax i.m. I generated the randomisation code with stratification by age and performed the vaccinations. All other study staff, including the medical officer and field workers who assessed systemic reactogenicity and lab staff who conducted safety and immunology assays, were blinded to vaccine allocation as were mothers and children. Mothers may have been aware that the two vaccines were given by different routes, but they could not have known which group

received the candidate malaria vaccine. Each child was observed for at least one hour after vaccination. Study visits were scheduled for day 1, 2, 7 and 28 days after vaccination and 6 months after vaccination.

9.1.4. Laboratory analysis

Each child had 5mls venous blood drawn on three occasions as follows: screening (day -28 to day -7); one and four weeks after vaccination(day 7, day 28). Full blood counts, ALT and creatinine assays were performed according to the standard operating procedures of the unit (see sections 5.4 and 5.5).

<u>ELISPOT Assays: Detection of Antigen-Specific Effector and Memory T cells.</u>

ex vivo ELISPOTs were performed as described in section 5.0 and 8.1.4. Cultured ELISPOTS were performed as described in section 5.1 with addition of Lymphocult-T on day 3 and day 7 and the γ-interferon ELISPOT assay on day 10. Due to extreme blood volume limitations very large pools of peptides were used in these experiments. In *ex vivo* assays three pools were assayed in duplicate containing all ME, 3D7 TRAP and T9/96 TRAP peptides respectively (up to 57 peptides in one pool). Identical pools were used for setting up short term cultures with smaller pools of 7-20 used for the cultured ELISPOT assay.

<u>Analysis of cultured ELISPOT data</u>

See Section 5.1.1. Cultured ELISPOT assays were conducted in the UK study VAC005 and the three Gambian studies VAC006, VAC011, VAC014. The same SOP was used, only differing in the size of the peptide pools and hence the number of test responses per individual due to blood volume restrictions in The Gambia and in children for VAC011.

9.2 Results

9.2.1 Safety and reactogenicity

56 children aged 1-5 were screened. 11 were excluded; 8 with a PCV of less than 30% and 3 with various medical conditions (1 with malnutrition, 2 with respiratory infections). 40 of the remaining eligible children were enrolled. The median age of the 20 MVA vaccinees was 37.8 months (range 12.5 – 70.1

months) compared to 36.0 months for the control group (range 14.3 – 66.4 months). There were 11 boys and 9 girls in the MVA groups and 14 boys and 6 girls in the control group.

At screening 12 of the 40 enrolled children had asexual *P. falciparum* parasites on thick malaria smears. 3 had clinical *P. falciparum* malaria, were treated with sulphadoxine/pyrimethamine and chloroquine and subsequently enrolled and vaccinated. Six parasitaemic volunteers received control vaccine, four received low dose MVA ME-TRAP and two received high dose MVA ME-TRAP. There were 7 episodes of clinical *P. falciparum* malaria during the four weeks after study vaccination, 3 and 1 in the low and high dose MVA ME-TRAP groups respectively and 3 in the control group. All of these children responded clinically and parasitologically to oral sulphadoxine/pyrimethamine and chloroquine treatment. One child with clinical malaria in the control group required admission to hospital for parenteral treatment due to persistent vomiting and recovered entirely over one week. There were no severe or serious adverse events related to vaccination during the 6 month follow-up period. 38 vaccinees completed the study protocol. 2 children in the control group left the study area between the day 28 and 6 month follow-ups.

The MVA ME-TRAP vaccine was well-tolerated at both doses. The mean PCV in the 2 MVA groups and control group were 32.9 (standard deviation 1.9) and 33.7 (2.1). The mean PCV at day 7 for these groups were 26.7 (4.1) and 28.9 (3.8). At day 28 the mean PCV were 27.2 (5.2) and 27.7 (4.3). There were no abnormalities of laboratory safety assays related to vaccination. There were similar frequencies of systemic adverse events in both MVA ME-TRAP groups and the control group (see table 9.1). Of the 7 episodes of axillary temperature >= 37.5°C during the 7 days after vaccination, 5 were associated with clinical malaria and one with a gluteal abscess unrelated to vaccination. The one remaining episode of fever occurred on the second day after vaccination with no apparent cause or associated symptoms or signs in a low dose MVA ME-TRAP vaccinee. The fever had resolved by day 3 and was

assessed as possibly related to vaccination. Discolouration and induration were seen most frequently in the higher dose MVA ME-TRAP vaccines, 3 of whom also developed blisters. These blisters were 1-5 mm in diameter and usually maximal size on day 2 after vaccination. They healed over 1-3 weeks without complications. Blisters were protected by non-adherent occlusive dressings and replaced daily until they were healing. 4/10 of the higher dose MVA vaccinees had visible faint marks at 6 months; none of the lower dose MVA vaccinees or the control vaccinees had visible marks at this time.

9.2.2 Ex vivo ELISPOT assays

There was no evidence of induction of antigen-specific T cells to 20-mers spanning either 3D7 or T9/96 TRAP after vaccination as assayed by *ex vivo* ELISPOT. There were no statistically significant changes in summed responses (geometric or arithmetic means or medians) to ME, 3D7 or T9/96 peptides in either MVA dose group between values at screening and at day 7.

9.2.3 Cultured ELISPOT assays

There were more positive γ-interferon responses in cultured ELISPOT 28 days after MVA vaccination than prior to vaccination (10/90 vs 2/90, p value = 0.04, χ^2 test) (Table 9.3). Similar increases were seen in malaria-naïve and malaria-exposed adults vaccinated with the same dose of MVA ME-TRAP. The analysis method renders questionable the value of magnitude in the responses defined as positive and magnitude is therefore not quoted .

9.3 Discussion

Ex vivo ELISPOT data presented indicate no induction of effector T cells as enumerated by this assay. One caveat is that much larger pools were used in this paediatric study than in adult studies. The size of these pools did not prevent cultured ELISPOT responses. This study demonstrates one of the applications of ELISPOT assays conducted on short-term cell lines. Cultured ELISPOT assays provided greater sensitivity to detect an induced immune response than *ex vivo* assays in this trial. A similar finding occurred during

description of both naturally acquired and RTS,S/AS02 induced immune responses to CS peptides in a field efficacy trial conducted in The Gambia in 1998. Indeed a cultured ELISPOT response to a CS peptide predicted protection from malaria infection in this study, whereas no responses in *ex vivo* assays were protective (Reece et al., 2004). The disadvantageous variability of cultured ELISPOT assays was minimized by using a robust, specific analysis method in the trial described in this chapter. A similar timecourse is seen in the groups vaccinated with MVA or DNA/MVA regimens in The Gambia and UK (table 9.3). Cultured ELISPOT responses increased from day 7 after MVA vaccination to day 28 in contrast to *ex vivo* responses which when induced (see chapters 7 and 8) contract considerably after day 7. Therefore based on this limited dataset for vaccination with ME-TRAP it appears that peripheral memory non-effector T cells as enumerated by the cultured ELISPOT assay have a more extended kinetic than the effector T cells enumerated by the *ex vivo* ELISPOT assay. Unfortunately cultured ELISPOT data are not available at higher doses of DNA or MVA ME-TRAP or at later timepoints, both of which may be informative. If the memory T cells assayed in the cultured assay were found to persist in the long term, duration of immunogenicity would be longer than previously seen in clinical trials of malaria subunit vaccines. Furthermore if the vaccine construct could be iteratively optimized to induce memory T cells that afforded protection against liver-stage malaria, long-lasting efficacy may be obtained.

Safety and reactogenicity of MVA ME-TRAP at doses of up to 3×10^7 pfu intradermally in Gambian children aged 1-5 is acceptable. The blisters seen will need to be carefully assessed in larger scale trials of MVA vaccines particularly at higher doses. However no infection-related complications of these blisters have been documented with MVA ME-TRAP. This study confirms the value of controls in phase I trials in endemic trials in populations in which the background rate of adverse events is high. Only 1 of the 7 documented febrile episodes during 7 days after vaccination was possibly related to vaccination and without the inclusion of control vaccines it would

have been difficult to determine whether or not MVA vaccination had contributed to these episodes. 3 of the 7 episodes occurred in control vaccinees and there is therefore little evidence that MVA ME-TRAP vaccination is associated with febrile episodes in this study.

The data in this study are consistent with the falsity of our hypothesis that enhanced immunogenicity seen in malaria-exposed Gambian adults would transfer to malaria-exposed Gambian children aged 1-5. It is not possible to determine the mean number of sporozoite inoculations the children in this study had received over their lifetime. However it is certain that they will have received less cumulative exposure than the Gambian adults described in chapter 8. Another factor is the relative lack of immune maturation in this paediatric population compared to adults, although there is little evidence that significant qualitative or quantitative immune maturation occurs beyond the age of 24 months.

As DNA/MVA proved more immunogenic than MVA alone in Gambian and UK adults why did we did not conduct a phase I trial of DNA/MVA in children? The theoretical risk of oncogenesis needed to be taken into account in the risk/benefit evaluation. No paediatric DNA vaccine trials have occurred for any disease and it could be argued that with such a theoretical risk for DNA vaccinations, as unlikely as it is that this will translate into adverse events, with the problem of consent for children and the lack of an expectation for an overriding medical benefit to study participant children, it would be preferable to await clear indication of efficacy prior to starting a paediatric DNA vaccine trial. This was my opinion, although not that of many malaria researchers.

This study provides encouragement for further work in paediatric populations with recombinant viral vaccines. Although enhanced immunogenicity was not observed, induction of T cell responses with a comparatively low dose of MVA ME-TRAP occurred in children aged 1-5. Use of higher doses and DNA-based prime-boost combinations will be likely to improve on the immunogenicity

seen. The results presented in this chapter are consistent with the expectation of broadly equivalent immunogenicity in children aged 1-5 in endemic countries and malaria-naïve adults and inform study progression of future candidate vaccine regimens.

Local Adverse Events	6 x 10^6 MVA (n=10)		3 x 10^7 MVA (n=10)		Men A,C (n=20)	
Discolouration	7		9		0	
Induration	5		8		0	
Pain	0		2		2	
Blisters	1		3		0	
Systemic Adverse Events	**Total**	**PB**	**Total**	**PB**	**Total**	**PB**
Temperature ≥ 37.5°C	2	0	2	0	3	0
Headache	2	0	1	0	1	0
Malaise	3	0	2	0	3	0
Vomiting	0	0	1	0	0	0

Table 9.1: Reactogenicity during the 7 days after MVA ME-TRAP or meningococcal A,C vaccination in VAC011. PB – probably related to vaccination.

	3 x 10^7 pfu MVA (n=10)			**6 x 10^6 pfu MVA (n=10)**			**Controls (n=20)**		
	ME	3D7	T/96	ME	3D7	T/96	ME	3D7	T/96
Screening	10.5	1.5	3.1	3.6	1.2	4.5	8.7	3.7	6.6
Day 7	6.9	3.2	2.7	8.7	2.2	3.7	4.4	4.5	3.7
Day 28	20.3	2	3.6	6.2	1.7	2.2	7.0	2.6	4.7

Table 9.2: Arithmetic mean summed *ex vivo* ELISPOT responses at screening and 7 and 28 days after vaccination in Gambian children receiving MVA ME-TRAP at one of two doses or controls receiving meningococcal polysaccharide vaccine. Trial code VAC011. ME – summed response to all ME string peptides. 3D7 – summed response to all 3D7 TRAP 20-mers. T9/96 – summed response to all T9/96 peptides. Units are SFCs per million PBMCs. The mean+/- standard error overlapped in all cases when comparing timepoints for a response within a group or when comparing between groups.

Regimen	UK or G	Timepoint	Age	Volunteers	Pos	Responses	Pos	χ^2
VAC006								
DDMM	G	Pre-vac	18-45	10	3 (30)	180	3 (1.7)	
DDMM	G	DDM+7	18-45	10	4 (40)	180	14 (7.8)	
DDMM	G	DDM+28	18-45	10	7 (70)	180	25 (13.9)	<0.001
VAC006								
MMM	G	Pre-vac	18-45	8	3 (37.5)	144	5 (3.47)	
MMM	G	M+7	18-45	8	3 (37.5)	144	10 (6.9)	
MMM	G	M+28	18-45	8	6 (75)	144	12 (8.3)	0.13
VAC011								
MMM(L)	G	Pre-vac	1-5	10	2 (20)	90	2 (2.2)	
MMM(L)	G	M+7	1-5	10	2 (20)	90	2 (2.2)	
MMM(L)	G	M+28	1-5	10	2 (20)	90	6 (6.6)	-
VAC011								
MMM	G	Pre-vac	1-5	10	2 (20)	90	2 (2.2)	
MMM	G	M+7	1-5	10	2 (20)	90	5 (5.5)	
MMM	G	M+28	1-5	10	5 (50)	90	10 (11)	0.04
VAC005								
GGMM	UK	Pre-vac	18-55	6	5 (83.3)	90	10 (11.1)	
GGMM	UK	GGM+7	18-55	6	6 (100)	90	16 (17.8)	
GGMM	UK	GGM+28	18-55	6	6 (100)	90	24 (26.7)	0.01

Table 9.3: Proportion of volunteers and of responses positive in cultured ELISPOT in adults in the UK and Gambia and Gambian children. Regimen: D – 1mg i.m. DNA ME-TRAP immunization, M – 3 x 10e7 pfu MVA ME-TRAP immunization, M(L) – 6 x 10e6 pfu MVA ME-TRAP immunization, G – 4 ug DNA ME-TRAP by needleless delivery. UK or G: UK – malaria-naïve British residents, G – malaria-exposed Gambian residents. Timepoint: + followed by a number indicates the number of days following immunization, hence DDM+28 indicates 28 days after a single MVA immunization which was given after two DNA immunizations. Age in years. Volunteers – number of volunteers per group. Responses – number of peptide pool responses assayed for the group. The two pos columns give the proportion of firstly volunteers and secondly responses which were positive as defined in section 9.1.4. χ^2 test values quoted include Yates' corrections and are comparisons between pre-vac and +28 proportions of positive responses.

Chapter 10

Higher Doses of DNA ME-TRAP and MVA ME-TRAP and a comparison of FP9 ME-TRAP with DNA ME-TRAP for priming in Gambian adults

10.0 Introduction

Priming with two 1mg i.m. DNA ME-TRAP immunisations followed by boosting with two 3 x 10^7 pfu i.d. MVA ME-TRAP, all at 3 week intervals, produced strong CD4+ and CD8+ T cell responses in the Gambian phase I study detailed in Chapter 8. Subsequent UK Phase I/IIa studies showed that increasing the dose of DNA ME-TRAP to 2mg and MVA ME-TRAP to 1.5 x 10^8 pfu dramatically increased the induced effector T cell frequency (Chapter 7). Another outcome of UK studies was the demonstration that DNA could be replaced by FP9 for priming with slightly lower immunogenicity but equivalent or perhaps somewhat higher efficacy as evidenced by partial protection against high-dose heterologous sporozoite challenge (D Webster, unpublished data).

Fowlpox is an avian poxvirus which causes disease in chickens but not mammals. Recombinant attenuated fowlpox has been used as a rabies vaccine in mammals, in which it shows good recombinant protein expression(Taylor et al., 1988). FP9 was derived from wild type fowlpox by 400 passages in tissue culture, leading to marked attenuation and loss of pathogenicity in chickens by all routes of administration to the extent of avirulence in one-day old chicks(Mayr and Malicki, 1966). Like all avipoxviruses, FP9 and FP9 ME-TRAP cannot replicate in mammalian cells.

Attenuated strains of fowlpox virus, such as TROVAC, and of another avipoxvirus, canarypox , named ALVAC, have been used safely as recombinant viral vaccines in many human clinical trials(Fries et al., 1996, Clements-Mann et al., 1998) and have been demonstrated to be non-virulent in a variety of immunosuppressed animals and human volunteers.

This Phase I study was designed to assess three issues: safety and immunogenicity of FP9 ME-TRAP priming followed by MVA ME-TRAP boosting in Gambian adults; safety and immunogenicity of the higher dose DNA ME-TRAP / MVA ME-TRAP regimen used in the UK; provision of data for two and three priming DNA ME-TRAP immunisations for subsequent MVA ME-TRAP boosting.

10.1 Methods

10.1.1 Study setting and volunteers

Volunteers were recruited from the town of Farafenni in The Gambia. The study occurred during the dry season when the background rate of adverse events in the study population is low. Approval was obtained from the Joint Gambian Government/ Medical Research Council Ethics Committee and the Oxfordshire Research Ethics Committee. Men living in Farafenni were recruited as follows. A general meeting was held in Farafenni during which the study was discussed in detail. Information sheets and consent forms were distributed. Field workers then held individual discussions with potential volunteers. A third discussion was then held with a study physician at least one week after first contact between study physician and volunteer. Written informed consent was obtained by a study physician from all volunteers during this third discussion. Potential volunteers underwent clinical evaluation including a full medical history and clinical examination and were screened for haematological (full blood count), renal (plasma creatinine) and hepatic (plasma alanine aminotransferase (ALT)) dysfunction. Of the 38 adults screened, five were excluded (1 with raised ALT, 2 with raised creatinine and 2 with low PCV). 29 healthy adults aged 18-45 were enrolled. The study was

monitored throughout by an independent safety monitor and was conducted according to the ethical priniciples of the Declaration of Helsinki and MRC clinical trial guidelines.

10.1.2 Vaccines

The three study vaccines were DNA ME-TRAP, FP9 ME-TRAP and MVA ME-TRAP. DNA ME-TRAP was prepared in vials of 2mg in 2ml. MVA ME-TRAP was prepared in vials of 10^8 pfu in 200 µl. FP9 ME-TRAP was prepared in vials of 10^8 pfu in 200 µl. The cold chain was maintained and monitored until vaccine administration.

10.1.3. Study Design

This was a phase I open-label study. All vaccinations were administered at 3 week intervals. Allocation to each of the three groups was by order of enrolment. The doses and routes were: $1x10^8$ pfu i.d. for FP9 ME-TRAP; 2mg i.m. for DNA ME-TRAP and $1.5x10^8$ pfu i.d. for MVA ME-TRAP. A group of 16 received FP9 ME-TRAP at weeks 0 and 3 followed by MVA ME-TRAP at week 6; a group of 8 received DNA ME-TRAP at weeks 0, 3 and 6 followed by MVA ME-TRAP at week 9 and a group of 5 received DNA ME-TRAP at weeks 0 and 3 followed by MVA ME-TRAP at week 6. Volunteers were observed for at least one hour after immunisation. Follow-up visits occurred on day 1, 2, 7, 21 for non-final immunisations and 1, 2, 7, 28 and 56 for final immunisations.

10.1.4 Laboratory Analysis

Each volunteer had 30ml venous blood drawn pre-vaccination, 7 days after penultimate vaccination and 7, 28 and 56 days after final vaccination. Full blood counts, plasma ALT and creatinine were performed according to the standard operating procedures of the unit. *Ex vivo* γ-interferon ELISPOT assays were performed as described in section 5.0. A single pool contained all ME peptides. 4 pools of 20-mer peptides spanned T9/96 and 3D7 strains of the entire TRAP antigen. Antibody and HLA typing data are not presented

from these volunteers. ELISPOT data were analysed as described in section 5.0.1. All values quoted in the text are geometric means.

10.2 Results

10.2.1 Safety and reactogenicity

Twenty five of 29 volunteers completed the protocol. Three volunteers migrated from the study area during the study period. One volunteer withdrew his consent. There were no laboratory abnormalities attributable to vaccination. The tabulated adverse events (table 10.2 and 10.3) are all mild or moderate. There were no severe or serious adverse events. Reactogenicity of DNA ME-TRAP was minimal, similar to that seen at lower doses. First dose FP9 ME-TRAP had slightly greater reactogenicity than MVA ME-TRAP with a proportion of volunteers experiencing mild to moderate malaise, headache and local pain of onset during the evening of vaccination with resolution during the two days after vaccination. In no cases did these episodes prevent the volunteers attending work or performing other activities of daily living. Second dose FP9 ME-TRAP was markedly less reactogenic than first dose FP9 ME-TRAP. There was induration, maximal at day 1, at the site of FP9 ME-TRAP and MVA ME-TRAP immunisations with range 1-9mm and 1-10mm and median 4mm and 5mm respectively. Discolouration was visible, maximal at day 2, and faded to a faint shiny plaque. The blisters were median 2mm, maximal on day 2 and resolved by day 21 without complication.

10.2.2. Optimised T9/96 TRAP-specific γ-interferon effector T cell induction after prime-boost immunisation with DNA/MVA

Pre-vaccination summed responses to T9/96 TRAP in volunteers from all 3 groups was geometric mean 8.6 SFCs per million PBMCs (range 2.5 – 80). There was a small increase after two or three DNA ME-TRAP immunisations (p values for increase above pre-vaccination – not significant). A single MVA immunisation after two DNA ME-TRAP immunisations (DDM group) induced a very large increase in effector T cell frequency to geometric mean 211.7 SFCs per 10^6 PBMCs (figure 10.1a). A single MVA boost after three DNA ME-TRAP

immunisations (DDDM group) induced a frequency of 331.0 SFCs per 10^6 PBMCs (figure 10.1b, p value for difference from two DNA immunisations – not significant). Geometric mean effector T cell frequency 4 weeks after final MVA ME-TRAP immunisation was 142.0 and 119.1 SFCs per 10^6 PBMCs for DDM and DDDM groups (figure 10.1a and b, 42.9% and 56.2% of the peak value). The corresponding values for 8 weeks after final immunization are 113.8 and 98.3 (34.4% and 46.4% of the peak value at the final timepoint in this study). Merging the DDM and DDDM groups to evaluate the decay during the plateau phase from 4 weeks to 8 weeks after final immunization, we find that the geometric mean at 8 weeks is 80.7% of the 4 week value (132.0 vs. 106.5).

10.2.3 Efficient priming and boosting with two recombinant viruses, FP9 ME-TRAP and MVA ME-TRAP

Effector T cell frequencies in this group increased after two FP9 ME-TRAP immunisations to 46.7 SFCs per 10^6 PBMCs. After a single MVA ME-TRAP boost frequencies had risen to a 156.3 SFCs per 10^6 PBMCs (figure 10.1c). At 4 and 8 weeks after the MVA immunisation geometric mean frequencies were 79.9 and 79.4 SFCs per 10^6 PBMCs (51.1% and 50.8% of the peak value). The decay kinetics for this group during the plateau phase are flat with geometric mean frequency at 8 weeks after final immunization being 99.4% of the 4 week value. Due to venesection limitations in this population no comparison is available between immunogenicity of first and second FP9 ME-TRAP immunisations.

10.2.4 T9/96 ME-TRAP prime-boost immunisation induces effector T cells cross-reactive for 3D7 TRAP

In order to assess the ability of induced T cells to recognise a non-vaccine strain of TRAP, cells were assayed with peptides encoding the 3D7 sequence for TRAP. 3D7 has 6% variance at the amino acid level with T9/96. In the DDM, DDDM and FFM groups, at the peak timepoint after MVA boosting the 3D7 TRAP-specific effector T cell frequencies were 274.6, 197.0 and 197.8

SFCs per 10^6 PBMCs (83.0, 93.1 and 126.5% of the T9/96-specific values, figure 10.1a-c). There is no statistical significance to the apparent difference between T9/96 and 3D7-specific frequencies, consistent with near to complete cross-reactivity. In all three groups both T9/96 and 3D7 specific frequencies are statistically significantly greater after MVA immunization compared to pre MVA immunization (p values<0.01).

10.2.5 Induction of effector T cells specific for epitopes from the polyepitope string

Responses to epitopes from the polyepitope string were present in many volunteers at lower frequencies than those to 20-mer peptides spanning TRAP. 4 of the 29 volunteers had frequencies greater than 50 SFCs per 10^6 PBMCs above the pre-vaccination values. The highest induced response to the ME string was 106.2 SFCs per 10^6 PBMCs (to the tetanus toxoid epitope). The geometric mean ME-specific frequency merging all groups at the peak timepoint (7 days after MVA immunization) was 13.4 SFCs per 10^6 PBMCs (p value <0.001 for increase above baseline).

10.2.6 Dose may affect immunogenicity in malaria-exposed adults

Increasing the dose of DNA to 2mg i.m. from 1mg i.m. and the dose of MVA from 3×10^7 pfu to 1.5×10^8 pfu i.d. increased geometric mean peak frequency from 69.8 to 211.7 SFCs per million PBMCs. A Mann-Whitney test returns a p value of 0.09 but it is likely that significance would be greater with larger numbers. Statistical significance is not seen for this comparison of dose increases between two groups of malaria-exposed individuals in contrast to the statistically significant difference seen for dose increases in malaria-naïve adults. There is also no statistical significance in the difference between the high dose DNA/MVA regimens in malaria-exposed and malaria-naïve individuals in contrast to the difference described in Chapter 8 for lower dose DNA/MVA in Gambians vs. UK volunteers.

10.3 Discussion

DNA has previously been administered at up to 2.5 mg doses in humans(Le et al., 2000); the safety of 2mg doses of DNA ME-TRAP is therefore unsurprising. We add to the body of data indicating that DNA has excellent safety profiles even at high doses. We have previously reported the safety of MVA at 3×10^7 pfu. Here we show that a fivefold increase in dose in malaria-exposed adults presents few safety issues. MVA ME-TRAP is more reactogenic at 1.5×10^8 pfu than at 3×10^7 pfu with local pain, headache and malaise being fairly common amongst volunteers over the 24-48 hour period after vaccination, but the safety profile is acceptable. The blisters seen were similar with FP9 and MVA and healed without complications in all cases over 1-3 weeks. These small blisters seen in a minority of volunteers would need to be assessed carefully in a paediatric population. The first FP9 ME-TRAP vaccinations were slightly more reactogenic than these higher dose MVA immunizations as detailed in table 10.2 but all MVA and FP9 related adverse events were mild to moderate in this study. Interestingly local adverse events were not only less frequent but resolved more quickly after second than first FP9 immunisation, possibly a manifestation of a host immune response to the FP9 vector (table 10.3). It may be possible to increase doses further if necessary for efficacy with these and other recombinant DNA, FP9 and MVA vaccines, although concentrations would need to be higher to make this practical.

DNA/MVA delivery of ME-TRAP is more immunogenic in Gambian adults at 2mg/ 1.5×10^8 pfu doses than 1mg/ 3×10^7 pfu doses although the increased immunogenicity is less marked than the same comparison in UK adults. There are several possible reasons for this. The high immunogenicity seen in UK individuals was in a single group of 4 volunteers (the DDDMM(15) group in chapter 7 in whom immunogenicity was greater than in the DDDM group in this chapter). Results from such a small group should be treated with caution. It is most likely that the enhanced immunogenicity seen at the lower 1mg/ 3×10^7 pfu doses in Gambian compared to UK adults only occurs at this lower

dose. At higher doses the available data suggest that the enhancement is lost because maximal effector T cell frequencies are reached whether malaria-naïve or malaria-exposed. It is conceivable that the prior exposure of Gambian adults to the many malaria antigens (and other genetic and environmental differences) could reduce the maximum immunogenicity possible compared to malaria-naïve adults but there are insufficient available data to address this question.

It is not possible to state whether two or three priming DNA immunizations offer greater immunogenicity for MVA boosting from this data. Any difference between the two regimens is highly unlikely to be significant for efficacy. We did not state limits for equivalence prior to onset of the study and this should be done prior to any future attempt to evaluate equivalence. High dose DDM delivery of ME-TRAP has not been evaluated in the UK and once this occurs further data for the comparison between two and three DNA primes will be available.

As a three dose regimen is far more practical for use in a non-research setting I believe DDM is preferable. The data in this thesis are consistent with high dose DDM being the best "real world" regimen for enhanced induction of effector T cells in humans and this finding should be applicable to other malaria constructs and vaccines for other infectious diseases and cancer immunotherapy. Most practical would be a three dose, four week interval regimen and future work will need to evaluate my belief that four rather than three weeks is unlikely to reduce immunogenicity or efficacy. This three dose, four week interval DDM regimen would allow administration at expanded programmes of immunisation (EPI) timepoints and therefore maximise the prospects for implementation of an effective vaccine within existing African public health frameworks.

Somewhat surprisingly FFM immunization is less immunogenic than DNA/MVA regimens in this study. This has also been seen in UK studies with

the ME-TRAP construct (D Webster, unpublished data). A possible reason is that there could be some cross-reactivity in the host immune responses to FP9 and MVA such that some MVA is neutralized before infection of cells and recombinant protein expression. Although FP9 and MVA are only distantly related phylogenetically, and so this was not expected to occur, the two poxviruses have moderate sequence homology. If the targets of the cross-reactive immunity could be characterized and removed from MVA by recombinant DNA techniques, the immunogenicity of FP9/MVA regimens could be increased substantially – in theory to greater frequencies than DNA/MVA regimens because of relative recombinant protein expression levels. This work would be a major undertaking however.

There are encouraging data from this study about the kinetics of induced T cell responses. As expected we see a substantial and rapid decline in frequency from 7 days to 4 weeks as apoptosis of effector T cells occurs but from 4 weeks to 8 weeks the decline is very gradual and flat in one group (the FFM group). I believe that an effective T-cell based malaria vaccine will not be developed until a relevant T cell response can be induced which is protective against natural infection at the frequencies induced *during the plateau phase* from 4 weeks onwards. It will be important to explore the kinetics of frequency decay further from 8 weeks to 12 months.

Our collaboration between the University of Oxford, UK and the Medical Research Council laboratories, The Gambia has facilitated the conduct of a series of phase I studies in the UK and The Gambia. Within an appropriately short timeframe, and therefore with the necessity of using small group sizes, we have optimised the following parameters: dose, route, interval, number of priming immunisations and choice of priming agent in terms of acceptable safety and reactogenicity and the highest possible immunogenicity. Prime-boost regimens showed a statistically significant delay in time to parasitaemia (figure 7.6) and this has been interpreted as possibly sufficient efficacy for complete protection in the field setting. We obtained funding for, designed and

conducted a field efficacy trial of high dose DDM delivery of ME-TRAP in The Gambia (see chapter 11).

It has been unclear whether it is possible to induce higher levels of immune responses through vaccination than occurs through natural infection. The requirement for such supra-natural immunogenicity is a key one for effective liver-stage malaria subunit vaccination. T cell responses to liver-stage antigens in semi-immune adults from sub-Saharan Africa in contrasting epidemiological settings are low(Lalvani et al., 1996, Plebanski et al., 1997). The pre-vaccination geometric mean effector T cell frequency to T9/96 TRAP in 43 healthy adult semi-immune volunteers enrolled into this study and the study described in chapter 8 is 6.1 SFCs per 10^6 PBMCs. Therefore the most immunogenic regime in this study induces responses fifty-fold higher than natural immunity.

Preliminary plans have been made to scale up recombinant MVA manufacture once a product is highlighted for licensure and widespread use. Currently DNA vaccines are considerably more expensive than recombinant poxviruses, but there are many examples of vaccines' costs coming down to affordable levels during the development process (notably the Hepatitis B vaccine now licensed and part of routine immunisations in the Gambian EPI). The technologies which the study vaccines represent are scaleable and suitable for licensure.

This study confirms that the increasing body of promising pre-clinical data with DNA/MVA vaccines translate into human immunity with a malaria antigen – a translation which has not held for several other approaches which were promising in pre-clinical studies(Doolan and Hoffman, 2001). The study provides strong rationale for further clinical evaluation of both DNA/MVA and FP9/MVA for diseases caused by intracellular pathogens. It provides hope that effective vaccines for liver-stage malaria, HIV and tuberculosis can be developed with heterologous DNA-based prime-boost combinations.

Table 10.1: Descriptive statistics for timecourses in the three vaccination groups in VAC14

	Screening	POST DNA+7	FF+7	DDM+7	DDDM+7	FFM+7
AM	14.8	18.9	78.9	671.0	627.5	289.7
GM	8.6	11.9	46.7	211.7	331.0	156.3
Median	10	14.4	57.5	101.25	458.75	165.6
IQR	2.5-13.75	3.4-31.2	29.4-128.4	55.6-1571.3	113.75-1053.8	120.3-450.9

Table 10.1: Summed *ex vivo* ELISPOT responses to T9/96 TRAP in Gambian adult males receiving three prime-boost malaria vaccination regimens. Units are SFCs per million PBMCs. The responses presented are the sum of the four T9/96 TRAP peptide pools presented in figure 10.2 with the negative control subtracted four times. Trial code VAC014. AM – Arithmetic mean. GM – geometric mean. IQR – interquartile range. POST DNA+7 includes volunteers in DDM group and DDDM group at 7 days after their final DNA ME-TRAP vaccination. FF+7 indicates volunteers in the FFM group at 7 days after their second and final FP9 ME-TRAP vaccination. DDM+7, DDDM+7 and FFM+7 indicates 7 days after final vaccination in the DDM, DDDM and FFM groups.

Table 10.2: Local and systemic adverse events with ME-TRAP vaccines in VAC14

	DNA N=13	FP9 dose 1 N=16	FP9 dose 2 N=16	MVA after DNA N=12	MVA after FP9 N=14
Headache	1	5	5	3	5
Malaise	1	7	4	3	4
Nausea/Vomiting	0	1	0	0	0
Local Adverse Events					
Pain	1	6	1	7	3
Itching	1	5	6	2	2
Blister	0	6	1	3	2

This table is a comparison of DNA, FP9 and MVA carriers and shows solicited adverse events over 7 days after immunisation. All adverse events were mild or moderate.

Table 10.3: Timecourse for local solicited adverse events after first and second FP9 and after MVA immunizations in VAC14.

FP9one	day 1	day 2	day 7	day 21	Size
Indurat^n	13	15	0	0	
Itching	5	6	1	0	
Pain	6	4	0	0	
blister	3	5	1	0	2-8 on day 2
LAM	0	0	0	0	
FP9two	**day1**	**day 2**	**day 7**	**day21**	**Size**
Indurat^n	7	2	0	0	
Itching	6	0	0	0	
Pain	1	0	0	0	
Blister	1	1	0	0	4 on day 2
LAM	0	0	0	0	
MVA	**day 1**	**day 2**	**day 7**	**day 21**	**Size**
Indurat^n	13	10	3	0	
Itching	4	3	8	0	
Pain	10	8	0	0	
Blister	6	8	0	0	1-10 on day 2
LAM	1	1	0	0	

n=16 for first and second FP9 immunisations. n=25 for MVA immunizations. Indurat^n – induration measured by palpation. Blister in either or both arms. LAM – limited arm motion. Size was measured in mm.

Figure 10.1 Timecourses for the three vaccination groups by *ex vivo* γ-interferon ELISPOT assay. (see page 146 below for legend)

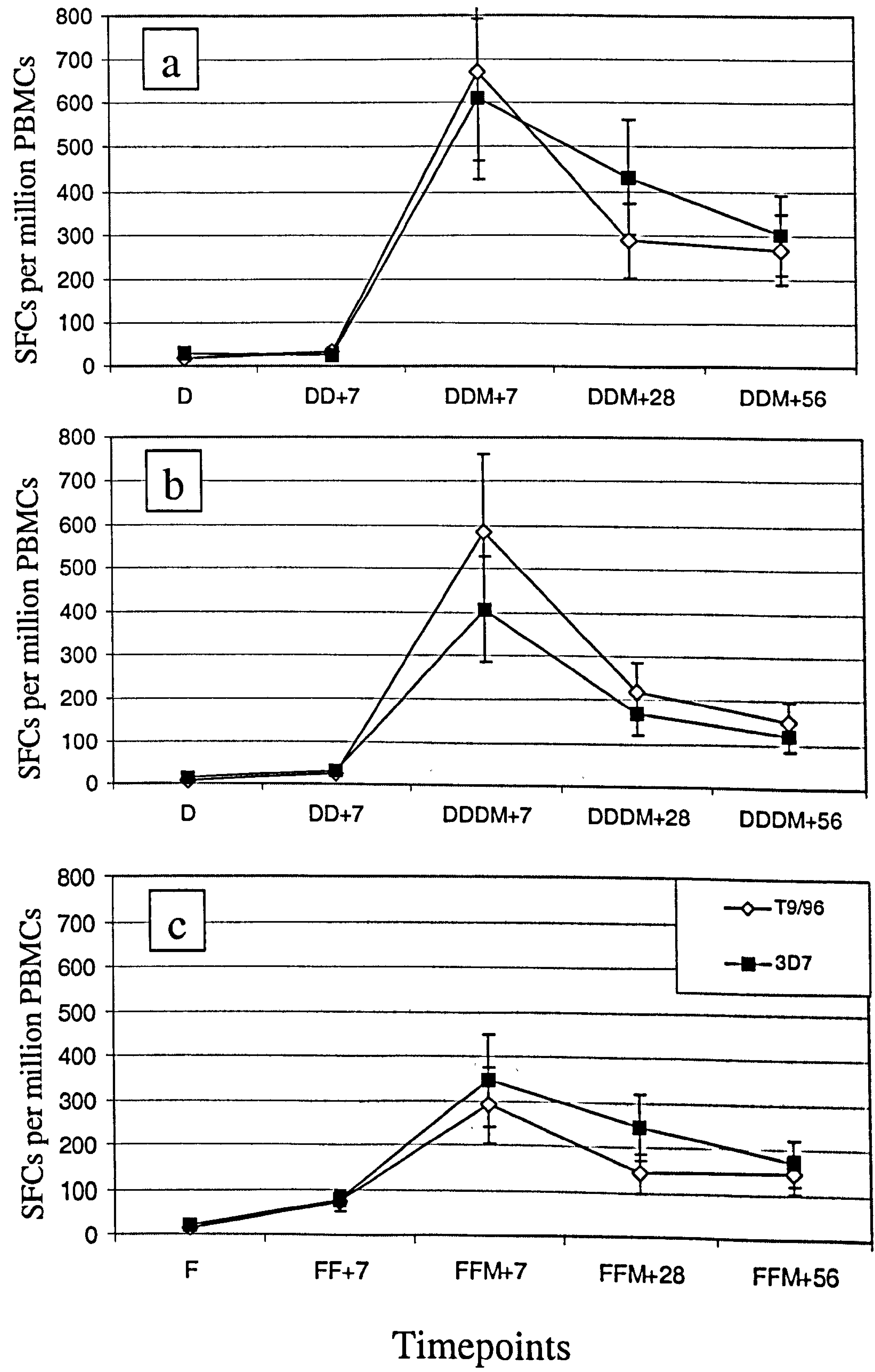


Figure 10.1 Magnitude, duration and 3D7 cross reactivity of γ–interferon responses by *ex vivo* ELISPOT. A) Group who received DNA immunizations at weeks 0 and 3 followed by an MVA immunization at week 6. B) Group who received DNA at weeks 0, 3 and 6 and MVA at week 9. C) Group who received FP9 at weeks 0 and 3 followed by MVA at week 6. Each point represents the arithmetic mean (standard error) of the summed *ex vivo* ELISPOT responses to T9/96 (or 3D7) TRAP. D and F are the first pre-vaccination timepoints. D- DNA ME-TRAP immunisation. M – MVA ME-TRAP immunisation. F – FP9 ME-TRAP immunisation. Numbers after plus signs represent the number of days after the most recent vaccination that blood was obtained. For example DDM+7 indicates the group of volunteers who received two DNA ME-TRAP immunisations followed by a MVA ME-TRAP immunisation with blood drawn seven days after the MVA immunisation. Y axis units are SFCs per million PBMCs. Trial code VAC14.

Figure 10.2 The breadth of responses across the ME-TRAP construct at seven days after MVA immunization in the three groups in VAC14

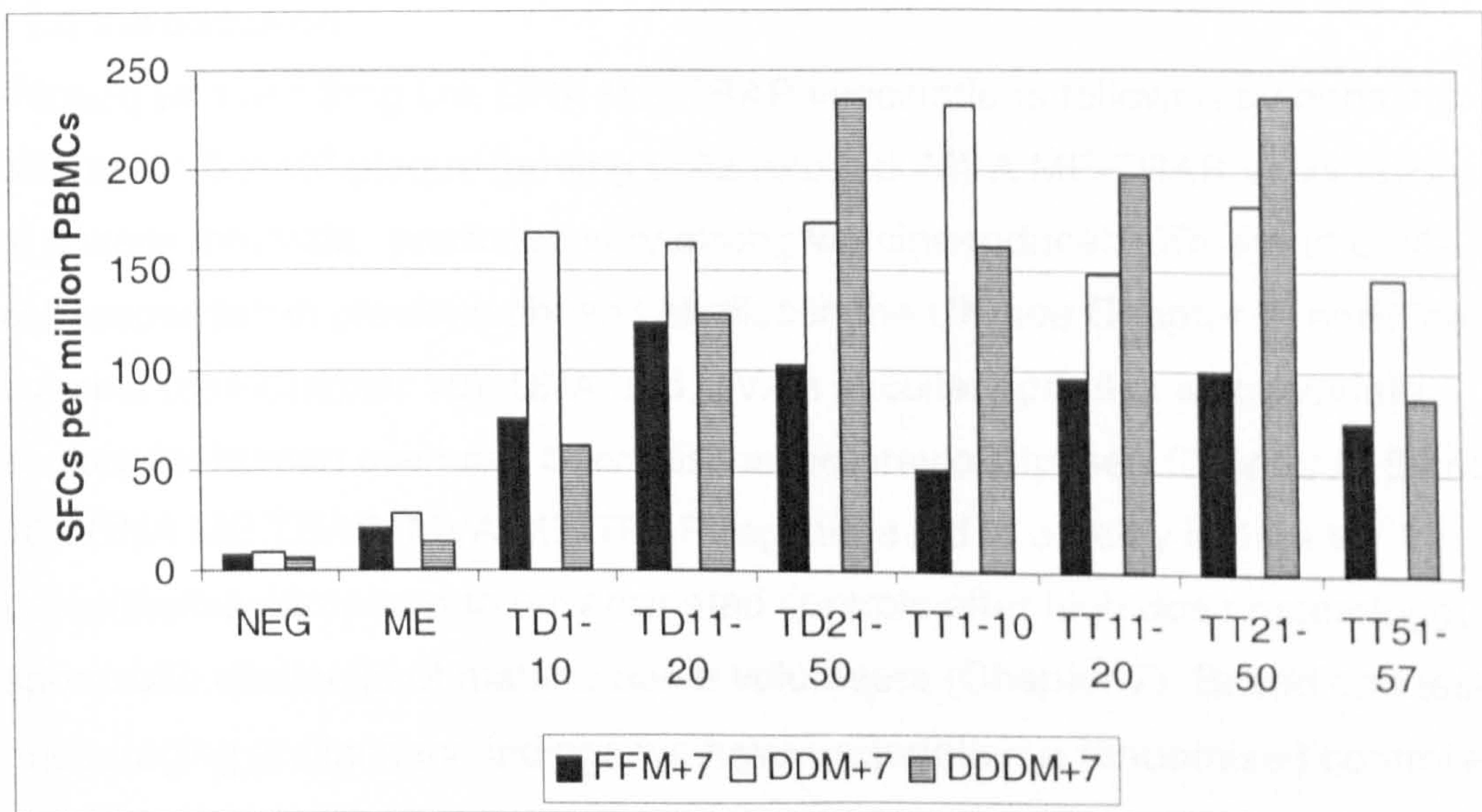


Figure 10.2 Arithmetic mean *ex vivo* ELISPOT responses at seven days after first MVA immunization in VAC14. ME – pool of all ME peptides. TD – 3D7 TRAP 20-mer peptides. TT – T9/96 TRAP 20-mer peptides.. 1-10 – pool of ten 20-mers spanning amino acids 1-110. 11-20 – pool spanning amino acids 100-210 etc. 51-57 pool completely conserved between 3D7 and T9/96

Chapter 11

Randomised, controlled, double-blind efficacy trial of DNA ME-TRAP in combination with MVA ME-TRAP in semi-immune males aged 15-45 in The Gambia

11.0 Introduction

Priming with two 2mg i.m. DNA ME-TRAP vaccinations followed by boosting with one 1.5 x 10^8 plaque forming units (pfu) i.d. MVA ME-TRAP vaccination, at 3 week intervals, produced very strong vaccine-induced CD4+ and CD8+ T cell responses in previous phase I studies in the UK(see Chapter 7) and The Gambia (see Chapter 10). DNA and MVA's excellent profiles as candidate vectors for human use have been discussed previously (see Chapter 6, 8 and 10). DNA ME-TRAP/ MVA ME-TRAP regimens led to a delay in time to parasitaemia compared to unvaccinated controls after high dose heterologous sporozoite challenge of malaria-naïve volunteers (Chapter 7). Based on these encouraging preliminary findings we have undertaken a randomised controlled trial of DNA ME-TRAP/ MVA ME-TRAP in a rural part of The Gambia to explore whether this vaccine combination could provide protection against natural *P. falciparum* infection.

11.1 Methods

11.1.1 *Study Setting and Volunteers*

Volunteers were recruited from 13 villages in the North Bank Division of The Gambia in July 2002 with follow-up to December 2002. Malaria incidence is highly seasonal in The Gambia with most infections occurring between August and December. In 2002, the malaria transmission season was delayed with

few disease episodes before October. The Entomological Inoculation Rate (EIR) varies between <1 and >100 in The Gambia(Hay et al., 2000). The villages were chosen for proximity to the alluvial flood plain . A strong association between distance from the flood plain and EIR has been seen in this part of The Gambia(Clarke et al., 2002) and thus the EIR in the study area is likely to have been in the range of 10 – 30 infectious bites during the period of follow-up surveillance.

Approval was obtained from the Joint Gambian Government/Medical Research Council Ethics Committee, the Oxford Tropical Research Ethics Committee, the London School of Hygiene and Tropical Medicine Ethics Committee and an independent data safety monitoring committee. Independent clinical trial monitors monitored for adherence to International Committee of Harmonisation Good Clinical Practice guidelines.

Before recruitment, meetings were held with village chiefs and elders followed by general village meetings at which the study was explained. Volunteers received information sheets and consent forms translated into the three local languages in Arabic script as well as in English. After written informed consent was obtained by a study physician, age and identity were checked, pre-test HIV counselling occurred and potential volunteers underwent clinical evaluation including a full medical history and clinical examination. They were screened for haematological (full blood count), renal (plasma creatinine) and hepatic (plasma alanine aminotransferase (ALT)) dysfunction and duplicate malaria smears were made. Exclusion criteria included clinically significant disease, ALT>42 (iu/l), creatinine>130 (μmol/l), PCV<30%, positive antibody ELISA to HIV-1 or HIV-2, simultaneous participation in another clinical trial, blood transfusion in the month prior to vaccination, previous experimental malaria vaccination, administration of another vaccine within 2 weeks of vaccination, previous rabies vaccination, allergy to any previous vaccine or to sulphadoxine/pyrimethamine, history of splenectomy and any treatment with immunosuppressive drugs. Eligible volunteers were given a unique study

number and a photographic identity card. Parental written informed consent was obtained for volunteers aged 15-17 years.

11.1.2 Procedures

Volunteers were randomly assigned to receive either two, 2mg doses of DNA ME-TRAP followed by a single 1.5×10^8 pfu dose of MVA ME-TRAP or three doses of rabies vaccine which were given on days 0, 21 and 42 timed to coincide with the start of the rainy season. A member of the data safety monitoring committee generated the randomisation list in London. A block procedure was used and whole villages were enrolled with sequential study numbers to ensure balanced numbers in each group. Local investigators did not know the size of the blocks. Non-translucent sealed envelopes were used for vaccine allocation. Study numbers were not pre-printed on vials but were written on vials at vaccination. Used vials were checked for correct allocation off-site. Vaccination was performed by nurses who played no other part in the trial. The first two doses of vaccine consisted of two intramuscular injections, one into each deltoid muscle. DNA ME-TRAP was given as 1ml and rabies vaccine as 0.5ml into each arm. The third dose of vaccine was given as four intradermal injections into the skin overlying the deltoid muscle; two injections into each arm. The malaria vaccine group received MVA ME-TRAP as four 0.1ml injections, two into each arm, whereas the control group received four 0.05ml injections of rabies vaccine. Two weeks before administration of the third dose all volunteers received three tablets of sulphadoxine/ pyrimethamine to clear blood-stage *P. falciparum* infections.

The individual epitopes making up the ME string are described in detail elsewhere(Gilbert et al., 1997). The strain of TRAP included in the vaccine construct is T9/96. The candidate malaria vaccines were manufactured to Good Manufacturing Practice by contract manufacturers (DNA ME-TRAP by Qiagen, Hilden, Germany and MVA ME-TRAP by IDT, Rosslau, Germany). DNA ME-TRAP was supplied as single dose 2mg in 2ml vials. MVA ME-TRAP was supplied as two dose vials each containing 3×10^8 pfu in 0.8ml. The

rabies vaccine (Chiron Behring GmbH, Marburg, Germany) was supplied as a lyophilised single dose vial with accompanying diluent and syringe. This vaccine was chosen because of its public health benefit in The Gambia.

After each vaccination, volunteers were observed for one hour and visited at home on the first, second and seventh day post-vaccination for assessment of local adverse events (discolouration, induration, blister formation, pain, limitation of arm motion), systemic adverse events (headache, nausea, malaise, axillary temperature) and unsolicited adverse events. One week and 13 weeks after the third vaccination venous blood was collected for measurement of full blood count, ALT and creatinine.

The local reactogenicity during the seven days after MVA vaccination could conceivably have unblinded its assessors. Therefore, we deployed different field workers to assess reactogenicity after dose 3 and to perform surveillance during the remaining period of follow-up. In this way we ensured that field workers making morbidity observations and taking blood smears after the period of vaccination were blind to the vaccine code. All slide readers had no contact with volunteers and were blind to the vaccine code.

During the surveillance period, starting two weeks after the third dose of vaccine, volunteers were visited twice weekly and asked whether they had attended a health centre. At the weekly visits, blood smears and axillary temperatures were taken. At midweek visits, blood smears and temperature were taken if symptoms compatible with malaria were present. Investigators and field supervisors did random visits to ensure accurate data collection. This active case detection was supplemented by passive case detection by study nurses to whom volunteers had 24 hour access at 3 of the study villages and by a clinic at MRC Farafenni (20km from the study villages). When blood smears were obtained, two sets of duplicate blood smears (four smears in total) were made. For possible clinical episodes an immediate Field's stain was performed and read. Two further smears ("A" and "B" slides) were stained

with Giemsa after overnight drying and 100 high power fields were read by different slide readers before being declared negative. *P. falciparum* parasites were confirmed by a supervisor before a slide was declared positive. The arithmetic mean of the A and B slides was used to determine parasite density and a volunteer was declared positive for asexual *P. falciparum* parasitaemia if either slide was positive. If A and B slides were markedly discrepant, a third read was performed by the supervisor and this read was used for analysis. Parasite density was expressed per μl (assuming one parasite per high power field = 500 parasites/μl). Full blood counts including packed cell volume were measured in a CA620 cell analyser (Medonic). ALT (iu/l) and creatinine (μmol/l) were measured in a Visual analyser (Biomerieux).

Effector T cell responses were assessed in *ex vivo* γ-interferon ELISPOT (enzyme-linked immunospot) assays for 98 volunteers randomly selected from a substudy list containing a 3 to 1 ratio of malaria vaccinees to control subjects. 4×10^5 peripheral blood mononuclear cells (PBMCs) were assayed as described(Moorthy et al., 2003) using Millipore MAIP S45 plates for 18-20 hours before being developed. Mabtech antibodies were used and counting of spots was performed blinded to vaccine allocation with the AutoImmun Diagnostika computerised system. All peptides were at 25 μg/ml concentration. A single pool contained all ME peptides. 4 pools each were used of 20-mer peptides, overlapping by 10, to span T9/96 and 3D7 strains of the entire TRAP antigen.

11.1.3 Statistical analysis

The primary endpoint was time to first infection with asexual *P. falciparum,* defined as the number of days from the start of the surveillance period to the date of the first positive slide. Vaccine efficacy was calculated from the hazard ratio estimated by Cox's regression, adjusting for the effects of prognostic variables. An analysis plan, written before unblinding, specified exclusion criteria, statistical methods and important covariates (age, village of residence and bednet use defined as sleeping nightly under an intact bednet). Ethnic

group, though not specified as a covariate in the analysis plan, was found on analysis to be associated with the risk of infection, and was included as a covariate. Because of small numbers in some villages and for presentation purposes only we describe village of residence as three groups of villages, divided geographically (see tables 10.1 and 10.4). There was no stratification by village. Volunteers who received fewer than three doses or who were parasitaemic both pre-vaccination and at the beginning of surveillance without an intervening negative blood smear were excluded from the primary analysis but included in a secondary analysis. Observations on individuals who were lost to follow-up or were missing from trial data for > 3 weeks were censored. The incidence of *P. falciparum* infection varies substantially within the study area from year to year. Based on data obtained over a period of 10 years it was calculated that the trial would have >80% power (with α=0.05) to detect a difference in the hazard of infection if vaccine efficacy was 40%. Symptomatic malaria was defined as presence of asexual *P. falciparum* parasites at any parasitaemia with either an axillary temperature of >= 37.5 °C or one or more of the following symptoms: headache, myalgia, arthralgia, malaise, nausea, dizziness or abdominal pain. Analyses were done with Stata version 7 (Stata Corporation, College Station, TX Station, USA).

ELISPOT responses were analysed as follows. After subtraction of medium alone values from each pooled peptide response, they were summed across T9/96 and 3D7 pools. Geometric means were calculated for T9/96 TRAP, 3D7 TRAPand ME string responses. Responses in the two groups were compared with the Mann-Whitney test.

11.2 Results

11.2.1 Safety and reactogenicity

489 volunteers aged 15-45 were screened of whom 113 were excluded. 46 could not be found on the day of vaccination, 40 were not eligible because of anaemia, an abnormal ALT or creatinine result, HIV, various medical conditions or because they were too young or too old and 27 withdrew consent. 372 were enrolled. 335 men (90%) received their second dose of

vaccine and 320 of these received the third dose. 52 who were randomised did not receive three doses (2 men received the wrong vaccine at dose 2, 26 left the study area, 23 withdrew consent and 1 was withdrawn because he developed pneumonia between dose 1 and dose 2). 296 (141 malaria, 155 rabies) received three doses and were followed up of whom 277 (74% of those initially enrolled) completed 11 weeks of surveillance. Additional data were available for 14 volunteers who did not receive all three vaccine doses (all 14 received dose 1 and dose 3), 2 (in the malaria vaccine group) who received the wrong vaccine at dose 2, and for 18 volunteers who were parasitaemic both before vaccination and at the start of surveillance. These individuals were included in a secondary analysis. Losses to follow-up were similar in the two groups. Prognostic variables were similarly distributed in the two groups at the start of surveillance (table 11.1).

11.2.2 Adverse events

No clinically significant differences in packed cell volume, ALT or creatinine were seen in either vaccine group. One volunteer who received rabies vaccine had a history of breathlessness and chest pain several years prior to enrolment, experienced a relapse of symptoms and deteriorated and died of likely cardiac pathology 3 months after the last vaccine dose. This event was regarded as unrelated to vaccination. There were no other serious adverse events. Adverse events were rare after first and second doses and were not increased in the DNA ME-TRAP group compared to the rabies vaccine group (data not shown). Injection site pain, limited arm motion, headache and malaise in the first 24 hours after vaccination were more common after MVA ME-TRAP vaccination than after rabies vaccination (table 11.2). Most volunteers developed an injection site blister 1-2 days after MVA ME-TRAP vaccination which healed over 1-3 weeks without complications. Induration (for 1-2 days) and discolouration (faint, shiny macular appearance for several weeks) was common after MVA ME-TRAP vaccination. There were severe adverse events in three individuals causally related to MVA ME-TRAP vaccination. These individuals experienced one or more of severe malaise,

headache, local pain or limited arm motion during the 24 hours after vaccination.

11.2.3 Effector T cell induction

Sixty-three and 30 volunteers from malaria and rabies vaccine groups respectively were tested for T cell responses 7 days after final vaccination (table 10.3). In the rabies vaccine group, geometric mean effector T cell responses to TRAP were 3.1, 3.9 and 1.4 spot forming cells (SFCs) per million PBMCs for T9/96 and 3D7 strains of TRAP and the ME string respectively (table 10.3). In the malaria vaccine group, the effector T cell frequency to the vaccine strain of the TRAP antigen, T9/96, was geometric mean 251.1 SFCs per million PBMCs (80-fold increase above control group, p value <0.001). 3D7 is a strain with 6% amino acid variance to T9/96 and is thought to have greater homology to locally circulating strains. The cross-reactive response to 3D7 TRAP was 197.5 SFCs per million PBMCs (50-fold increase above control group, p value <0.001). The ME string response in the malaria vaccine group was 4.7 SFCs per million PBMCs (3 fold increase, p value 0.03).

11.2.4. Time to first P. falciparum *infection*

171 subjects developed parasitaemia at least once during the surveillance period, 80/141 (57%) in DNA/MVA group and 91/155 (59%) in the rabies group. The distribution of time to first infection was similar in the two groups as seen in the graphical representation of the Kaplan Meier Survival curve (Fig 2). Vaccine efficacy among subjects in the according to protocol group who received 3 doses, adjusted for age, bednet use, ethnic group and village of residence was 10.3% (95%CI -22% to +34%) p=0.49. Similar results were obtained when all subjects who received at least one dose of vaccine were included in the analysis (efficacy 0.95% (95%CI -32% to +25%) p=0.95.

Geometric mean *P.falciparum* densities in first infections were similar in the two groups (31 (IQR 5-154)/μl in the malaria vaccine group compared to 24 (IQR 5-69)/μl in the rabies group, p=0.79 (Mann-Whitney test)).

During surveillance there were 10 episodes of symptomatic malaria in the malaria vaccine group and 13 in the rabies group. The risk of malaria-related symptoms during an episode of parasitaemia was similar in both vaccine groups.

Within the ELISPOT substudy group, the risk of developing parasitaemia was not associated with the magnitude of the summed effector T cell response by *ex vivo* ELISPOT to the 3D7 strain of TRAP. The 80 men from the substudy group who received 3 doses of either malaria vaccine (55 men) or rabies vaccine (25 men), completed 11 weeks of surveillance, and had complete ELISPOT data after dose 3, were divided into four quartiles according to the magnitude of their summed response to 3D7 TRAP 20-mer peptides. Men with the highest effector T cell responses had similar hazard ratios for incidence of patent blood stage infection, as estimated by Cox's regression, compared to those with the lowest effector T cell responses after adjustment for age, bednet use and village of residence. However, the small numbers limits the power of this analysis.

The incidence of parasitaemia decreased with increasing age, and was decreased in those of Fula ethnicity compared to Mandinka and Wollof ethnic groups (Table 11.4).

11.3 Discussion

This trial demonstrates that vaccination with two doses of DNA ME-TRAP followed by a single dose of MVA ME-TRAP is safe and highly immunogenic for effector T cell induction but is unlikely to protect against natural *P. falciparum* infection. This provides a second comparison between protection in malaria-naive and malaria-experienced adults. RTS,S/AS02 provides 30-

60% complete protection in the artificial challenge model and 71% short-term protection against natural infection(Bojang et al., 2001). The lack of field efficacy in this paper suggests the use of complete, not partial, protection in the artificial challenge model as a screening measure for candidate pre-erythrocytic vaccines. However some vaccines are known to prevent disease but not infection, as is also the case for naturally acquired immunity to malaria. This study does not exclude the possibility that the candidate regimen could provide significant anti-disease immunity. Paediatric study designs may be required to show this.

The candidate regimen represents a new method for induction of very high effector T cell frequencies, which are about 50 fold higher than those induced by lifelong natural exposure. The failure of these effector T cells to kill infected hepatocytes is intriguing. Perhaps in infected hepatocytes *in vivo* ME-TRAP epitopes are not expressed, processed or presented adequately for the required T cell-hepatocyte synapse formation. The predominance of CD4+ over CD8+ T cells in the induced responses was unexpected and could be relevant for efficacy(McConkey et al., 2003, Moorthy et al., 2003). Both considerations may be addressed by altering the malarial construct to include different antigen(s) or epitopes. For example DNA/MVA delivery of a circumsporozoite (CS) antigen based construct may allow translation of the strong effector T cell responses now possible through vaccination to protection against natural *P. falciparum* infection. Other heterologous combinations (for example replacement of DNA priming with another attenuated viral vector) may be necessary for efficacy.

DNA/MVA vaccination has not to date induced significant antibody titres in humans(McConkey et al., 2003); trials are underway of combination with recombinant protein vaccines for dual antibody/effector T cell induction. Combination with protectively immunogenic blood-stage candidates is also desirable. Determining methods for the successful combining of different

candidate vaccine regimens (whether within or between parasite stages) will be one of the important challenges of coming years.

Even for an adult population, the incidence of clinical disease was lower than expected. Sulphadoxine/pyrimethamine was administered four weeks before the start of surveillance in this study and in an RTS,S field efficacy study(Bojang et al., 2001). There is some evidence that pre-treatment with this antimalarial reduces incidence of clinical malaria for longer than four weeks(Genton et al., 2000, Coulibaly et al., 2002). However there was also less clinical disease than in recent years in paediatric cohorts recruited for other studies in 2002 at the study setting and so there may have been a general reduction for climatic reasons.

This study highlights North Bank Division in The Gambia as an excellent malaria vaccine field trial site both for adults and by extrapolation for children. In a low transmission year cumulative incidence overall in men aged 15-45 was 72% over 11 weeks, higher than expected, compliance was good despite a demanding study design and migration from the study area was acceptable.

This paper adds to the body of data detailing the very gradual acquisition of anti-infection immunity in adults resident in sub-Saharan Africa. While substantial immunity to severe malaria is acquired after only a few infections and anti-disease immunity is acquired in childhood, we saw statistically significant decreases in incidence of infection with increasing age in the 15-45 age range (table 4) as was observed in the previous Gambian RTS, S adult efficacy trial(Bojang et al., 2001). The protection against infection for those with Fula ethnicity in this trial is consistent with a report from Burkina Faso(Modiano et al., 1996) and with an earlier study in the Farafenni area (Greenwood et al., 1987).

Effector T cell induction 50 fold above that induced by natural infection is now possible through DNA-based prime boost vaccination. Further candidate

vaccine evaluation is required to assess which candidate immunogens will confer efficacy in this system and to identify methods for dual high frequency induction of CD8+ T cells and antibody-producing plasma cells.

Table 11.1: Characteristics of the trial cohorts at the start of surveillance in VAC20 (296 subjects who received 3 doses of vaccine and were followed up).

	Malaria vaccine (n=141)	Rabies vaccine (n=155)
Median age (years) (interquartile range)	20 (17-32)	21 (18-30)
Village group 1 (Alkali Kunda, Chamen, Yallal)	26 (18%)	29 (19%)
Village group 2*	79 (56%)	93 (60%)
Village group 3 (Bambali)	36 (26%)	33 (21%)
Ethnic group: Fula	12 (9%)	19 (12%)
Mandinka	81 (57%)	82 (53%)
Wollof	48 (34%)	54 (35%)
Sleeps nightly under intact bednet	76 (54%)	83 (54%)

*Village group 2 consisted of 9 closely situated villages

Table 11.2: Frequency of solicited symptoms during the 7 days after the third dose of vaccine in VAC20.

	i.d. MVA ME-TRAP			**i.d. RABIES VACCINE**		
	N	**%**	**95% CI**	**N**	**%**	**95% CI**
Local symptoms						
Limited arm motion	28/152	18%	(13-26)	2/158	1.3%	(0.2-4.5)
Pain	103/152	68%	(60-75)	19/157	12%	(7.4-18)
Discolouration	116/151	77%	(69-83)	26/156	17%	(11-23)
Induration	138/152	91%	(85-94)	86/158	54%	(46-62)
Blister	89/151	59%	(51-67)	14/156	9.0%	(5.0-15)
General symptoms						
Headache	45/161	28%	(21-36)	19/168	11%	(6.9-17)
Objective Fever	6/161	3.7%	(1.4-7.9)	0/168	0%	(0-2.0)
Malaise	40/161	25%	(18-32)	14/168	8.3%	(4.6-14)
Nausea	3/161	1.9%	(0.4-5.3)	1/168	0.6%	(0-3.3)

Notes: N = total number of subjects who received the third dose. n (%) = number (percentage) of subjects who had at least one report of the symptom.

Table 11.3: Effector T cell responses one week after the third vaccination in VAC20*

Stimulant	Malaria vaccine (n=55)	Rabies vaccine (n=25)	P value
	Geometric mean SFCs per million PBMCs	Geometric mean SFCs per million PBMCs	
ME peptides	4.70	1.39	0.10
3D7 TRAP peptides	198	3.91	<0.001
T9/96 TRAP peptides	251	3.30	<0.001

*80 of the 93 individuals identified for testing T cell responses received three doses of vaccine according to protocol and gave analysable responses. Groups of summed responses were compared by the Mann-Whitney test in order to obtain p values.

Table 11.4: Results of Cox proportional hazards regression analysis for the risk of developing parasitaemia after 3 doses of vaccine in VAC20

Variable	No. developing parasitaemia/total	Crude hazard ratio	Adjusted hazard ratio
Vaccine Group			
Malaria vaccine	80/141 (57%)	1	1
Control	91/155 (59%)	1.07 (0.79-1.45)	1.12 (0.82-1.52)
Village group			
1	29/55 (53%)	1	1
2	109/172 (63%)	1.18 (0.78-1.78)	0.92 (0.54-1.55)
3	33/69 (48%)	0.93 (0.56-1.54)	0.94 (0.54-1.66)
Age at enrolment (years)			
15-17	48/69 (70%)	1	1
18-20	39/72 (54%)	0.66 (0.43-1.01)	0.56 (0.36-0.87)
21-29	41/71 (58%)	0.74 (0.49-1.13)	0.61 (0.39-0.95)
30-45	43/84 (51%)	0.56 (0.37-0.85)	0.46 (0.29-0.73)
Bednet use			
Yes	89/159 (56%)	0.93 (0.69-1.26)	1.07 (0.75-1.53)
No	82/137 (60%)	1	1
Ethnic group			
Fula	15/31 (48%)	1	1
Mandinka	86/163 (53%)	1.20 (0.69-2.07)	1.16 (0.65-2.1)
Wollof	70/102 (69%)	1.60 (0.91-2.79)	1.92 (1.02-3.60)

Figure 11.1 Trial Profile for VAC20

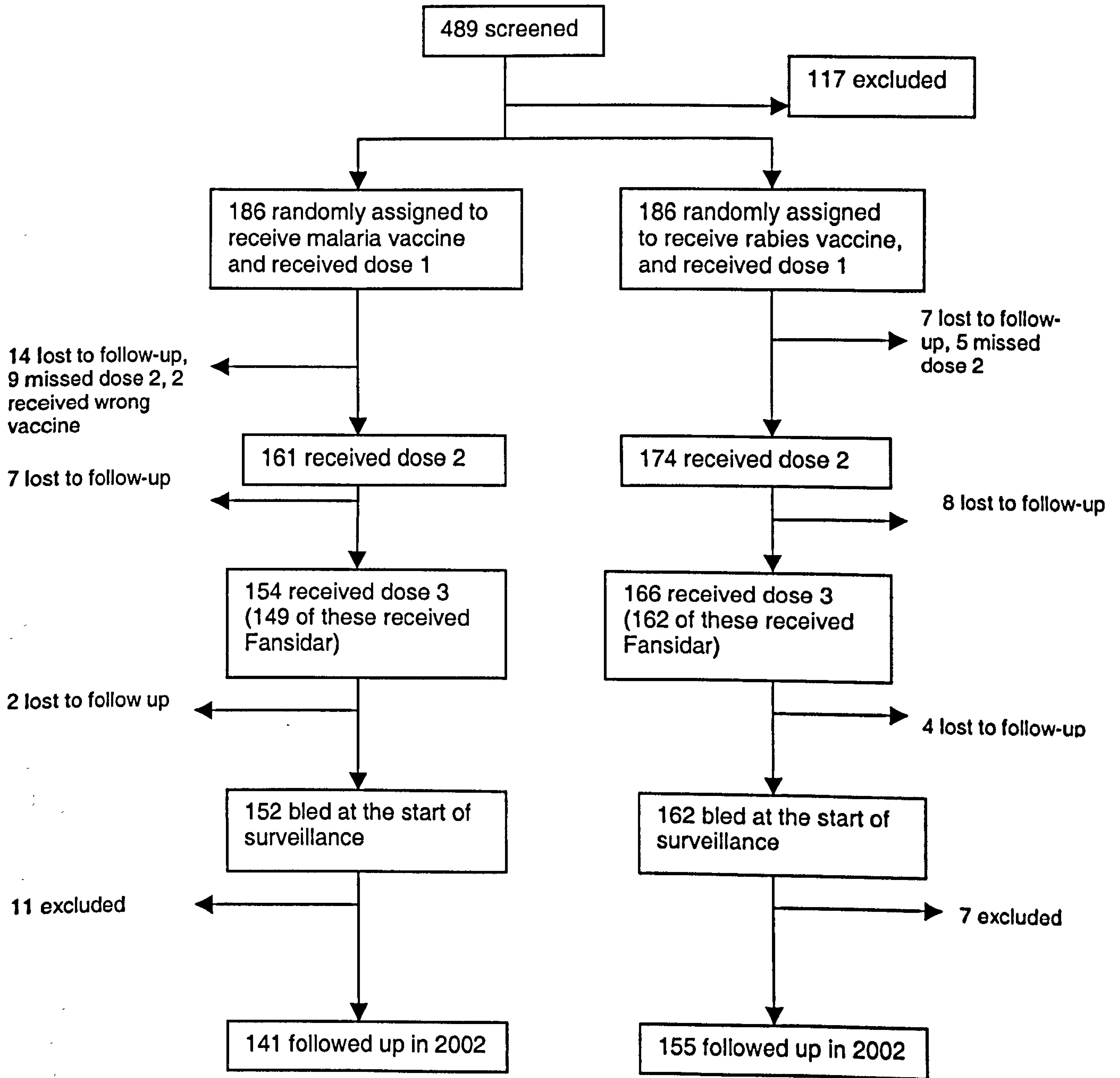


Figure 11.2: Kaplan-Meier survival curves showing the probability of remaining free of *P. falciparum* infection during the 11 weeks of surveillance in VAC20. Week 0 of surveillance began in October 2002, 14 days after dose 3 of vaccine was administered.

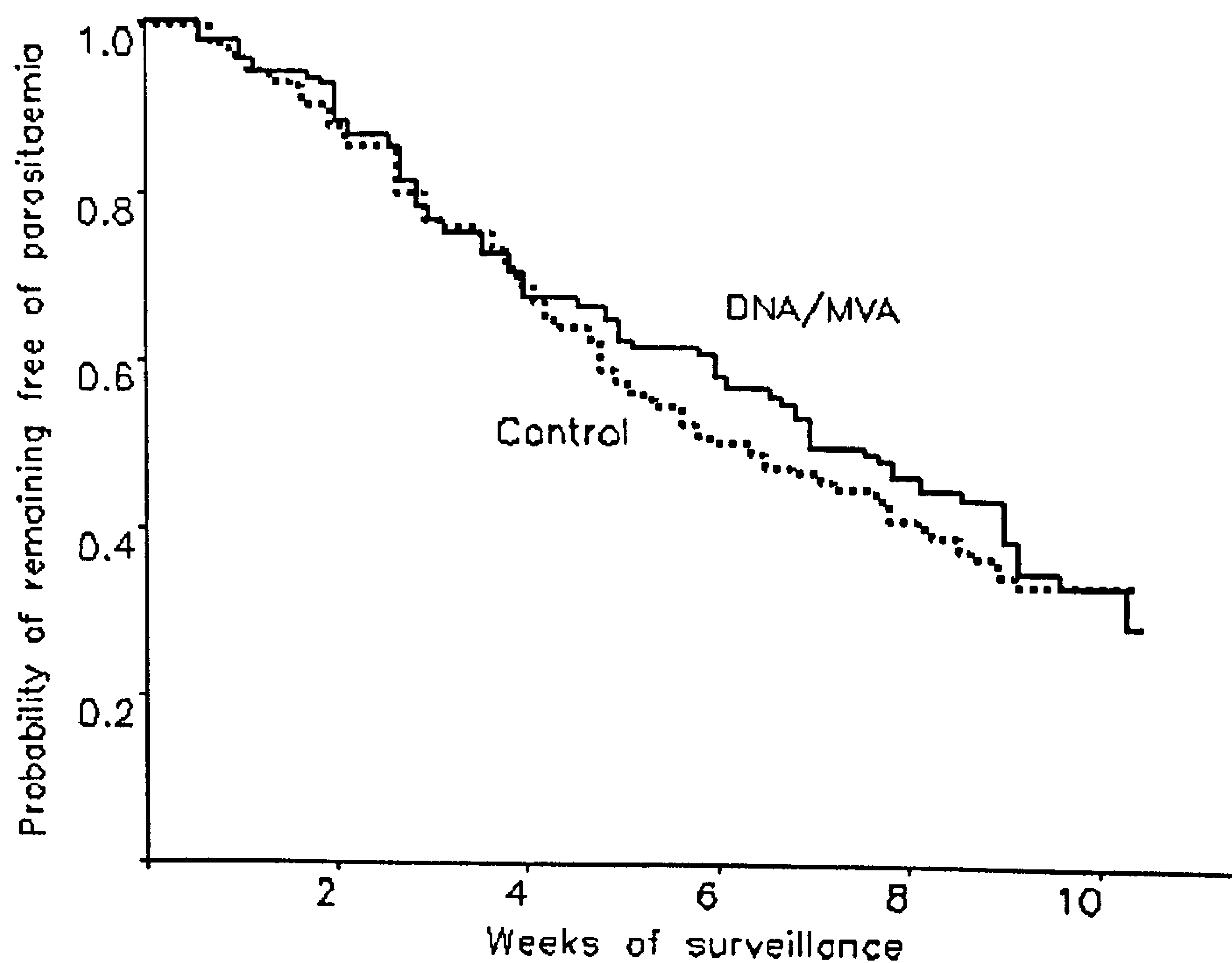
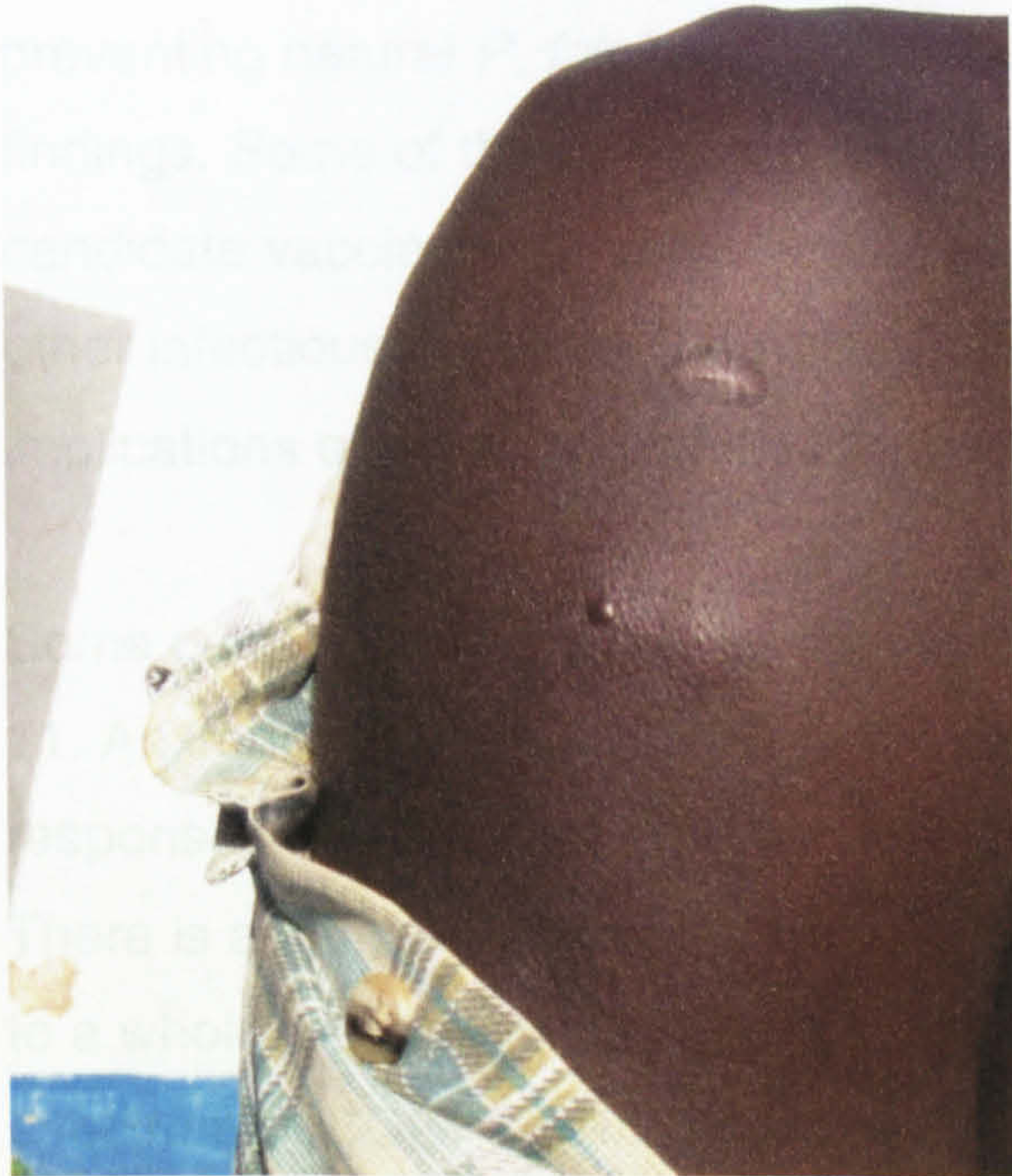


Figure 11.3: Local reactogenicity two days after injection of MVA ME-TRAP in VAC20. Two areas of induration are seen at the two adjacent injection sites. Note a 3mm blister at the centre of one injection site. The topmost lesion is an unrelated old scar.



Chapter 12

Conclusions

This thesis describes a series of studies culminating in a field efficacy trial. Although the intervention evaluated in the efficacy trial did not succeed in preventing natural *P. falciparum* blood-stage infection there were several notable findings. Some of these findings will have applications to development of candidate vaccine regimens by other groups both against liver-stage malaria and other infectious diseases. The thesis will therefore conclude by discussing the implications of these results for future vaccine research and development.

Some possible reasons for the failure of the intervention are described in Chaper 11. An important consideration is the apparent predominance of CD4+ T cell responses over CD8+ T cell responses with DNA/MVA ME-TRAP vaccination. There is a difficulty in determining the true magnitude of CD8+ T cell responses to a whole antigen, in this case TRAP, in an outbred human population. As the spectrum of CD8+ T cell epitopes to which volunteers will respond cannot be predicted precisely it is not possible to assay with selected CD8+ T cell epitopes. We used 20-mers overlapping by ten to span the whole protein. The high concentration used should have maximized the ability to detect CD8+ T cells but there is a possibility that some populations of such cells are not enumerated by this approach. In our laboratory experiments using recombinant viruses to infect presenters as an alternative way of providing MHC classI/epitope complexes for T cell activation have occurred. However the frequency of non-specific activation of T cells by non-recombinant virus has been problematic to date using human cells even though this method works adequately in murine cell assays. Optimisation of such a system should enable improvements in CD8+ T cell detection above current assays used.

Another possibility for the low CD8+ T cell frequencies seen to date is that insufficient APC transfection occurs with DNA/MVA vaccination. In this scenario antigen transfer from transfected cells to APCs occurs with bias towards the HLA class II presentation pathway. De novo synthesis within APCs is likely to be necessary for sufficient entry into the HLA class I presentation pathway. A third possibility is that processing of expressed antigen does not lead to generation of HLA class I epitopes after vaccination.

If there are sufficient CD8+ T cells, but the assays described in this thesis have not enumerated them, there are several reasons why they may not have been protective. Frequencies of effector T cells at the time of exposure may have been inadequate and a population of non-effector T cells with a memory phenotype may have been unable to expand and differentiate to a population with effector function within the timeframe required by the expression profile of TRAP. Locally circulating strains may have been sufficiently different to T9/96 strain to prevent T-cell synapse formation or activation. TRAP may not be expressed *in vivo* sufficiently in infected hepatocytes. Induced memory or effector T cells may not have the necessary migration phenotypes to be able to survey infected hepatocytes.

Because there is no satisfactory method of distinguishing between these reasons for failure using *in vitro* systems, the reason will not be known until a related DNA-based regimen is shown to protect against malaria. At this point detailed comparisons between protective and non-protective T-cell responses should determine the immune correlates of protection not induced by DNA ME-TRAP in combination by MVA ME-TRAP vaccination. If human hepatocytes could be maintained in culture this would allow characterization of vaccine-induced T cell/infected hepatocyte interactions.

How should we design a construct which will induce protective CD8+ T cell responses? The next pragmatic step is to attain the very high immunogenicity

seen to ME-TRAP by prime boost delivery of another antigen. If CD8+ T cell frequencies are higher with DNA/MVA delivery of for example CS then it could simply be that processing and/or presentation of TRAP within the ME-TRAP construct favours CD4+ T cell induction in a construct specific manner. It may also be the case, however, that a bias towards CD4+ T cell induction is a general feature of DNA/MVA vaccination in humans. This would appear to be very unlikely from considerations arising from basic immunology. DNA vaccination induces CD8+ T cells as assayed by conventional chromium release lysis assays (Wang et. al., 1998). Such assays have not been conducted after DNA/MVA vaccination, so a direct comparison is not available. 1 x 10^8 pfu FP9/ 1.5 x 10^8 pfu MVA delivery of ME-TRAP led to 2/5 complete protection against sporozoite challenge 2 weeks after final vaccination in one phase IIa trial (D Webster, unpublished data). In a follow-up IIa trial using the same immunization regime statistically significant delay in time to parasitaemia was observed with a 7 week challenge. Interestingly *ex vivo* ELISPOT responses appeared to be of lower magnitude than after 2mg DNA/ 1.5 x 10^8 pfu MVA vaccination. Detailed characterization of T cell responses induced by FP9/MVA ME-TRAP vaccination may determine the immunological mechanism of protection. It may be that the CD8/CD4 ratio is higher after FP9/MVA compared to DNA/MVA vaccination.

The poor induction of antibodies by DNA/MVA vaccination may not preclude successful development of a liver-stage *P.falciparum* candidate. However it does indicate that DNA/MVA regimens and other regimens with poor antibody induction will require combination with other platforms which are strong antibody-inducers for formulation of an effective deployable vaccine. The CS antigen offers an excellent opportunity for evaluation of such combination strategies. Apovia has developed a hepatitis B core platform which to date has been used to deliver CS B cell epitopes together with a single CD4 T cell epitope in the candidate ICC-1132. This induces high titres of antibody by ELISA and sporozoite IFA. RTS,S/AS02 is an alternative CS-based recombinant protein particle vaccine.

The following questions need to be answered with regard to combination of the DNA/MVA platform (or FP9/MVA) with recombinant protein based platforms. When the constructs contain significant overlap of malaria T cell epitopes can a protein vaccine either replace the DNA prime or the MVA boost for T cell amplification? What is the most effective method of simultaneous vaccination with antibody-inducing vaccines and DNA/MVA prime-boost regimens? (i.e. how does mixing of vaccine in the same syringe compare to concomitant vaccination in the same or opposite arms). Interference between antibody-inducing vaccines and DNA/MVA regimens may well occur but be surmountable by modifying vaccine regimens (for example by staggering vaccinations by a week or so if necessary).

A related question is how to combine two antigens delivered by the same platform. A great deal of work will be necessary to determine the mechanisms for combination, i.e. prevention of interference and competition. It is unclear how cocktails of constructs encoding two antigens individually will compare with a single construct encoding a multi-antigen. The order of encoding within the multi-antigen may also be important. Should emphasis be placed on determining the mechanisms for interference and competition in animal models? Or should we essentially perform clinical research to determine empirically certain successful candidate vaccine combinations? I believe the two must run in parallel. The risk with waiting for results for animal models is that this may be a very long term outcome of one or more decades and that particular successful combinations in a *P. berghei*/ BALB/c model for example will not transfer to humans. The true goal of the animal model research would be an understanding of mechanisms for interference and competition which may well transfer to human infection with *P. falciparum* even though particular examples of successful antigen combinations do not transfer, but this appears to be a long way ahead. Some insight into the challenging complexity of immunogenicity with multi-antigen constructs has emerged from years of murine research by researchers in Professor Hill's

laboratory. This work has indicated that insertion of an antigen into a multi-antigen can remove immunogenicity to other antigens present with the construct pre-insertion. An example of this in human trials using two recombinant proteins was the combination of RTS,S/AS02 with TRAP. Protection conferred by RTS,S/AS02 was completely removed by combination vaccination with the TRAP protein vaccine.

Combination of antigens between stages should prevent issues no different to those discussed above for within-stage combination in terms of immunogenicity but the complexity which arises is in study design for evaluation of efficacy of such combination vaccines. Data from efficacy studies will need to enable evaluation of the efficacy of individual components as well as the combination and in the case of between-stage combination, this will require inclusion of mutiple endpoints. For example both time to infection and parasite density may independently give indications of efficacy of pre-erythrocytic and blood-stage components, but components of the combination vaccine may act as confounders for the efficacy of each other. Factorial designs may be able to address such considerations and so transmission settings will need to be chosen carefully to minimize the increases in sample size such designs will require. Evaluation of combinations of sexual stage vaccines with other stages would probably require a community randomized design and a factorial design. There may be some benefit to conducting mathematical modeling experiments of various between-stage combination vaccine efficacy study designs.

One positive outcome of this thesis was the ability to evaluate an efficacy hypothesis in adults living in endemic areas within an appropriately short timeframe. Funding was obtained in April 2002 for the efficacy trial and within 9 months the primary endpoint reported in chapter 11 was determined. However this highlights one tension in choices for vaccine development plans in the malaria field. We promptly determined that DNA ME-TRAP in combination with MVA ME-TRAP did not protect men aged 15-45 living in villages close to

Farafenni in The Gambia from infection with *P. falciparum*. But we did not answer the more relevant questions as to whether protection against mild disease, severe disease or death could be afforded in children living in endemic countries. The more relevant the endpoint the more subjects would be required and paediatric study designs are required for anti-disease endpoints. I believe this work therefore should encourage the use of complete protection in the IIa artificial challenge model as a valid screening tool for candidate pre-erythrocytic malaria vaccines. The decision about when to proceed directly to paediatric study designs from IIa studies with only intervening adult phase I studies in endemic countries will be aided by the knowledge of efficacy of RTS,S/AS02 in children aged 1-5, which is currently being evaluated in Mozambique. This will provide the first comparison between IIa efficacy and anti-disease efficacy in children aged 1-5 living in endemic countries.

Chapter 13

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