Identification and characterization of *Plasmodium falciparum* and *Mycobacterium ulcerans* antigens as potential vaccine components and targets for serological test and molecular typing methods

INAUGURALDISSERTATION

Zur

Erlangung der Würde einer Doktorin der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

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aus

Manizales (Kolumbien)

Basel, 2006

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
Der Universität Basel auf Antrag der

Herren Prof. Dr. Thomas Bickle, Dr. Thomas Bodmer und Prof. Dr. Gerd Pluschke

Basel, 8 Dezember 2005

Prof. Dr. Hans-Jakob Wirz
Dekan

Dedicado a mi mamá (la Silvia), por darme todo el amor y apoyarme en todos los momentos. En mi corazón aun está una de tus frases "con honestidad, respeto y amor se puede conseguir lo que uno se propone", he seguido tus consejos y he logrado escalar un paso más en mi vida, disfrutando y creciendo. También dedico esta tesis a: mis hermanos, mis sobrinos y mi familia, por su constante apoyo. Igualmente a mi papá y a Oscar Orozco, mi maestro y amigo; donde quiera que estén saben que siempre estarán en mi corazón

TABLE OF CONTENTS **i**

ACKNOWLEDGEMENTS **iii**

SUMMARY **vi**

ZUSAMMENFASSUNG **viii**

ABRREVIATIONS **x**

CHAPTER 1: Introduction **1**

1.1. History And Epidemiology 2

1.2. Causative Organism 3

1.2.1 M. ulcerans Toxin 4

1.3. Pathology And Clinical Presentation 6

1.3.1 Clinical Features 6

1.3.2 Histopathology 7

1.4. Diagnosis 8

1.5. Treatment 9

1.5.1 Surgery 9

1.5.2 Drug Treatment 9

1.5.3 Heat Treatment 10

1.6. Transmission 10

1.7. Prevention 12

1.8. Genome And Bacterial Population Structure 12

1.9. Immune Response 14

1.10. Aim Of The Thesis 17

1.10.1 Buruli Ulcer 17

1.10.2 Malaria 17

1.11. References 19

CHAPTER 2: *M. ulcerans* Laminin-Binding Protein 26

CHAPTER 3: *M. ulcerans* 18kDa *shsp* 47

CHAPTER 4: *M. ulcerans*' Immunodominant Antigens 71

CHAPTER 5: D13, A Conserved-Stage-Specific Antigen of *P. falciparum* 92

CHAPTER 6: *P. falciparum*'s GAPDH: Potential Drug Target 119

CHAPTER 7: Discussion 151

7.1. Monoclonal Antibodies: Important Tool In Biomedical Research And Applications 152

7.2. Buruli Ulcer 153

7.2.1. Immune Protection And Vaccine Design 153

7.2.2. Identification Of Polymorphic Immunodominant Proteins And Molecular Fingerprinting Of M. ulcerans Isolates 155

7.2.3. Identification Of Target Antigens For Serological Analysis 156

7.2.4. Antigen Detection In Environmental Samples 157

7.3. Malaria 159

7.3.1. Characterization Of Potentially Novel Vaccine Candidate By Mabs 159

7.3.2. Characterization Of Potentially Drug Target By Mabs 160

7.4. Concluding Remarks 162

7.5. References 163

CURRICULUM VITAE 170

Acknowledgments

I am very grateful to Prof. Dr. Gerd Pluschke for this PhD thesis amongst his group in Switzerland, for his personal and scientific support, his encouragement, his advices and for always having time for me. His confidence never decreased and motivated me to proceed also through difficult, result-less time.

I am very thankful to Dr. Claudia Daubenberger, who introduced me to the work at the Swiss Tropical Institute, and gave me her constant support. Her advice encouraged me to work efficiently. I am very grateful for her taking care of me during of one of the most difficult times of my life.

I would also like to express my gratitude to Prof. Dr. Niklaus Weiss, and Prof. Dr. Marcel Tanner for their support and acceptance me at the Swiss Tropical Institute. I am grateful to Prof. Thomas Bickle and Dr. Thomas Bodmer for being part of my thesis committee.

My sincere thanks go to Dr. Heinz Döbeli, Nicole Soder, Arno Friedlein and Dr. Axel Ducret at Hoffman-La-Roche. Their generous scientific and technical contributions were essential to identify and characterize *Mycobacterium ulcerans* proteins for the development of this project.

I would like to acknowledge Dr. Ernestina Mensah-Quainoo, Tema District Health Director, and Mrs Dorothy Yeboah-Manu from the Noguchi Memorial Institute for Medical Research, for providing serum samples from Buruli patients and household contacts.

I would like to thank Prof. Dr. Francoise Portaels, Dr. Thomas Bodmer, and Gisela Bretzel for supplying us with *M. ulcerans* isolates and other mycobacterium strains, without which this PhD thesis would never have been possible.

I would also like to thank the “protozoologist”, first of all Christian Scheurer for all the support in the *Plasmodium falciparum* culture, and Prof. Dr. Brun for allowing me to use their culture facilities.

I would like to thank Till Voss, Tobias Spielmann and Igor Niederwieser for their technical and scientific advices which contributed in the very nice and clean Northern blots obtained during this research.

A special thank goes to Dan Anderegg for his critical reading of the thesis, his patience, his correcting from “Spanglish” to English, and all his help to improve this thesis.

I shared wonderful and few sad moments with the STI and ex-STI people, my very warm thanks go to my friends and dear colleagues: Marija Curcic (my angel in Basel) who always took care of me and helped with all the administrative documents; Shinji Okitsu ("mi hermanito") for all the support and the silly discussions about science and life in the evening and on the way home after billiard, when we were tired and emotional; Verena Christen and Tobias Jäggi, (my diploma students), for their enthusiasm and a great source of new ideas; Valentin Pflüger (Valentino), for the nice discussions about the life at the smoking corner; Elisabetta Peduzzi, for her support mainly at the scientific meetings; Jean Pierre Dangy (dear French man), Martin Nägeli (dear Swiss man), Denise Vogel, Sybille Siegrist (my dear Swiss girls), Marco Tamborrini (new sister), Julia Leimkugel, Markus Müller, Michael Käser, Max Bastian (my dear Germans), Simona Rondini (from the Italian team), Tatjana Zalac (the sporting girl), Daniela Schütte (the new German girl), Therese Ruf, Rolf Spirig (for Long Island Ice Tea), Christine Banholzer and Niels Pfeiffer for all the help, support, patience in desperate moments of no-perfect results and also for their friendship; Frieda Poeltl-Frank, Rafael Moreno, Bea Nickel, for their continuous friendship; Lucy Ochola and Dorothy Yeboah-Manu (my African friends) for their kind heart, and support in critical and nice moments.

I am also thankful to my neighbours: Christian Flück, for his kind heart, all the science discussions and his sincerely friendship; Selina Bopp (the Blonde girl) her friendship and the nice chatting nights with a beer; Cornelia Spycher, for helping me to remember the Spanish words; Mathias Rottmann, Anouk Müller, Sebastian Rusch, Mirjam Kästli, Hans-Peter Beck, and Ingrid Felger for the good time; Jutta Marfurt, for the chatting (about science and life) at the STI smoking corner. I would also like to thank the Irish Pub and pool-crew for many great nights: Roland Degen, Christian Scheurer, Michael Oberle, Moni Fasler, Igor Niederwieser, Sonja Bernhard, and those mentioned above.

Special thanks go to Martin Raab and Birgit Schmid for the nice weekends on the mountains and their friendship.

For good times and helpful scientific input I would like to thank all members from the lab, ex-lab, neighbouring labs, ex-STI and STI, particularly: Werner Rudin, Yvette Endriss (for all "the alarm" help, nice food and support), Guy Hutton, Sergio Wittlin, Kerstin Gillingwater, Markus Hilty, Amanda Ross, Christian Lengeler, Angelica Barry, Jakob Zinsstag and Thomas Smith. I extend many thanks to those who helped me in one way or

another: Martin Baumann, Simon Roelly, Christine Walliser, Isabelle Bolliger, Eliane Ghilardi, Agnès Doré, Madleine Buholzer, Beatrice Waeckerlin, Heidi Immler, Fabienne Fust, Annina Isler and Elisabeth Sedlmeier.
I am very grateful to all of you; it was very nice to meet you. All of you contribute to making this time special and wonderful.

I am grateful to the Amt für Ausbildunsbeiträge of the county of Basel-Stadt and Aide aux Lépreux Emmaüs–Suisse from which I received financial support.

Finally, my warmest thanks go to: my family, in special my mother for all the support, care and love; my ex-colleagues and friends in Colombia (Jimena Cortez, Luis Eduardo Rodríguez, Manuel Alfonso Patarroyo, Marisol Ocampo, Magnolia Vanegas, Fanny Guzmán, Gabriela Delgado, Yolanda López, Yolanda Silva), Rosita Moya, Javier Niño, Pilar Alcala, Alberto Moreno, and. Prof. Manuel Elkin Patarroyo

Summary

The increase of *Plasmodium falciparum*'s drug resistance and the resurgence of *Mycobacterium ulcerans* disease through environmental changes aggravate health problems caused by these pathogens.

Buruli ulcer, caused by *M. ulcerans*, is after tuberculosis and leprosy the third most common mycobacterial human infection and the most poorly understood of these three diseases. It is characterized by chronic, necrotizing ulceration of subcutaneous tissues and the overlying skin. *M. ulcerans* is a slow-growing mycobacterium which multiplies extra-cellularly in Buruli ulcer lesions. There is a broad antigenic overlap between mycobacterial species, which complicates the analysis of adaptive immune responses and hampers the development of specific sero-diagnostic tests for *M. ulcerans* in areas where BCG vaccination has been implemented and tuberculosis is endemic. In an effort to identify immunodominant antigens of *M. ulcerans,* we have generated panels of monoclonal antibodies from mice immunized with this pathogen. Cross-reactivity studies with other mycobacterial species performed by Western blot and immunofluorescence assays have identified immunodominant epitopes with a limited cross-species distribution (18kDa and the 34-37kDa proteins). In contrast, the majority of antigens were spread widely amongst different mycobacterial species. One set of non-crossreactive monoclonal antibodies recognized an 18kDa protein of *M. ulcerans* that is associated with the cell-wall fraction, and expressed in Buruli ulcer lesions. The target protein was identified by mass-spectroscopy as the *M. ulcerans* orthologue of the *M. leprae* 18kDa small heat shock protein, which has no orthologues in the genomes of *M. bovis* and *M. tuberculosis*. Human anti-18kDa small heat shock protein antibodies were found in the serum of all Buruli ulcer patients tested, but not in sera from Europeans volunteers and only rarely in sera from Africans living in Buruli ulcer non-endemic regions. Reactivity of sera from a large proportion of people living in a Buruli ulcer endemic area and in contact with Buruli ulcer patients indicated that an 18kDa small heat shock protein-based serological test is suitable to detect exposure to *M. ulcerans.*

Since *M. ulcerans* shows only very limited genetic diversity, standard multi-locus sequence typing of housekeeping genes is not a suitable tool for molecular epidemiological

analysis of Buruli ulcer. Among the monoclonal antibodies exhibiting broad inter-species cross-reactivity, one group recognized the *M. ulcerans* orthologue of mycobacterial laminin-binding protein. DNA sequence analysis demonstrated that the corresponding *hupB* gene from *M. ulcerans* isolates of diverse geographical origin exhibited considerable diversity based both on insertional/deletional polymorphism and on single base exchanges. Dominance of non-conservative exchanges was indicative of a diversifying selection pressure. Sequences analysis of a set of such variable genes may develop into a new tool for genetic fingerprinting of isolates.

There is great need to identify new malaria vaccine and drug targets. Monoclonal antibodies were used to characterize a novel conserved protein of *P. falciparum* designated D13. Western blot analysis demonstrated that D13 is stage-specifically expressed during schizogony in asexual blood stages of the parasite. It has a functionally essential role in parasite biology, since anti-D13 monoclonal antibodies have parasite growth inhibitory activity. The D13 protein may represent a suitable target for a malaria vaccine design.

Immunofluorescence analysis with monoclonal antibodies specific for glyceraldehydes-3-phosphate dehydrogenase (pfGAPDH) and pfAldolase showed that pfGAPDH and pfAldolase colocalise in early stages of both liver and asexual blood stage parasite development. However, during schizogony, unlike pfAldolase, pfGAPDH was enriched in the apical region of the parasites. In addition, Western blot analyses demonstrate that pfGAPDH is in both the membrane-containing pellet and supernatant fractions. These results have provided evidence that pfGAPDH exerts non-glycolytic function(s) in *P. falciparum*; including possibly a role in vesicular transport and biogenesis of apical organelles. This data together with the limited amino acid sequence identity with human GAPDH suggest that the pfGAPDH could be a promising safe target for drug treatment.

Zusammenfassung

Die Ausbreitung der Medikamentenresistenz bei *Plasmodium falciparum* und die mit Umweltveränderungen assoziierte zunehmende Ausbreitung der *Mycobacterium ulcerans* Infektion verschärft die durch diese beiden Pathogene hervorgerufenen Gesundheitsprobleme.

Der von *M. ulcerans* hervorgerufene Buruli-Ulkus stellt nach Tuberkulose und Lepra, die dritthäufigste mykobakterielle Infektion des Menschen und die am wenigsten verstandene dieser drei Infektionskrankheit dar. Charakteristisch für die Erkrankung sind chronisch nekrotisierende Ulzerationen des subkutanen Gewebes und der darüber liegenden Haut. *M. ulcerans* ist ein langsam wachsendes Mykobakterium, das sich in Buruli Ulkus Läsionen extrazellulär vermehrt. Breite antigenische Kreuzreaktivität zwischen verschiedenen mykobakteriellen Spezies erschwert die Analyse der adaptiven Immunantworten und hat die Entwicklung spezifischer serodiagnostischer Nachweismethoden für *M. ulcerans* in Regionen, in denen mit BCG geimpft wird und Tuberkulose endemisch ist, bislang verhindert. Zur Identifizierung immundominanter Antigene von *M. ulcerans* haben wir Sätze von monoklonalen Antikörpern mit Mäusen hergestellt, die mit diesem Pathogen immunisiert worden waren. Auf Western blotting und Immunfluoreszenz-Anfärbung basierende Kreuzreaktivitäts-Studien mit anderen mycobakteriellen Spezies haben immundominante Epitope mit begrenzter inter-Spezies Verbreitung identifizieren. Der grösste Teil der Antigene hingegen war bei verschiedenen Mycobakterien weit verbreitet. Eine Gruppe von nicht-kreuzreaktiven monoklonalen Antikörpern erkannte ein 18KDa grosses *M. ulcerans* Protein, das mit der Zellwand assoziiert und in Buruli Ulkus Läsionen exprimiert war. Das Zielantigen konnte durch massenspektroskopische Analyse als *M. ulcerans* Ortholog des 18KDa small heat shock Proteins (shsp) von *M. leprae* identifiziert werden, welches keine Orthologe im Genom von *M. tuberculosis* und *M. bovis* hat. Humane anti-18KDa shsp Antikörper wurden im Serum aller untersuchten Buruli Ulkus Patienten, aber nicht im Serum von Europäern, und nur selten im Serum von Afrikanern gefunden, die nicht in einer Buruli Ulkus endemischen Region leben. Reaktivität eines grossen Teils der Seren von Menschen aus Buruli Ulkus endemischen Gebieten und von Kontaktpersonen von Patienten lässt vermuten, dass ein auf dem 18KDa shsp basierender serologischer Test geeignet ist, die *M. ulcerans* Exposition einer Population zu erfassen.

Da *M. ulcerans* nur eine sehr begrenzte genetische Diversität aufweist, ist die gängige Multi-Lokus-Sequenztypisierung keine geeignete Methode für mikroepidemiologische Analysen der *M. ulcerans* Infektion. Eine Gruppe der monoklonalen Antikörper, die eine breite inter-Spezies Kreuzreaktivität aufwies, erkannte das *M. ulcerans* Ortholog des mykobakteriellen Laminin-bindenden Proteins. DNA-Sequenzanalysen zeigten, dass das korrespondierende *hup*B Gen von *M. ulcerans* Isolaten unterschiedlichen geographischen Ursprungs einen beträchtlichen Polymorphismus aufwiesen. Dieser basierte sowohl auf Punktmutationen als auch auf der Insertion und Deletion von Sequenzabschnitten. Eine Dominanz von nicht-konservativen Punktmutationen deutete auf Diversifizierung durch Selektion hin. Sequnzanalysen mit einem Satz solcher polymorpher Gene könnte sich zu einem neuen Ansatz zur Feindifferenzierung von *M. ulcerans* Isolaten entwickeln.

Es besteht grosses Interesse, neue Zielstrukturen für Malaria-Medikamente und einen Malaria-Impfstoff zu identifizieren. Wir haben monoklonale Antikörper eingesetzt, um ein neues konserviertes Protein von *P. falciparum* zu charakterisieren. Western blot Analysen zeigten, dass das D13 genannte Protein während der Schizogonie Stadien-spezifisch exprimiert wird. Anti-D13 monoklonale Antikörper wiesen Wachstums-inhibierende Aktivität auf. Dies lässt vermuten, dass D13 eine essentielle biologische Funktion erfüllt und ein für die Impfstoffentwicklung geeignetes Antigen darstellen könnte.

Immunfluoreszenzanalysen mit monoklonalen Antikörpern gegen die Glycerinaldehyd-3-phosphat Dehydrogenase (pfGAPDH) und pfAldolase zeigten, dass beide Enzyme in frühen Entwicklungsphasen der asexuellen Blutstadien und der Leberstadien kolokalisiert sind. Hingegen war die pfGAPDH verglichen mit der pfAldolase während der Schizogonie in der apikalen Region des Parasiten angereichert. Weiterhin war pfGAPDH sowohl mit der löslichen als auch mit der Membran-Fraktion von aufgeschlossenen Parasiten assoziiert. Diese Ergebnisse weisen darauf hin, dass pfGAPDH nicht-glykolytische Zusatzfunktionen erfüllt, die möglicherweise mit dem vesikulärem Transport und der Biogenese der apikalen Organellen assoziiert sind. Zusammen mit der relativ geringen Sequenzidentität mit der humanen GAPDH weisen diese Ergebnisse darauf hin, dass pfGAPDH ein geeignetes Zielenzym für eine Medikamentenentwicklung sein könnte.

ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
BCG	Bacillus Calmette-Guèrin
CFU	Colony Forming Unit
DRC	Democratic Republic of Congo
DTH	Delayed Hypersensitivity
ELISA	Enzyme-linked Immunoabsorbent Assay
ELISPOT	Enzyme-Linked Immunospot Assay
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
IFA	Immunofluorescence Assay
IFN-γ	Gamma Interferon
IgG, M	Immunoglobulin G, M
IL-4, 5,..	Interleukin-4, 5,…
IS	Insertion Sequences
kDa	Kilo Dalton
LBP	Laminin-Binding Protein
MAbs	Monoclonal Antibodies
MLST	Multilocus Sequence Typing
mRNA	Messenger Ribonucleic Acid
PCR	Polymerase Chain Reaction
PNG	Papua New Guinea
PPD	Purified Protein Derivative
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal Ribonucleic Acid
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
sHSP	Small Heat Shock Protein
STI	Swiss Tropical Institute
TNF-α	Tumor Necrosis Factor Alpha
VNTR	Variable Number Tandem Repeat
WHO	World Health Organization
ZN	Ziehl-Neelsen

Chapter 1

INTRODUCTION

Buruli ulcer, caused by the environmental pathogen *Mycobacterium ulcerans,* is disease of skin and soft tissue with the potential to leave scarring and deformities. The causative organism is from the bacterial family which causes tuberculosis and leprosy, and produces a dermonecrotic toxin. Most patients are children who live in rural sub-Saharan Africa; however, healthy people of all age, race and socio-economic class are susceptible.

1.1 History and epidemiology

Buruli ulcer is the third most important mycobacterial disease after tuberculosis and leprosy in humid tropical countries of West Africa[1]. The definitive description of *M. ulcerans* was published in 1948 by MacCallum *et al*. where they reported 6 cases of an unusual skin infection in Australia, caused by a mycobacterium that could only be cultured in Löwenstein-Jensen medium when the incubation temperature was set lower than for *M. tuberculosis*[2]. Before, large skin ulcers almost certainly caused by *M. ulcerans* were previously described in Uganda in 1897 by Sir Albert Cook, and by Kleinschmidt in northeast Congo during the 1920s[3], however these cases were not published in the medical literature.

Prior to 1980, *M. ulcerans* infections were reported in several African countries: Congo, Uganda, Gabon, Nigeria, Cameroon and Ghana[4]. The term "Buruli ulcer" was chosen by the Uganda Buruli Group after they studied the clinic-pathological and epidemiological aspects of the disease extensively in a county called Buruli, near Lake Kyoga[5].

Since the 1980s, Buruli ulcer has emerged as a serious public health problem in an increasing number of countries. West Africa thus far appears to be the most affected area, especially Côte d'Ivoire, Benin[6] and Ghana[7]. New foci were discovered recently in Togo[8], Angola[9] and Guinea[4]. In Ghana, the overall crude prevalence rate was 20.7 per 100,000 persons in 1999, but rose to 150.8 per 100,000 in the most disease-endemic district[7]. In southern Benin, Buruli ulcer had a higher detection rate (21.5 per 100,000 per year) than leprosy (13.4 per 100,000) and tuberculosis (20.0 per 100,000)[6]. In Côte d'Ivoire, over 15,000 cases were recorded between 1978 and 1999[10].

In West Africa, about 70% of affected individuals are children under the age of 15 years, and between 20 to 25 percent of those with healed lesions are left with disabilities.

Several other countries outside Africa are also endemic such as rural areas of Papua New Guinea, Australia, Malaysia, French Guyana and Mexico. French Guiana has the highest prevalence of Buruli ulcer cases among Latin American countries with 193 cases reported, whereas only 8 cases were registered in Peru, 8 in Mexico[11], 1 in Bolivia and 1 in Suriname[12].

Since the first Australian cases reported in 1948, until the 1990s, one or two cases have occurred annually. In the 1990s, however, incidence of the disease increased suddenly with the development of new foci on Phillip Island and the Frankston/Langwarrin district south of suburban Melbourne. There are now 20–30 cases per annum, a 10-fold increase over the past 15 years[12]. Few cases have been reported in non-endemic areas in North America and Europe as a consequence of international travel[10,13].The worldwide distribution of Buruli ulcer disease is shown in figure 1.

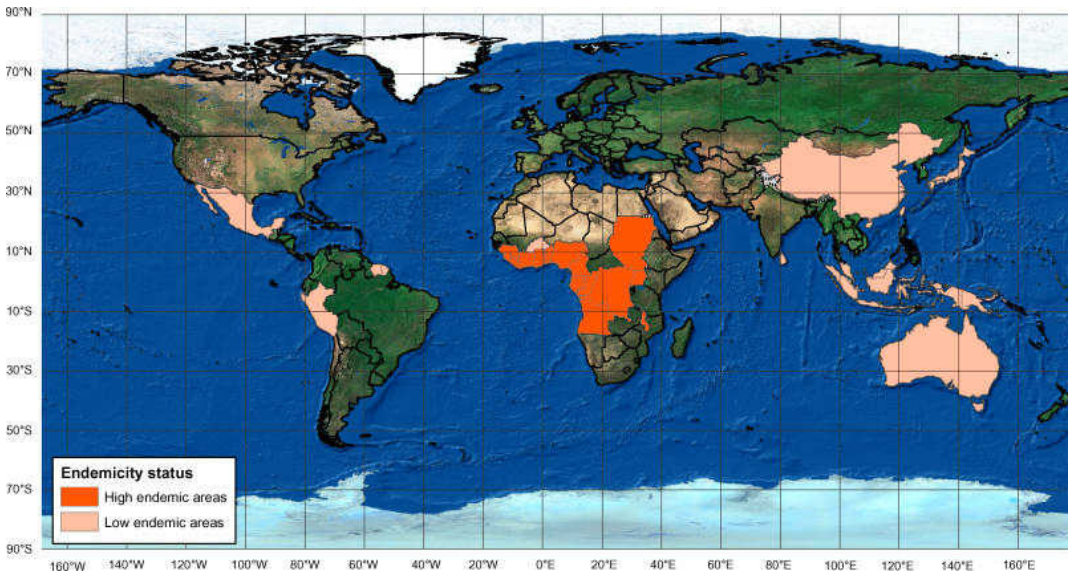


Figure 1. Countries reporting Buruli ulcer (Source: Johnson *et al., PLoS Med*. 2005)

1.2 Causative Organism

M. ulcerans, together with the other mycobacteria, corynebacteria and nocordia form a monophyletic taxon within the family of actinomycetes[13]. The slowly growing *M. ulcerans* belongs to a group of mycobacteria that are potentially pathogenic for humans and animals. These are sometimes called “opportunistic mycobacteria” or “occasional pathogens” to

distinguish them from strict pathogens. Most species belonging to this group are widespread in the environment and may become pathogenic under specific circumstances[14].

The generation time of *M. ulcerans in vitro* is 20 hours, as is the case for the *M. tuberculosis*-complex organisms, and a positive culture requires an incubation time of 6 to 8 weeks[3]. *M. ulcerans* is possibly the only pathogenic mycobacterium species that does not have a significant intracellular existence[15]. However, recent studies showed that *M. ulcerans* proceeds through an initial phase where bacilli are internalized by phagocytic cells, like most mycobacterial species. The transition to a second phase, where the bacteria are extracellular, occurs by action of mycolactone[16]. The best growing rates are achieved at low temperature (32ºC) on Lowenstein-Jensen medium. It grows best in microaerophilic conditions[17].

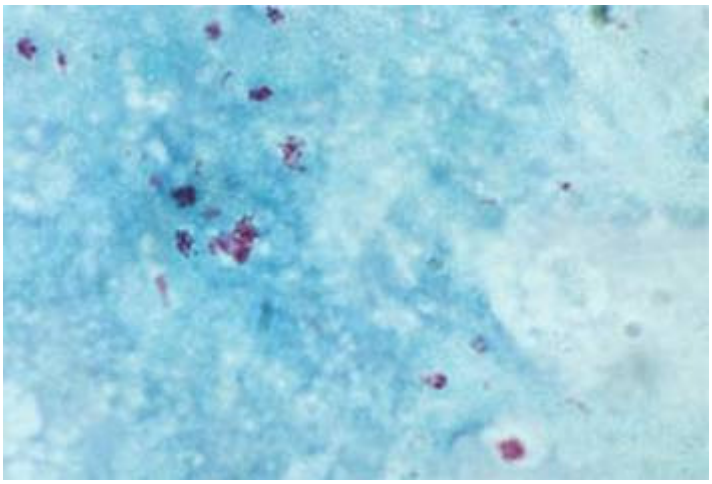


Figure 2. Acid fast bacilli (*M. ulcerans*) in a smear taken from an ulcer. The bacteria can be clearly seen as red clumps on a blue background. Oil immersion microscopy (1000x).

1.2.1. M. ulcerans toxin

The pathogenesis of *M. ulcerans* is closely associated with expression of a macrolide toxin, mycolactone. Mycolactone was identified by two-dimensional nuclear magnetic resonance spectral analysis as a polyketide-derived 12-membered ring macrolide ($C_{44}H_{70}O_9$) (fig. 3). The mycolactone is a major component of acetone-soluble lipids (ASL) present in an

organic extract from an *M. ulcerans* sterile filtrate. The toxin was named mycolactone to reflect its mycobacterial source and chemical structure.

Mycolactone isolated from *M. ulcerans* cultures has been shown to be immunosuppressive and cytotoxic *in vitro*, as addition of mycolactone to macrophages and fibroblast has effects on the cytoskeleton and leads to cell growth arrest in the G_0/G_1 stage and apoptosis[15]. Injection of mycolactone into the dermis of guinea pigs is sufficient to induce ulcers[18], and natural mycolactone deficient mutants failed to induce ulcers in these animals[15]. Mycolactones induce cell death by apoptosis, which may explain the absence of an inflammatory immune response despite extensive tissue damage[18].

Until now, no cell receptor has been found to explain the cascade of effects induced by mycolactones[19]. The lipid toxin mycolactone is synthesized by the giant polyketide synthases, which are encoded by three very large and homologous genes, harboured in the 174-kb virulence plasmid pMUM001[20].

In contrast to the wild type *M. ulcerans,* mycolactone negative mutants fail to colonize the salivary glands of water insects, suggesting that these molecules may play a role in the ability of *M. ulcerans* to colonize reservoir species[15,21].

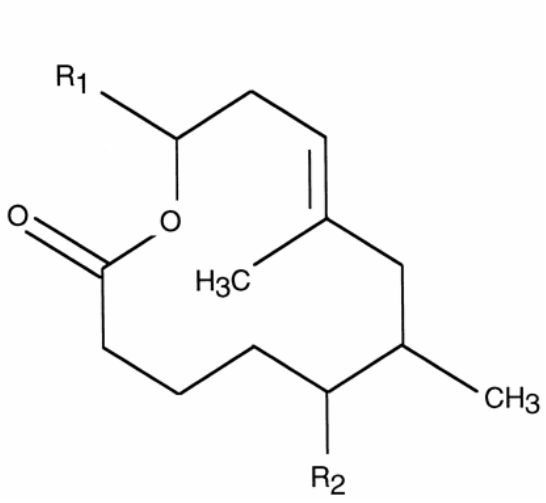
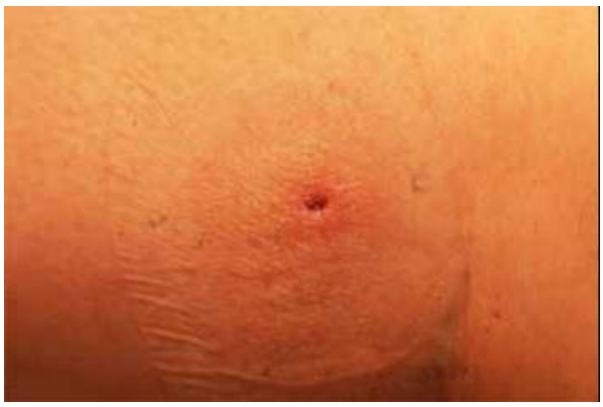


Figure 3. Mycolactone is composed of a 12-membered ring to which two polyketide-derived side chains (R1 and R2) are attached. (Source: George, *et al.*,, *Science* 1999).

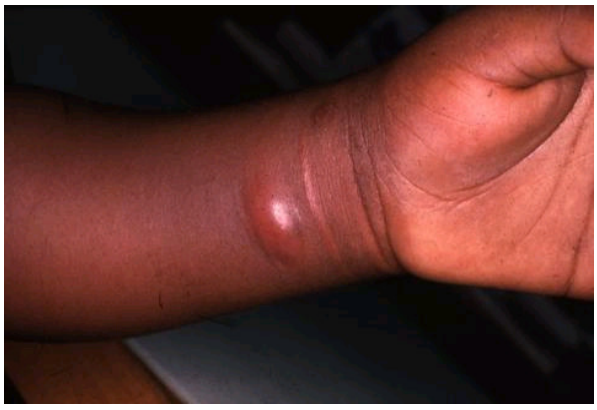
1.3 Pathology and Clinical Presentation

1.3.1 Clinical features

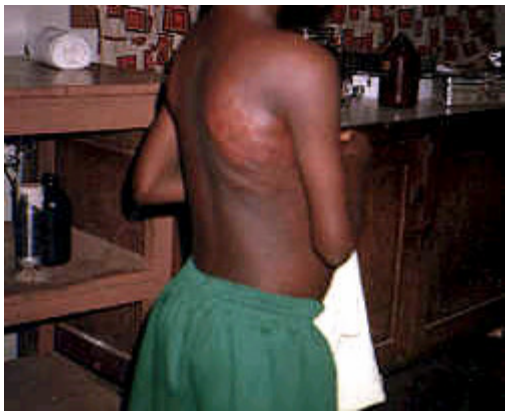
A papule or a firm nodule indicates the first stage of the disease. A papule is defined as a painless, raised skin lesion surrounded by reddened skin (Fig. 4a). The nodule is characterized as an extended lesion from the skin into the subcutaneous tissue. It is usually painless as a papule but may be itchy while the surrounding skin may be discoloured compared to adjacent areas (Fig.4b). Occasionally, some patients develop extensive indurate lesions or plaques that are painless and present irregular edges (Fig.4c). In the second stage, ulceration takes place on the floor of the ulcer which displays a white cotton wool-like appearance in the necrotic slough (Fig.4d). Many acid-fast bacilli are present in the slough, and the necrosis can extend away from the site where *M. ulcerans* is placed. The ulcer is usually painless, unless there is a secondary bacterial infection. Complications include loss of organs such as the eye and breast, amputation of limbs and other permanent disabilities[22,23]. In advanced cases the bone may also become involved[23].



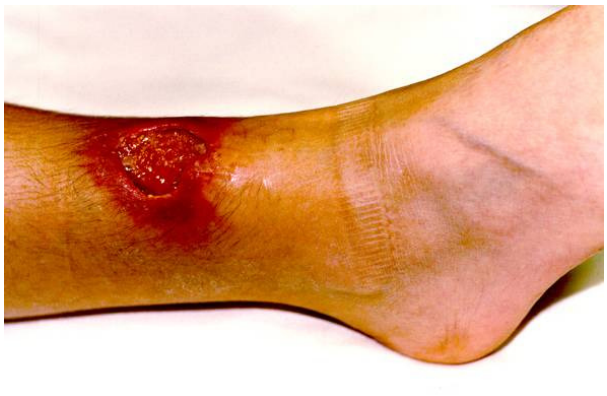
Papule (a)



Nodule (b)



Plaque (c)



Ulcerative form (d)

Figure 4. Clinical forms of Buruli ulcer. (Source: Portaels F. Johnson P, Meyers WM editors, 2001. World Health Organisation)

1.3.2 Histopathology

Progressive *M. ulcerans* infection causes characteristic tissue changes. The first stage of the disease (papule, nodule) presents coagulative necrosis of the lower dermis and subcutaneous fat. The organisms are present in clumps or in smaller microcolonies in the centre of the lesion. In these early lesions there is little cellular reaction despite the presence of large clumps of extracellular organisms and there is no evidence of an inflammatory response or the development of granulomas. The second stage of the disease (ulcerative form) produces granulomatous reactions with epithelioid macrophages, a variable numbers of giant cells of the Langhans type, and relatively few acid-fast organisms[24]. As the disease progresses, all elements of the skin are affected including nerves and blood vessels. Later, during the natural course of the disease, the immunosuppressive effect of the toxin is somehow overcome by the host, allowing then immunity to develop and healing to commence.

This may account for the observation that patients with active lesions are often unresponsive to *M. ulcerans*-derived antigens (burulin) on skin testing[25]. Later, during the healing phase, characterized by the appearance of granulomas, there is conversion to a positive burulin test indicating that a specific cellular response develops.

1.4 Diagnosis

In endemic areas, the clinical diagnosis of ulcerating lesions by experienced clinicians is straightforward. The painless ulcer with undermined edges and a necrotic slough can be recognised easily. Patients with Buruli ulcer have no clinically detectable lymphadenitis, no systemic symptoms such a fever or malaise suggesting a staphylococcal or streptococcal infection. Since early and healing lesions may be confused with other skin diseases endemic in tropical areas, probable cases defined clinically have to be confirmed. Commonly used diagnostic laboratory tests are: i) detection of mycobacteria by Ziehl-Neelsen (ZN) staining, a technique which lacks sensitivity and specificity, ii) culture of *M. ulcerans*, which may take several months, iii) detection of characteristic histopathological changes in excised tissue, iv) detection of *M. ulcerans* DNA by polymerase chain reaction (PCR); PCR is a rapid, sensitive and specific diagnostic method but requires advanced technical expertise and is not always available in developing countries. A dry-reagent-based PCR formulation using lyophilized, room-temperature-stable PCR reagents[26], and a real-time PCR using the TaqMan system (IS2404 TaqMan) to quantify *M. ulcerans* DNA by monitoring the real-time amplification of IS2404 represent modifications of the conventional IS2404 PCR method[27]. Real-time PCR offers the possibility to measure the starting amount of target DNA in clinical specimens and other samples, thus providing a measure of mycobacterial burden as well[28].

Although molecular biological methods are quickly able to confirm the clinical diagnosis, high laboratory standards are needed to avoid the contamination risk and thus false positive results. While swabs can be taken to test the undefined edges of ulcerative lesions, it is much more problematic to take punch biopsies from pre-ulcerative lesions, since this technique promotes the spread of the mycobacterium.

The World Health Organization (WHO) Global Buruli Ulcer Initiative has asked the research community to develop a simple and rapid diagnostic test which could be used to identify patients early during the course of the infection, so that the detection rate of patients with Buruli ulcer could be improved and preventive therapy and early treatment options could be fully implemented. Humoral immunity has been studied for the diagnosis of the disease, since serum samples from infected individuals from different regions of Buruli ulcer endemicity have shown high titers of antibodies to *M. ulcerans* antigens.

Three *M. ulcerans* antigens of 70, 38/36 and 5 kDa of *M. ulcerans* culture filtrate have been commonly recognized by Buruli ulcer patient antibodies[29,30]. However, sera of household contacts and tuberculosis patients from endemic areas showed cross-reactivity to *M. ulcerans* antigens[31]. Recent studies showed that IgM antibody responses from Buruli ulcer patients recognized antigens from *M. ulcerans* culture filtrate. A total of 84.8% of the Buruli ulcer patients present IgM antibody responses, whereas only 4.5% of household contacts exhibited such responses[32]. The publication of the genomic sequence of *M. ulcerans* will be soon available and could assist the development of a non-invasive serodiagnostic assay based on *M. ulcerans*-specific antigens.

1.5 Treatment

1.5.1 Surgery

The standard treatment is limited to surgical excision, followed by skin grafting, but this intensive therapy and the required need for long-term care results in great economic impact on affected communities. The aim of the treatment is to halt the infection and repair existing damage. After the early excision of small pre-ulcerative lesions (papules and nodules) the skin can often be closed requiring no skin grafting. Necrotic ulcers are easily recognised and should be carefully removed, with excision extending into healthy tissue, to prevent persistent subcutaneous infection from residual bacilli[10]. Relapse after surgery may occur in 5%-47% of the cases[33,34]. Early excision can prevent development of the large and disfiguring ulcers often associated with persistent deformity after healing[35].

1.5.2 Drug treatment

Treatment of *M. ulcerans* infections with antimycobacterial agents has generally been disappointing, especially in extensive ulcers. The only published controlled trials in humans suggest that both clofazamine[36] and cotrimoxazole[37] are ineffective and that rifampicin and dapsone combined have limited efficacy for ulcers[38]. Anecdotal reports of antibiotic administration have been discouraging, and it has been postulated that antibiotics fail to penetrate *M. ulcerans* lesions because of the extensive necrosis caused by

mycolactone. *M. ulcerans* has been shown *in vitro* to be susceptible to rifampicin, some amino glycosides, macrolides and quinolones[39,40]. *M. ulcerans* was susceptible to the same drugs in the mouse footpad model[41], where the size of the mouse's footpad lesions treated with rifampicin and amikacin together for 12 weeks decreased progressively, reducing the mean CFU counts of *M. ulcerans* while no relapse occurred[42]. Recent studies suggest that a combination of anti-mycobacterial antibiotics that include rifampicin and either streptomycin or amikacin for 4 weeks are able to kill *M. ulcerans* in human lesions. In this study, no lesion became enlarged during antibiotic treatment and most became smaller[43]. Recent, successful results have encouraged the WHO to recommend the use of this combination for the treatment of small early *M. ulcerans* lesions. The treatment (duration) and the doses of the antibiotics depend of the size of the lesion and other complications. For example, small early lesions should be treated after surgery with this combination for 4 weeks, and ulcerative plaque or oedematous forms should be treated at least 4 weeks with antibiotics before and after surgery[44].

1.5.3 Heat treatment

One study following eight patients showed that continuous local heating to 40° C for 4-6 weeks promotes healing even without excision. In addition, heat treatment may improve blood flow, antibiotic penetration and phagocytosis[45].

1.6 Transmission

The exact mode of transmission of *M. ulcerans* is an enigma. Epidemiological studies demonstrated that *M. ulcerans* is strongly associated with swampy areas[21,23,46-50]. Changes in the environment, such as the construction of irrigation systems and dams, seem to play a role in the resurgence of the disease. In Nigeria, infections have emerged when a small stream was dammed to make an artificial lake[51]. In Phillip Island, Australia, a recent outbreak was associated with the formation of a swampy area and the outbreak stopped once the swamp's drainage was improved[52].

M. ulcerans is thought to reach the human dermis through wounds or skin abrasions via contact with *M. ulcerans*-containing environmental reservoirs. In addition, *M. ulcerans*

was detected in aquatic insects obtained from endemic areas in Africa by Polymerase Chain Reaction (PCR)[48], leading to the possibility that *M. ulcerans* may be transmitted by bites of the insect order Hemiptera (fig 5).

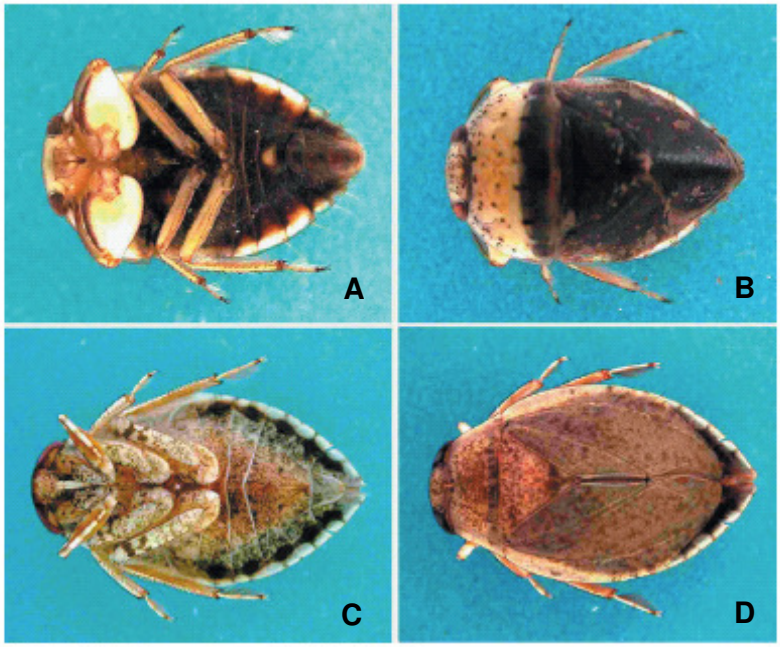


Figure 5. Semi-Aquatic Hemiptera positive for *M. ulcerans* by PCR. The top row is *Macrocoris sp*., 1.0 body length (Naucoridae), and the bottom row is *Appassus sp*., about 2.5 cm body lengths (Belastomatidae). Ventral view (A, C) and dorsal view (B, D) (Source: Johnson, *et al.*, *PLoS Med*. 2005)

In support of this hypothesis, *M. ulcerans* has been detected in the salivary glands of *Naucoris sp*., and a mouse tail exposed to this aquatic insect (Naucoridae) that had ingested a *M. ulcerans*-loaded prey, displayed cutaneous lesions from which *M. ulcerans* was isolated[21]. There is additional evidence that *M. ulcerans* DNA can be detected by PCR in other aquatic insect predators (Odonata and Coleoptera), as well as in aquatic snails, small fish and the biofilms of aquatic plants[50].

The possible mechanisms through which *M. ulcerans* infects water bugs that may act as hosts and vectors have been describe recently. Coelomic plasmatocytes could be the first cells of *Naucoris cimicoide* to be involved in the infection process, acting as shuttle cells that deliver *M. ulcerans* to the salivary glands. After ingestion of *M. ulcerans*-loaded food, the accessory salivary glands were shown to contain increasing numbers of bacilli,

indicating that these metabolically active tissues can be weakened/adopted as optimal niches for bacteria multiplication. This process is strictly dependent on mycolactone as mycolactone deficient bacilli were unavailable to establish long-term infectious niches. In addition, the bacilli can be rapidly detected within the cavity of raptorial legs. The setae of these appendages are covered by *M. ulcerans*-containing material resembling biofilms[53].
Other transmission pathways have been suggested in Australia: Aerosols arising from contaminated water may disseminate *M. ulcerans* and infect humans via the respiratory tract, or through contamination of skin lesions and minor abrasions[52,54], however this has yet to be demonstrated.
The discovery of the IS2406 and IS2606 sequences, used for epidemiological investigation of Buruli ulcer disease in *M. liflandi*[55], was a major finding that made the interpretation of environmental studies of *M. ulcerans* questionable.

1.7 Prevention

There is no specific vaccine against *M. ulcerans*, but Bacillus Calmette-Guèrin (BCG) vaccination has an incomplete but significantly protective effect against the most severe forms of Buruli ulcer[56]. Mechanical protection of exposed areas of the body, such as wearing trousers, shirt-sleeves and shoes, may also protect individuals at risk[57,58].
Additional studies to identify modifiable risk factors for infection and disease are needed.

1.8 Genome and bacterial population structure

M. ulcerans is an emerging pathogen that seems to have evolved from *M. marinum* by the acquisition and concomitant loss of DNA, in a manner analogous to the emergence of *M. tuberculosis*, where species diversity is being driven mainly by the activity of mobile DNA elements. A hallmark is the acquisition of a virulence plasmid, which encodes a polyketide synthase responsible for the synthesis of mycolactone[20,49]. This plasmid has a common evolutionary origin in *M. ulcerans* isolates from diverse geographical locations around the world[59]. Several conventional and newly developed typing techniques have attempted to describe the population structure of *M. ulcerans* and to investigate its evolution.

Comparing the sequences of housekeeping genes by Multilocus Sequence Typing (MLST), 6 genotypes were identified from 18 different *M. ulcerans* strains. The 6 genotypes were related to the 6 geographical areas of Suriname, Mexico, China/Japan, Africa and Australia (Victoria). Comparative analysis between *M. marinum* and *M. ulcerans* confirmed their relatedness, suggesting a recent divergence of *M. ulcerans,* by the acquisition and concomitant loss of DNA, such as the specific insertions sequences IS2404 and IS2606[60].

Differentiation of phylogenetically related mycobacteria based on 16S-23S rRNA gene internal transcribed spacer sequences showed identical sequences for *M. ulcerans* and *M. marinum*[61], supporting the hypothesis that both species share common ancestors.

Analysis of the 3'-terminal region of the 16S rRNA sequences of seventeen isolates of *M. ulcerans* from Africa, the Americas and Australia revealed three subgroups corresponding to the continent of origin[62].

The analysis of 12 *M. ulcerans* strains from Australia, Malaysia and Africa by Amplified Fragment Length Polymorphism (AFLP) could discriminate just two groups of isolates: the African group and the Australian (and Malaysian) group[63].

IS2404 Restriction Fragment Length Polymorphism (RFLP) subtyping divided *M. ulcerans* isolates into six groups related to six geographical regions, including Africa, Australia, Mexico, South America, Asia and Southeast Asia[64].

PCR amplification with primers based on IS2404 and GC-rich repeat sequence of 32 *M. ulcerans* isolates revealed 10 different patterns corresponding to the geographic origin of the isolates. All of the 16 isolates from six different African countries produced identical profile; two different genotypes were identified in East Asia (Japan and China), Australia (Victorian and Queensland) and in Papua New Guinea (PNG I and II genotypes), the Malaysian genotype, South America (French Guiana and Suriname with identical profile) and the Mexican genotype[65].

Eight different *M. ulcerans* genotypes were found by Variable Number Tandem Repeat (VNTR) typing, including Australia, Africa, Southeast Asia, Papua New Guinea, Asia, and Mexico. This technique made it also possible to differentiate between the South American strains (French Guiana and Suriname)[66].

M. ulcerans DNA harbours a circular plasmid (pMUM001) which comprises 81 protein-coding sequences. The primary function of this plasmid is mycolactone toxin production[20]. The occurrence of inter-strain variability was also discovered at the plasmid level, which

was related to the mycolactone structure[67] and to frequent genetic rearrangements that render the virulence plasmid particularly unstable[59].

The clonal population structure of *M. ulcerans* is reflected by the fact that all genetic fingerprinting methods applied so far for *M. ulcerans* have resolved only a limited number of geographical types, which is particularly insufficient to differentiate among isolates from the same area. This makes the methods unsuitable to perform micro-epidemiological studies, where the fingerprinting of the strains is aimed at revealing transmission pathways and environmental reservoirs. The expected publication of the entire *M. ulcerans* genome sequence in 2005 will provide an opportunity to discriminate *M. ulcerans* strains by differential genomic hybridisation using microarrays.

1.9 Immune responses

The immune mechanisms involved in protection against Buruli ulcer are largely unknown at present. Buruli ulcer disease follows an indolent course, with a lack of immflamatory cells in lesions and predominantly negative *M. ulcerans* and *M. bovis* purified protein derivative (PPD) skin tests[22]. Tuberculin or burulin skin tests switch positive over time[25], and intralesional influx of leucocytes has been reported in late stages, suggesting a change in inflammatory response[29,68]. A cellular Th-1 immune response with high levels of gamma interferon (IFN-γ) is regarded as crucial for the host defence against mycobacteria[69]. *In vitro* immune analysis has confirmed the notion of a systematic T-cell anergy, as peripheral blood mononuclear cells from patients with active disease or whom had recovered from surgical excision of Buruli ulcer showed significantly reduced lymphoproliferation and IFN-γ production in response to stimulation with *M. bovis* BCG or *M. ulcerans*, and a Th-2-type (interleukin-4 (IL-4), IL-5, and IL-10) cytokine mRNA pattern was present[30,31], suggesting Th-2-mediated Th-1 down-regulation. In one case study, it has been shown that the development of ulcerative *M. ulcerans* disease is associated with a shift from the Th-1 to Th-2 phenotype[70].

Other studies have shown that IL-10 may be a key cytokine that mediates local Th-phenotype switching within nodules and ulcers. IL-10 can facilitate both Th-2 and Th-1 down-regulation and is not exclusively produced by Th-2 cells[71,72]. Recently, Prévot *et al.,* evaluated cytokine production by peripheral whole-blood mononuclear cells and detected

high IFN-γ but low IL-10 mRNA levels in nodular lesions, whereas high IL-10 but low IFN-γ mRNA levels were present in ulcerative lesions. Moreover, tumor necrosis factor alpha (TNF-α) as expressed in lesions from both patients groups, and levels of TNF-α expression were higher in nodular than in ulcerative lesions. TNF-α could be one of the additional factors involved in the pathology of Buruli disease, but its precise role in the development of the skin lesions remains to be elucidated[73]. In contrast, a study of cytokine responses of peripheral whole-blood mononuclear cells from patients with Buruli ulcer disease in Ghana showed Th-1 down-regulation in early Buruli ulcer and down-regulation reversed in later stages of the disease without association with IL-10 or IL-4 production[74]. Whether Buruli ulcer disease is associated with a shift in T-helper subset responses is still unclear.

A mouse model has been used to analyse the primary immune response against *M. ulcerans*[13]. Histopathological analysis of the lesions induced by *M. ulcerans* infections showed comparable necrosis and changes in vasculature and collagen degeneration as the ulcerative lesions in Buruli ulcer patients. Similar to most mycobacterial species, *M. ulcerans* infection proceeds through an initial phase, where bacilli are internalized by phagocytic cells. Transition to an extracellular phase is caused by mycolactone, which induces host cell death within days of infection *in vitro*. Several lines of evidence suggest that this transient intracellular step may contribute to the successful establishment of a chronic extracellular infection. First, the uptake of *M. ulcerans* by phagocytes may induce Th-1 host immune responses, ineffective for clearance of extracellular bacteria. Second, the suppression of TNF-α production together with stimulation of inflammatory chemokines by infected phagocytes may prevent the organization of granulomas able to control the infection[16].

Other evidence exists for the protective role of acquired cellular immunity: bacillus Calmette-Guèrin vaccination is protective in mice against low-dose inoculation and to some extent in man[75]. Finally, data from Benin suggest that there is a second peak of incidence in the elderly, which may correspond with declining immunity[76].

Although, cell-mediated immunity is considered to be the major component of the host response against *M. ulcerans*, antibodies may also have a protective role against the bacteria, as the pathogen is extracellular during active disease. Several studies have shown that serum samples of infected individuals from geographically distinct regions have high

antibody titers to *M. ulcerans* antigens, and that antibody responses are not correlated with disease stage[29-31]. The nature of the bacterial antigens to region which these antibodies are directed is unknown, but the range of molecular weights, together with the smeared appearance of the antigens suggest that both protein and non-protein antigens are recognized[77].

In recent years, studies from several groups have challenged the traditional dogma that cell-mediated immunity is the major component of the host response against *M. tuberculosis*, and have demonstrated that monoclonal antibodies (mAbs) can modify various aspects of mycobacterial infection. The studies performed using mAbs against various mycobacterial epitopes suggest that certain antibodies present during infection can affect the course of the disease to benefit the host. One of the studies with mAbs showed that mAbs directed to arabinomannan and the carbohydrate portion of lipoarabinomannan induced protection[78,79].

The role of the antibodies in protection against Buruli ulcer disease has not been studied. Experimental infection of mice genetically inactivated in various compartments of the immune response (B lymphocytes, Th-cells, and cytolytic T lymphocytes, cytokines, and monokines) will help to understand how host immunity is acquired.

There is no specific vaccine against *M. ulcerans* available, but *M. bovis* - BCG offers some protection, albeit short lived[75]. BCG may also provide more enduring protection against the most severe forms of Buruli[56].

Current prospects for better vaccines include improved or repeated BCG vaccination, attenuation of an *M. ulcerans* isolate, and subunit vaccines aimed at immunodominant protein antigens or the toxin itself[80].

10.1 Aim of the thesis

10.1.1 Buruli ulcer

In May 2004, the World Health Assembly adopted a resolution on Buruli ulcer that called for research in areas that provide direct benefit to Buruli ulcer patients:
These include:

1. Studies on the transmission of Buruli ulcer
2. Development of methods for early diagnosis
3. Vaccine development

Within the framework of these priorities, the aim of this thesis was to identify, clone, recombinant express, purify and characterize immunodominant antigens of *M. ulcerans* using mAbs. This approach was based on the hypothesis that some of the immunodominant antigens might be suitable targets for serological tests, and micro-epidemiology studies based on polymorphism.

10.1.2 Malaria

The drive to identify novel vaccine candidates or drug targets has in part focussed on identifying genes coding for transmembrane or secreted proteins of *Plasmodium falciparum*[81,82]. Access to the sequence of the entire genome of *P. falciparum* has provided the opportunity to deduce the function of many of the predicted proteins through the identification of orthologue genes and motifs in other organisms[81]. However, a large proportion of the predicted genes have no detectable orthologues in other organism, reminding researchers that many aspects of parasite biology still have to be uncovered[83]. The elucidation of molecular mechanisms responsible for recognition and the subsequent invasion of erythrocytes by malaria parasites are of central importance towards the development of new intervention strategies. Novel proteins encoded by open reading frames designated D13 and glyceraldehyde-3-phosphate dehydrogenase (pfGAPDH) were identified functionally by an *in vitro* transcription-translation-translocation system.

The aim of the project was to generate mAbs against the two recombinantly expressed proteins and to use them to describe the sub-cellular localisation, the stage-specific expression and the biological function, particularly the interaction with host cell.

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Laminin-binding protein of *Mycobacterium ulcerans* is polymorphic, surface exposed and expressed in Buruli ulcer lesions

Running title: Laminin-binding protein of *Mycobacterium ulcerans*

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Abstract

Mycobacterium ulcerans is an emerging pathogen which has diverged from *M. marinum* by acquisition of a virulence plasmid bearing a cluster of genes necessary for the synthesis of the macrolide toxin mycolactone. This slow growing mycobacterium causes an infectious disease characterized by chronic, necrotizing ulceration of subcutaneous tissues and the overlying skin, commonly designated as Buruli ulcer. Standard genetic fingerprinting methods including multi-locus sequence typing have resolved only a limited number of geographical types of *M. ulcerans*. We reasoned that immunodominant proteins may be under selection pressure and exhibit more diversity than most other *M. ulcerans* proteins. Among immunodominant proteins recognized by monoclonal antibodies, one was identified by mass spectrometric analysis as the *M. ulcerans* homolog of the mycobacterial laminin-binding protein. DNA sequence analysis demonstrated that the corresponding *hupB* gene represents a relatively variable element within the highly conserved genetic background of *M. ulcerans*. Sequence typing based on a set of such variable genes may develop into a new tool for molecular epidemiological studies. Diversity was based both on insertional/deletional polymorphism and on single base exchanges. Dominance of non-conservative exchanges was indicative for a diversifying selection pressure. We demonstrate that the *M. ulcerans* laminin-binding protein is associated with the cell wall fraction and expressed in Buruli ulcer lesions. The Laminin-binding protein is involved in the adherence of mycobacteria to target tissues and it remains to be elucidated whether the *M. ulcerans* homologue plays a role in host-pathogen interaction and/or persistence in an environmental habitat.

Introduction

Buruli ulcer is a devastating human disease caused by *Mycobacterium ulcerans* and characterized by chronic, necrotizing ulceration of subcutaneous tissues and the overlying skin (1). It has been observed in more than 30 tropical and subtropical countries, but the main burden of disease falls on children living in sub-Saharan Africa (33). *M. ulcerans* is a slow-growing environmental mycobacterium, which can be cultured from infected human tissue on mycobacterial media at 30-32 ˚C. In Buruli ulcer lesions clumps of extracellular acid-fast organisms surrounded by areas of necrosis are found in particular in subcutaneous fat tissue (8). A diffusible toxic macrolide, mycolactone, plays a key role in the massive tissue destruction seen in Buruli ulcer (7). The toxin causes mammalian cells to undergo apoptosis and its action explains at least in part that in Buruli ulcer lesions inflammatory response are poor (8). Genetic analyses suggest the recent divergence of *M. ulcerans* from *M. marinum* (30), which is well known as fish pathogen and can cause limited granulomatous skin infections in humans. One of the hallmarks of the emergence of *M. ulcerans* as a more severe pathogen is the acquisition of a 174-kb plasmid bearing a cluster of genes necessary for the synthesis of mycolactone (29); (31).

While it is well established that proximity to marshes and wetlands is a risk factor for contracting Buruli ulcer, the exact mode of transmission of *M. ulcerans* is incompletely understood (1). *M. ulcerans* DNA has been detected in aquatic organisms and biofilms (14); (19); (23); (28). Since many patients have had antecedent trauma at the site where the lesion later occurred (16), contamination of lesions by direct contact with environmental reservoirs, such as water or mud may play an important role. Since *M. ulcerans* has been detected in the salivary glands of carnivorous aquatic insects it has been hypothesized that it may be transmitted by water bug bites (11-13); (19). Currently no molecular fingerprinting method for *M. ulcerans* is available, that has a sufficiently high resolution for micro-epidemiological analyses. The apparent lack of genetic diversity of *M. ulcerans* within individual geographical regions (3); (4); (20); (28); (32); (30) is indicative for a clonal population structure. We reasoned that immunodominant proteins may be under selection pressure and exhibit more diversity than the housekeeping genes used for multi-locus sequence typing (30). Here we demonstrate that the *M. ulcerans* homologue of the mycobacterial laminin binding protein (LBP) is highly immunogenic and shows

considerable diversity based both on insertional/deletional polymorphism and on point mutations.

Materials and Methods

Mycobacterial lysates and subcellular fractions

M. ulcerans isolates from diverse geographical origins used in this study are listed in Table 1. Other mycobacterial species included in the analyses are *M. abscessus* (ATCC 19977), *M. avium* subsp. *avium* (MAC101), *M. bohemicum* (clinical isolate), *M. bovis* biovar. BCG *(*ATCC 35734), *M. chelonae* (DSM 43804), *M. fortuitum* (ATCC 49403), *M. gordonae* (Pasteur 14021.001), *M. haemophilum* (ATCC 29548), *M. intracellulare* (clinical isolate), *M. kansasii* (NCTC 10268), *M. lentiflavum* (clinical isolate), *M. malmoense* (NCTC 11298), *M. marinum* (ATCC 927), *M. scrofulaceum* (Pasteur 14022.0031), *M. simiae* (clinical isolate), *M. smegmatis* (Pasteur 14133.0001), *M. terrae* (clinical isolate) and *M. tuberculosis* (Pasteur 14001.0001).

For the preparation of lysates mycobacterial cells were heat-inactivated at 80°C for 1 hour and suspended in PBS (50mM sodium phosphate, 150mM sodium chloride, pH 7.4) containing 5% SDS and 1mM phenylmethyl-sulphonyl fluoride (PMFS), and 10 µg/ml each of leupeptin and trypsin inhibitor (Sigma, St. Louis, Mo). 200mg of cell suspension (wet weight) was subjected to a bead beater (Mikro-Dismembrator, Braun Biotech International) treatment with 400µl of 0.1mm zirconia beads (BioSpec Products) at 2300 rpm for 15 minutes. Beads and unbroken cells were removed by centrifugation at 10000g for 10 min. Protein content of the lysate was quantified using the BCA protein assay (Pierce).

For the preparation of sub cellular fractions 400 mg of heat inactivated *M. ulcerans* cells were suspended in 3 ml of PBS containing 0.1% Tween 80 and a proteinase inhibitor cocktail described above. Cells were broken by three cycles of ultrasonic disruption (Branson sonifier 250) on ice for 10 min with 50% Duty cycle and 40% output using a microtip probe. Unbroken cells were removed by centrifugation at 3000g for 10 min. A cell wall fraction was prepared from the supernatant by centrifugation at 27000g for 1 hour and was washed twice with PBS. The supernatant was subjected to a 100000g centrifugation for 4h; a cytosol fraction was obtained from the supernatant and the membrane fraction from the pellet. The membrane fraction was washed with PBS and suspended in 0.01M ammonium bicarbonate and the supernatant was dialyzed against 0.01M ammonium bicarbonate (2).

Western blots analysis

Mycobacterial lysates and sub-cellular fractions (10µg of total protein/lane) were separated on 12% SDS PAGE gels under reducing conditions in Laemmli buffer (24). Proteins were transferred to nitrocellulose paper (BioRad) in Tris glycine buffer (25 mM Tris and 192 mM Glycine pH 8.3). Filters were blocked in 5% skim milk in PBS with 0.1% Tween 20 overnight. Sera or monoclonal antibodies diluted in blocking solution were incubated with membranes for two hours. Bound antibodies were detected using alkaline phosphatase conjugated goat anti-mouse IgG (Sigma Chemicals, St. Louis, Mo) as secondary antibodies and 5-bromo-4-chloro-3-indolylphosphate (BCIP)-nitro blue tetrazolium (BioRad) as substrate.

Generation of monoclonal antibodies

Hybridoma cell lines were generated as described (25) from mice immunized intraperitoneally three times with 20µg of a lysate of the *M. ulcerans* strain 97-610 from Ghana formulated in the MPL +TDM adjuvant (Sigma Chemicals, St. Louis, Mo) and immune sera were analyzed by Western blotting with the same lysates. Three days before cell fusion, mice that recognized a broad range of mycobacterial proteins received an intravenous booster injection with 20µg of *M. ulcerans* lysate in PBS. Antibodies specific for *M. ulcerans* antigens were identified by ELISA using Immunolon 4 plates (Dynes Technologies Inc., Chantilly, Va.) coated with *M. ulcerans* lysate. From a panel of hybridomas generated (manuscript in preparation), two (designated DD2.2 and DD2.3) that secrete mAbs specific for a 27 kDa protein were identified by Western blotting.

Partial purification and identification of the mAb DD2.2/3 reactive protein from *M. tuberculosis*

A lysate of *M. tuberculosis* cells (strain QK 228) in 8M urea was fractionated by reverse-phase HPLC using a C8 column (Nucleosil 300-5 C; Agilent Technologies). Fractions containing the mAb DD2.2/.3-reactive protein were identified by Western blot analysis. Proteins in the peak fraction were separated by 12% SDS-PAGE and a band of apparent molecular mass of 27 kDa was cut out and digested with trypsin as described (6). The protein was identified by matrix assisted laser desorption ionization-mass spectrometry

following in-gel digestion in low-salt, non-volatile buffer and simplified peptide recovery as described (6).

DNA extraction and sequence analysis of the *M. ulcerans hup*B gene

For DNA extraction 100µl of a 100mg/ml lysozyme solution were added to a suspension of heat inactivated *M. ulcerans* cells in 500µl of extraction buffer (50mM tris-HCl, 25mM EDTA, 5% monosodium glutamate). After 2 hours of incubation at 37°C, 70µl proteinase K-10X buffer (100mM Tris-HCl, 50mM EDTA, 5% SDS, pH 7.8) and 10µl of a 20mg/ml proteinase K solution were added. After incubation at 45°C overnight, the samples were subjected to bead beater treatment with 300µl of 0.1mm zirconia beads (BioSpec Products) at 3000 rpm for 7 minutes. Beads and unbroken bacteria were removed by centrifugation and the supernatant was transferred to fresh tubes. An equal volume of phenol-chloroform (Fluka) was added and the DNA contained in the upper phase was precipitated and washed with ethanol.

For sequences analysis two overlapping fragments of the *hupB* gene were amplified by PCR using the primer combinations 5'-CCATAAACGAGGACCGC-3' and 5'-TCTTTGCCGCAGCCTTCTTGG-3' or 5'-GCCAAGAAGGTGACCAA-3' and 5'-TGCGGGCCTAACGCACGAATA-3', respectively. Amplifications were performed with the following profile: 5 min 96°C; 30 x (1min 96°C, 1 min 63°C, 1 min 72°C), 7 min 72°C. Amplicons were purified using a PCR product purification kit (Qiagen) and sequenced using an ABI PRISM 310 genetic analyzer (Perkin-Elmer). All sequences were reconfirmed at least twice using independent PCR products.

Nucleotide sequences

The nucleotide sequences reported in this paper have been submitted to the GenBank with the accession numbers: AY954292 to AY954299, corresponding to the *hup*B alleles 1 to 8.

Results

Identification of an immunodominant protein of *M. ulcerans* that shows size variation

Sera from *M. ulcerans* immunized mice showed reactivity with a broad range of mycobacterial antigens in Western blot analysis. The bands stained by all mouse sera included a protein with an apparent molecular mass of 27 kDa (data not shown). Within a panel of mAbs generated from the spleen cells of a *M. ulcerans* immunized mouse, two mAbs (designated DD2.2 and DD2.3) were identified, which reacted with this 27 kDa protein. Both mAbs stained one additional band with an apparent molecular mass of 32 kDa. Western blot analysis with a spectrum of *M. ulcerans* isolates of geographically diverse origins revealed size variation in the stained protein bands (Fig. 1).

A mAb DD2.2/3-reactive homologue was found in whole cell lysates of all 20 mycobacterial species tested (Fig. 2). Variation in the number and size of the stained bands may reflect differences in length of the coding gene sequences and/or in posttranslational modification. In indirect immunofluorescence assays all mycobacterial species tested were stained by mAbs DD2.2 and DD2.3 (data not shown).

Identification of the *M. tuberculosis* homolog of the mAb DD2.2/3-reactive protein

Since a completely annotated *M. ulcerans* genome was not available, we identified the mAb DD2.2/3-reactive homolog of *M. tuberculosis* instead of the *M. ulcerans* protein itself. A *M. tuberculosis* whole cell lysate was fractionated by reverse-phase chromatography on a C8 column. Fractions were analyzed by Western blotting, and a band of apparent molecular mass of 27KDa was cut out from a SDS-PAGE lane loaded with the peak fraction. The excised protein was digested with trypsin, and the total peptide mixture was extracted and analyzed by MALDI-TOF. Eight peptides sequences obtained (MNKAELIDVLTQK, QATAAVENVVDTIVR, GDSVTITGFGVFEQR, AVVSGAQRLPAEGPAVK, VKPTSVPAFRPGAQFK, AVHKGDSVTITGFGVFEQR, TGETVKVKPTSVPAFRPGAQFK, and VKPTSVPAFRPGAQFKAVVSGAQR) allowed to identify the mAb DD2.2/3-reactive protein as laminin binding protein (LBP) encoded by the *hupB* gene. Reactivity of mAbs DD2.2 and DD2.3 with a GST-LBP fusion protein of *M. tuberculosis* (5) reconfirmed the identification (data not shown).

Localization and expression of *M. ulcerans* LBP

The two mAb DD2.2/3-reactive bands were primarily present in *M. ulcerans* cell wall fractions (Fig. 3B), but undetectable in culture filtrate (Fig. 3A) and in a cytosol fraction (Fig. 3B). The *M. ulcerans* LBP is expressed *in vivo*, as it was detectable in a tissue lysate from the centre of an excised Buruli ulcer lesion (Fig. 3C). No staining was observed in lysates from the healthy margins of the excised tissue.

Diversity of the *hupB* gene in *M. ulcerans* isolates

The *hup*B gene of *M. ulcerans* was identified by homology search in the *M. ulcerans* genome project database (http: genopole.pasteur.fr/Mulc/BuruList.html). Primers located in the 3' and 5' untranslated regions flanking the *hup*B gene were used to analyze *hup*B gene diversity in *M. ulcerans* by PCR amplification and DNA sequence analysis. Significant diversity based both on insertional/deletional polymorphism and on point mutations was observed. In addition to three different types of deletions in the LBP repeat region, point mutations at altogether 15 positions were found (Table 1). Based on the sequence of their *hup*B genes the 24 *M. ulcerans* strains analyzed could be subdivided into eight groups (Table 1). While all nine African isolates (from six countries) analyzed had the same allele, three different alleles were found among the six Australian strains included. Allele 2 differed in one and allele 3 in three non-synonymous point mutations from the *hup*B gene sequence (allele 1) of the African isolates. Compared to allele 1, allele 4 had a 27 base deletion in the repeat region of *hup*B. Of the two isolates from PNG, one had allele 1 and one allele 4. Allele 5 found in the two Mexican isolates analyzed differed from allele 1 in seven non-synonymous point mutations plus a 27 base deletion located at another position than that of allele 4. While the same deletion was also found in the isolates from China and Japan, the three isolates from Malaysia, French Guiana and Suriname shared another (54 bp) deletion. While allele 6 from Japan and China differed in six point mutations form allele 5 (Mexico), there was a difference in only one position between allele 7 (Malaysia) and allele 8 (French Guiana and Suriname). While all Asian and American isolates had a T^{509} and a C^{593}, all African, Australian and PNG isolates had a C^{509} and a G^{593}.

All three types of deletions were located in a region of LBP containing AKKA and ATKAP repeats (Fig. 4). Compared to allele 1, allele 4 was shortened by a nine amino acid (ATKAP-AKKA) block. In alleles 5 and 6 a variant block (ATKA**R**-AKKA) present in alleles 1 to 4 was missing. Alleles 7 and 8 shared an 18 amino acid deletion (AKKA-ATKA**R**-AKKA-ATKAP). Twelve of the 15 single nucleotide polymorphisms found were non-synonymous. While all but one (C/G^{593}) of these non-synonymous polymorphisms were located within the repeat encoding sequence stretches, two of the three synonymous polymorphisms (C/T^{102} and C/T^{117}) were located outside the repeats. An alignment of deduced amino acid sequences of LBP from other mycobacterial species revealed that the N- terminal portion of the protein is highly conserved between species. Like the intra-species diversity in *M. ulcerans,* the inter-species diversity is based both on deletional/insertional and on point mutational changes and is focused in the C-terminal portion of the protein (Fig. 4).

Discussion

M. ulcerans is an emerging pathogen that seems to have evolved from *M. marinum* by acquiring a virulence plasmid encoding a polyketide synthase responsible for the synthesis of mycolactone (30, 31). This plasmid has a common evolutionary origin in *M. ulcerans* isolates from diverse geographical locations around the world (29). The clonal population structure of *M. ulcerans* is reflected by the fact, that all genetic fingerprinting method applied so far for *M. ulcerans* have resolved only a limited number of geographical types (3, 4); (20); (28, 28, 30). To date, the highest resolution, nine distinct profiles related to geographical regions, was obtained by a PCR method that captures differences in regions between the high-copy-number insertion sequences IS*2404* and IS*2606* (28). Only six multi-locus sequence types were found among 18 *M. ulcerans* isolates of diverse geographical origin when seven unlinked gene loci were sequenced (30). In view of this extremely low genetic diversity, it is highly remarkable, that sequencing of the *hupB* gene of 24 *M. ulcerans* isolates has identified eight alleles. Interestingly, three different *hupB* alleles were found among the six Australian isolates analyzed here. Two of these alleles (2 and 4) were found in isolates coming from the Victoria region, and allele 3 was found in a strain from the Queensland area. Of the two analyzed isolates from Papua New Guinea one had an allele 4 found in three isolates from the Victoria region, while the other one had allele 1, which was found in all African isolates, irrespective of the country of origin. Only a single base-exchange distinguished allele 8 found in isolates from Suriname and French Guiana from allele 7 found in a Malaysian isolate. Both alleles share a unique deletion (bp 445-498) which may be indicative for import of the pathogen by Asian contract workers in the last century.

The polymorphism of the highly immunogenic LBP may reflect inherent instability of the repetitive sequence stretch or diversifying selection pressure. In addition to three different types of deletion in the repeat region, point mutations at altogether 15 positions were found. Twelve of these 15 types of point mutation were non-synonymous, which indicates that this diversity does not represent accumulation of neutral mutations but is rather the result of some type of selection. The predicted size of LBPs is smaller than the observed size in SDS/PAGE, which may be related to the high content of lysine and arginine. The observed variation in protein size and the occurrence of a second protein band in many of

the mycobacterial lysates suggest posttranslational modification, such as methylation of the lysine-rich repeats (17).

Consistent with our finding of an association of *M. ulcerans* LBP with the cell wall fraction, homologues of this protein in other mycobacterial species are localized on the mycobacterial surface (26), (15), (18). LBPs seem to play an important role in the adherence of pathogenic mycobacteria to their target tissues within the infected host. LBP of *M. leprae* binds to the peripheral nerve laminin-2 isoform and may be involved in the invasion of Schwann cells (26), (22). LBP of *M. smegmatis* has been shown to promote mycobacterial adherence to pneumocytes and macrophages (18). It remains to be elucidated, whether LBP of the extracellular pathogen *M. ulcerans* plays a role in host-pathogen interaction, persistence in insect salivary glands and/or establishment of biofilms on plant surfaces (14). In *M. smegmatis* LBP is upregulated by cold-shock stress (27) and in the anaerobiosis-induced dormant state (9). In view of these findings it is interesting that the *M. ulcerans* LBP was detectable in lysates of Buruli ulcer lesions. Currently it is not clear whether *M. ulcerans* can enter into a dormant state and persist in this stage in the mammalian host. LBPs represent immunodominant antigens (21), (10) and their potential as vaccine components or target structures for immunodiagnostic tests should be evaluated further.

Acknowledgements

We thank Tim Stinear for helpful discussions and reconfirmation of genetic data, the heads of the *M. ulcerans* and *M. marinum* genome sequencing projects for the permission to use the sequence data. The work of Diana Diaz was supported by a grant of the Amt für Ausbildunsbeiträge of the county of Basel.

Allele	***M .ulcerans* strain**	**Position of point mutation or deletion**														
		P102	P117	P364	P384	P385	P387	P399	P411	P412	P414	P424	P426	Deletion	P509	P593	P647
1	ITM 97-680 Togo	C	C	A	C	G	T	C	T	C	C	G	C	-	C	G	C
	ITM 96-657 Angola	C	C	A	C	G	T	C	T	C	C	G	C	-	C	G	C
	ITM 97–104 Benin	C	C	A	C	G	T	C	T	C	C	G	C	-	C	G	C
	ITM 97–111 Benin	C	C	A	C	G	T	C	T	C	C	G	C	-	C	G	C
	ITM 5150 DRC	C	C	A	C	G	T	C	T	C	C	G	C	-	C	G	C
	ITM 5151 DRC	C	C	A	C	G	T	C	T	C	C	G	C	-	C	G	C
	001441 Ghana	C	C	A	C	G	T	C	T	C	C	G	C	-	C	G	C
	ITM 94-511 Ivory Coast	C	C	A	C	G	T	C	T	C	C	G	C	-	C	G	C
	ITM 97-483 Ghana	C	C	A	C	G	T	C	T	C	C	G	C	-	C	G	C
	ITM 94-1331 PNG	C	C	A	C	G	T	C	T	C	C	G	C	-	C	G	C
2	ITM 9550 Australia	C	C	G*	C	G	T	C	T	C	C	G	C	-	C	G	C
	ITM 8849 Australia	C	C	G*	C	G	T	C	T	C	C	G	C	-	C	G	C
3	ITM 9540 Australia	C	C	A	T*	C*	C*	C	T	C	C	G	C	-	C	G	C
4	ITM 94–339 Australia	C	C	A	C	G	T	C	T	C	C	G	C	P427-453	C	G	C
	ITM 5147 Australia	C	C	A	C	G	T	C	T	C	C	G	C	P427-453	C	G	C
	ITM 5142 Australia	C	C	A	C	G	T	C	T	C	C	G	C	P427-453	C	G	C
	ITM 9537 PNG	C	C	A	C	G	T	C	T	C	C	G	C	P427-453	C	G	C
5	ITM 5114 Mexico	C	C	A	C	G	T	C	C*	G*	T*	C*	T*	P454-480	T*	C*	C
	ITM 5143 Mexico	C	C	A	C	G	T	C	C*	G*	T*	C*	T*	P454-480	T*	C*	C
6	ITM 98-912 China	C	C	A	C	G	T	T	T	C	C	G	C	P454-480	T*	C*	C
	ITM 8756 Japan	C	C	A	C	G	T	T	T	C	C	G	C	P454-480	T*	C*	C
7	ITM 94-1328 Malaysia	T	T	A	C	G	T	T	T	C	C	G	C	P445-498	T*	C*	C
8	ITM 7922 French Guiana	T	T	A	C	G	T	T	T	C	C	G	C	P445-498	T*	C*	T*
	ITM 842 Suriname	T	T	A	C	G	T	T	T	C	C	G	C	P445-498	T*	C*	T*

Table 1. Sequence diversity of the *hupB gene* of *M. ulcerans*. Non-synonymous base exchanges are indicated by asterisks

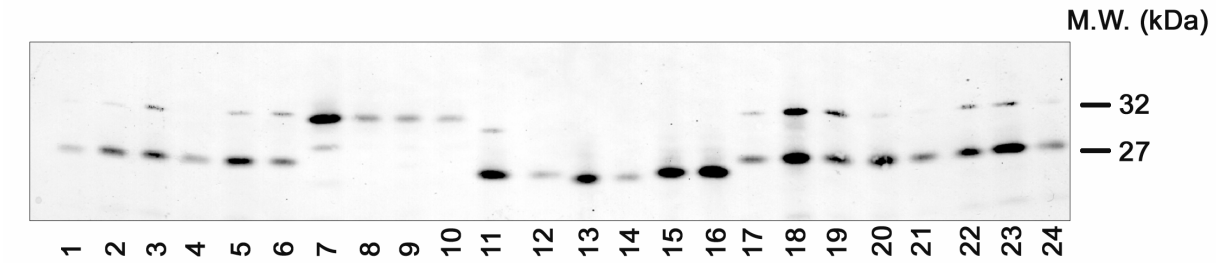

Figure 1: Western blot analysis of lysates of *M. ulcerans* strains of diverse geographical origin with mAb DD2.2. 1) Democratic Republic of Congo (5151), 2) Democratic Republic of Congo (5150), 3) Benin (ITM 97–111), 4) Benin (ITM 97–104), 5) Australia (ITM 9540), 6) Australia (ITM 8849), 7) Australia (ITM 9550), 8) Australia (ITM 94–339), 9) Australia (5142), 10) Australia (ITM 5147), 11) Mexico (ITM 5114), 12) Mexico (5143), 13) Suriname (ITM 842), 14) French Guiana (ITM 7922), 15) China (980912), 16) Japan (ITM 8756), 17) Malaysia (941328), 18) Papua New Guinea (9357), 19) Papua New Guinea (ITM 94-1331), 20) Ghana (97-483), 21) Ghana* (001441), 22) Ivory Coast (940511), 23) Togo (ITM 97-680), 24) Angola (960657).

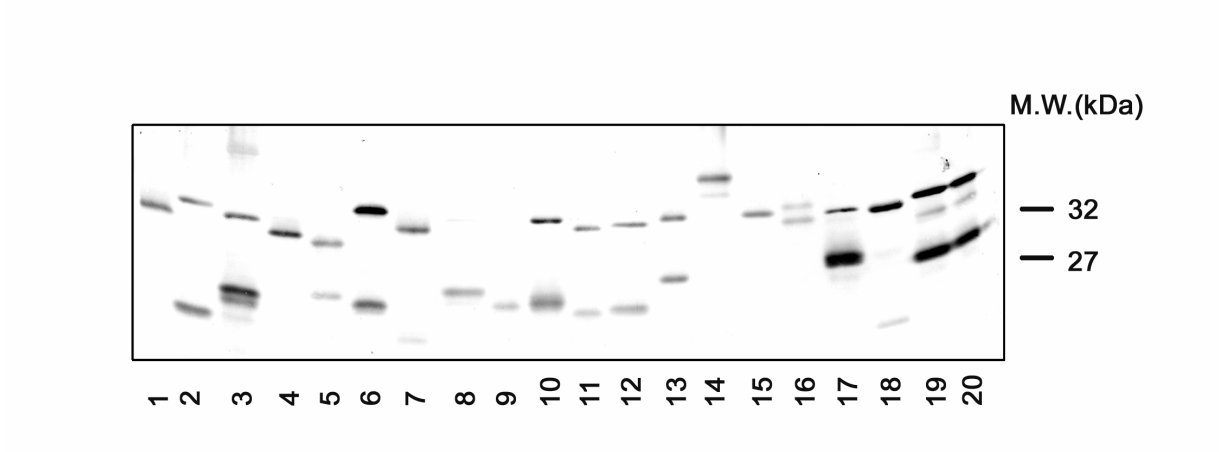

Figure 2. Western blot analysis of sub-cellular fractions and of *M. ulcerans* infected tissue with mAb DD2.2. A: 1) Culture medium, 2) *M. ulcerans* culture filtrate, 3) *M. ulcerans* lysate. B: 1) *M. ulcerans* lysate, 2) membrane fraction of *M. ulcerans, 3)* cytosol fraction of *M. ulcerans, 4)* cell wall fraction of *M. ulcerans*. C: 1) tissue lysate from the centre of a Buruli ulcer lesion 2) tissue lysate from the healthy margin of a Buruli ulcer lesion 3) lysate of *in vitro* cultivated *M. ulcerans* cells.

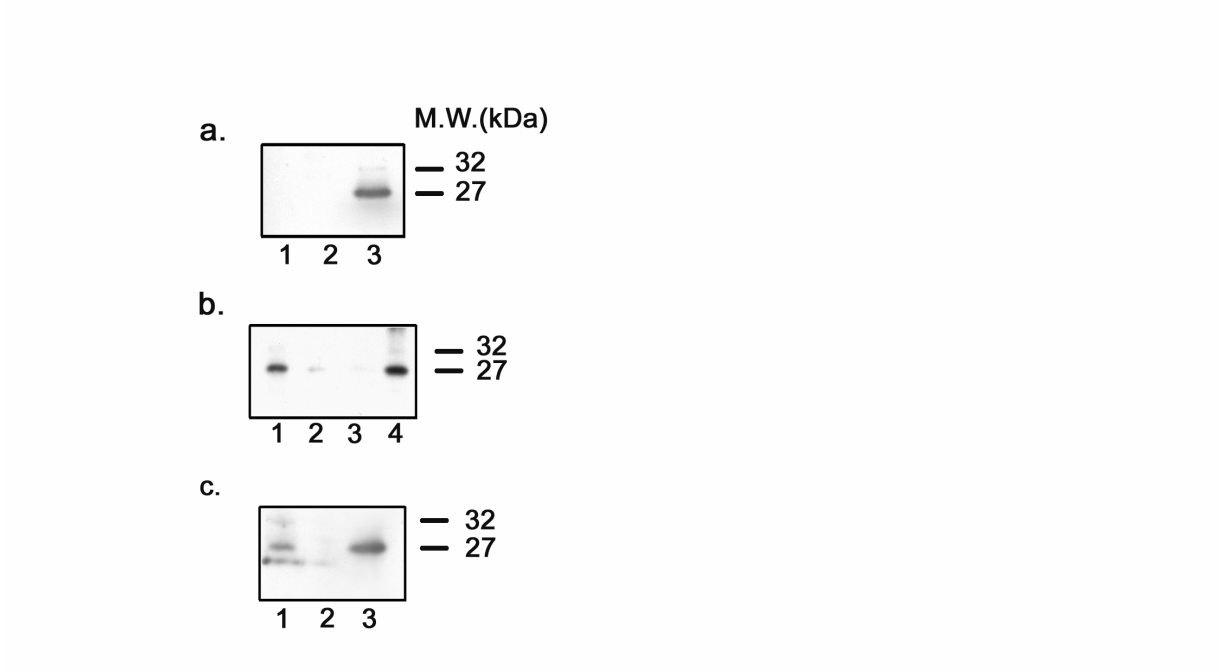

Figure 3. Western blot analysis of lysates of different mycobacterium species with mAb DD2.2. *1) M. abscessus, 2) M. avium ssp avium, 3) M. bohemicum, 4) M. chelonae, 5) M. fortuitum, 6) M. gordonae, 7) M. haemophilum 8) M. intracellulare, 9) M. kansasii, 10) M. lentiflavum, 11) M. malmoense, 12) M. scrofulaceum, 13) M. simiae, 14) M. Smegmatis, 15) M. terrae, 16) M. xenopi, 17) M. ulcerans, 18). M. tuberculosis, 19) M. marinum, 20) M. bovis.*

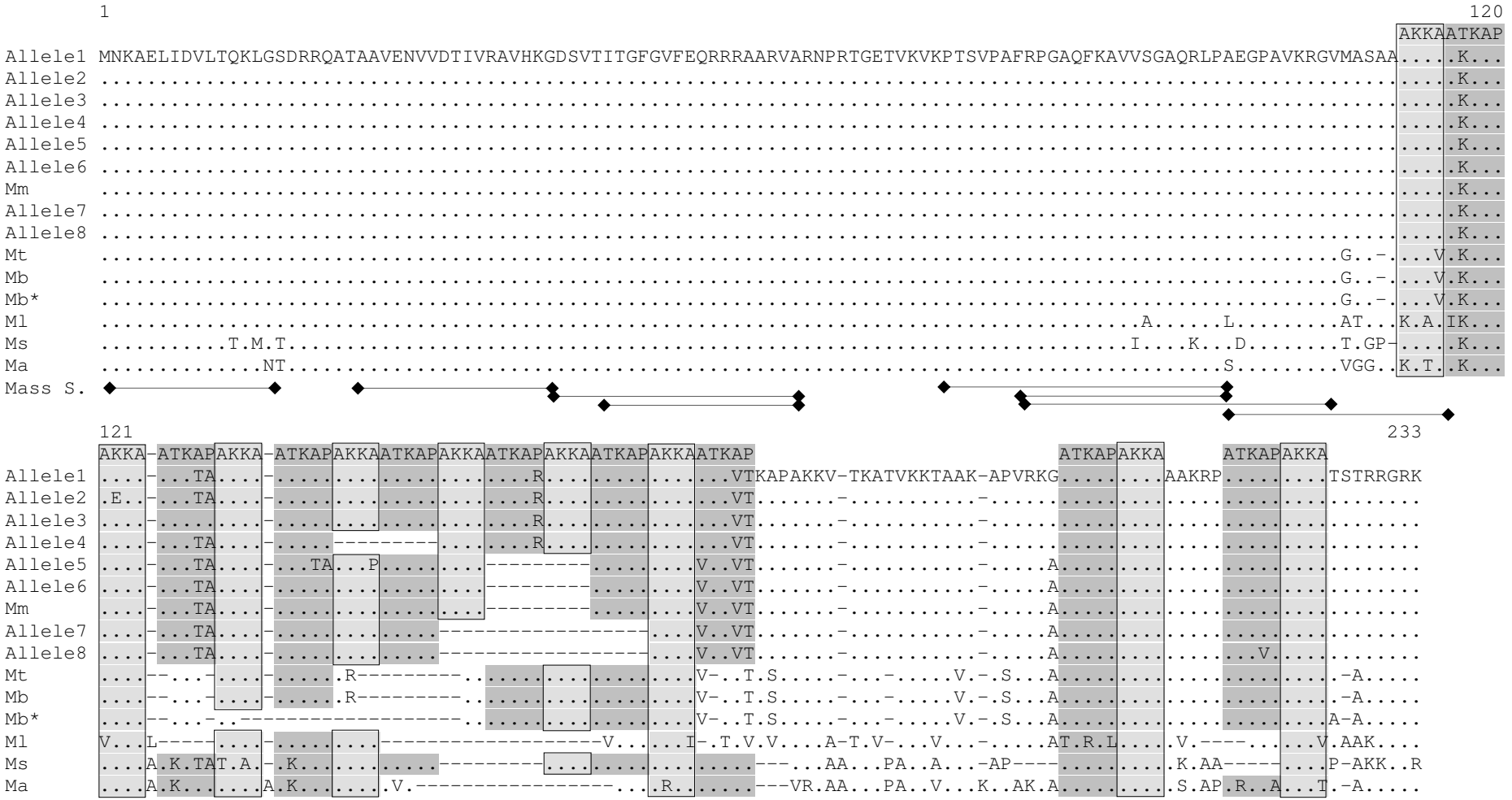

Figure 4. Comparison of the predicted amino acid sequence of LBP of *Mycobacterium ulcerans* isolates of diverse geographical origin. Identity with the reference sequence is indicated by a dot (.), deletions are indicated by dashes (-). AKKA repeats are shown in light grey boxes and ATKAP repeats in dark grey boxes. Mm: *M. marinum* [sequence obtained by blast search of data publicly available on *M. marinum* genome sequencing project web site (http://www.sanger.ac.uk/Projects/M_marinum/)]; Mt: *M. tuberculosis* (NC_000962); Mb: *M. bovis* (NP_856655); Mb*: *M. bovis* (Y18421); Ms: *M. smegmatis* (AF068138); Ma: *M. avium* (AE017238); Ml: *M. leprae* (AB022517). Continuous lines indicate the position of peptides identified by MALDI-TOF.

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Use of the immunodominant 18kDa small heat shock protein as serological marker for exposure to *Mycobacterium ulcerans*

Running title: Immunodominant small heat shock protein of *Mycobacterium ulcerans*

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Submitted

Abstract

While it is well established that proximity to wetlands is a risk factor for contracting Buruli ulcer, it is not clear, which proportion of a population living in an endemic area is exposed to *M. ulcerans*. Immunological cross-reactivity among mycobacterial species complicates development of a specific serological test. Among immunodominant proteins recognized by a panel of anti-*M. ulcerans* monoclonal antibodies, the *M. ulcerans* homologue of the *M. leprae* 18 kDa small heat shock protein (shsp) was identified. Since this shsp has no homologues in *M. bovis* and *M. tuberculosis*, we evaluated its use as target antigen for a serological test. Anti-18 kDa shsp antibodies were frequently found in the serum of Buruli ulcer patients and healthy household contacts, but rarely in controls from non-endemic regions. Results indicate that only a small proportion of *M. ulcerans* infected individuals develop the clinical disease.

Introduction

Buruli ulcer is a chronic necrotizing disease of skin and soft tissue caused by *M. ulcerans*. The disease starts as a subcutaneous nodule, papule or plaque that eventually ulcerates and progresses over months to years. After tuberculosis and leprosy, Buruli ulcer is the third most common mycobacterial infection in immunocompetent humans. The main burden of disease falls on children living in sub-Saharan Africa, but healthy people of all ages, races and socioeconomic class are susceptible as well (2).

In Buruli ulcer lesions, clumps of extra-cellular acid-fast organisms surrounded by areas of necrosis are often found in subcutaneous fat tissue (19). *M ulcerans* produces a family of macrolide toxin molecules, the mycolactones, which are associated with tissue destruction and local immunosuppression (13). In cell culture experiments, mycolactones produce apoptosis and necrosis in many human cell types (8,14). The toxin appears to play a role in inhibiting the recruitment of inflammatory cells to the site of infection, which explains at least in part why inflammatory responses are poor in Buruli ulcer lesions (19). Down-regulation of Th-1 responses may play a role in the progression of early Buruli ulcer disease (15-17,34), but may reverse in later stages (11). Intralesional influx of leukocytes and granulomatous responses in the dermis and panniculus has been reported in late stages of the disease (11,18). Spontaneous healing can occur and is often accompanied by a conversion of the Burulin (*M. ulcerans* sonicate) skin test from negative to positive.

In spite of some degree of local and peripheral T cell anergy, Buruli ulcer patients seem to be able to raise a humoral immune response against *M. ulcerans* antigens (15), and the analysis of serological responses to culture filtrate antigens of *M. ulcerans* has suggested that serological tests may be useful in the diagnosis and surveillance of the disease (9,31). Broad antigenic cross-reactivity between mycobacterial species represents a major problem for the development of a serological test that is specific and sensitive enough to monitor immune responses against *M. ulcerans* in populations where exposure to *M. tuberculosis* and BCG vaccination is common.

We reasoned that the identification, recombinant expression and immunological profiling of immunodominant proteins will provide target structures for analyzing protective

immune mechanisms and for the development of a serological test suitable for detecting *M. ulcerans* exposure and/or disease. Here, we describe serological responses against a highly immunogenic 18 kDa shsp of *M. ulcerans*, which has no homologue in *M. bovis* and *M. tuberculosis*. Serological analysis indicates that this protein represents a suitable target antigen for monitoring exposure to *M. ulcerans.*

Materials and Methods

Mycobacterial isolates

Mycobacterial species included in the study are *M. abscessus* (ATCC 19977), *M. avium* subsp. *avium* (MAC101), *M. bohemicum* (clinical isolate), *M. bovis* biovar. BCG *(*ATCC 35734), *M. chelonae* (DSM 43804), *M. fortuitum* (ATCC 49403), *M. gordonae* (Pasteur 14021.001), *M. haemophilum* (ATCC 29548), *M. intracellulare* (clinical isolate), *M. kansasii* (NCTC 10268), *M. lentiflavum* (clinical isolate), *M. malmoense* (NCTC 11298), *M. marinum* (ATCC 927), *M. scrofulaceum* (Pasteur 14022.0031), *M. simiae* (clinical isolate), *M. smegmatis* (Pasteur 14133.0001), *M. terrae* (clinical isolate), *M. tuberculosis* (Pasteur 14001.0001) and *M. leprae* (offered kindly by Dr. Brennan). *M. ulcerans* isolates of diverse geographical origin analyzed in this study are: Democratic Republic of Congo (5151), Angola (960657), Ghana (97-483), Australia (ITM 5147), Australia (ITM 9540), Mexico (ITM 5114), Australia (ITM 9550), Malaysia (941328), French Guiana (ITM 7922), Japan (ITM 8756), and Australia (94-1324). The mycobacteria were cultured as described (37).

Mycobacterial lysates and sub cellular fractions

Mycobacterial cells were heat-inactivated at 80°C for 1 hour and suspended in PBS (50 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) containing 5% SDS and 1 mM phenylmethyl-sulphonyl fluoride (PMFS), and 10 µg/ml each of leupeptine and soybean trypsin inhibitor (Sigma, St. Louis, Mo). Two hundred mg of cell suspension was subjected to a bead beater (Mikro-Dismembrator, Braun Biotech International) treatment with 400 µl of 0.1 mm zirconia beads (Bio Spec Products) at 2300 rpm for 15 minutes. Beads and unbroken cells were removed by centrifugation at 10000 x g for 10 min. Protein content of the lysate was quantified using the BCA protein assay (Pierce).

For the preparation of sub-cellular fractions, 400 mg of heat inactivated *M. ulcerans* cells were suspended in 3 ml of PBS containing 0.1% Tween 80 and the proteinase inhibitor cocktail described above. Cells were broken by three cycles of ultrasonic disruption (Branson sonifier 250) on ice for 10 min with 50% Duty cycle and 40% output using a microtip probe. Unbroken cells were removed by centrifugation at 3000 x g for 10 min. A cell-wall fraction was prepared from the supernatant by centrifugation at 27000 x g for 1

hour and was washed twice with PBS. The supernatant was subjected to a 100000 x g centrifugation for 4 h; a cytosol fraction was obtained from the supernatant and the membrane fraction from the pellet. The membrane fraction was washed with PBS and dialyzed against 0.01 M ammonium bicarbonate (3).

Western blots analysis

Mycobacterial lysates and sub-cellular fractions (10 µg of total protein/lane) were separated on 12% SDS PAGE gels under reducing conditions in Laemmli buffer. Proteins were transferred to nitrocellulose membranes (BioRad) in tris glycine buffer (25 mM Tris and 192 mM glycine pH 8.3). Filters were blocked with 5% skim-milk in PBS with 0.1% Tween 20 overnight. Mouse sera or monoclonal antibodies diluted in blocking solution were incubated with membranes for two hours. Bound antibodies were detected using alkaline phosphatase conjugated goat anti-mouse IgG (Sigma Chemicals) as secondary antibodies and 5-bromo-4-chloro-3-indolylphosphate (BCIP)-nitro blue tetrazolium (BioRad) as substrate.

For the analysis of human sera, 1 µg of recombinant 18 kDa shsp of *M. ulcerans* was separated on 12% SDS PAGE preparative gels and transferred as described above. Human sera diluted 1:100 were incubated with the antigen strips for 1 h. These were washed five times with either non-stringent (0.15 M PBS pH 7.2, 0.1% Tween 20) or stringent wash buffer (0.3 M PBS pH 7.2, 1% Tween 20). After incubation for 1 h with alkaline phosphatase–conjugated AffiniPure F(ab')$_2$ fragment Goat anti-human IgG (Jackson ImmunoResearch laboratories), 5-bromo-4-chloro-3-indolylphosphate (BCIP)-nitro blue tetrazolium (BioRad) was used as substrate.

Generation of monoclonal antibodies

Hybridoma cell lines were generated as described (32) from mice immunized intra-peritoneally three times with 20 µg of a lysate of the *M. ulcerans* strain 97-610 from Ghana formulated in the MPL +TDM adjuvant (Sigma Chemicals). Three days before cell fusion, mice received an intravenous booster injection with 20 µg of *M. ulcerans* lysate in PBS. Antibodies specific for *M. ulcerans* antigens were identified by ELISA using Immunolon 4 plates (Dynes Technologies Inc., Chantilly, Va.) coated with *M. ulcerans* lysate. From the panel of hybridomas generated, Western blotting analysis identified three

(designated DD2.5, DD2.6 and DD3.6), which secreted mAbs specific for an 18 kDa protein.

Partial purification and identification of the mAb DD2.5/6/3.6 reactive protein

300 mg of heat inactivate *M. ulcerans* cells (wet weight) were washed with 1% sodium sarcosylat, five times with PBS and dissolved in 20 ml 8 M urea. After centrifugation at 20000 x g for 30 min the supernatant was applied onto a RP-8 HPLC column (Nucleosil 300-5 C8). The column was washed with 0.1% trifluoroacetic acid in water and then eluted with a gradient of acetonitril. After separation on a 12% SDS-PAGE gel, a band with an apparent molecular weight of 18 kDa was excised and digested with trypsin as described (12). For nanoelectrospray ionization tandem mass spectrometry, the peptides obtained were desalted and concentrated on POROS R2 reverse phase material (Applied Biosystems, Foster City, CA). They were eluted with 60% acetonitrile in 5% formic acid directly into a nanoelectrospray capillary needle. Mass spectra were acquired on a QSTAR Pulsar i quadrupole TOF tandem mass spectrometer (Applied Biosystems/MDS-Sciex, Toronto, Canada) equipped with a nano electrospray ion source (Proxeon, Odense, Denmark) as described (42). Fragmentation by tandem MS yielded a stretch of amino acid sequence together with its location in the peptide (sequence tag). With this sequence tag information, appropriate protein databases were searched using MASCOT Search software (Matrix Science, London, UK). These searches were not successful because the corresponding protein from *M. ulcerans* was not in the database. Therefore, the amino acid sequences of the peptides were determined (*de novo* tandem MS sequencing) to perform database searches based on sequence homology using FastA software (Genetics computer group, Madison, WI).

Sequence analysis of the *M. ulcerans* 18 kDa shsp encoding gene

For sequence analysis, the 18 kDa shsp encoding gene was amplified by PCR using the primers 5'-CCATGGTGATGCGTACCGACCCG-3' and 5'-CTCGAGGGCTTCTATCACCTCAGG-3'. DNA was extracted as described ((37)) and amplifications were performed with the following profile: 5 min 96°C; 30 x (1 min 96°C, 1 min 63°C, 1 min 72°C), 7 min 72°C. Amplicons were purified using a PCR product purification kit (Qiagen), and then sequenced using an ABI PRISM 310 genetic analyzer

(Perkin-Elmer). All sequences were reconfirmed at least twice using independent PCR products.

Recombinant expression of the *M. ulcerans* 18 kDa shsp

The *M. ulcerans* 18 kDa shsp was recombinantly expressed in *Escherichia coli* as his-tagged fusion protein ($M^1V^2M^3$---$A^{149}LEH_6$), comprising the entire open-reading frame of 149 codons with one amino acid exchange (L^2 to V^2). Briefly, the 453-bp PCR product generated from genomic DNA of the Ghanaian *M. ulcerans* isolate ITM 97-483, using the primers described above, was digested with the restriction enzymes *Nco*I and *Xho*I and cloned into the pETBLUE2 vector employing its *Nco*I and *Xho*I sites. Competent *E. coli* Tuner cells (pLac) (Novagen) were transformed and expression of the fusion protein was induced by addition of 1 mM isopropyl thiogalactoside (IPTG) (Calbiochem) for 4 h at 37°C. Cells were lysed on ice for 30 min with 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris/HCl (pH 8.0). After centrifugation at 10000 x g, the protein was purified from the supernatant by nitrilotriacetic acid column (Qiagen) chromatography and subsequent reverse-phase HPLC, using a C8 column (Nucleosil 300-5 C; Agilent Technologies).

Immunization of mice with *M. ulcerans* r18 kDa shsp

Antiserum was obtained from mice immunized three times with 20 µg of *M. ulcerans* r18 kDa shsp formulated in the MPL +TDM adjuvant (Sigma Chemicals, St. Louis, Mo).

Human sera and tissue lysates

Tissue specimens were taken from the IS2404 PCR positive center and the PCR negative margin of an excised Buruli ulcer lesion described by Rondini et al. ((37)). Samples of about 100 mg were heat inactivated at 80°C for 1 h, suspended in reducing Laemmli buffer (39) and subjected to bead beater treatment as describe above. Sera from BU patients and household contacts residing in the Buruli ulcer endemic Ga district of Ghana were collected after informed consent was obtained. Ethical approval for the study was obtained from the local ethical review board of the Noguchi Memorial Institute for Medical Research, Legon, Ghana.

Results

Inter-species cross-reactivity patterns of mAbs raised against a whole cell lysate of *M. ulcerans*

To identify immunodominant antigens of *M. ulcerans,* mice were immunized with a complete lysate of the Ghanaian *M. ulcerans* strain 97-610. In Western blot analysis, the generated antisera exhibited cross-reactivity with a broad range of *M. bovis* and *M. tuberculosis* antigens (data not shown). In order to identify *M. ulcerans* antigens not present in these two mycobacterial pathogens, B cell hybridomas were generated with spleen cells of the immunized mice. While some of the obtained mAbs showed cross-reactivity with all 21 mycobacterial species tested, others were more selective (Fig.1). The highest selectivity was observed with three mAbs, designated DD2.5, 2.6 and 3.6, which stained a *M. ulcerans* protein with an apparent molecular mass of 18 kDa. Although generated from two different mice, all three mAbs shared the same limited inter-species cross-reactivity pattern, i.e. they only reacted with *M. chelonae,* where a protein with an apparent mass of 20 kDa was recognized. In indirect immunofluorescence assays only *M. ulcerans* was stained by mAbs DD2.5, DD2.6 and DD3.6 (data not shown), indicating that cross-reactivity with *M. chelonae* was too low in affinity to be detectable in this assay. The mAbs stained an 18 kDa band in all ten *M. ulcerans* isolates of different geographical origins tested (data not shown).

Identification of the mAb DD2.5/2.6/3.6 reactive protein

For the identification of the mAb DD2.5/2.6/3.6 reactive 18 kDa protein, a *M. ulcerans* whole-cell lysate was fractionated by reverse-phase HPLC. Fractions in which the immune reactive protein was enriched were identified by Western blot analysis, peak fractions were pooled, and the 18 kDa band cut-out from a SDS-PAGE gel loaded with the pooled material. The excised protein was analyzed by matrix-assisted laser desorption ionization-mass spectrometry (Fig. 2). The three peptide sequences obtained matched to sequence stretches (Table 1) of a *M. leprae* protein (Swissprot:18kd_mycle) designed 18 kDa shsp (HSP 16.5) (5). A *M. ulcerans* homologue with 79 % protein identity (Fig. 3) and 85 % identity at the DNA sequence level was identified by homology search in the *M. ulcerans* genome project database (http: genopole.pasteur.fr/Mulc/BuruList.html). Codon analysis of the *M. ulcerans* gene predicted a functional open-reading frame of 149 amino acids and

a molecular mass of 16,556 Da. A gene bank Blast search identified homologues of 18 kDa shsp in *M. intracellulare*, *M. avium*, and *M. leprae* with protein sequence identity of 79.5%, 71.5% and 78.8%, respectively, and extensive inter-species diversity focused on the carboxyl-terminus (Fig.3). In contrast, no homologues are present in the *M. tuberculosis* and *M. bovis* genomes.

To confirm the identification of the 18 kDa shsp as target for mAbs DD2.5, DD2.6 and DD3.6, the complete coding sequence of the *M. ulcerans* homologue was expressed as carboxy-terminally hexa-histidine tagged fusion protein in *E. coli.* All three mAbs showed reactivity with the affinity-purified recombinant protein in Western blot analysis (data not shown).

Inter-species immunological cross-reactivity of the 18 kDa shsp was analyzed further by Western blot analysis with mouse antisera raised against the recombinant *M. ulcerans* 18 kDa shsp. Like the three mAbs DD2.5, DD2.6 and DD3.6, all six antisera tested exhibited cross-reactivity with *M. chelonae* (Fig. 4). In addition, antisera cross-reacted with an 18 kDa *M. leprae* protein, which was only stained by mAbs DD2.5, DD2.6 and DD3.6 when they were used at very high ($\geq$ 5 µg/ml) concentrations (data not shown). When a set of ten overlapping synthetic 20mer peptides spanning the entire sequence of the *M. ulcerans* 18 kDa shsp was tested in ELISA, mAbs DD2.5, DD2.6 and DD3.6 showed no reactivity and only some of the antisera bound weakly to the C-terminal peptide.

Sequence conservation, sub-cellular localization and expression of the *M. ulcerans* 18 kDa shsp in Buruli ulcer lesions

Only four single nucleotide polymorphisms were detected when the 18 kDa shsp encoding genes of ten *M. ulcerans* isolates of diverse geographical origin were compared by PCR amplification and DNA sequence analysis of PCR products (Fig. 3). Two single nucleotide polymorphisms, one non-synonymous (G/T^{424}) and one synonymous (T/C^{374}) distinguished the sequence of strain 7922 from French Guiana from that of the Ghanaian genome project reference strain Agy-99. Strain 8756 from Japan exhibited two synonymous single nucleotide polymorphisms (G/A^{278} and C/T^{395}) with respect to the reference sequence.

Sequences from the other eight *M. ulcerans* strains analyzed were identical with the Agy-99 reference sequence.

The mAb DD2.5/6/3.6-reactive band was primarily found in *M. ulcerans* cell-wall fractions (Fig. 5A), but was undetectable in cytosol fractions (Fig. 5A) and in culture filtrate (not shown). The *M. ulcerans* 18 kDa shsp was detectable in human tissue lysates from the centre of excised Buruli ulcer lesions (Fig. 5B). No staining was observed in lysates from the healthy margins of the excised tissue (Fig. 5B).

Reactivity of human sera with the recombinant 18 kDa shsp of *M. ulcerans*

In Western blot analyses, the majority of sera from pre-ulcerative (Fig. 6A), early-ulcerative (Fig. 6B) and late-ulcerative (Fig. 6C) Buruli ulcer patients showed reactivity with the recombinant 18 kDa protein of *M. ulcerans*. While 75% (24/32) of the patient sera were tested positive, 38% (9/24) of sera from household contacts also showed reactivity (Fig. 6D). Samples from Europeans (Fig 6F) and from the vast majority of Africans living in Buruli ulcer non-endemic regions (Fig. 6E) were negative.

Discussion

Currently it is not clear, which proportion of a population living in an African Buruli ulcer focus area is exposed to *M. ulcerans*. In the case of *M. tuberculosis*, an infection remains latent in 90–95% of individuals and progressive disease development is only observed in a minority of infected individuals. Likewise a significant proportion of individuals infected by *M. ulcerans* may not develop the disease. Few data are available on co-infections of HIV and *M. ulcerans* and it is not entirely clear whether HIV infection is a risk factor for Buruli ulcer (1). Anecdotal evidence indicates that HIV infection affects the outcome of Buruli ulcer disease (21).

For the assessment of the prevalence of exposure, a test is required that is negative for non-exposed persons from non-endemic regions and positive for a significant proportion of exposed individuals in an endemic region. The test should discriminate between immune responses against *M. ulcerans* and other mycobacteria, in particular *M. tuberculosis* and *M. bovis* BCG. Serological studies performed with complex antigen preparations have suggested that serological tests may be useful in the diagnosis and surveillance of Buruli ulcer (9,16,31). In view of the presence of species cross-reactive antibodies in sera of Africans living in Buruli ulcer endemic regions, thoroughly selected recombinantly expressed target antigens are required. Our search for immunodominant proteins of *M. ulcerans* has identified the 18 kDa shsp as promising candidate for a serological test suitable to monitor the exposure of a population to *M. ulcerans.* While the 18 kDa shsp has no homologue in the genomes of *M. bovis* and *M. tuberculosis,* homologues have been described in *M. leprae*, *M. intracellulare*, and *M. avium*. Mouse sera raised against the recombinantly expressed *M. ulcerans* protein showed cross-reactivity with a 18 kDa protein of *M. leprae* and a 20 kDa protein of *M. chelonae*, but not with *M. intracellulare*, and *M. avium* lysates. The 18 kDa shsp of *M. leprae* has been evaluated as target antigen for serological and cellular diagnostic tests for leprosy (10,29,43). While the *M. leprae* protein seems to share epitopes with an unidentified *M. tuberculosis* antigen (30,36,41), our mAbs and mouse sera specific for the *M. ulcerans* 18 kDa shsp did not cross-react with *M. tuberculosis* and *M. bovis* BCG lysates. Furthermore, sera from individuals living in Buruli ulcer non-endemic regions were largely negative. These results indicate, that immune responses against environmental mycobacteria, such *M. chelonae,* which is widely

distributed in the environment (4,22) and expresses a cross-reactive homologue of the 18 kDa shsp, do not obscure results with the *M. ulcerans* 18 kDa shsp based serological test.

Like its homologue in *M. leprae* (23), the 18 kDa shsp of *M. ulcerans* is associated with the cell-wall fraction. It has been postulated, that the 18 kDa shsp of *M. leprae* is relevant for the survival of the mycobacteria within macrophages (7). While *M. ulcerans* is largely an extracellular pathogen, it appears to be captured by phagocytes and transported to draining lymph nodes within host cells during the early stage of infection (6). The *M. ulcerans* 18 kDa shsp may play a role in this early intracellular stage of the infection and protect the mycobacteria in extreme environmental conditions by stabilizing the cell wall (25,26).

While it is clear from many epidemiological studies, that proximity to wetlands is a risk factor for *M. ulcerans* infection, the exact mode of transmission is not clear (20). Recent field and laboratory studies have implicated aquatic insects in the transmission of the pathogen (25,33) and is has been demonstrated that mycolactone toxin-producing *M. ulcerans* isolates are able to invade the salivary glands of water insects (24). *M. ulcerans* DNA has also been detected by PCR in aquatic snails, fish, and the biofilm of aquatic plants (27,38,40), but the contributions of these elements of the environment in transmission has remained largely unknown. *M. ulcerans* may often reach the human dermis through minor wounds or skin abrasions via contact with *M. ulcerans* containing environmental reservoirs (28). Our analysis of sera from healthy household contacts indicates that exposure to *M. ulcerans* leads only in a minority of exposed individuals to clinical disease. Most of the others may only develop transient infection foci and even nodular lesions may resolve spontaneously (35). Immune responses in healthy household contacts have also been described in an Australian study (16), where a lower background staining than with African sera facilitated analysis with cellular extracts. Our preliminary analysis of sera from Africans living in Buruli ulcer endemic regions indicate that exposure is common in these environments also among non-household contacts. Future prospective analysis of cellular and humoral immune responses with recombinant *M. ulcerans* proteins in a population living in a highly endemic region of Africa should give better insight into

patterns of exposure. Such studies may also lead to the identification of surrogate markers of protection crucial for the development of a vaccine against *M. ulcerans* infection.

Acknowledgments

We thank Dan Anderegg for his critical reading of the manuscript, Martin Naegeli for support with DNA sequencing, and Francoise Portaels and Gisela Bretzel for supplying us with *M. ulcerans* isolates. We acknowledge the BuruList World-Wide Web Server for making available *M. ulcerans* genome sequencing data. *M. leprae* cell lysate was kindly provided by Drs. Patrick J. Brennan and John S. Spencer at Colorado State University, Department of Microbiology, Immunology, and Pathology, Fort Collins, Colorado, through the NIH/NIAID contract AI-25469. Diana Diaz and Dorothy Yeboah-Manu received supported from the Amt für Ausbildunsbeiträge of the county of Basel-Stadt. This work was in part supported by Aide aux Lépreux Emmaüs–Suisse.

Peptide Mass $[M+H]^+$	*M. ulcerans* sequence determined by MS/MS	corresponding *M. leprae* 18kDa shsp sequence
$M_{1:}$ 1151.64 Da	I**A**ASY**T**EGVLK	ILASYQEGVLK
M_2: 1177.66 Da	FA**Q**QVLGTSAR	FAEQVLGTSAR
M_3: 1328.75 Da	QLVLGENLDT**A**R	QLVLGENLDTER

Table 1

Amino acid sequences of the *M. ulcerans* 18kDa protein-derived peptides determined by *de novo* tandem MS sequencing. Leu and Ile can not be discriminated by the technology applied and are therefore interchangeable.

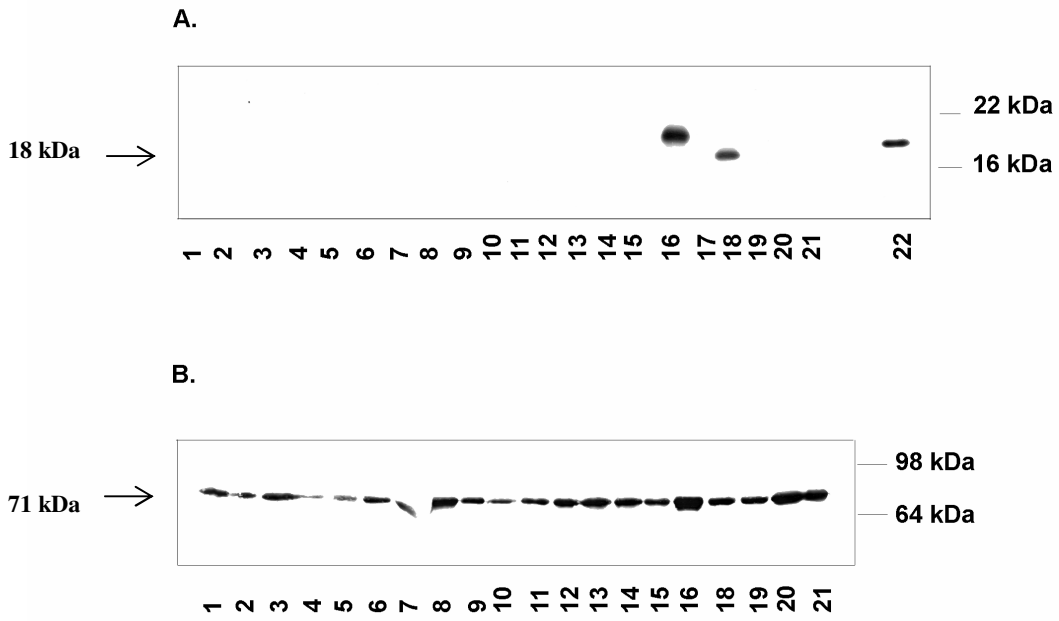

Figure 1. Western blot analysis of lysates of different mycobacterial species with mAbs A) DD3.4 and B) DD3.7. *1) M. abscessus, 2) M. avium, 3) M. bohemicum, 4) M. fortuitum, 5) M. gordonae, 6) M. haemophilum, 7) M. intracellulare, 8) M. kansasii, 9) M. scrofulaceum, 10) M. malmoense, 11) M. lentiflavum, 12) M. simiae, 13) M. smegmatis, 14) M. terrae, 15) M. xenopi, 16) M. chelonae , 17) M. leprae, 18) M. ulcerans, 19) M. tuberculosis, 20) M. marinum, 21) M. bovis, 22)* recombinant *M. ulcerans* 18kDa shsp

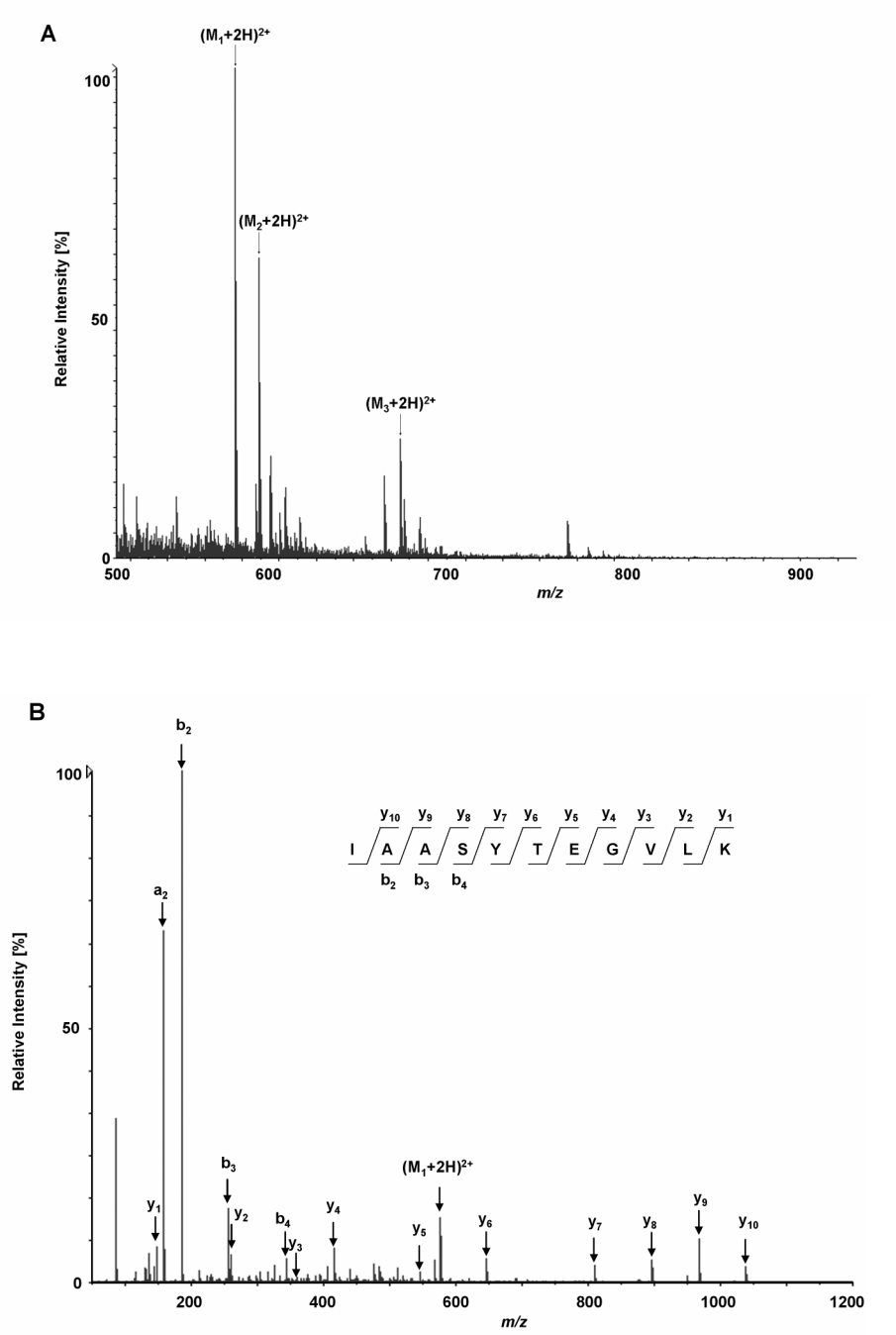

Figure 2. Analysis of the in-gel digested band of apparent molecular weight of 18 kDa. (A) Survey mass spectrum of the tryptic in-gel digest. The arrows indicate the doubly-charged ion signals of the three peptides, which were *de novo* sequenced by tandem MS. (B) Tandem mass spectrum of the $[M+2H]2^+$ precursor ion (m/z 576.32) of peptide M_1 (mass determined = 1150.64 Da). The database search was performed with the singly-charged fragment ions labeled (y1) to (y10).

```
                          1                                                                                80
M. ulcerans Ghana Agy-99  MLMRTDPFRD LDRFAQQVLG TSARPAVMPM DAWREGDKFV VEFDLPGIDA DSLDIDIERN VVTVRAERPA VDPNREMLAS
M. ulcerans French Guiana    ------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
M. leprae                 ---------E -----E---- ---------- ------EE-- --------K- ---------- ---------G ---D-----A
M. avium                  ----S----E ---LTN---- -AT------- ------EH-- ---------- E--------- -L-------- L--S-----T
M. intracellulare         ----S----E -----H---- -A-------- ----Q-EE-- ---------- ---------- ---------- L--------T

                          81                                                                     149
M. ulcerans               ERPRGVFSRQ LVLGENLDTA RIAASYTEGV LKLQIPVAEK AKPRKISITR GAGDKTISEN VAHPEVIEA
M. ulcerans French Guiana ---------- ---------- ---------- ---------- ---------- ---------- G--------
M. leprae                 -------N-- ---------E --L---Q--- ---S-----R -------VD- -NNGHQTINK T--.-I-D-
M. avium                  ---------E ----D----D K-E---RD-- -S-H------ ------AVG- -EAPRAVT-T A..R--VN-
M. intracellulare         ---------- ---------D K-Q---S--- -S-H------ ------AVG- -D-HHAVA-G A-QR---N-
```

Figure 3. Comparison of the predicted amino acid sequence of 18kDa shsp of different mycobacterial species. Identity with the *M. ulcerans* reference sequence is indicated by dashes (-), deletions are indicated by a dot (.). Grey boxes indicate the position of peptides identified by ms-ms MALDI-TOF. GenBank accession numbers: *M. leprae*: AL583923, *M. intracellulare*: LI2240, *M. avium*: AE017238

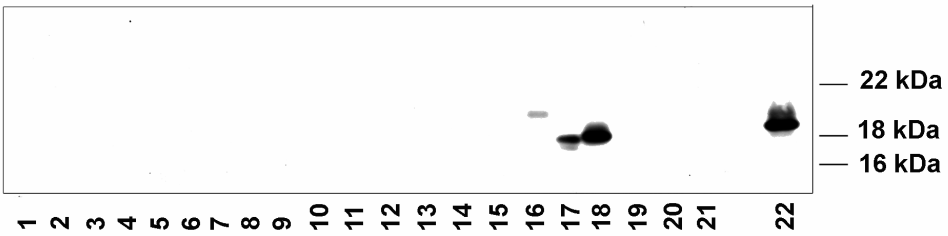

Figure 4. Western blot analysis of lysates of different mycobacterial species with mouse anti-sera raised against the recombinant *M. ulcerans* 18 KDa shsp. Numbering of samples is the same as in Figure 1.

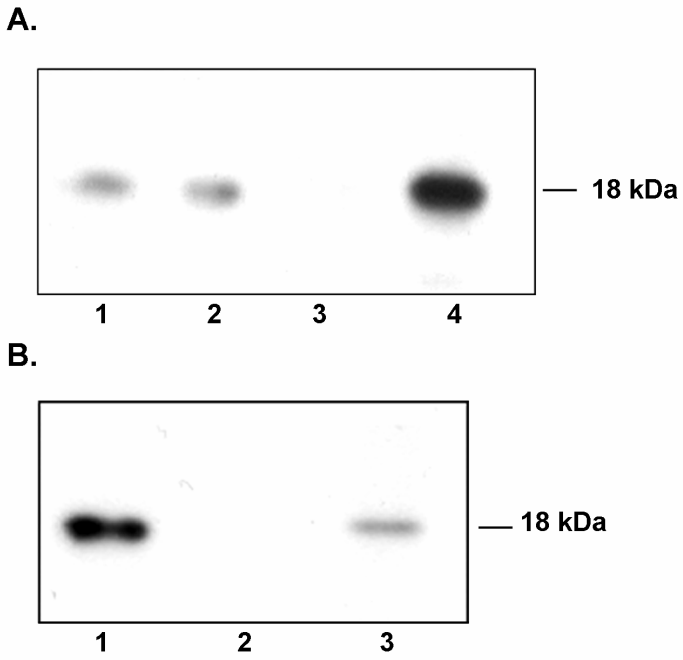

Figure 5. Western blot analysis of sub-cellular fractions and of *M. ulcerans* infected tissue with mAb DD3.6. A: 1) *M. ulcerans* lysate, 2) membrane fraction of *M. ulcerans*, 3) cytosol fraction of *M. ulcerans*, 4) cell wall fraction of *M. ulcerans*. B: 1) lysate of in vitro cultivated *M. ulcerans* cells 2) human tissue lysate from the healthy margin of a Buruli ulcer lesion 3) human tissue lysate from the centre of a Buruli ulcer lesion

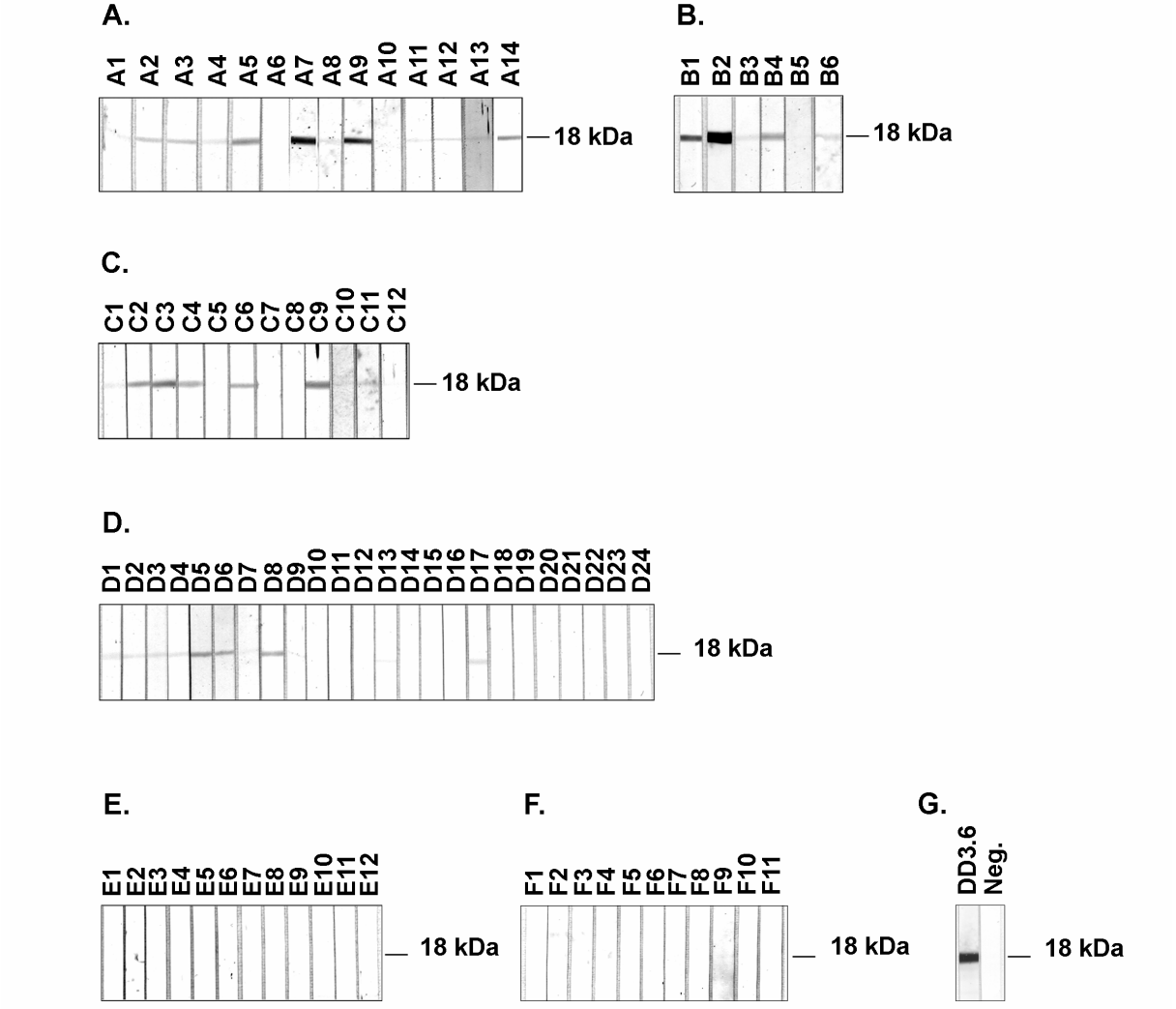

Figure 6. Western blot analysis of human sera with the recombinant *M. ulcerans* 18KDa shsp using stringent washing conditions. Sera from Ghanaian patients with pre-ulcerative (A), early-ulcerative (B) and late-ulcerative (C) lesions and from their healthy household contacts (D), from Africans living in Buruli ulcer non-endemic regions (E) and from Europeans (F) were analyzed at a dilution of 1:100. G: mAb DD3.6 and negative control.

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Generation of monoclonal antibodies against immunodominant antigens of *Mycobacterium ulcerans*

Running title: Immunodominant antigens of *Mycobacterium ulcerans*

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Abstract

Buruli ulcer caused by *Mycobacterium ulcerans* is characterized by chronic, necrotizing ulceration of subcutaneous tissues and the overlying skin. *M. ulcerans* produces a family of macrolide toxin molecules, the mycolactones, which are associated with tissue destruction and local down-regulation of Th-1 responses. Since *M. ulcerans* multiplies extra-cellularly in Buruli ulcer lesions, antibody-mediated immune effector functions may be of great importance for immune protection. By generating panels of monoclonal antibodies from *M. ulcerans* antigen immunized mice we identified a range of immunodominant mycobacterial antigens. Some of these may have potential as vaccine candidate antigens or as target structures for serological or cellular diagnostic test systems.

Introduction

Buruli ulcer caused by the slow growing environmental pathogen *M. ulcerans* is characterized by the development of chronic, necrotizing skin and soft tissue ulcers[1]. The disease starts as a subcutaneous nodule or plaque that eventually ulcerates and progresses over weeks to months until surgical excision or spontaneous healing occurs. In endemic regions, BU is the most common mycobacterial disease after tuberculosis and leprosy among immunocompetent people. It has been observed in over 30 tropical and subtropical countries, but the main burden of disease falls on children living in sub-Saharan Africa[2]. Although it is well established that proximity to marshes and wetlands is a risk factor for contracting BU, the natural reservoir and the exact mode of transmission is unclear[1].

In BU lesion, clumps of extra-cellular acid-fast organisms surrounded by areas of necrosis are found primarily in subcutaneous fat tissue[3]. A diffusible toxic macrolide, mycolactone, plays a key role in the pathogenesis[4]. Since the toxin causes mammalian cells to undergo apoptosis, its action explains at least in part that inflammatory responses in BU lesions are poor[3]. Th-1 cell driven production of gamma interferon appear to be down-regulated in Buruli ulcer patients in the early stage of the disease[5-7]. This down-regulation may reverse in later stages[8]. *M. ulcerans* infection leads to the generation of antibodies against a variety of mycobacterial antigens[5-7,9,10]. Inter-species cross-reactivity of antibodies complicate the analysis of anti-*M. ulcerans* immune responses. All humans are exposed to environmental mycobacteria which frequently come into the contact with the skin and mucous membranes. In addition, in areas where *M. ulcerans* is endemic, the majority of children are vaccinated with BCG and exposed to *M. tuberculosis*. In the last 20 years, the isolation and characterization of antigens from complex mixtures such as parasites and bacteria, including *M. leprae* and *M. tuberculosis*, have been vastly facilitated by monoclonal antibodies technology[11-14]. In the present report we describe a panel of monoclonal antibodies generated from mice immunized with *M. ulcerans* cell lysates. While our primary interest was to identify antigens suitable as target structures for *M. ulcerans* specific immunodiagnostic assays, some of the immunodominat antigens identified may also have potential as vaccine components or as target structures for genetic fingerprinting methods required for micro-epidemiological studies.

Materials and Methods

Mycobacterial lysates and sub cellular fractions

M. ulcerans isolates of diverse geographical origin used in this study are: Suriname (ITM 842), Democratic Republic of Congo (5150), Mexico (ITM 5114), Australia (ITM 5142), Mexico (ITM 5143), Australia (ITM 5147), Democratic Republic of Congo (5151), Japan (ITM 8756), Australia (ITM 9540), Ivory Coast (ITM 94-511), Papua New Guinea (ITM 94-1331), Benin (ITM 97-104), Ghana (97-483), China (ITM 98-912), and Ghana (001441). All strains except for 001441 (isolated from an infected insect) were clinical isolates. Other mycobacterial species included in the analyses are *M. abscessus* (ATCC 19977), *M. avium* subsp. *avium* (MAC101), *M. bohemicum* (clinical isolate), *M. bovis* biovar. BCG *(*ATCC 35734), *M. chelonae* (DSM 43804), *M. fortuitum* (ATCC 49403), *M. gordonae* (Pasteur 14021.001), *M. haemophilum* (ATCC 29548), *M. intracellulare* (clinical isolate), *M. kansasii* (NCTC 10268), *M. lentiflavum* (clinical isolate), *M. malmoense* (NCTC 11298), *M. marinum* (ATCC 927), *M. scrofulaceum* (Pasteur 14022.0031), *M. simiae* (clinical isolate), *M. smegmatis* (Pasteur 14133.0001), *M. terrae* (clinical isolate), and *M. tuberculosis* (Pasteur 14001.0001).

For the preparation of lysates, mycobacterial cells were heat-inactivated at 80°C for 1 hour and suspended in PBS (50mM sodium phosphate, 150mM sodium chloride, pH 7.4) containing 5% SDS and 1mM phenylmethyl-sulphonyl fluoride (PMFS), and 10 µg/ml each of leupeptine and trypsin inhibitor (Sigma, St. Louis, Mo). Two hundred mg of cell suspension was subjected to a bead beater (Mikro-Dismembrator, Braun Biotech International) treatment with 400µl of 0.1mm zirconia beads (Bio Spec Products) at 2300 rpm for 15 minutes. Beads and unbroken cells were removed by centrifugation at 10000g for 10 min. Protein content of the lysate was quantified using the BCA protein assay (Pierce).

For the preparation of sub-cellular fractions, 400 mg of heat inactivated *M. ulcerans* cells were suspended in 3 ml of PBS containing 0.1% Tween 80 and the proteinase inhibitor cocktail described above. Cells were broken by three cycles of ultrasonic disruption (Branson sonifier 250) on ice for 10 min with 50% Duty cycle and 40% output using a microtip probe. Unbroken cells were removed by centrifugation at 3000g for 10 min. A cell wall fraction was prepared from the supernatant by centrifugation at 27000g for 1 hour

and was washed twice with PBS. The supernatant was subjected to a 100,000g centrifugation for 4h; a cytosol fraction was obtained from the supernatant and the membrane fraction from the pellet. The membrane fraction was washed with PBS and suspended in 0.01M ammonium bicarbonate and the supernatant was dialyzed against 0.01M ammonium bicarbonate[15].

Immunization with *M. ulcerans* lysate and whole cells

Groups of mice carrying immunoglobulin heavy chain γ2a and κ light chain replacement mutations[16] were used for intraperitoneal immunization with *M. ulcerans* antigens. Group I was immunized with approximately 2 x 10^8 heat-inactivated *M. ulcerans* 97-610 cells emulsified in incomplete Freund's adjuvant. Group II received 20 μg of a lysate of strain 97-610 in incomplete Freund's adjuvant. Group III was immunized with 20 μg of the lysate in MPL +TDM adjuvant (Sigma Chemicals, St. Louis, Mo). Humoral immune responses against *M. ulcerans* antigens were evaluated by ELISA and Western blot analyses.

Generation of monoclonal antibodies

Hybridoma cell lines were generated as described[17]. Three days before cell fusion, mice received an intravenous booster injection with *M. ulcerans* lysate or capsular material in PBS. Hybridomas secreting antibodies specific for *M. ulcerans* lysate antigens were identified by ELISA using Immunolon 4 plates (Dynes Technologies Inc., Chantilly, Va.) coated with *M. ulcerans* lysate.

Indirect immunofluorescence test

Heat inactivated *M. ulcerans* cells were placed on a poly-L-Lysine-coated glass microscope slide (Erie-Scientific ES-242B-AD) and fixed by drying over night at room temperature. Slides were incubated for 30 min at room temperature with sera or mAbs diluted in 1% skim milk in water and washed five times with distilled water. Bound antibodies were detected using FITC- or Cy3-conjugated goat anti-mouse IgG (Sigma Chemicals, St. Louis, Mo) as secondary antibodies. The DNA of *M. ulcerans* was label with Hoechst dye no. 33256 (bisBenzimidine, Sigma Chemicals, St. Louis, Mo). After washing with water slides were sealed with mounting medium [o-phenydiamine

dihydrochloride (Sigma, Chemicals. St. Louis, Mo), 0.1M tris and Glycerol (Sigma, Chemicals, St. Louis, Mo)].

Western blot analysis

Mycobacterial lysates and sub-cellular fractions (10µg of total protein/lane) were separated on 12% SDS PAGE gels under reducing conditions in Laemmli buffer[18]. Proteins were transferred to nitrocellulose paper (BioRad) in Tris glycine buffer (25 mM Tris and 192 mM glycine pH 8.3). Filters were blocked overnight with PBS containing 5% skim milk and 0.1% Tween 20. Mouse sera or monoclonal antibodies diluted in blocking solution were incubated with membranes for two hours. Bound antibodies were detected using alkaline phosphatase conjugated goat anti-mouse IgG (Sigma Chemicals, St. Louis, Mo) as secondary antibodies and 5-bromo-4-chloro-3-indolylphosphate (BCIP)-nitro blue tetrazolium (BioRad) as substrate.

Results

Dissection of the complex anti-*M. ulcerans* antibody responses of mice by generation of mAbs

Complex antibody responses were observed in Western blotting analysis of sera from mice immunized with whole heat inactivated *M. ulcerans* cells or with lysates. Sera of mice immunized with whole heat inactivated mycobacterial cells (Fig. 1A) showed reactivity against a broad-range of antigens. The most prominent bands had an apparent molecular mass of about 250kDa and 71kDa and were also found in lysates of the three other mycobacterial species (*M. tuberculosis*, *M. marinum* and *M. bovis*) tested. Antibodies elicited by *M. ulcerans* lysate in combination with Freund's (Fig. 1B) or MPL-KLP adjuvant (Fig. 1C) showed predominantly inter-species cross-reactivity with *M. marinum.* Reactivity with protein bands of apparent molecular masses between 36 and 32kDa dominated. Antigens recognized by antisera elicited with *M .ulcerans* lysate and MPL-KLP included a 35kDa band present in all four mycobacterial species tested and an 18kDa band only found with *M. ulcerans* (Fig. 1C). Immunofluorescence analysis also demonstrated broad cross-reactivity of all sera with all four mycobacterial species tested (data not shown).

Monoclonal antibodies were generated from mice immunized with a lysate of *M. ulcerans* strain 97-610 in incomplete Freund's adjuvant and with the lysate in MPL +TDM adjuvant. Eighteen mAbs were selected for further analysis; their isotypes and binding pattern to a range of *M. ulcerans* proteins are summarized in table 1. Fifteen of the eighteen mAbs reacted against *M. ulcerans* antigens in Western blot analysis (Fig. 2). MAbs VC1.3 and DD2.4 were negative in Western blots, but strongly positive in immunofluorescence analysis. Fourteen of the 18 mAbs showed reactivity against *M. ulcerans* by immunofluorescence analyses. The mAbs DD2.7/3.1/2/4 were IFA negative, however, they bond to denature proteins by Western blot analyses. Different staining patterns were observed, including cellular and diffuse extra-cellular staining patterns (Fig 3).

Association of the mAb-reactive antigens with subcellular fractions of *M. ulcerans* was analyzed by Western blot analysis (Fig. 4). The 50kDa protein recognized by mAbs DD2.1/VC1.1/2, the 27-32 kDa laminin binding protein recognized by mAbs DD2.2/3 and

the 18kDa small heat shock protein (shsp) recognized by mAbs DD2.5/6/3.5/6 were primarily present in the cell wall fraction. Identification of the latter two proteins will be described elsewhere. Two other immunodominant antigens, the 71 kDa protein recognized by mAbs DD3.1/2/7 and the 34-37 kDa proteins recognized by mAbs DD3.3/4 were detected in more than one subcellular fraction. Staining of the 34-37 kDa protein was observed in extracts obtained with 1% SDS or 1% Sarcosyl, but not with other detergents or with 8M urea (data not shown). None of the immunodominant antigens were detectable in culture filtrate (data not shown).

Inter-species cross- reactivity of anti- *M. ulcerans* mAbs and size variation in *M. ulcerans* isolates of diverse geographical origin

Cross-reactivity of mAbs was analysed by Western blotting with lysates from 20 mycobacterial species. Orthologues of the 71 kDa protein (Fig 5D), and the laminin-binding protein (data not shown) were detectable in all 20 mycobacterium species tested. In contrast, mAb DD3.3/4 stained 34-37 kDa protein bands only in a subset (*M. gordonae*, *M. kansasii*, *M. lentiflavum*, *M. ulcerans* and *M. marinum*) of the additional mycobacterial species tested (Fig. 5C). MAbs DD2.1 and VC1.1/2 stained a 50 kDa protein in a broader spectrum of mycobacterial species (Fig. 5A and 5B, respectively). MAbs DD2.5/6/3.5/6 stained an orthologue of the *M. ulcerans* 18kDa shsp only in *M. chelonae* (data not shown). Immunofluorescence analysis reconfirmed the cross-reactivity results, except for the 18kDa protein reactive mAbs DD2.5/6/3.5/6 that recognized only *M. ulcerans* (data no shown).

Western blot analysis with a spectrum of *M. ulcerans* isolates of geographically diverse origin revealed slight size variation in the stained 50kDa, 34-37kDa and 27-32kDa protein bands. The 50kDa protein (fig. 8D and 8E) was slightly larger in American than in African strains. In the case of the 34-37 kDa double bands, the bands were slightly smaller in isolates from China and Japan (Fig.8A-8C). No size variation was found in the 18kDa shsp and the 71kDa protein (data not shown). Variation in the number and size of the stained bands may reflect differences in length of the coding gene sequences and/or in posttranslational modification.

Discussion

Analysis of immune responses to *M. ulcerans* may contribute to a more effective control of Buruli ulcer by identifying antigens suitable as targets for a specific diagnostic test or a vaccine. Studies of immunodominant antigens of *M. tuberculosis* and *M. leprae* have provided such candidate target structures including antigens belonging to the family of heat shock proteins[11], secreted antigens [ESAT-6[19] Ag85B[20], CFP-10[21], MPT64 and MPB70], associated membrane proteins [heparin-binding hemagglutinin (hbha)[22], 38kDa protein, 30kDa and 16kDa[13]], and carbohydrates-glicolipids [arabinomannan[23], lipoarabinomannan[24]]. While various techniques have been used for the identification of protective antigens and targets for diagnostic tests, most structures have been identified and characterized with mAbs[11,25,26] or by characterization of T-cell responses in mouse models[27].

Since our knowledge of immune responses against *M. ulcerans* antigens is very limited, we have started to identify immunodominant antigens by generating a panel of mAbs from *M. ulcerans* immunized mice. The nature of the humoral immune response of mice to *M. ulcerans* antigens was strongly dependant on the immunisation procedure, facilitating the generation of mAbs against a broad range of antigens. We observed a broad inter-species cross-reactivity of most mAbs within the non-tuberculous and the tuberculous group. This antigenic overlap complicates the development of a *M. ulcerans* serodiagnostic test in endemic areas where BCG vaccination has been implemented and tuberculosis is also endemic. On the other hand, it may facilitate the development of a vaccine which can protect simultaneously against several mycobacterial diseases. In this context, it is of interest that BCG offers some protection against Buruli ulcer in general and in particular against the most severe forms of the disease[28]. The cell surface associated immunodominant antigens of *M. ulcerans* identified here (50kDa, 71kDa, 18kDa shsp, laminin binding protein) may have potential for subunit vaccine development. In the case of tuberculosis, surface exposed and secreted antigens recognized by antibodies and IFN-γ secreting T-cells have been considered promising candidate antigens. The most extensively studied candidate antigens for a tuberculosis subunit vaccine include hsp65[29], Ag85[30,31], MPT51[32], ESAT-6[33] and the hbha protein[22]. Subunit vaccine formulations incorporating recombinant proteins, synthetic peptides or DNA vaccines have conferred partial

protection against tuberculosis in experimental animals. The most common approach is now to test more than one antigen in a single vaccine to ensure broad coverage of an immunogenetically heterogeneous population. In the case of the extracellularly multiplying pathogen *M. ulcerans,* antibodies may play a key role in immunoprotection and a combination of several surface exposed antigens may be suitable for vaccine development. Future passive immune protection experiments with the mAbs described in this study may help to identify antigens suitable for inclusion into a subunit vaccine.

MAb	Isotype	ELISA*	Western blot (kD)*	IFA**
VC1.3	IgG2b/ λ	++	-	+++
VC1.1a	IgG1/ λ	++	50	+
VC1.2	IgG1/ λ	++	120, 50	+
VC1.4	IgG2b/ λ	++	-	+/-
DD2.1a	IgG2b/ λ	+++	50	++
DD2.2	IgG2b/ λ	+++	27-32	+
DD2.3	IgG2b/ λ	++	27-32	+
DD2.4a	IgG3/ λ	+	-	+/-
DD2.5	IgG2b/ λ	++	18	++
DD2..6	IgG2b/ λ	++	18	++
DD2.7	IgM/ λ	+	71	-
DD3.1a	IgG3/ λ	+++	71	-
DD3.2a	IgG3/ λ	+++	71	-
DD3.3a	IgG2b/ λ	++	34, 37	++
DD3.4a	IgG2b/ λ	++	34, 37	-
DD3.5a	IgG2b/ λ	++	18	+
DD3.6a	IgG2b/ λ	++	18	++
DD3.7	IgG2b/ λ	+++	71	++++

Table 1: Characterization of anti-*M. ulcerans* monoclonal antibodies.

* Lysate of *M. ulcerans*

**Immunofluorescence assay (heat inactivate *M. ulcerans* cells)

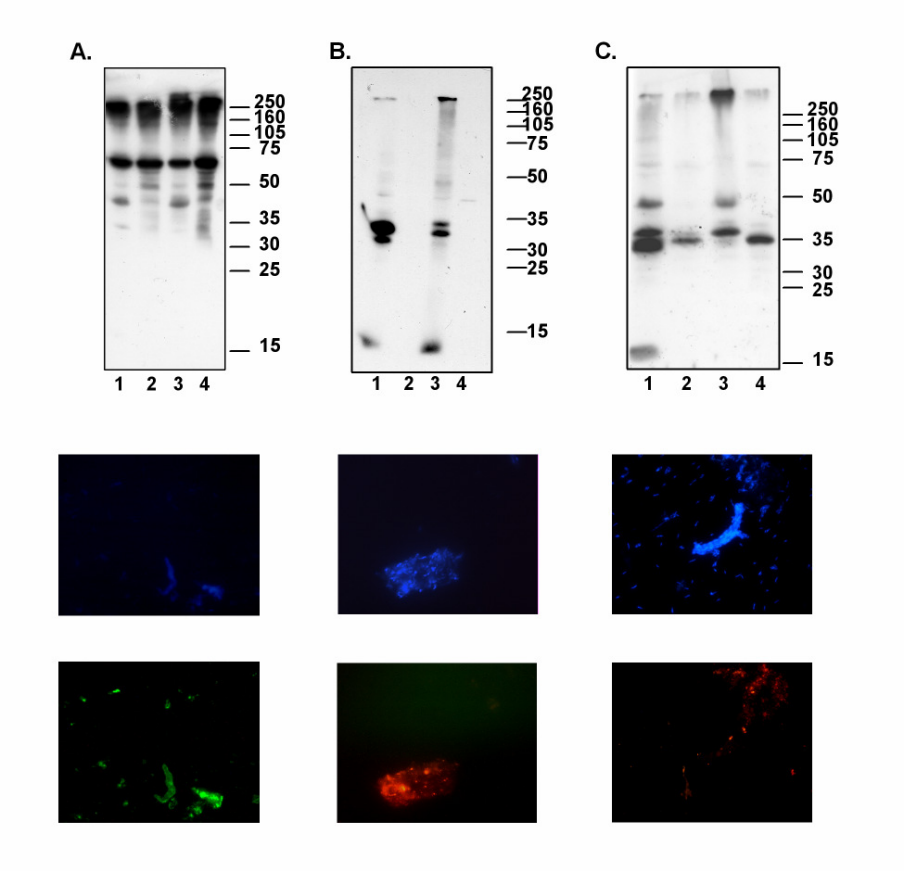

Figure 1: Reactivity of sera from mice immunized with *M. ulcerans* analyse by Western blotting with mycobacterial lysates (1: *M. ulcerans*, 2: *M. tuberculosis*, 3: *M. marinum*, 4: *M. bovis*) and by immunofluorescence staining of heat inactivated *M. ulcerans* cells. Representative results with individual sera are shown. Mice were immunized with A: heat inactivated *M. ulcerans*/incomplete Freund's adjuvant (group I), B: *M. ulcerans* lysate/incomplete Freund's adjuvant (group II), C: *M. ulcerans* lysate/MPL-KLP (group III).

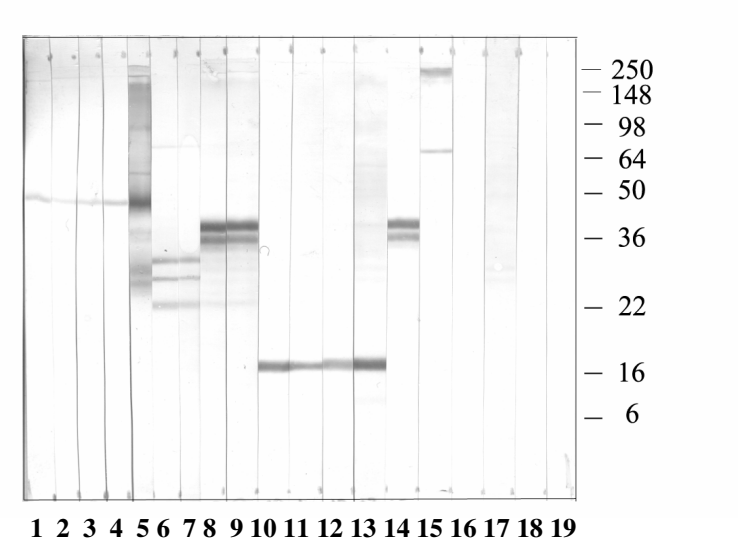

Figure 2: Staining patterns obtained in Western blot analysis with *M. ulcerans* lysate using mAbs (1: VC1.1, 2: VC1.1a, 3: VC1.1c, 4: VC1.2, 5: DD2.1a, 6: DD2.2a, 7: DD2.3a, 8: DD3.3a, 9: DD3.4a, 10: DD2.5, 11: DD2.6, 12: DD3.5, 13: DD3.6, 14: DD3.2 15: DD3.7, 16: VC1.4, 17: DD2.4a, 18: VC1.3, 19: negative control).

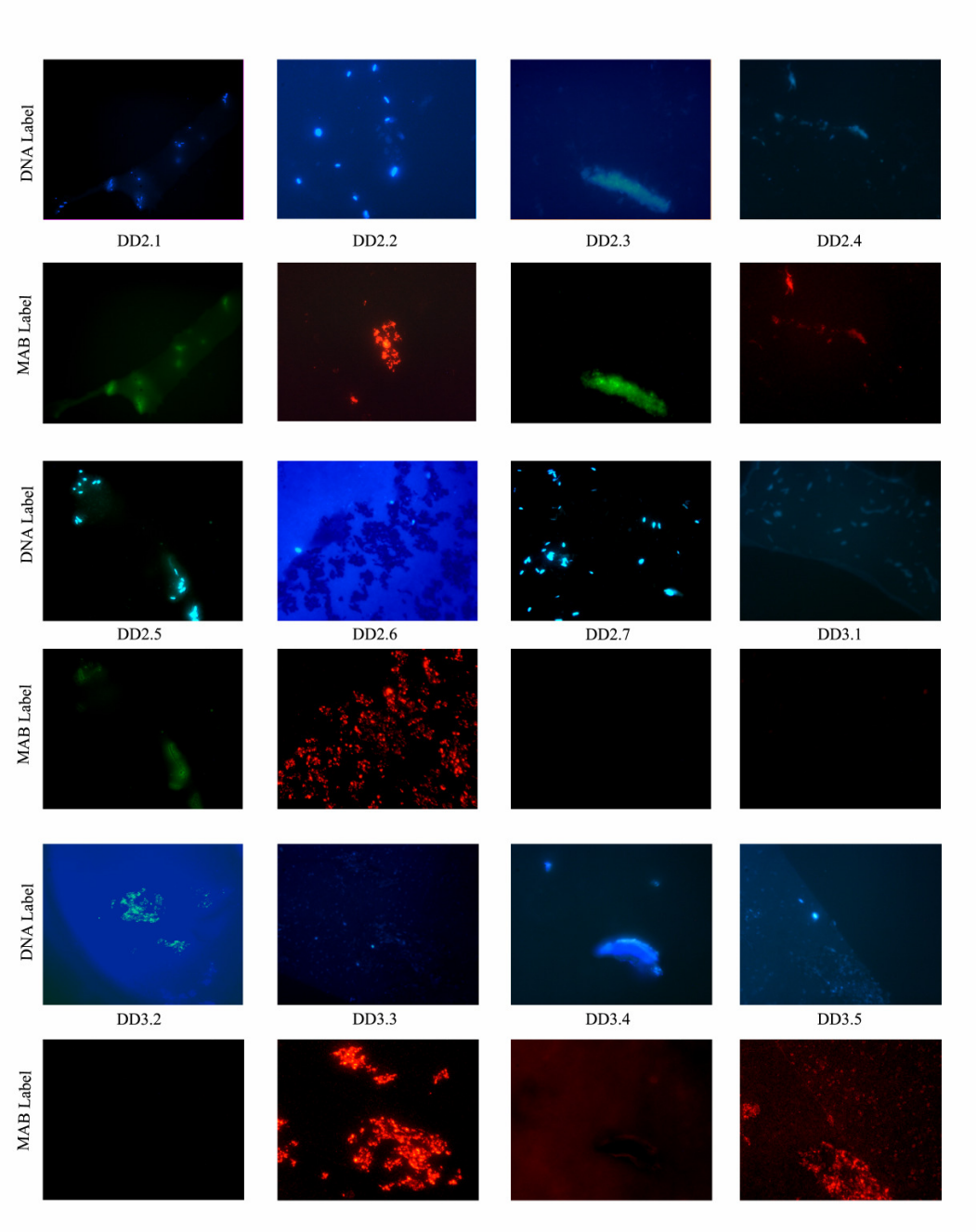

Figure 3a: Immunofluorescence staining patterns obtained with heat-inactivated *M. ulcerans* cells and the mAbs indicated. DNA was stained with Hoechst dye no.33256 reagent (blue). Second antibodies: anti-mouse IgG-FITC (Green), or anti-mouse IgG CY3 (Red).

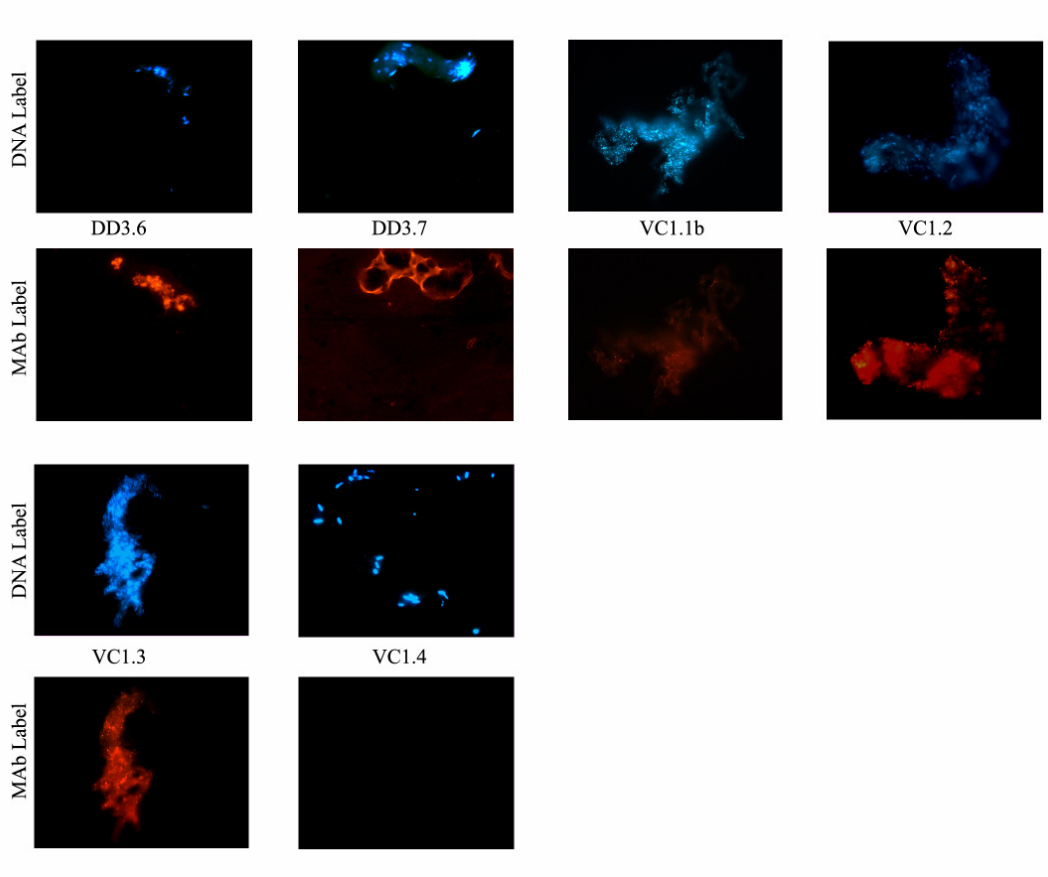

Figure 3b: Immunofluorescence staining patterns obtained with heat-inactivated *M. ulcerans* cells and the mAbs indicated. DNA was stained with Hoechst dye no.33256 reagent (blue). Second antibodies: anti-mouse IgG-FITC (Green), or anti-mouse IgG CY3 (Red).

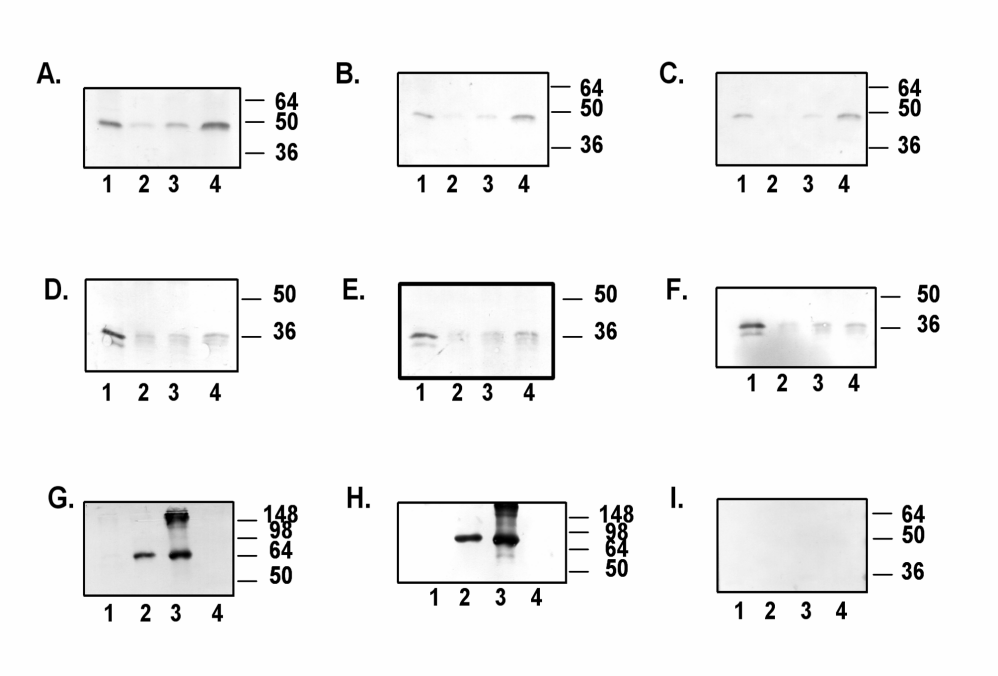

Figure 4: Western blot analysis of mAbs with sub-cellular fractions (1: lysate, 2: cytosol fraction*, 3:* membrane fraction*, 4:* cell wall fraction. The mAbs analysed are: A: DD2.1, B: VC1.1, C: VC1.2, D: DD3.3, E: DD3.4, F: DD3.2, G: DD3.7, H: DD3.1, I: VC1.3.

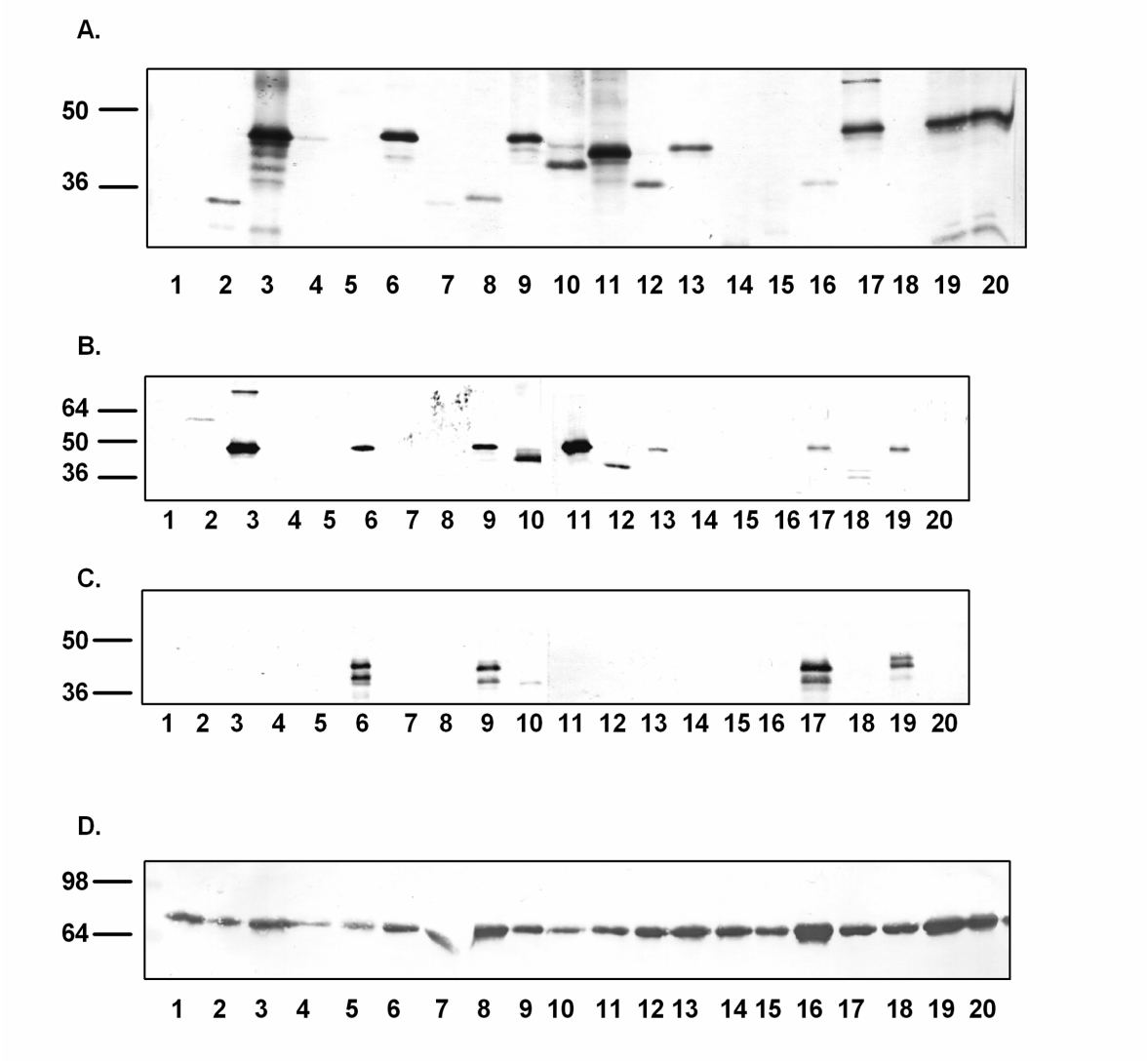

Figure 5: Western blot analysis of inter-species cross-reactivities of mAbs (A: DD2.1, B: VC1.1, C: DD3.3, D: DD3.7) with mycobacterial lysates (*1: M. abscessus, 2: M. avium ssp avium, 3: M. bohemicum, 4: M. chelonae, 5: M. fortuitum, 6: M. gordonae, 7: M. haemophilum 8: M. intracellulare, 9: M. kansasii, 10: M. lentiflavum, 11: M. malmoense, 12: M. scrofulaceum, 13: M. simiae, 14: M. smegmatis, 15: M. terrae, 16: M. xenopi, 17: M. ulcerans, 18: M. tuberculosis, 19: M. marinum, 20: M. bovis*).

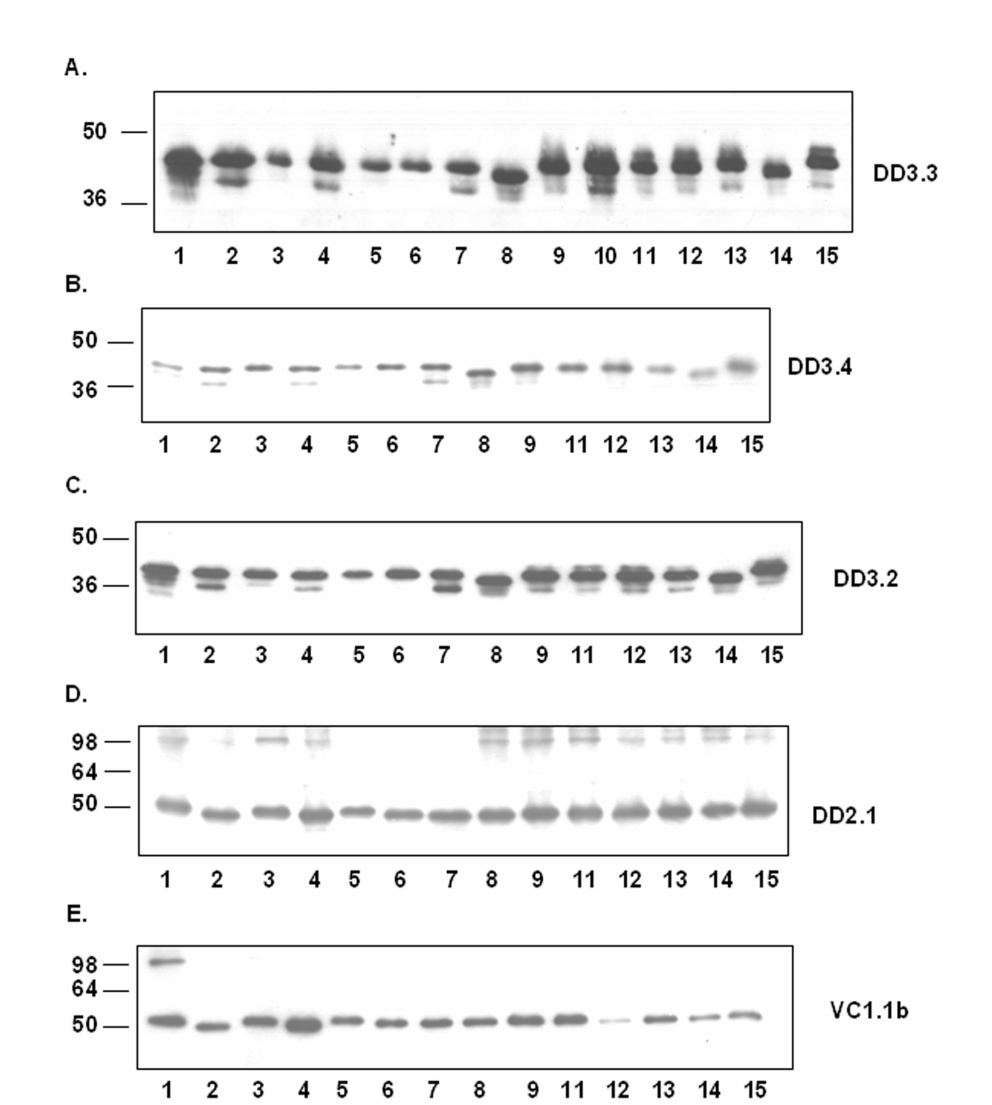

Figure 6: Western blot analysis of lysates of *M. ulcerans* strains of diverse geographical origin with mAbs DD3.3, DD3.4, DD3.2; DD2.1, VC1.1b. 1: Suriname (ITM 842), 2: Democratic Republic of Congo (5150), 3: Mexico (ITM 5114), 4: Australia (5142), 5: Mexico (5143), 6: Australia (ITM 5147), 7: Democratic Republic of Congo (5151), 8: Japan (ITM 8756), 9: Australia (ITM 9540), 10: Ivory Coast (940511), 11: Papua New Guinea (ITM 94-1331), 12: Benin (ITM 97–104), 13: Ghana (97-483), 14: China (980912), 15: Ghana (001441).

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Identification and Characterization of a Conserved, Stage-Specific Gene Product of *Plasmodium falciparum* Recognized by Parasite Growth Inhibitory Antibodies

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This article has been published in Infection and Immunity, April 2003, p. 2173-2181, Vol. 71, No. 4

Abstract

We have identified a novel conserved protein of *Plasmodium falciparum*, designated D13, that is stage-specifically expressed in asexual blood stages of the parasite. The predicted open reading frame (ORF) D13 contains 863 amino acids with a calculated molecular mass of 99.7 kDa and displays a repeat region composed of pentapeptide motives. Northern blot analysis with lysates of synchronized blood stage parasites showed that D13 is highly expressed at the mRNA level during schizogony. The first N'-terminal 138 amino acids of D13 were expressed in *Escherichia coli* and the purified protein was used to generate anti-D13 monoclonal antibodies (MAbs). Using total lysates of blood stage parasites and Western blot analysis, these MAbs stained one single band of ~100 kDa, corresponding to the predicted molecular mass of ORF D13. Western blot analysis demonstrated further that D13 is expressed during schizogony, declines rapidly in early ring stages and is undetectable in trophozoites. D13 protein is localized in individual merozoites in a distinct area, as demonstrated by indirect immunofluorescence analysis. After subcellular fractionation, D13 was confined to the pelleted fraction of the parasite lysate and its extraction by alkaline carbonate buffer treatment indicated that D13 is not a membrane-integral protein. Inclusion of certain anti-D13 MAbs into in vitro cultures of blood stage parasites resulted in considerable reduction in parasite growth. The N'-terminal domain encompassing 158 amino acids is 94 and 95%, respectively, identical at the amino acid level between *Plasmodium knowlesi*, *Plasmodium yoelii*, and *P. falciparum*. In summary, we describe a novel stage-specifically expressed, highly conserved gene product of *P. falciparum* that is recognized by parasite growth inhibitory antibodies.

Introduction

Malaria is a debilitating and frequently fatal disease of the tropics caused by parasites of the genus *Plasmodium*. Four different species cause human malaria, *Plasmodium falciparum* and *Plasmodium vivax* accounting for the majority of the problem. The former causes widespread mortality, and the latter is most prevalent outside Africa. Annually 300 million clinical malaria cases are reported, with over 1 million deaths in sub-Saharan Africa alone (23). Widespread and increasing drug and insecticide resistance has exacerbated the situation, undermining the effectiveness of malaria control methods that depend on chemotherapy and vector control, respectively (21). Novel means to fight the disease are urgently needed, and a vaccine is predicted to have the greatest impact in addition to being the most cost-effective control measure (11,20).

Access to the sequence of the entire genome of *P. falciparum* has provided the opportunity to deduce the function of many of the predicted proteins through the identification of orthologue genes and motifs in other organism (11). However, as with annotation of the human genome, the annotation of the complete *P. falciparum* genome represents a major challenge. Almost two-thirds of the predicted genes of the published chromosomes 2 and 3 had no detectable orthologues in other organisms, suggesting that many aspects of parasite biology has still to be discovered (9).

P. falciparum has a complex life cycle involving transmission within and between vertebrate and invertebrate hosts by specialized cell-invasive stages, termed zoites. Sporozoites injected into a human host by the bite of an infective mosquito invade hepatocytes and, after schizogony, release thousands of merozoites capable of invading red blood cells (RBC). All of the clinical symptoms and pathogenic manifestations associated with mammalian malaria infections are caused by the asexual erythrocytic phase of the Plasmodium life cycle. After invasion of erythrocytes, merozoites develop into trophozoites, and multiply further within these cells, forming multinucleated blood stage schizonts. These infected RBC rupture, releasing newly formed merozoites into the circulation that can invade new erythrocytes. An intricate series of biological events and developmental processes must occur for this cyclical erythrocytic stage of the infection to continue in a vertebrate host. Thus, the elucidation of molecular mechanisms responsible for recognition and subsequent invasion of erythrocytes by the malaria parasite is of central importance towards the development of new intervention strategies.

In this study properties of a novel protein encoded by an open reading frame (ORF), designated D13, is described. The sequence of D13 is highly conserved in several *P. falciparum* isolates and orthologues of it are identified in the genome of *Plasmodium knowlesi* and *Plasmodium yoelii*. The parasite growth inhibitory activity of anti-D13 monoclonal antibodies (MAbs) is indicative for a functionally essential role of this protein in parasite biology.

Materials and Methods

Identification of ORF D13

A cDNA library was constructed from total RNA isolated from *P. falciparum* strain K1 employing the SMART PCR cDNA Library Construction Kit (Clontech) as described (5). Briefly, 2 µg of total RNA was reverse transcribed using a modified oligo(dT) primer, and the SMART oligonucleotide was added to the reaction to serve as a short, extended template at the 5' end of the RNA for reverse transcription. To select for PCR products longer than 0.7 kb, PCR products were run on a 1% agarose gel, selectively excised, and purified. PCR products were ligated into pGem 5 T vector (Promega). DH125 cells (BRL-Life Technologies) were transformed by electroporation with the *P. falciparum* cDNA library. Plasmid DNA of randomly picked clones was digested with enzymes *Not*I/*Nco*I, and the insert size was analyzed on 1% agarose gels. Clones carrying inserts of more than 1 kb were chosen for further analysis. Linearized DNA was transcribed and translated in vitro as described (6) and positive cDNA clones were characterized further by sequencing employing an ABI 310 automatic sequencer (Perkin-Elmer).

P. falciparum culture and Northern blot analysis

P. falciparum strains K1 and FVO were cultured in RPMI 1640 medium (Life Technologies, Inc.) containing gentamicin (50 mg/liter) and 10% A^+ human serum at a hematocrit of 5%, essentially as described previously (14). In some experiments, the cultures were synchronized by hemolysis of mature trophozoite stage-parasitized erythrocytes in a 5% (wt/vol) sorbitol solution with two sorbitol synchronization steps one cycle before harvesting. Synchronization was confirmed and the level of parasitemia estimated by standard microscopy. Aliquots of cells were taken every 6 h postsynchronization. Cells were washed and total RNA prepared using TRIzol reagent (Gibco-BRL) as described previously (25). Total RNA (25 µg) was separated on 0.8% agarose gel and transferred to Hybond-XL nylon membranes (Amersham Pharmacia Biotech) using a vacuum blotter (Appligene) as described elsewhere (25). Hybridization was performed in an UltraHyb device (Ambion) at 42°C with the [α-^{32}P]dCTP-labeled D13 probe (5'-terminal 422-bp PCR product) and the pfGAPDH probe (complete cDNA; AF03044) (5) generated by random priming using High Prime solution (Roche Biochemicals). High stringency washes were performed at 42°C. Membranes were

subjected to autoradiography using X-ray films. Loading of equal amounts of RNA on agarose gels was confirmed by comparison of the intensity of ethidium bromide stained bands of 18S and 28S RNA.

Recombinant expression of N'-terminal fragment of D13

The N'-terminal 139 amino acid residues were recombinant expressed in *Escherichia coli* using the pETBlue2 expression system (Novagen). Briefly, PCR product of D13 was generated from a cDNA library of *P. falciparum* strain K1 employing the following primer combination: 5'-CAACACCATGGTTTATGCCACACTTTTGAGTG-3' and 5'-CGTTTGCTCGAGTGGCAACTTGTAAGTACCAGGG-3', containing *Nco*I and *Xho*I sites, respectively. The 422-bp amplicon was digested with the restriction enzymes and cloned into the pETBlue2 vector employing its *Nco*I and *Xho*I sites. Recombinant plasmids were sequenced to ensure that the D13 fragment was in the correct reading frame and to exclude PCR errors. Competent *E. coli* Tuner cells (pLys) (Novagen) were transformed with the recombinant plasmid and expression of the fusion protein was induced by the addition of 1 mM isopropyl thiogalactoside (IPTG) (Calbiochem) after the A_{600} reached 0.6. The cells were induced at 37°C for 4 h and were harvested by centrifugation and lysed on ice for 30 min with 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris/HCl (pH 8.0) and sonicated. After centrifugation at 10,000 x *g*, the supernatant was loaded onto an nitrilotriacetic acid column (Qiagen) and purified according to manufacturer's instructions. The hexahistidine-tagged recombinant protein was recovered using elution buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl [pH 4.5], 500 mM imidazole). Purified protein was analyzed by sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis (SDS-15% PAGE), and the protein concentration was determined according to the method of Bradford using bovine serum albumin as the standard. The purified recombinant protein was identified as the expected D13 protein by matrix-assisted laser desorption ionization-time of flight mass spectrometry after tryptic digestion.

Generation of hybridoma cell lines producing anti-D13 MAbs

Hybridoma cell lines were generated from mice immunized essentially as described (18). A group of five mice was immunized subcutaneously three times with 50 µg recombinant D13 protein isolated under denaturing conditions and formulated in MPL+TDM adjuvant (Sigma Chemicals, St. Louis, Mo.). Three days before cell fusion, the mice received an

intravenous booster injection with 20 µg of recombinant D13 in phosphate-buffered saline (PBS). Cells were fused with PAI mouse myeloma cells as a fusion partner (19). Hybrids were selected in hypoxanthine-aminopterin-thymidine (HAT) medium, and cells secreting D13-specific immunoglobulin (IgG) were identified by enzyme-linked immunosorbent assay (ELISA) using Immunolon 4 plates (Dynex Technologies Inc., Chantilly, Va.) coated with D13 protein. Four hybridoma cell lines (named DD1.1, DD1.2, DD1.3, and DD1.4) specific for the D13 N'-terminal fragment could be established and characterized.

Western blot analysis and fractionation of infected erythrocytes

SDS-PAGE was performed essentially as described (5). Briefly, total cell lysates of asynchronous or synchronous cultures of *P. falciparum* (strain FVO) were run on 10% gels. For time course analyses, aliquots of synchronous cultures of malaria-infected erythrocytes were harvested at 6-h intervals and washed with PBS, pH 8.0. The cells were lysed in 10 ml of 0.15% saponin in H_2O, and hemoglobin-depleted infected erythrocytes were collected by centrifugation. Aliquots of the samples were mixed with loading buffer and heated 5 min at 95°C before loading on the gels. As a molecular weight marker, SeeBluePlus (Invitrogen) was used. Separated proteins were transferred electrophoretically to nitrocellulose filter (Protean Nitrocellulose, BA 85; Schleicher & Schuell). Blots were blocked and then incubated with hybridoma supernatant for 1 h. After several washing steps, blots were incubated with goat anti-mouse IgG horseradish peroxidase conjugated Ig (Bio-Rad Laboratories, Hercules, Calif.) for 1 h. Blots were developed using the ECL system according to manufacturer's instructions.

For subcellular fractionation experiments, late stage infected erythrocytes were enriched to 95 to 99% using 60% Percoll gradient essentially as described previously (29). Cells were washed three times in Hanks buffered salt solution and lysed by three cycles of freeze-thaw in 10 volumes of double-distilled water containing protease inhibitors (100 µM phenylmethylsulfonyl fluoride, leupeptin [10 µg/ml], aprotinin [0.4 U/ml], 1 µM pepstatin, 2 µM EDTA [pH 8.0]). A total membrane fraction was collected by ultracentrifugation at 100,000 x *g* for 1 h at 4°C. For certain experiments, equivalent amounts of the sediments were treated with 100 mM Na_2CO_3, pH 11.3, or with distilled H_2O containing protease inhibitors for 30 min on ice prior to further centrifugation for 1 h at 100,000 x *g* (6). Samples were solubilized in denaturing SDS sample buffer and equivalent amounts of each

sample were subjected to Western blot analysis. Western blot analysis was conducted with anti-D13 MAb DD1.1 and anti-merozoite surface protein 1 (anti-MSP-1) MAb 7/27. MAb 7/27 recognizes the N'-terminal block 1 of MSP-1 (Helg et al., submitted for publication). The blots were developed using the ECL system.

Immunofluorescence analysis of asexual blood stage parasites

Immunofluorescence analysis of blood stage parasites was conducted essentially as described (5). Briefly, multitest immunofluorescence microscopy slides (Flow Labs, Switzerland) were pretreated with 0.01% (wt/vol) poly-L-lysine (Sigma) for 30 min at room temperature and washed. Erythrocytes from in vitro cultivated *P. falciparum* (strain FVO) were washed and mixed with 2 volumes of a solution containing 4% paraformaldehyde and 0.1% Triton X-100. Droplets of 30 µl of cell suspension were added to each well and incubated for 30 min at room temperature. Cells were blocked with blocking solution containing 100-mg/ml fatty acid-free bovine serum albumin in PBS. Cells were incubated with hybridoma supernatants for 1 h. After several washing steps, cells were incubated with secondary antibodies specific for mouse IgG conjugated with Cy3. The immunoreactivity was observed using a Leica TCS NT confocal microscope. Images were acquired with a 63x Plan-Apochromat oil immersion objective (NA 1.32). Pinhole settings were 1 airy unit for all images that were processed with Imaris (Bitplane, Switzerland) and Adobe Photoshop (Mountain View, Calif.).

Sequence analysis of D13 derived from parasite strains. Genomic DNA was prepared from *P. falciparum* strains K1, IFA9, and MAD20 and used for PCR amplification of D13 in order to gain insight into possible nucleotide sequence polymorphism. For sequence analysis, D13 was amplified in two overlapping fragments with the following primer combinations: forward (p17), 5'-CAACAAAATGGTTTATGCCACACTTTTGAGTGAAG-3', and reverse (p19) 5'-CAGGAATTCACATTTGAACAATTGGATTG-3'; forward (p29), 5'-CCTACTCAAGAAATAGCATG-3', and reverse (p25), 5'-GTATAGACATGTTTTGTTTCATATTATTATATAG-3'. Amplifications were performed with the following profile: 5 min 94°C; (25 x 20 s 94°C, 30 s 44°C, 2 min 68°C) 7 min 72°C, soak at 4°C. Amplicons were purified using a PCR product purification kit (Roche

Molecular Biochemicals) according to manufacturer's protocol and cloned into the pGEM5 T-vector (Promega, Catalys AG). After isolation of plasmids using the NucleoSpin kit (Macherey-Nagel AG), double-stranded plasmid DNA was sequenced and analyzed employing an ABI PRISM 310 genetic analyzer (Perkin-Elmer). All strains were amplified twice and several independent plasmids were sequenced on both strands using internal primers designed according to the D13 sequence available from the genome project.

In vitro parasite growth inhibition assays

In vitro growth inhibition assays with *P. falciparum* strain K1 were conducted essentially as described (16). Briefly, synchronous late trophozoites were diluted with fresh RBC to give a parasitemia of 0.5% and were mixed with purified MAbs at the indicated concentrations. Parasites were cultivated under an atmosphere of 4% CO_2, 3% O_2, and 93% N_2 at 37°C. Final hematocrit in cultures was adjusted to 0.5%. Each culture was set up in sextuplicate in 96-well flat-bottom culture plates. After 96 h plates were centrifuged at 180 x *g* for 5 min and culture supernatants were discarded. Pelleted erythrocytes were resuspended in 200 µl of PBS supplemented with hydroethidine fluorescent vital stain (15 µg/ml; Polyscience Inc., Warrington, Pa.) and incubated at room temperature for 45 min. The erythrocytes were washed twice with PBS, resuspended in 400 µl of PBS, and analyzed in a FACScan flow cytometer (Becton-Dickinson, San Jose, Calif.) with CELLQuest program. The hydroethidine emission was detected in the FL2 channel by logarithmic amplification, and the erythrocytes were gated on the basis of their forward and sideward scatters. A total of 30,000 cells per sample were analyzed. Percent inhibition was calculated from the geometric mean parasitemias of sextuplicate test and control wells as 100 x (control - test)/control. Statistical significance was calculated by a two-sided *t* test. Confidence intervals ($P < 95\%$) were calculated by antilogging the confidence limits calculated on the log scale.

Nucleotide sequence accession numbers

The nucleotide sequences reported in this paper have been submitted to the GenBank with the accession numbers AF491296 to AF491298.

Results

Identification and sequence analysis of ORF D13 of *P. falciparum*

In order to identify novel secreted and transmembrane gene products of *P. falciparum* involved in host-parasite interactions, a *P. falciparum* cDNA library was screened in an in vitro transcription-translation-translocation assay for genes encoding protein products that are translocated into microsomes and are therefore protected from proteinase K digestion (6). One of the plasmids, designated D13, gave rise to a 37-kDa protein product that resisted proteinase K digestion indicative for membrane association or translocation (data not shown). The 1.2-kb insert of this plasmid was sequenced and results compared with sequence data of the ongoing *P. falciparum* Genome Project available at PlasmoDB database [http://plasmodb.org]) (27). One predicted ORF (chr14_1.glm_722) that encompassed the partial sequence of plasmid D13 was identified. The complete predicted ORF D13 is 2,586 bp and codes for 862 amino acid residues, with a calculated molecular mass of 99.7 kDa and an pI of 5.30. The three gene prediction algorithms used by the genome project, Glimmer, Genefinder, and Path, predicted identically D13. Screening of the PlasmoDB database using the Blast program showed that D13 is present on chromosome 14 as a single-copy gene. The identification of the predicted initiation codon is supported by the nucleotide context of the ATG start codon, AAATGG, found in other genes of *P. falciparum* and the lack of alternative start codons anywhere in vicinity (4). D13 is intron-less and the putative protein is rich in asparagine (19.3%), lysine (9.5%), glutamic acid (7.7%), and aspartic acid (7.2%) (Fig. 1). D13 has no predicted N'-terminal signal sequence and other primary structural characteristics of an integral membrane protein are also lacking. Analysis of the predicted secondary structure of D13 with the Predictprotein program package (http://bioc.cubic.colombia.edu/predictprotein) identified several low-complexity, nonglobular regions separating two globular domains located each at the N'- and C'-terminal ends. Searching databases of sequenced genomes of eukaryotes and prokaryotes demonstrated no significant homology to functional domains of other characterized gene products and hence no functional classification can currently be assigned (17).

D13 nucleotide sequences of *P. falciparum* strains K1, MAD20, and IFA9 (13) were compared with the 3D7 sequence available from the genome project. D13 was amplified by

PCR from genomic DNA of in vitro-grown blood stage parasites, cloned into pGem5T vector and sequenced as described previously (13). All sequences obtained were identical to the *P. falciparum* 3D7 sequence apart from one nonsynonymous base exchange at nucleotide position 1444 in *P. falciparum* K1. This exchange resulted in a conservative amino acid exchange of D to E and was reconfirmed by a second analysis using an independent PCR product. The nucleotide sequences were deposited in the GenBank with the accession numbers AF491296 to AF491298.

Sequence comparison of D13 of *P. falciparum* using Blast program and the PlasmoDB database [http://plasmoDB.org]) revealed that full-length putative orthologues of D13 are present in the genome of *P. knowlesi* (chrPkn_008795-6-7284-4963) and *P. yoelii* (chrPyl_cpy805-2-1337-3844) (Fig. 1). When the D13 amino acid sequence of *P. falciparum* was compared using the Blast program to putative orthologues in *P. knowlesi* and *P. yoelii*, sequence identities were 38 and 41%, respectively. Alignment of the deduced amino acid sequences revealed that a repeat region with twelve consecutive repeats of the pentapeptide motif (K/R)(N/S)(D/E)N(I/M/T) is unique for D13 of *P. falciparum* and is absent in the orthologues sequences of the other Plasmodium spp. aligned. This repeat region constituted 8% of the molecule. In contrast to the low overall conservation of the D13 sequence, the N'-terminal domain encompassing the first 158 amino acid residues is highly conserved between the three Plasmodium spp. aligned displaying sequence identities of 94 and 95%, respectively, between *P. knowlesi*, *P. yoelii*, and *P. falciparum* (Fig. 1). Ten strictly conserved cysteine residues with the spacing H_2N-19-C-7-C-6-C-11-C-16-C-14-C-5-C-20-C-7-C-4 are present. Shorter stretches of conserved sequence motifs at the C'-terminal end of the D13 amino acid sequence were also found (Fig. 1).

Stage-specific expression of ORF D13 mRNA in synchronized blood stage parasites

In order to define the transcription pattern of D13, Northern blot analysis was performed using total RNA isolated from asexual blood stage parasites. After hybridization with a radiolabeled PCR product representing the 5'-terminal 422 bp of D13, one specific signal was detected in RNA from unsynchronized blood stage parasites (data not shown). The size of the transcript was about 7.5 kb by comparison with the RNA size marker, indicating the presence of extensive 5'- and 3'-untranslated regions. In parasites from synchronized cultures, this transcript was only detected in RNA of schizonts collected 48 h after

synchronization (Fig. 2A, panel I, lane 8). In order to verify the integrity of the RNA preparations isolated at different times after synchronization, the blot was rehybridized with a radiolabeled cDNA of pfGAPDH (5). The pfGAPDH-specific hybridization signal of 3.6 kb was observed in each lane confirming that the RNA was intact and comparable amounts of RNA were present for hybridization (Fig. 2A, panel I and II).

Establishment of D13-specific MAbs and analysis of the expression of D13 protein in blood stage parasites

After having established that D13 transcription is largely confined to the schizont stage, we wanted to analyze next whether, when and at what size D13 protein is expressed. The N'-terminal 139 amino acids of D13 were expressed in *E. coli* as hexahistidine tagged fusion protein using the pETBlue2 expression system. Purification by nitrilotriacetic acid-affinity chromatography under denaturing conditions yielded a recombinant protein of the predicted molecular mass of 17 kDa in SDS-PAGE (data not shown). The purified recombinant protein was identified as the expected D13 protein by matrix-assisted laser desorption ionization-time of flight mass spectrometry. D13-specific MAbs were generated from mice carrying human heavy and light immunoglobulin chain replacement mutations (18). After repeated immunization with the recombinant fragment of D13 delivered with MPL+TDM as adjuvant, four anti-D13 MAbs were generated that reacted with the recombinant 17 kDa fragment in ELISA (Table 1). DD1.1 and DD1.2, but not DD1.3 and DD1.4, recognized in Western blot analysis of total lysates of unsynchronized blood stage parasites one distinct band of ~100 kDa (Fig. 2B and data not shown). The size of the identified band corresponded to the predicted molecular weight of 99,7 kDa of ORF D13 (chr14_1.glm_722). As a representative example, results obtained with MAb DD1.1 are shown in Fig. 2B, lane 9.

In the light of the highly regulated transcription of the D13 gene, we investigated the presence of D13 protein during asexual blood stage development. Aliquots of the lysates from the identical synchronized cultures that had been used for Northern blot analysis were separated by SDS-PAGE and probed with MAb DD1.1. D13 protein was detectable in ring stage parasites (Fig. 2B, lanes 1 to 4), disappeared during the trophozoite stage (Fig. 2B, lanes 5 and 6), and reappeared in the schizont stage (Fig. 2B, lanes 7 and 8). In the late schizont stage at 48 h postsynchronization, the relative abundance was highest (Fig. 2B,

lane 8). Uninfected RBC yielded no detectable signal, confirming the specificity of MAbs DD1.1 for a parasite-encoded protein (Fig. 2B, lane 10). Results obtained with MAb DD1.2 were comparable to DD1.1, although considerably weaker signals were obtained (data not shown). Protein staining of SDS-PAGE demonstrated that comparable amounts of total cell lysates from synchronized blood stage parasites were present on nitrocellulose membranes (data not shown).

Immunolocalization of D13 in asexual blood stages of *P. falciparum*

All anti-D13 MAbs were tested for parasite binding using indirect immunofluorescence assay (IFA). DD1.1 bound strongest to the parasite in IFA, while DD1.2 and DD1.4 yielded only weak signals at high antibody concentrations indicating that their affinity for the native D13 protein was considerably lower compared to DD1.1 (Table 1). Therefore, MAb DD1.1 was used for the characterization of the subcellular localization of D13 protein by IFA and confocal microscopy. In column 1 of Fig. 3, typical results of the indirect IFA are depicted, while in column 2, phase contrast pictures of the corresponding parasites are shown. Specific staining of schizonts (first row), segmenters (second row) and released merozoites (third row) are presented. Interestingly, in the merozoite stage, D13 protein seemed to be concentrated in a distinct area of the cell, while during schizont stages the protein was more evenly distributed in the parasite.

Subcellular fractionation of *P. falciparum* infected erythrocytes

In order to investigate whether D13 protein is associated with parasite membranes, cell fractionation experiments were performed and the distribution of D13 protein into sediment and supernatant fractions examined using the anti-D13 MAb DD1.1 for Western blot analysis. Infected erythrocytes were Percoll-purified to maximize parasite protein content, washed thoroughly and hypotonically lysed in water. A membrane-containing fraction was pelleted by ultracentrifugation and analyzed. In the sediment fraction derived from infected erythrocytes, a specific band corresponding to the D13 protein could be detected (Fig. 4A, lane 1). This band was considerably weaker in the supernatant fraction of infected erythrocytes (Fig. 4A, lane 2). To analyze the nature of the association of D13 with the pellet fraction, total membrane fractions were stripped of peripherally attached proteins by treatment at high pH with sodium carbonate. After centrifugation, D13 protein could be

detected in both the pellet and supernatant fractions, indicating that D13 is not a membrane-integral protein (Fig. 4A, lanes 3 and 4). In pellets incubated with water, D13 protein remained in the sediment and was not detected in the supernatant fraction (Fig. 4A, lanes 5 and 6). Aliquots of the same samples were blotted onto nitrocellulose and probed with MAb specific for the abundant membrane-integral protein MSP-1. MSP-1 is synthesized as a large (~195 kDa) precursor that is held by a glycosyl phosphatidyl inositol anchor on the parasite membrane. It undergoes posttranslational proteolytic processing to produce fragments of 83, 42, 38, and 28 to 30-kDa, which persist as a noncovalently linked complex on the surface of mature merozoites (12). The anti-MSP-1 antibody 7/27 is specific for block 1 of MSP-1 present on both the precursor molecule and the processed 83 kDa fragment. As expected, MAb 7/27 detected a major band of ~195 kDa in the sediment of untreated, alkaline carbonate and water treated pellets but not in the supernatants (Fig. 4B). The smaller band of ~83 kDa represent processing products of MSP-1 (Fig. 4B). These results indicated that D13 is pelleted with the membrane-fraction but it is not a membrane-integral protein.

P. falciparum in vitro growth inhibition assays with anti-D13 MAbs

After having established that MAb DD1.1, DD1.2, and DD1.4 but not DD1.3 bind to native D13 protein expressed in the invasive stage of the parasite blood stage cycle, we conducted in vitro growth inhibition assays for two cycles of merozoite invasion. At 500 µg/ml the anti-D13 MAb DD1.1 showed growth inhibitory effects in three independent experiments (47.6% average growth inhibition) conducted with two different batches of antibody preparations (Fig. 5). This inhibition was statistically significant as judged by a two-sided *t* test. At an antibody concentration of 250 µg/ml, the measured inhibition remained statistically significant, while at 100 µg/ml the effect was diminished by dilution. In contrast to the good binding MAb DD1.1, the IFA-negative anti-D13 MAb DD1.3 did not interfere with the growth of the parasite in vitro (Fig. 5).

Discussion

Research conducted in recent years identified the importance of both antibody-dependent and cell-mediated mechanisms of immunity to the erythrocytic stage of P. falciparum (15). A protective role for antimalaria antibodies was shown by the pioneering studies of Cohen et al. (3), who showed that protective immunity could be transferred by using the IgG fraction of sera from immune West African adults. Administration of large doses of antimalaria antibodies to children with acute infection resulted in reduction of parasitemia and recovery from clinical illness (3). It was thought that these antibodies interacted with either late-stage schizonts or free merozoites (2). Further studies showed that antimalarial IgG from immune sera of West Africans protected against P. falciparum infection in East Africa (2). Additionally, transfer of purified, pooled hyperimmune IgG from African adults to Thai patients was found to reduce parasite level. These transfer experiments suggest that geographically diverse parasite strains may share antigens important in inducing protection (22). Hence, there is considerable interest in the molecular identification of parasite proteins as potential targets of vaccine-induced antibodies preventing invasion of erythrocytes.

We are currently characterizing secretory and transmembrane gene products of P. falciparum stage-specifically expressed during schizogony. Expression screening of a P. falciparum cDNA library for in vitro-translated and -translocated products yielded the cDNA clone D13 encompassing the N'-terminal 1.2-kb fragment of a novel predicted ORF (chr14_1.glm_722). The expression of D13 mRNA turned out to be highly regulated and detectable by Northern blot analysis only in schizont development. Therefore, we decided to characterize D13 in more detail. D13 is a single-copy gene localized on chromosome 14 according to the ongoing P. falciparum genome project and is predicted to code for a 99.7-kDa protein. D13 contains a region of low-complexity constituted by 12 tandem repeats of a pentapeptide sequence motif that is followed by shorter homopolymer runs of asparagine residues. Many of the malaria antigens that have been characterized in P. falciparum contain tandem arrays of relatively short sequences. A number of characteristics allow distinctions to be drawn among such malaria antigens. One group is characterized by one centrally located block of tandem repeats that constitutes a significant proportion of the polypeptide chain. This group includes the S antigens, MSP-2 and the circumsporozoite protein (1). Other antigens contain a single set of repeats comprising a minor segment of

the polypeptide chain. This group includes the thrombospondin-related adhesive protein, the exported protein 1, and also D13, since the repeat region constitutes about 8% of the whole molecule (1). Other characteristics that distinguish between different repeat-containing antigens are diversity of repeat sequences and variation in the number of tandemly repeated sequences. Sequence diversity can be observed both within blocks of repeats and between equivalent repeat segments in allelic gene products (1). In D13, there is variation between the 12 sequence repeats that are based mainly on single base pair exchanges (data not shown). Surprisingly, sequence polymorphism of the low-complexity region of D13 of P. falciparum in the laboratory isolates 3D7, K1, and MAD20 and the field isolate IFA9 was not detected although polymorphism of antigens of P. falciparum is usually particularly extensive in repeat regions of the molecules (1).

In order to study whether and when D13 protein is expressed during asexual blood stages, the N'-terminal 138 amino acids were expressed in E. coli as hexahistidine-tagged protein, purified, and used to raise specific MAbs in mice. Immunogenicity of the recombinant protein was low but we were able to establish several anti-D13 MAbs. The anti-D13 MAbs DD1.1 and DD1.2 recognized a band of 100 kDa in lysates of blood stage parasites in Western blot analysis. This size of the band corresponded to the predicted molecular weight of D13. In IFA, the cross-reactivity of MAb DD1.1, DD1.2, and DD1.4 with the native parasite protein was established. Western blotting and IFA both showed that the abundance of D13 protein was highest in schizonts. It declined during ring stage while D13 was undetectable in trophozoites. D13 protein was detectable for about 30 h during the asexual blood stage cycle, while D13 specific mRNA was present only during the last 6 h of schizont development. Hence, the D13 protein was detectable four to five times longer than the D13-specific mRNA during one asexual developmental cycle.

The strict regulation of D13 mRNA expression together with the highest expression of D13 protein in schizonts suggested an involvement of the protein in the complex biological processes of merozoite development, rupture of mature schizonts, release of merozoites and invasion of fresh erythrocytes. Therefore, we used an in vitro parasite growth inhibition assay to test whether anti-D13 MAb influence the progression of the infective cycle. The results showed that incorporation of the strongest parasite binding anti-D13 MAb DD1.1 inhibited parasite growth by an average of 47.6% in several independent experiments. The non-parasite-binding anti-D13 MAb DD1.3 had no effect on parasite growth. These results

indicate that the affinity and/or the epitope recognized by the anti-D13 MAbs might be important for the inhibitory function. Currently, it is not possible to assign exactly the subcellular localization of D13 by IFA using confocal microscopy. D13 is not obviously expressed on the surface of merozoites like the MSPs but might be rather concentrated on one distinct pole of the merozoite. The combined results of the in vitro parasite growth inhibition assays with the cell fractionation experiments indicate that D13 is during blood stage development accessible to antibodies in solution and becomes enriched in the pelleted fraction of malaria parasites. However, more detailed analyses, including immuno-electron microscopy, are essential to determine the exact subcellular localization of D13 protein.

Currently, the correlation of in vitro growth inhibitory activities of antibodies with their potential protective capacity in vivo is incompletely understood. Active immunization studies in animal models are therefore essential to demonstrate unequivocally that the N'-terminal domain of D13 might be a target of protective antibody responses in vivo.

The fact that D13 is conserved in four parasite isolates may suggest that it is not under immune pressure, although sera from donors naturally exposed to malaria contain antibodies reacting with the recombinant N'-terminal domain of D13 in ELISA (unpublished observation). The high level of sequence conservation is in marked contrast to MSPs like MSP-1 and MSP-2 (7, 8, 10). In contrast, the rhoptry-associated protein 2 displays very limited sequence diversity (24). It has been demonstrated that antibodies raised against rhoptry-associated protein 2-derived peptides reduce parasite growth in vitro, indicating that conserved proteins can be targeted by parasite inhibitory antibodies (26).

Besides offering the possibility to improve the annotations of the P. falciparum genome through comparative analysis, animal models represent a potent source of information concerning protein function within the context of the infected host or vector (30). Alignment of the deduced amino acid sequences of putative D13 orthologues in P. falciparum, P. knowlesi, and P. yoelii showed that the N'-terminal domain is highly conserved, while the rest of the molecule displayed extensive sequence variation. The high degree of amino acid sequence conservation between rodent, monkey and human malaria species suggests a conserved biological role of the N'-terminal domain in malaria. The prominence of synonymous versus nonsynonymous base exchanges in this domain (data not shown) suggests that apart from differences in the codon usage in different Plasmodium

species (28) a negative selection pressure might be operating to preserve a distinct three-dimensional structure. The conserved N'-terminal domain of D13 might bind to and interact tightly with other proteins to conduct its biological function(s). Functional investigations of the biological role(s) of D13 will probably further our understanding of molecular mechanism(s) mediating evasion, recognition, invasion and subsequent establishment of the parasites in host cells.

Acknowledgments

This work was supported by a grant from the Swiss National Science Foundation to C. A. Daubenberger (3100-061513.00). The Plasmodium Genome Database, a collaborative effort of investigators at the University of Pennsylvania and Monash University Melbourne, Australia, is supported by the Burroughs-Wellcome Fund. We thank Shinji Okitsu for in vitro cultivation of *P. falciparum* and Bernd Bohrmann for support in confocal microscopy. We thank the scientists and funding agencies comprising the international Malaria Genome Project for making sequence data from the genome of *P. falciparum* (3D7) public prior to publication and the completed sequence.

TABLE 1. Reactivities of anti-D13 MAbs used in this study

Designation of anti-D13 MAb	Isotype	Reactivity in ELISA to D13 N'-terminal fragment (µg/ml)[a]	Concn µg/ml) of anti-D13 MAbs yielding a positive result in indirect IFA[b]
DD1.1	IgG1/λ	0.08	0.15
DD1.2	IgG1/λ	0.06	5
DD1.3	IgG2b/λ	0.1	—[c]
DD1.4	IgG2b/λ	0.04	5

[a] Purified anti-D13 MAbs were serially diluted and tested for reactivity in ELISA with plates coated with recombinant D13 N'-terminal fragment at a concentration of 5 µg/ml. Given are the concentrations yielding half-maximal binding as measured by optical density at 405 nm.

[b] Purified anti-D13 MAbs were serially diluted and used for indirect IFA of unsynchronized parasites fixed onto slides. Antibody binding was assessed by fluorescence microscopy on a Leitz Dialux 20 fluorescence microscope and documented with a Leica DC200 digital camera system. The lowest concentration of anti-D13 MAbs yielding a positive signal in indirect IFA is given.

[c] MAb DD1.3 showed no binding in IFA.

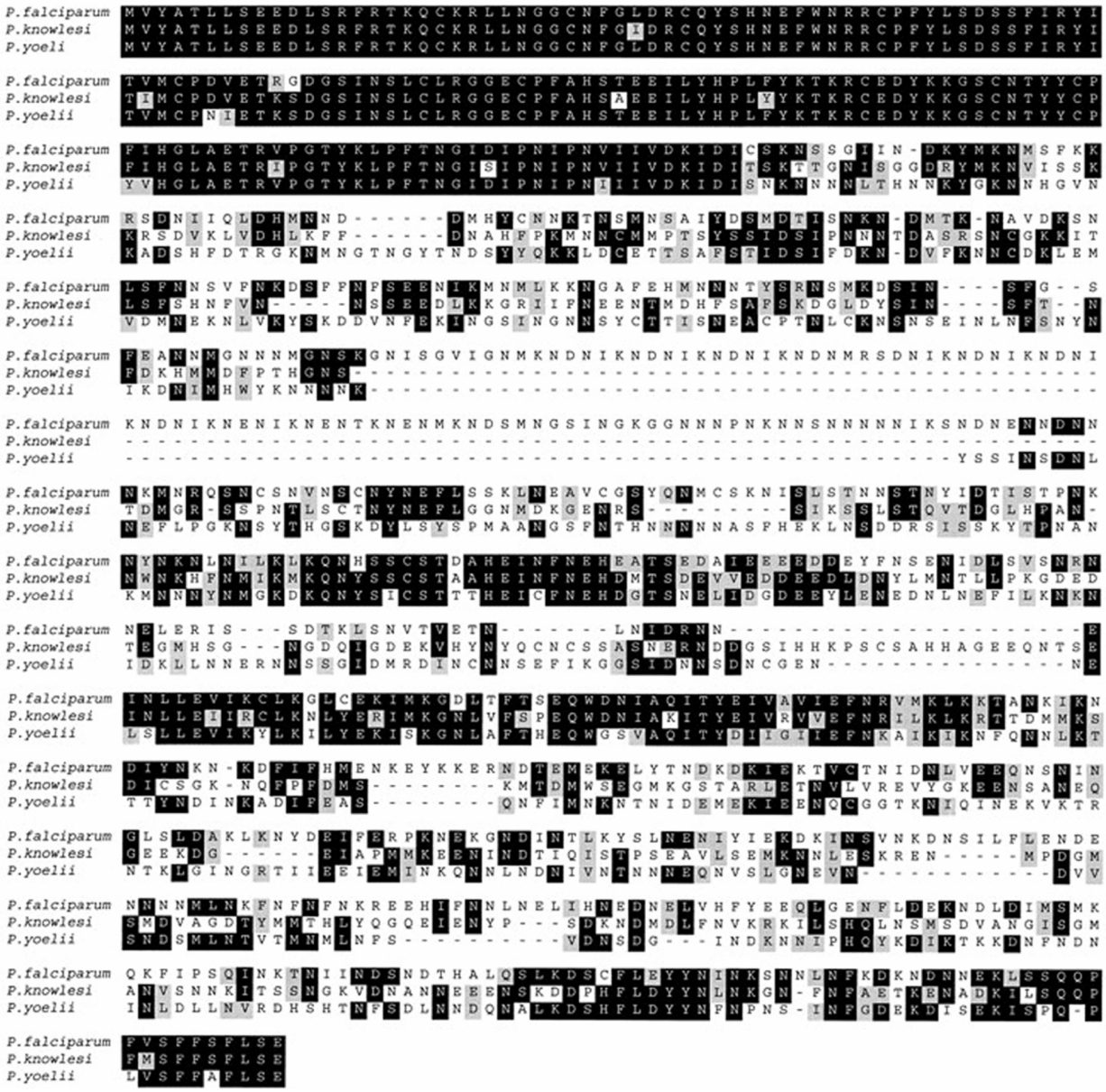

Figure 1. Alignment of deduced amino acid sequences of D13 orthologues of *P. falciparum*, *P. knowlesi*, and *P. yoelii*. The alignment was done with ClustalW and prepared for display using BOXSHADE (http://bioweb.pasteur.fr). Gaps were inserted to give the best fit. (Sequences for *Plasmodium* spp. are available from the PlasmoDB database [http://www.plasmoDB.org].)

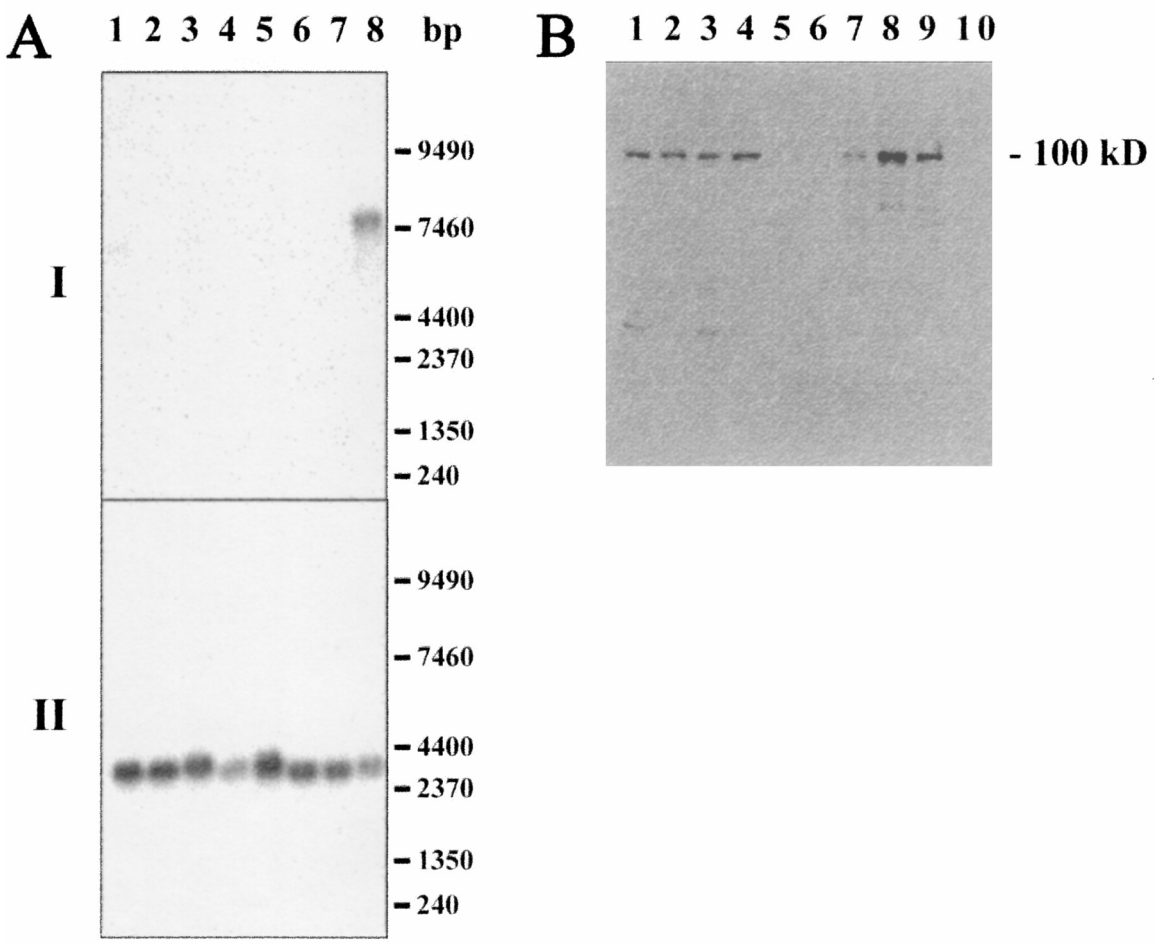

Figure 2. (A) Northern blot analysis of synchronized asexual blood stage of *P. falciparum*. Panel I shows total RNA (25 µg) from in vitro-grown synchronized *P. falciparum* blood stage parasites that was separated on agarose gels, blotted onto nylon membrane, and hybridized to an [α-^{32}P]-dCTP-labeled probe corresponding to the 5'terminal 422-bp fragment of D13. Time points analyzed were 0 to 6 h, 6 to 12 h, 12 to 18 h, 18 to 24 h, 24 to 30 h, 30 to 36 h, 36 to 42 h and 42 to 48 h (lanes 1 to 8) after synchronization. Panel II shows the blot rehybridized with an [α-^{32}P]-dCTP-labeled probe of cDNA of pfGAPDH (5). (B) Western blot analysis of total lysates of blood stage parasites. Total lysates of infected RBC were separated by SDS-10% PAGE under reducing conditions and blotted onto a nitrocellulose membrane. Samples were taken at 6, 12, 18, 24, 30, 36, 42, and 48 h (lanes 1 to 8) after synchronization. Lysates of unsynchronized infected RBC (lane 9) and uninfected RBC (lane 10) were also loaded. The blot was incubated with anti-D13 MAb DD1.1 and developed using the ECL system. MAb DD1.1 recognized a single protein band of about 100 kDa in most lanes containing lysates of infected, but not in uninfected RBC.

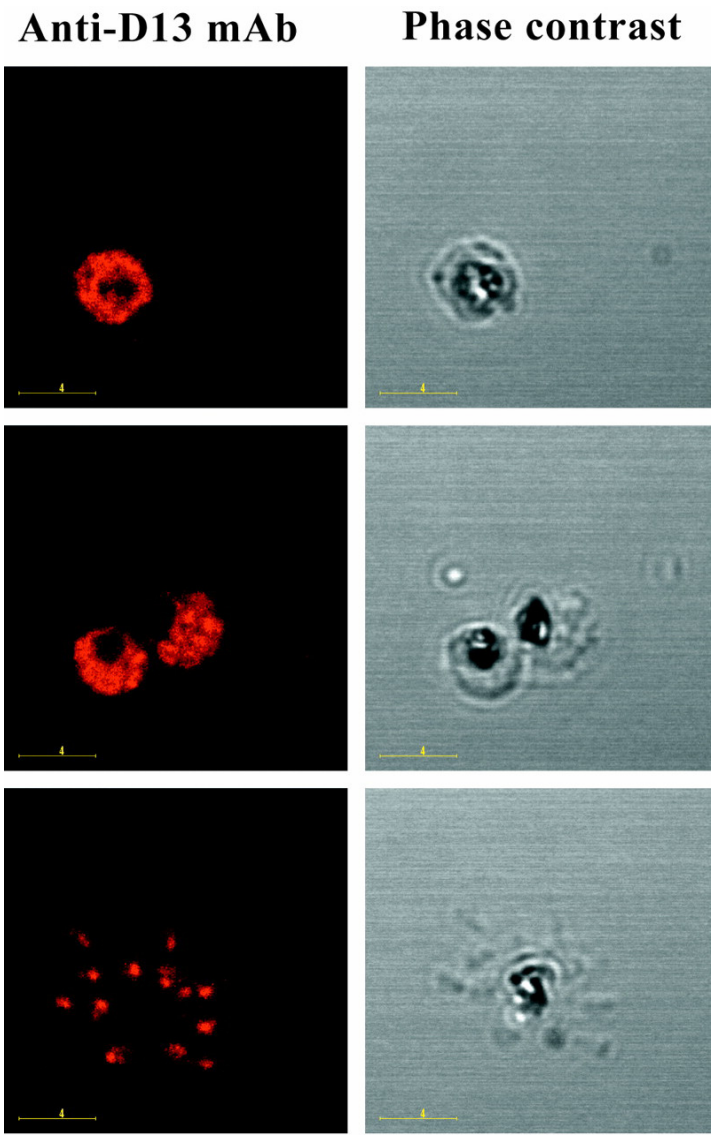

Figure 3. Immunofluorescence analysis of schizonts, segmenter, and released merozoites of *P. falciparum* using anti-D13 MAb DD1.1 and confocal microscopy. In column I, staining with MAb DD1.1 is shown, while in column II the phase contrast of the corresponding parasites is presented.

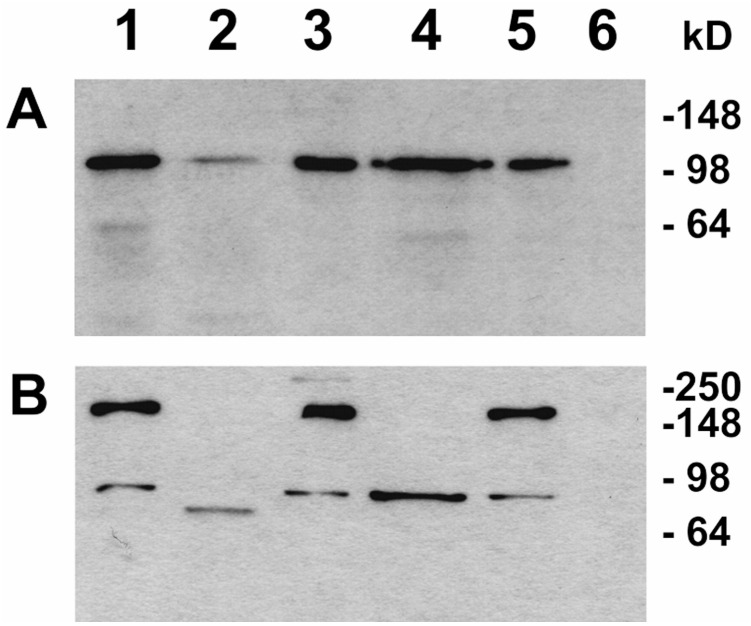

Figure 4. Association of D13 with the sediment fraction of infected erythrocytes. Schizonts were enriched by Percoll-gradient centrifugation and hypotonically lysed by repeated cycles of freeze-thaw in water. The sediment (lane 1) and supernatant (lane 2) fractions were obtained by centrifugation. Aliquots of membrane fractions were further processed by incubation with alkaline carbonate (lanes 3 and 4) or water (lanes 5 and 6), respectively, and separated by ultracentrifugation in sediment lanes (lanes 3 and 5) and supernatant lanes (lanes 4 and 6) fractions, respectively. The samples were electrophoresed and probed with anti-D13 MAb DD1.1 (Fig. 4A) or anti-MSP-1 MAb 7/27 (Fig. 4B).

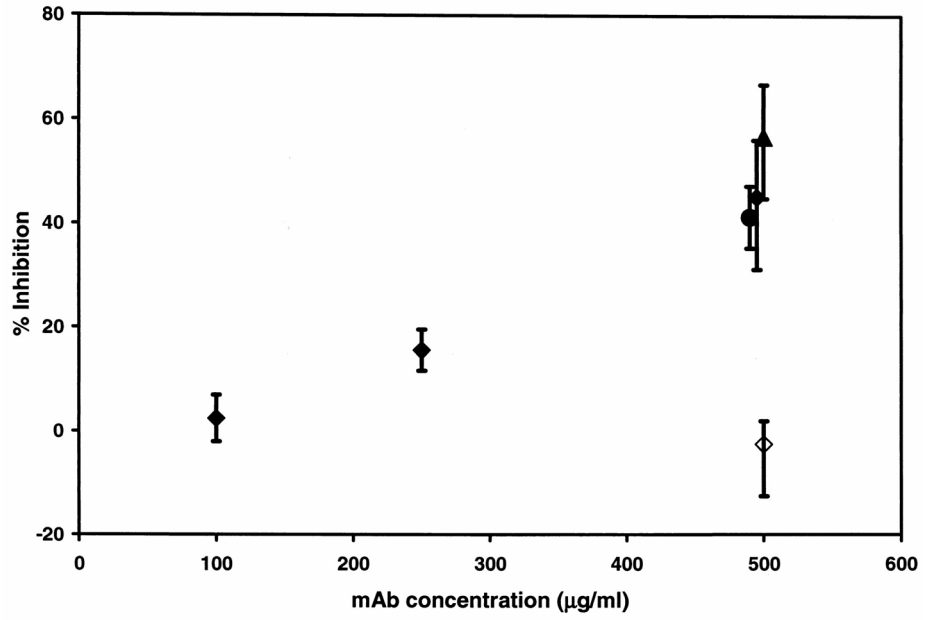

Figure 5. Parasite growth inhibitory activity of anti-D13 MAbs. The vertical bars indicate the 95% confidence intervals. The filled symbols represent results of three separate experiments conducted with MAb DD1.1, while results with the parasite nonbinding MAb DD1.3 are represented by the open symbol (◇).

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The N'-Terminal Domain of Glyceraldehyde-3-phosphate Dehydrogenase of the Apicomplexan *Plasmodium falciparum* Mediates GTPase Rab2-Dependent Recruitment to Membranes

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This article has been published in Journal of Biological Chemistry. 2003 August; 384 (8):1227-37

Summary

Spatial and temporal distribution of the glycolytic enzymes glyceraldehydes-3- phosphate dehydrogenase (pfGAPDH) and aldolase (pfAldolase) of *Plasmodium falciparum* were investigated using specific mAbs and indirect immunofluorescence analysis (IFA). Both glycolytic enzymes co-localized during ring and trophozoite stages of both liver and asexual blood stage parasites. During schizogony, pfGAPDH became associated with the periphery of the parasites and eventually accumulated in the apical region of merozoites while pfAldolase showed no segregation. Sub-cellular fractionation experiments demonstrated that pfGAPDH was found in both the membrane-containing pellet and the supernatant fraction of parasite lysates. In contrast, pfAldolase was only found in the supernatant fraction. A quantitative binding assay showed that pfGAPDH could be recruited to HeLa cell microsomal membranes in response to mammalian GTPase Rab2 indicating that Rab2-dependent recruitment of cytosolic components to membranes is conserved in evolution. Two overlapping fragments of pfGAPDH (residues 1 - 192 and 133 - 337) were evaluated in the microsomal binding assay. We found that the N'-terminal fragment competitively inhibited Rab2-stimulated pfGAPDH recruitment. Thus, the domain mediating the evolutionary conserved Rab2-dependent membrane-recruitment is located in the N'-terminus of GAPDH. These combined results suggest that pfGAPDH exerts non-glycolytic function(s) in *P. falciparum*, possibly including a role in vesicular transport and biogenesis of apical organelles.

Key words: *Plasmodium falciparum* / glyceraldehyde-3-phosphate dehydrogenase / membrane association / non-glycolytic function / GTPase Rab2 /evolution

Introduction

Malaria caused by the apicomplexan parasite *Plasmodium falciparum* is one of the most important parasitic diseases in man (World Health Organization, 2001) (Sachs and Malaney, 2002) Sporozoites injected into a human host by the bite of an infective mosquito invade hepatocytes and, after schizogony, release thousands of merozoites capable of invading red blood cells (RBC). Malaria pathogenesis is associated with this intracellular blood-stage of the parasite's life cycle involving repeated rounds of invasion, growth and schizogony in the erythrocyte. Trophozoite, schizont and merozoite stages develop during the liver stage that lasts ~ 5 - 7 days in man, while ring, trophozoite, schizont and merozoite stages are distinguished during asexual blood stage development that takes ~ 48 h.

P. falciparum targets a distinct set of proteins to each of the apical secretory organelles (Preiser *et al.,* 2000). These proteins enable merozoites to selectively adhere and invade host cells and, once within, to cause modifications of the host cell (Preiser *et al.,* 2000; Preiser *et al.,* 2002). Rhoptries appear to be formed by fusion of vesicles derived from the Golgi cisternae (Bannister *et al.,* 2000). The rhoptry associated protein 1 (RAP-1) has been localized exclusively to rhoptries (Clark *et al.,* 1987) and can be used as a marker for the localization of the apical complex (Howard *et al.,* 1998; Moreno *et al.,* 2001). Recent results suggest that the parasite uses a "just in time" developmental strategy where compartments of the secretory pathway are strategically positioned when and where they are needed during organelle biogenesis (Kocken *et al.,* 1998; Noe *et al.,* 2000). However, there are still major unanswered questions related to the components involved in the secretory pathway of *P. falciparum* (Ward *et al.,* 1997; Albano *et al.,* 1999; Wiser *et al.,* 1999).

In mammalian cells and in yeast, a multitude of proteins have been shown to play a role in sub-cellular trafficking including the Rab protein family. The Rab proteins are GTPases that regulate vesicular traffic between specific compartments of the endocytic and exocytic pathways (Nuoffer and Balch, 1994). When these proteins are membrane associated, they promote recruitment of cytosolic components that function in vesicle formation, docking and fusion. For example, the activated form of Rab2 initiates in mammalian cells a cascade of events leading to the recruitment of soluble factors to pre-

Golgi intermediates that ultimately leads to the release of retrograde-directed vesicles (Tisdale *et al.,* 1992; Tisdale and Balch, 1996; Tisdale, 1999). Interestingly, one of the soluble factors recruited has been identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) (Tisdale, 2001). GAPDH is commonly known as a key enzyme in glycolysis and catalyses the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3 diphosphoglycerate in the presence of NAD^+ and inorganic phosphate. There is substantial evidence suggesting that GAPDH might be involved in additional cellular activities unrelated to its role in glycolysis (Sirover, 1999). Multiple studies from different groups employing independent methods have demonstrated the participation of one or more GAPDH isoforms in membrane fusion and trafficking in biological systems (Lopez Vinals *et al.,* 1987; Robbins *et al.,* 1995; Volker and Knull, 1997; Han *et al.,* 1998; Hessler *et al.,* 1998; de Arcuri *et al.,* 1999; Tisdale, 2001; Bressi *et al.,* 2001; Glaser *et al.,* 2002). Guided by the crystal structures of Trypanosomatidae GAPDH's in comparison with human GAPDH, an structure-based approach of designing competitive and selective inhibitors of Glycolysis was successfully followed (Verlinde *et al.,* 2001).

In the current study we have established monoclonal antibodies (mAbs) specific for pfGAPDH and pfAldolase. Co-localization studies by indirect immunofluorescence analysis (IFA) and con-focal microscopy and sub-cellular fractionation experiments in combination with a quantitative microsomal recruitment assay provide evidence that pfGAPDH exerts non-glycolytic functions.

Results

Generation and Characterization of Monoclonal Antibodies Specific for GAPDH and Aldolase of *P. falciparum*

After having produced enzymatically active recombinant pfGAPDH (Daubenberger *et al.,* 2000), we wanted to investigate whether this enzyme is involved in additional non-glycolytic functions. Therefore, mAbs specific for pfAldolase and pfGAPDH were established from mice immunized with the respective recombinant proteins (Döbeli *et al.,* 1990; Daubenberger *et al.,* 2000). These mAbs were tested in Western blot analysis for their antigen specificity (Figure 1) using infected red blood cells (RBC) (lanes 1), uninfected RBC (lanes 2), recombinant pfAldolase, (lanes 3) and pfGAPDH (lanes 4). SDS-PAGE followed by Coomassie blue staining demonstrated the purity of the preparations of recombinant proteins (Figure 1 A).

Out of a panel of anti-pfAldolase mAbs, two, designated P.41-2/3-7 and P.41-1/2-7, recognized in total lysates of infected but not uninfected RBC a band of 41 kDa, representing the predicted molecular weight of pfAldolase (Figure 1 B, lanes 1, 2). Additionally, the recombinant pfAldolase but not pfGAPDH was identified by P41-2/3-7 (Figure 1 B, lanes 3, 4). Results obtained with the second anti-pfAldolase mAb P.41-1/2-7 reactive in Western blot analysis were comparable (data not shown). The other anti-pfAldolase mAbs were not reactive in Western blot analysis but precipitated from metabolically labeled blood stage parasites one major band of 41 kDa. All anti-pfAldolase mAbs stained infected RBC in IFA (data not shown).

Two anti-pfGAPDH mAbs, designated 1.4 and 1.10, were isolated from mice immunized with recombinant pfGAPDH (Daubenberger *et al.,* 2000). Western blot analysis showed that both mAbs recognized the recombinant pfGAPDH but not pfAldolase (Figure 1, C, D, lanes 3, 4). Also, in total lysates of infected RBC (Figure 1, C, D, lane 1) but not in uninfected RBC (Figure 1, C, D, lane 2) a protein of 36 kDa, the predicted molecular weight of pfGAPDH, was detected. Taken together, data presented in Figure 1 showed that we obtained mAbs specific for the glycolytic enzymes Aldolase and GAPDH of *P. falciparum* that are not cross-reactive with human erythrocyte proteins. In order to

delineate closer the epitope recognized by the anti-pfGAPDH mAbs, we produced two fragments of recombinant proteins in *E. coli* encompassing the first 192 N'-terminal or the C'-terminal 204 amino acids of pfGAPDH, respectively. Both fragments overlapped by a stretch of 59 amino acids. Separation by SDS-PAGE and staining with Coomassie blue showed that the affinity purified recombinant pfGAPDH-fragments were of the predicted molecular sizes of 24,2 kDa and 23,0 kDa, (Figure 2, lanes 2, 3). Immuno-detection with mAb 1.4 and mAb 1.10 (data not shown) demonstrated that both recognized the complete pfGAPDH (lane 1) and the N'-terminal fragment thereof (lane 3), whereas the C'-terminal fragment (lane 2) was not detected. Hence, the anti-pfGAPDH mAbs 1.4 and 1.10 recognized an epitope located within the first 192 amino acid residues of pfGAPDH.

Sub-Cellular Distribution of pfGAPDH and pfAldolase During Asexual Blood-Stage Parasite Development

In order to assess whether the two glycolytic enzymes co-localize during *P. falciparum* asexual parasite development, indirect IFA and con-focal microscopy was performed. In columns I and II of Figure 3 A, results obtained with the anti-pfGAPDH and anti-pfAldolase mAbs are shown in green and red, respectively. In column III, the super-positioning of both images given in columns 1 and 2 and in column 4 the phase contrast of the parasites is depicted. Cultured parasites were synchronized and at 10 h (row 1), 20 h (row 2), 30 h (row 3) and 40 h (row 4) post synchronization aliquots of cells were removed and analyzed. The pictures obtained at time points 10 h and 20 h demonstrated that pfAldolase and pfGAPDH were co-localized in the early stages of parasite development since both, the green and the red signals were super-imposed resulting in orange signals. Cells harvested 40 h post synchronization displayed a different distribution pattern. The green signal of the anti-pfGAPDH staining was largely confined to the periphery of the schizont while the red signal of the anti-pfAldolase staining remained distributed equally throughout the schizont. In column III of row 4, the overlay of the images showed a distinct central region stained in red and a peripheral region stained in orange. Therefore, pfGAPDH seemed to be enriched in the periphery of the developing schizont relative to pfAldolase. At the time point of 30 h this segregation process has started already as indicated by the red signal in the center of the parasites (column III, row 3).

Since the spatial separation of pfGAPDH and pfAldolase could be observed in the late schizont stage, we wanted to see whether pfGAPDH was enriched in the sub-cellular region containing the apical complex of *P. falciparum*. Single merozoites after schizont rupture were analyzed similarly as described above. In row 1 of Figure 2 B, results of IFA and con-focal microscopy using anti-pfGAPDH (green) and anti-pfAldolase (red) mAbs are shown. The overlay in Figure 3 B, column III, row 1, shows that regions stained in green and red appeared indicating that pfGAPDH and pfAldolase were preferentially localized at opposite cell poles. Additionally, regions of overlapping fluorescence signals (orange) were present. In Figure 3 B, row 2, results obtained with anti-pfGAPDH (green) and anti-RAP-1 mAbs (red) demonstrated that pfGAPDH was preferentially co-localized with RAP-1 that is associated with the apical complex of the merozoites (Clark *et al.,* 1987).

Immunolocalization Studies of pfGAPDH and pfAldolase in Liver Stage Parasites

In order to analyze whether the spatial and temporal changes in the sub-cellular distribution of pfAldolase and pfGAPDH is comparable between liver and asexual blood-stages of *P. falciparum*, human liver cells were infected with sporozoites isolated from salivary glands of infected Anopheles mosquitoes. The liver parenchymal cells were stained on days 2, 4 and 7 after infection with anti-pfGAPDH and anti-pfAldolase mAbs and analyzed by con-focal microscopy (Figure 4). In non-mature liver schizonts (days 2 and 4), both glycolytic enzymes were co-localized in the cytosol (Figure 4, rows 1 and 2). In contrast, in maturing schizonts (day 7), pfGAPDH seemed to be localized at a higher concentration in the periphery of the schizont while pfAldolase remained evenly distributed (Figure 4, row 3). Hence, the enrichment of pfGAPDH in the periphery of the developing schizont in comparison to pfAldolase was comparable to the results obtained with asexual blood stage parasites.

Sub-Cellular Fractionation of *P. falciparum* Infected Erythrocytes

The combined results obtained by IFA and con-focal microscopy of liver and asexual blood stage parasites strongly indicated that pfGAPDH and pfAldolase are not distributed

equally during schizont and merozoite stages. In order to verify these observations, cell fractionation experiments of blood stage schizonts were performed and the distribution of pfGAPDH and pfAldolase in sediment and supernatant fractions examined. Schizonts of blood stage parasites of strains K1 and FVO were enriched up to 95 - 99% purity using Percoll gradient centrifugation as described (Wahlgren *et al.,* 1983). Purified parasites were washed thoroughly, lysed by repeated cycles of freeze/thaw in water and the membrane and sediment fractions were separated by ultracentrifugation. Aliquots of these fractions were subjected to SDS-PAGE, blotted onto nitrocellulose and probed with anti-pfGAPDH and anti-pfAldolase mAbs, respectively. One specific band corresponding to pfGAPDH could be detected in both the pellet (Figure 5 A, lanes 1 and 3) and supernatant fractions (Figure 5 A, lanes 2 and 4). In contrast, the anti-pfAldolase mAb detected exclusively in the supernatant fraction one single band (Figure 5 B). Similar results were obtained in three independent experiments and indicated that a substantial fraction of pfGAPDH was associated with the membrane-containing, pelleted sub-cellular fraction of the parasite obtained after ultracentrifugation, while pfAldolase resided in the supernatant fraction.

Mammalian Rab2 Stimulates pfGAPDH Recruitment to HeLa Cell Derived Microsomal Membranes

After having established that pfGAPDH was detectable in the membrane-containing sub-cellular fraction, we wanted to investigate the functional basis of membrane recruitment of pfGAPDH. Rab2 protein and a peptide representing the N'-terminal thirteen amino acids of Rab2 (Rab2 13-mer) have been reported previously to stimulate mammalian GAPDH binding to membranes (Tisdale, 2001). Using sequence data from the *P. falciparum* genome project (http://plasmodb.org), a potential Rab2 homologue (GenBank accession number AJ 308736) was identified. This pfRab2 homologue displayed an overall 75% identity at the amino acid level with human Rab2 and was highly conserved in the domain that stimulates Rab2 dependent recruitment of soluble factors (AYAYLFKYIIIGD of human Rab2 versus PYEYLFKYIIIGD of pfRab2). We performed a quantitative binding assay to measure pfGAPDH recruitment using microsomes prepared from whole cell homogenates of HeLa cells as described (Tisdale and Balch, 1996; Tisdale, 2001). Briefly,

salt-washed HeLa microsomes were pre-incubated with increasing concentrations of Rab2 peptide or Rab2 protein supplemented with recombinant pfGAPDH for 10 min on ice. Cytosol and GTPγS were added and the membranes incubated for 15 min at 32 ºC. The membranes were collected by centrifugation and then analyzed by SDS-PAGE and Western blotting using anti-pfGAPDH mAb 1.4 to assess the level of membrane-bound pfGAPDH. As shown in Figure 6 A, Rab2 peptide and Rab2 protein promoted pfGAPDH membrane association in a dose-dependent manner. The amount of membrane bound pfGAPDH increased ~ 5-fold when incubated with 100 μM of Rab2 peptide. In order to confirm that mAb 1.4 does not cross-react with human or rat GAPDH also present in the assay, HeLa microsomes, rat liver cytosol and recombinant pfGAPDH were separated by SDS-PAGE, transferred to nitrocellulose and immuno-blotted. The results confirmed that mAb 1.4 lacks cross-reactivity with rat liver or human GAPDH (Figure 6 B) and indicated that Rab2 protein and Rab2 peptide stimulated pfGAPDH recruitment to membrane in a dose-dependent manner.

The N'-Terminal Fragment of pfGAPDH Binds to Microsomal Membranes in Response to Rab2

After establishing that pfGAPDH was recruited to microsomal membranes in response to Rab2 and Rab2 peptide, we investigated which domain of pfGAPDH was involved in this interaction. Salt-washed HeLa microsomes were pre-incubated with increasing concentrations of Rab2 peptide and Rab2 protein supplemented with recombinant pfGAPDH N'-terminal domain (residues 1 - 192) for 10 min on ice. Cytosol and GTPγS were then added, and the microsomes incubated. The membranes were collected by centrifugation, separated by SDS-PAGE and immuno-blotted. Results shown in Figure 7 A demonstrated that Rab2 and Rab2 peptide stimulated membrane recruitment of the N'-terminal fragment in a dose-dependent manner. Next we wanted to learn whether the N'-terminal fragment could effectively compete for membrane binding with the full-length recombinant pfGAPDH. Therefore, salt-washed microsomes were pre-incubated with Rab2 peptide or Rab2 protein and supplemented with the indicated concentrations of N'-terminal fragment for 10 min on ice (Figure 7 B). Recombinant pfGAPDH at a constant concentration, cytosol and GTPγS were added and the membranes incubated as described

above. To terminate the reaction, membranes were collected by centrifugation and then analyzed as above. Results demonstrated that the N'-terminal fragment competed with recruitment of the full-length pfGAPDH (Figure 7 B). In contrast, the C'-terminal fragment of pfGAPDH (encompassing residues 133 - 337) did not interfere with the ability of Rab2 or Rab2 peptide to recruit full-length recombinant pfGAPDH to membrane (Figure 7 C). In summary, these results indicated that the N'-terminal fragment of pfGAPDH contains the domain leading to Rab2 dependent recruitment to HeLa cell derived microsomal membranes.

Discussion

In this report we provide evidence that GAPDH of *P. falciparum* is partially segregated in the late stages of parasite development from the cytosol, suggesting additional non-glycolytic function(s) of this enzyme. First, using IFA and con-focal microscopy, we found that the two glycolytic enzymes pfGAPDH and pfAldolase co-localize in early stages of both liver and asexual blood-stage parasite development. However, during schizogony pfGAPDH was in comparison to pfAldolase enriched in the periphery of the developing parasites. Hence, pfGAPDH might be concentrated during schizogony in the same sub-cellular region where vesicular-tubular clusters and the developing apical complex are observed (Bannister *et al.,* 2000). Second, sub-cellular fractionation experiments demonstrated that pfGAPDH was in contrast to pfAldolase easily detected in both the membrane-containing pellet and the supernatant fractions. Third, a quantitative microsomal recruitment assay showed that pfGAPDH and the N'-terminal domain thereof were recruited to HeLa cell derived microsomes in a Rab2 dependent manner.

These results are consistent with other reports showing that GAPDH exerts non-glycolytic functions in mammalian cells (Sirover, 1999). Protein transport in the early secretory pathway of mammalian cells requires Rab2 (Tisdale *et al.,* 1992; Tisdale and Balch, 1996; Tisdale and Jackson, 1998; Tisdale, 1999). This protein immuno-localizes to pre-Golgi intermediates and mediates the recruitment of soluble factors including GAPDH to these structures (Tisdale, 2001). The amino terminus of Rab2 (residues 1 - 13) is essential for Rab2 activity. Deletion of these residues result in loss of function, whereas the Rab2 peptide mimics the downstream enlistment of accessory proteins similar to the intact Rab2 protein (Tisdale and Balch, 1996; Tisdale and Jackson, 1998). In the light of the sub-cellular fractionation experiments and the evolutionary conservation of the functional domain of pfRab2, we investigated whether recombinant pfGAPDH could be recruited to HeLa cell microsomes after incubation with mammalian Rab2. Our results suggested that the Rab2 mediated recruitment of GAPDH to membrane is also operating in *P. falciparum*. Results with fragments of pfGAPDH demonstrated further that the N'-terminal fragment of pfGAPDH encompass the domain interacting with Rab2 leading to membrane recruitment.

Several independent groups reported that GAPDH is involved in membrane fusion and trafficking (Lopez Vinals *et al.,* 1987; Han *et al.,* 1998; Hessler *et al.,* 1998; de Arcuri

et al., 1999; Bressi *et al.,* 2001; Glaser *et al.,* 2002) and in the modulation of the cytoskeleton by promoting actin polymerization and microtubule bundling (Kumagai and Sakai, 1983; Reiss et al., 1996; Tisdale, 2002). These observations lead to the hypothesis that mammalian GAPDH is recruited to tubulin associated vesicles in a Rab- and GTP-dependent manner. Subsequently, phosphorylation results in a conformational change of GAPDH altering its interactions with tubulin and facilitating membrane fusion by de-inhibition of GAPDH. After membrane fusion, GAPDH is released from the vesicles in an ATP- dependent manner followed by de-phosphorylation of GAPDH (Glaser et al., 2002). Our data indicate that the proposed mechanism of GAPDH involvement in the secretory system of mammalian cells might be conserved in apicomplexan organisms. This is particularly interesting since the phylum apicomplexa is most closely related to the ciliates and dinoflagellates, branching off from the eukaryotic lineage prior to the divergence of animals, fungi and plants (Roos *et al.,* 1999).

What could be the possible non-glycolytic role of pfGAPDH during parasite development? It is tempting to speculate that the distribution of pfGAPDH in schizont and merozoite stages in the sub-cellular region close to the apical complex implicates that pfGAPDH could be involved in the fusion of vesicles during biogenesis of the organelles of the apical complex. Alternatively, pfGAPDH might participate in the interaction between vesicular or intracellular compartment(s) with the cytoskeleton mediating the positioning of the apical complex during its biogenesis.

In malaria parasites, no energy reserves are stored and almost all metabolized glucose passes through the anaerobic Embden-Meyerhoff-Parnas pathway resulting in a 100-fold increase in glucose consumption of infected RBC versus uninfected cells (Roth, Jr. *et al.,* 1988). The obligate dependence on glycolysis for ATP production combined with the possible involvement of pfGAPDH in other biological functions renders this conserved, single copy gene product a particularly attractive target for malaria drug development. PfGAPDH has only limited (63.5%) amino acid sequence identity with human erythrocytic GAPDH. Certain sequence features at the active site (an insertion at position 206 and an amino acid exchange at position 198) are unique for the parasite enzyme and suggest that selective inhibitors of pfGAPDH could be developed (Daubenberger *et al.,* 2000). This is supported by the successful development of anti-trypanosomatid parasite drugs like

adenosine analogues as potent and selective GAPDH inhibitors following a structure based drug design approach (Bressi *et al.,* 2001; Verlinde *et al.,* 2001).

Materials and Methods

Expression and Purification of Recombinant pfGAPDH and N'- and C'-Terminal Fragments Thereof

The complete cDNA of pfGAPDH was amplified from a cDNA library of *P. falciparum* strain K1 by PCR using primers 5'-GATGATCGCCCATATGGCAGTAACAAAACTTGGAATTAATGG-3' and 5'-GCCGTACACATTACTAACAACTAACTCGAGTAAACATG-3' containing *Nde*I and *Xho*I sites as described (Daubenberger *et al.,* 2000). For the amplification of the N- and C'-terminal fragments of pfGAPDH the following primer combinations were used: 5'-GATGATCGCCCATATGGCAGTAACAAAACTTGGAATTAATGG-3' and 5'-TTGTTGATGGTCCATAACTCGAGGGTAAGGACTGGAGAGC-3'; 5'-CCCCAATTTATCATATGGGTATTAACCACCACC-3' and 5'-GCCGTACACATTACTAACAACTAACTCGAGTAAACATG-3', respectively. The amplicons were digested with *Nde*I and *Xho*I, gel purified and then cloned in frame into the bacterial expression vector pET28a+ (Novagen). Competent *E. coli* BL21 (DE3) cells (Novagen) were transformed with the recombinant pET28a+ plasmids and expression of the His^6-tagged fusion protein was induced by the addition of 1 mM isopropyl thiogalactoside (IPTG) (Calbiochem) at 0.6 OD_{600}. *E. coli* cells expressing recombinant protein were collected by centrifugation and resuspended in lysis buffer (50 mM NaH_2P0_4, 300 mM NaCl, 10 mM imidazole) with 1 mg/ml lysozyme (Appligene Oncor) for 30 min. on ice and sonicated. After centrifugation at 10 000 g the supernatant was loaded onto a Ni^{2+}-NTA agarose column (Qiagen) and purified according to the manufacturer's instructions. The recombinant protein was recovered using elution buffer (50 mM NaH_2P04, pH 8.0, 300 mM NaCl, 500 mM imidazole). After analysis by SDS-PAGE and Western blotting, purified proteins were pooled, dialyzed against a buffer containing 25 mM Tris / 500 mM $(NH_4)_2SO_4$ / 0,1 mM NAD^+ / 2 mM TCEP / 2 mM DTT and stored at 4 ºC. The recombinant proteins differed from the predicted natural parasite protein by the additional N'-terminal amino acid sequence MGSSHHHHHHSSGLVPRGSH.

Generation of Hybridoma Cell Lines Producing anti-pfGAPDH and anti-pfAldolase Antibodies

Mice were immunized with recombinantly expressed pfGAPDH (Daubenberger *et al.,* 2000) or pfAldolase (Döbeli *et al.,* 1990) and hybridoma cell lines were generated essentially as described (Pluschke *et al.,* 1998). The hybridoma cell lines secreting anti-pfAldolase mAb were generated from Balb/c mice and contained a κ light and γ1 heavy chains. In contrast, hybridoma cell lines producing anti-pfGAPDH antibodies were generated from mice carrying immunoglobulin heavy chain γ2a and light chain κ replacement mutations (Pluschke *et al.,* 1998). Fusion with these mice yielded either mouse λ light chain or human κ light chain containing mAbs. These mAbs are ideally suited for double staining immunofluorescence analyses in conjunction with mouse κ chain carrying mAbs obtained from un-mutated mouse strains. Accordingly, the anti-pfGAPDH mAbs 1.4 and 1.10 express IgG2a:λ and the anti-pfAldolase mAbs P.41-1/2-7 and P.41-2/3-7 carry IgG1: κ chains, respectively. MAbs were purified from hybridoma supernatant by affinity chromatography using HighTrap™ Protein A following the manufacturer's instructions (Amersham Pharmacia Biotech). The anti-RAP-1 mAb 5-2 (IgG1:κ) has been described previously (Moreno *et al.,* 2001).

Cultivation and Sub-Cellular Fractionation of *P. falciparum* Blood Stage Parasites and Western Blot Analysis

P. falciparum strain 3D7 parasites were cultured and synchronized using standard methods (Matile and Pink, 1990) with two sorbitol synchronization steps one cycle before harvesting. Synchronization was confirmed and the level of parasitemia estimated by standard microscopy. SDS-PAGE was performed essentially as described (Daubenberger *et al.,* 2000). Briefly, total cell lysates of cultures of *P. falciparum* were harvested and washed with phosphate-buffered saline, pH 8,0. The cells were lysed in 10 ml of 0.15% saponin in SSC (150 mM, NaCl, 15 mM sodium citrate) and haemoglobin-depleted iRBC were collected by centrifugation at 2000 *g* for 20 min and separated on 12% SDS-PAGE, and then transferred electrophoretically to nitrocellulose membrane (Protean Nitrocellulose, BA 85, Schleicher & Schuell). Blots were incubated with blocking buffer

(0.1% Tween / 5% skimmed milk powder in PBS) and then incubated with purified anti-GAPDH mAbs 1.4, mAb 1.10 and anti-pfAldolase mAb P.41-2/3-7 for 1 h. After several washing steps, blots were incubated with appropriately diluted goat anti-mouse IgG alkaline phosphatase conjugated antibodies and developed using BCIP (5-bromo-chloro-3-indolylphosphate, Biorad) and NBT (nitrobluetetrazolium, Biorad) (Figure 1). Alternatively, blots were incubated with appropriately diluted goat anti-mouse peroxidase conjugated antibodies (Sigma) developed using ECL system following manufacturer's instructions (Figure 2).

For sub-cellular fractionation experiments, late stage infected erythrocytes were enriched to 95 - 99% using 60% Percoll gradient as described (Wahlgren *et al.,* 1983). Cells were washed three times in phosphate-buffered saline and lysed by three cycles of freeze/thaw in 10 volumes of double-distilled water. Total pellet and supernatant fractions were collected by ultracentrifugation at 100 000 g for 1 h at 4°C. Samples were harvested and solubilized in denaturing SDS sample buffer and equivalent amounts of each sample were subjected to Western blot analysis. Western blot analysis was conducted with anti-pfGAPDH mAb 1.4 and anti-pfAldolase mAb P.41-2/3-7. The blots were developed using the ECL system.

Indirect Immunofluorescence Analysis of Asexual Blood-Stage Parasites

IFA of blood-stage parasites was conducted essentially as described (Daubenberger *et al.,* 2000). *P. falciparum* cultures were synchronized twice and aliquots of cells were removed for staining every 10 h. Multi-test immunofluorescence microscopy slides (Flow Labs, Switzerland) were pre-treated with 0.01% (w/v) poly L-lysine (Sigma) for 30 min at room temperature and washed. Infected RBC were washed and mixed with two volumes of a solution containing 4% paraformaldehyde and 0.1% Triton X-100. Droplets of 30 µl cell suspension were added to each well and incubated for 30 min at room temperature. Cells were blocked with 100 mg/ml fatty acid-free BSA in PBS. Cells were incubated with an appropriate dilution of anti-GAPDH mAb 1.4 and anti-pfAldolase mAb P.41-2/3-7 and after several washing steps, cells were incubated with secondary antibodies specific for mouse κ or λ immunglobulin light chains conjugated with TXRD and FITC, respectively (Southern Biotechnology Associates). The immuno-reactivity was observed using a Leica

TCS NT con-focal microscope. Images were acquired with a 63x Plan-Apochromat oil immersion objective (NA 1.32). Pinhole settings were 1 airy unit for all images that were processed with Imaris (Bitplane, Switzerland) and Adobe Illustrator and Adobe Photoshop (Mountainview, USA).

Immunofluorescence Analysis of Liver Stage Parasites

Primary cultures of human hepatocytes were prepared from liver segments taken from adult patients during partial hepatectomy. Hepatocytes were isolated using the two-step enzymatic perfusion technique with minor modifications (Guguen-Guillouzo *et al.,* 1982). Briefly, the hepatic cells were successively perfused with HEPES buffer and 0.05% collagenase D (Roche), dissociated, and viable cells were isolated on a 36% Percoll gradient. Cells were seeded at density of 1.4 x 10^5 per cm^2 in eight-chamber permanox Lab-Tek culture slides (Nalge Nunc International) coated with rat tail collagen I (Beckton-Dickinson) and incubated at 37°C in 4% CO_2 atmosphere. Hepatocytes were cultivated in Williams medium E (Life Technologies) supplemented with 10% fetal calf serum (Life Technologies), 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (Bio-Whittaker), 10 mg l^{-1} insulin (Sigma), 200 U ml^{-1} penicillin and 200 µg ml^{-1} streptomycin (Life Technologies). After complete adherence of the cells, culture medium was replaced by fresh medium supplemented with 10^{-7} M dexamethasone (Sigma). *P. falciparum* NF54 strain sporozoites obtained by aseptic dissection of infected *Anopheles stephensi* salivary glands were co-cultured with hepatocytes essentially as described (Ponnudurai *et al.,* 1982). After the indicated time periods cultures were washed, fixed in cold methanol and the liver schizonts were labeled with mAb as described for the blood-stage parasites. Samples were analyzed by con-focal microscopy.

Quantitative Microsomal-Binding Reaction

HeLa cells were washed three times with ice-cold PBS. The cells were scraped off the dish with a rubber policeman into 10 mM Hepes (pH 7.2) and 250 mM mannitol, then broken with 15 passes of a 27 gauge syringe. The broken cells were pelleted at 500 *g* for 10 min at 4°C, and the supernatant removed and re-centrifuged at 20,000 *g* for 20 min at

4°C. The pellet containing ER, pre-Golgi, and Golgi membranes was washed with 1 M KCl in 10 mM Hepes, (pH 7.2) for 15 min on ice to remove peripherally associated proteins, then centrifuged at 20,000 *g* for 20 min at 4ºC. The membranes were resuspended in 10 mM Hepes (pH 7.2) and 250 mM mannitol and employed in the binding reaction (Tisdale, 2001). Membranes (30 µg of total protein) were added to a reaction mixture that contained 27.5 mM Hepes (pH 7.2), 2.75 mM MgOAc, 65 mM KOAc, 5 mM EGTA, 1.8 mM $CaCl_2$, 1 mM ATP, 5 mM creatine phosphate, and 0.2 U of rabbit muscle creatine kinase. Recombinant Rab2, Rab2 (13-mer), recombinant pfGAPDH, and N'-terminal and C'-terminal pfGAPDH domains were added at the concentrations indicated under "Results" and the reaction mix incubated on ice for 10 min. Rat liver cytosol (50 µg) and 2.0 µM GTPγS were then added, and the reactions shifted to 32 °C and incubated for 15 min. The binding reaction was terminated by transferring the samples to ice and then centrifuged at 20,000 *g* for 10 min at 4 °C. The pellet was resuspended in sample buffer, separated by SDS-PAGE and transferred to nitrocellulose in 25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol. The blot was blocked in TBS which contained 5% non-fat dry milk and 0.5% Tween-20, incubated with a mAb 1.4 made to pfGAPDH, washed, further incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse antibody, developed with enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL), and then quantified by densitometry.

Acknowledgments

We acknowledge the organizations involved in generating the Plasmodium genome sequence and for making their data available in public databases prior to publication. We thank Christian Scheurer for supply of parasite cultures, Laurent Hannoun and Jean-François Franetich for the hepatocyte cultures, and Eric Rubinstein for con-focal microscopy of liver stages. This work was supported by a grant from the Swiss National Science Foundation to C.A. Daubenberger (3100-061513.00). EJT is supported by a grant from the American Heart Association-Midwest Affiliate (0030385Z).

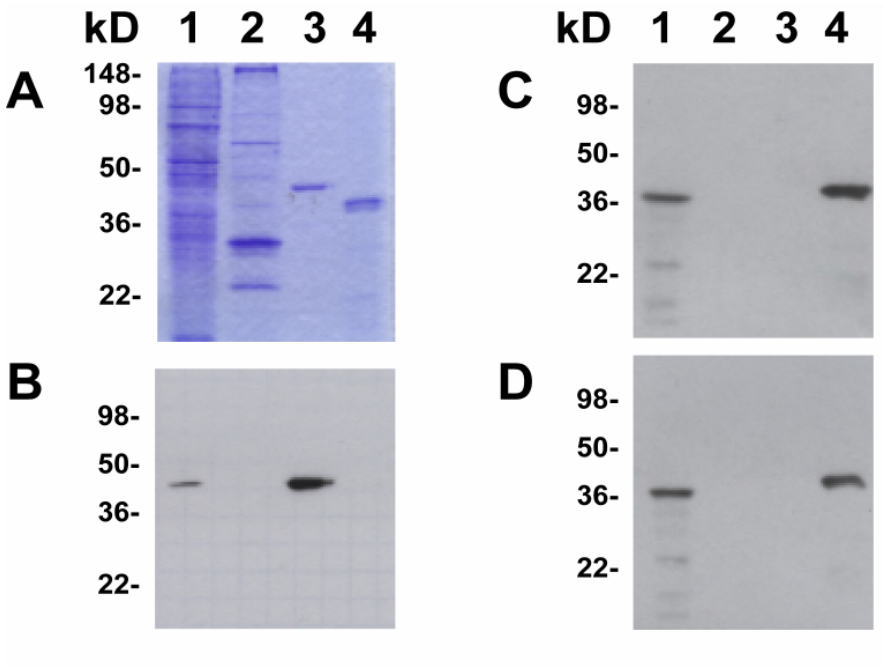


Figure 1 Specificity of anti-pfGAPDH and anti-pfAldolase monoclonal antibodies

The specific reactivity of the antibodies was analyzed with total lysates of infected (lane 1), and uninfected RBC (lane 2), purified recombinant pfAldolase (lane 3) and recombinant pfGAPDH (lane 4).

(A) Proteins were separated by SDS-PAGE and stained with Coomassie blue. The molecular weight in kDa is given on the left. (B) Western blot analysis with anti-pfAldolase mAb P.41-2/3-7. MAb P41-2/3-7 recognized a single band of ~ 41 kDa in iRBC (lane 1) and the recombinant pfAldolase protein (lane 3). In contrast, no band was detected in lysates of uninfected RBC and recombinant pfGAPDH, respectively (lanes 2, 4). Data obtained with anti-pfAldolase mAb P.41-1/2-7 were identical (data not shown). (C) and (D) Western blot analysis with anti-pfGAPDH mAbs 1.4 (C) and 1.10 (D). Both mAbs recognized a single band of ~ 36 kDa in iRBC (lane 1) and the slightly larger hexahistidine-tagged recombinant pfGAPDH protein (lane 4). In contrast, no band was detected in uninfected RBC (lane 2) and no cross-reactivity was observed with the recombinant pfAldolase protein (lane 3).

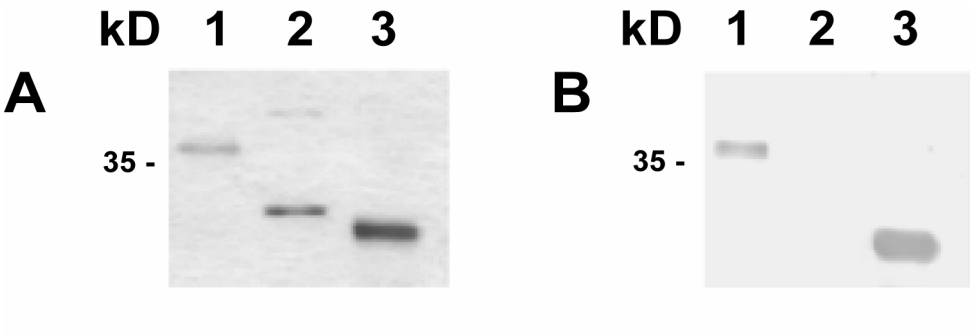


Figure 2 Recombinant expression of a C'-terminal and a N'-terminal fragments of pfGAPDH.

(A) Complete recombinant pfGAPDH (lane 1), a C'-terminal fragment (lane 2) and a N'-terminal fragment (lane 3) was fractionated by SDS-PAGE and stained with Coomassie blue. The molecular weight of the proteins representing the complete pfGAPDH and the fragments thereof were 36 kDa, 24,2 kDa and 23,0 kDa as predicted. The molecular weight in kDa is given on the left. (B) Western blot analysis using anti-pfGAPDH mAb 1.4. This mAb recognized the complete pfGAPDH and its N'-terminal fragment (lanes 1, 3), but not the C'-terminal fragment (lane 2).

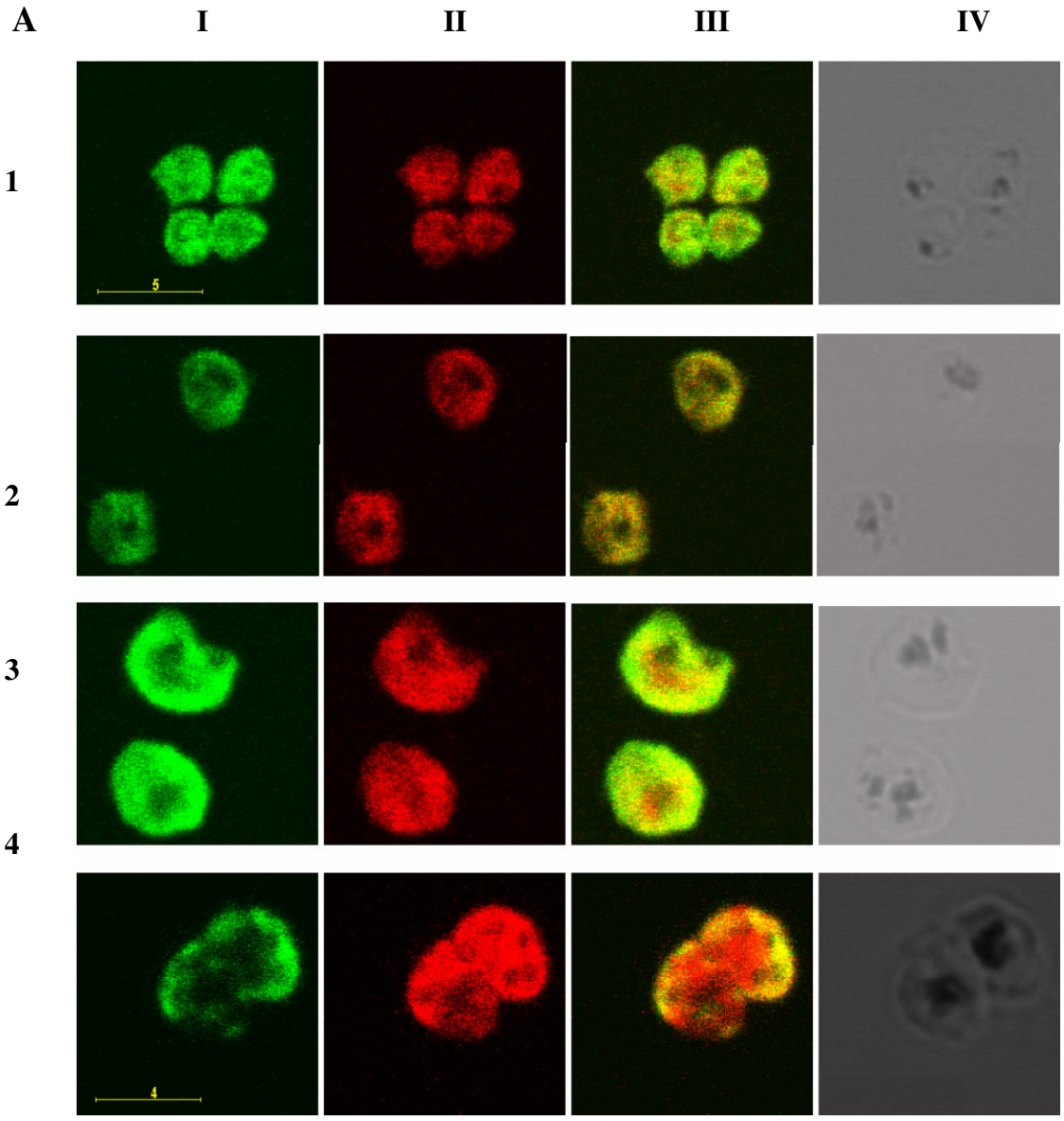
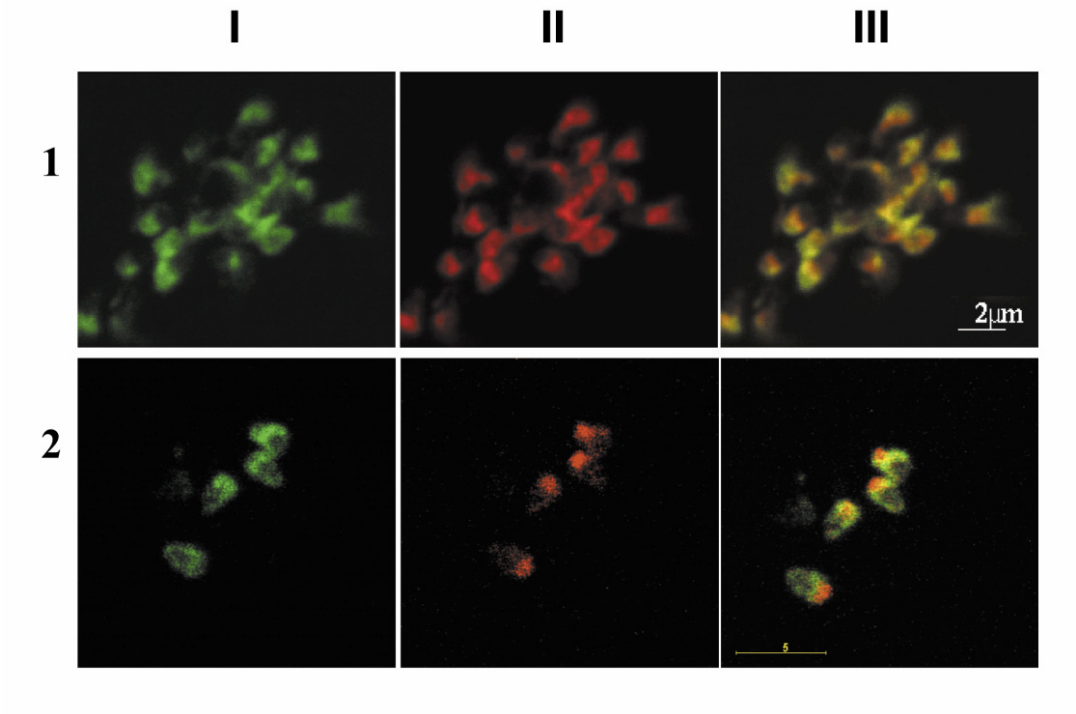


Figure3 Temporal and spatial changes in sub-cellular distribution of pfAldolase and pfGAPDH during asexual blood-stage development.

(A) IFA of synchronized blood-stage parasites using anti-pfGAPDH and anti-pfAldolase mAbs. Cells were removed from culture at 10 h (row 1), 20 h (row 2), 30 h (row 3) and 40 h (row 4) after synchronization and stained with anti-pfGAPDH and anti-pfAldolase mAbs followed by incubation with FITC-labeled or Texas red-labeled secondary antibodies specific for λ or κ mouse immunglobulin light chains, respectively. The images are: column I, anti-pfGAPDH (green); column II, anti-pfAldolase (red), column III, merged images derived from columns I and II; column IV, phase contrast images of the parasites.

B



(B) IFA of merozoites stained with mAb specific for pfGAPDH, pfAldolase and RAP-1. Row 1 demonstrates merozoites stained with anti-pfGAPDH mAb (green, column I), anti-pfAldolase mAb (red, column II) and the overlay of both images (column III).

Row 2 shows merozoites stained with anti-pfGAPDH mAb (green, column I) and anti-RAP-1 mAb 5-2 (red, column II) and the overlay of both images (column III).

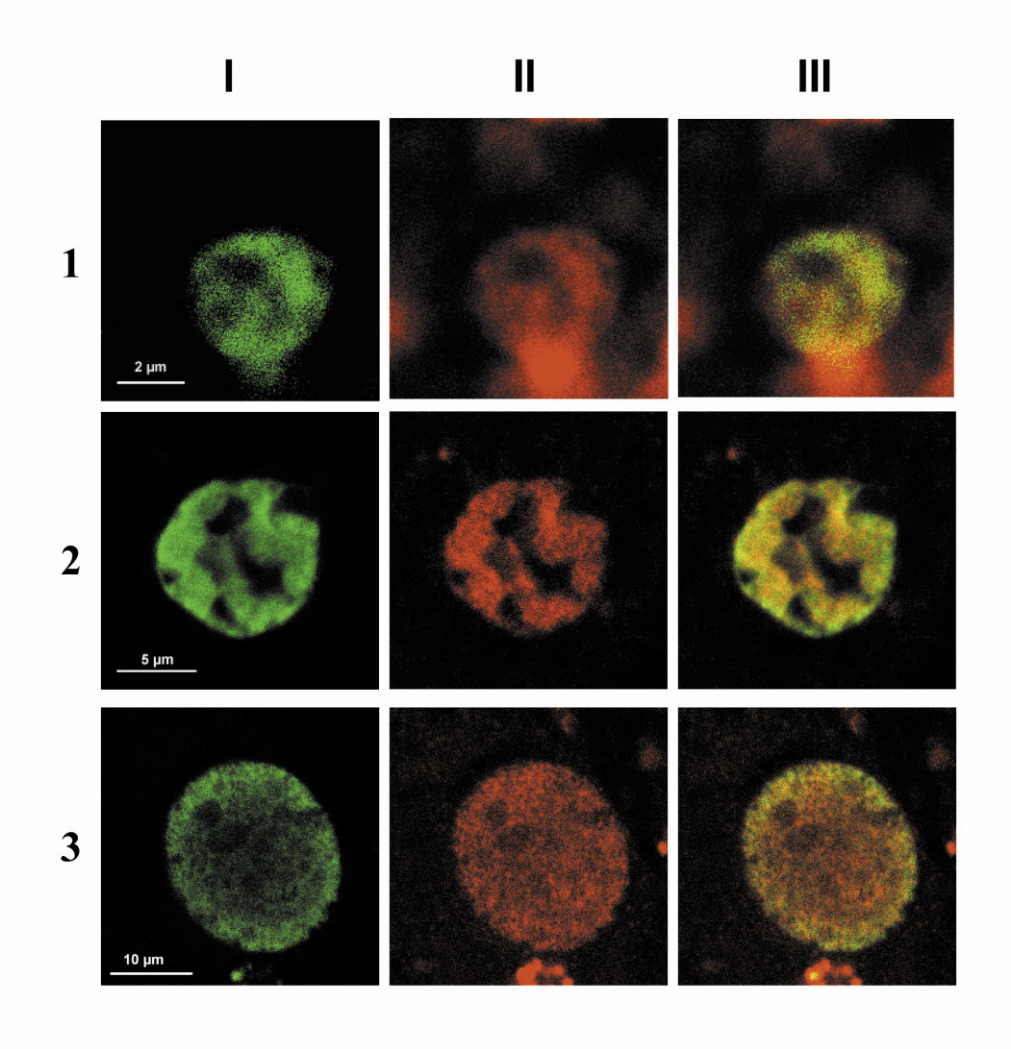


Figure 4 Spatial and temporal distribution of pfAldolase and pfGAPDH during liver stage development.

Human hepatocytes were infected with sporozoites of *P. falciparum* and incubated for 2 (row 1), 4 (row 2) or 7 (row 3) days *in vitro*. Cells were stained with mAbs as described in Figure 3 A. In column I, staining with anti-pfGAPDH mAb (green), in column II with anti-pfAldolase mAb (red) and in column III the super-positioning of both images is depicted.

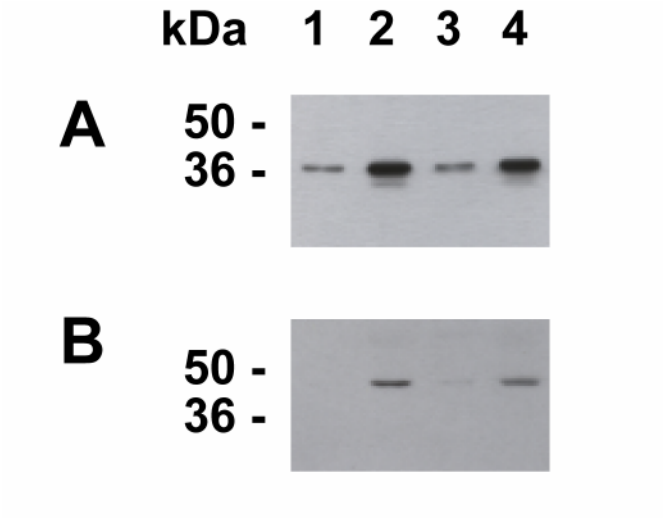


Figure 5 Association of pfGAPDH but not pfAldolase with the sediment fraction of blood stage parasite lysates.

Schizonts of *P. falciparum* strains K1 and FVO were enriched by Percoll-gradient centrifugation and hypotonically lysed by repeated cycles of freeze/thaw in water. The sediment fractions of K1 (lane 1) and FVO (lane 3) and the corresponding supernatant fractions of K1 (lane 2) and FVO (lane 4) were obtained by ultracentrifugation. Aliquots of the samples were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-pfGAPDH mAb 1.4 (Figure 5 A) or anti-pfAldolase mAb P.41-2/3-7 (Figure 5 B).

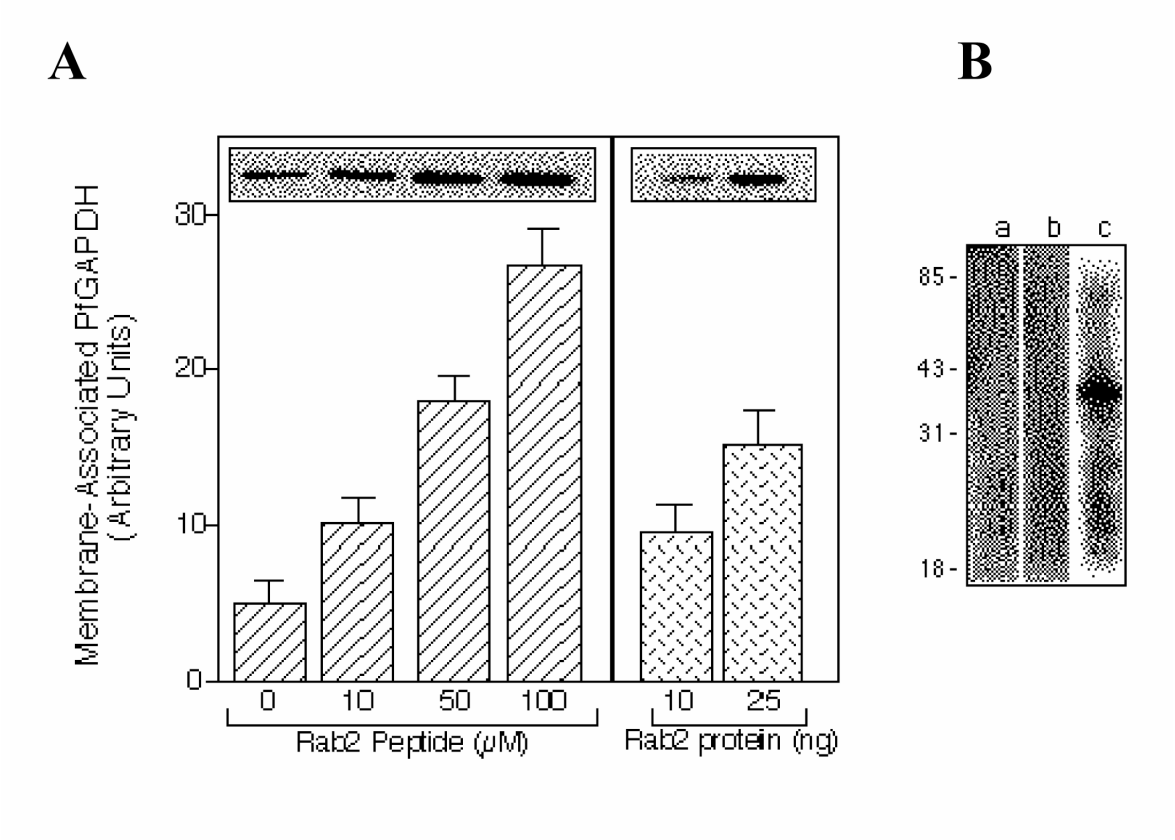


Figure 6 Rab2 (13-mer) and Rab2 protein stimulate pfGAPDH recruitment to HeLa microsomes.

(A) Rab2 peptide and Rab2 protein promote pfGAPDH membrane association in a dose-dependent manner. Salt-washed HeLa microsomes were pre-incubated with increasing concentrations of Rab2 (13-mer) or Rab2 protein supplemented with 10 ng recombinant pfGAPDH for 10 min on ice. Cytosol and GTPγS were then added and the membranes incubated. Membranes were collected, separated on SDS-PAGE and transferred to nitrocellulose. The blot was probed with anti-pfGAPDH mAb 1.4. The amount of recruited pfGAPDH was quantified by densitometry and the results are the mean $\pm$ S.D. of three independent experiments performed in duplicate.

(B) The anti-pfGAPDH mAb 1.4 does not cross-react with human or rat GAPDH. HeLa cell microsomes (a), rat liver cytosol (b), and recombinant pfGAPDH (c) were separated by SDS-PAGE, transferred to nitrocellulose membrane and the blot probed with anti-pfGAPDH mAb 1.4.

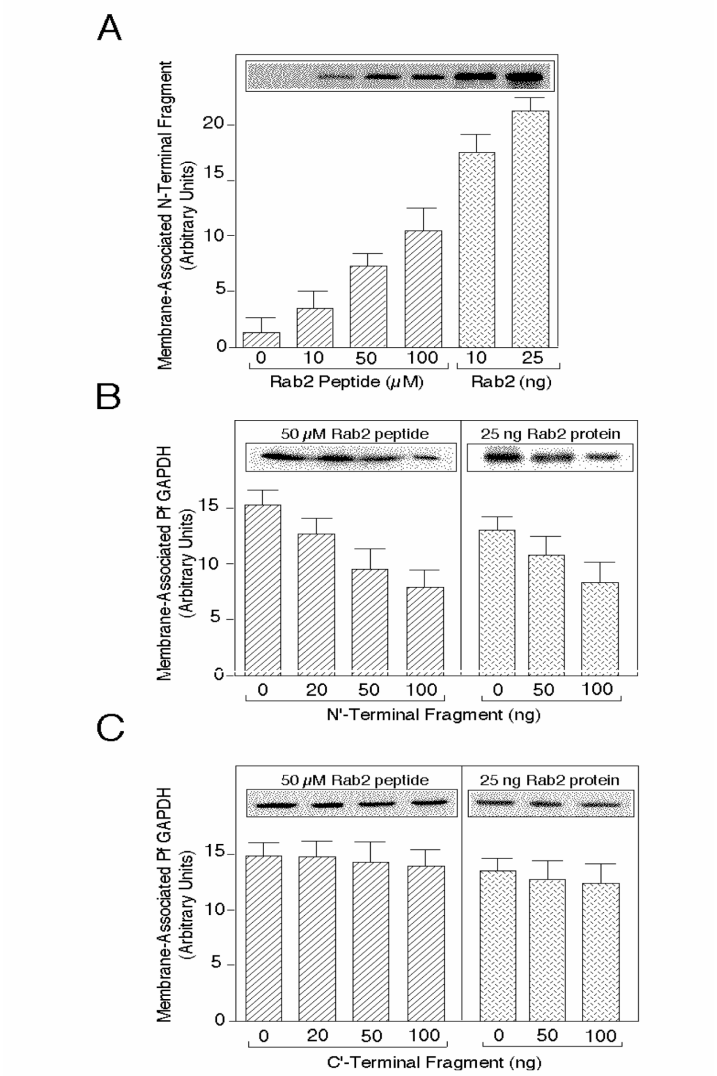


Figure 7 The N'-terminal pfGAPDH fragment binds to HeLa microsomes and blocks Rab2 dependent recruitment of pfGAPDH.

(A) Rab2 and Rab2 peptide promote recruitment of N'-terminal pfGAPDH fragment to HeLa cell membrane. Salt-washed HeLa microsomes were pre-incubated with increasing concentrations of Rab2 peptide and Rab2 protein supplemented with 10 ng of the recombinant N'-terminal pfGAPDH fragment (residues 1 - 192). Cytosol and GTPγS were then added and the membranes incubated for 15 min. To terminate binding, the membranes were centrifuged, and the membrane pellet separated by SDS-PAGE and transferred to nitrocellulose. The blot was probed with anti-pfGAPDH mAb 1.4. The amount of recruited N'-terminal pfGAPDH fragment was quantified by densitometry and the results

represent the results represent the mean ± S.D. of three independent experiments performed in duplicate.

(B) The N'-terminal pfGAPDH fragment competes with full-length pfGAPDH for membrane-association in response to Rab2.

Salt-washed HeLa microsomes were pre-incubated with Rab2 peptide or Rab2 protein, 10 ng recombinant pfGAPDH and increasing concentrations of the N'-terminal pfGAPDH fragment for 10 min. Cytosol and GTPγS were added and membranes incubated for 15 min, then analyzed as above. The amount of membrane-associated pfGAPDH was detected by mAb 1.4.

(C) The C'-terminal pfGAPDH fragment does not interfere with Rab2-stimulated membrane recruitment of pfGAPDH. Same protocol as described in (B) was applied using the C'-terminal pfGAPDH fragment. The amount of membrane-associated pfGAPDH was detected with mAb 1.4.

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Chapter 7

DISCUSSION

7. Discussion

7. 1 Monoclonal antibodies: important tool in biomedical research and applications

Antibodies are host proteins that represent one of the principal effectors of the adaptive immune system. They are produced in response to molecules or organisms which they eliminate and/or neutralize. The ability of antibodies to bind to the antigen with a high degree of affinity and specificity has led to their use in research, diagnostics and in the treatment of diverse diseases.

Most antigens are highly complex, and present various epitopes recognized by a large number of lymphocyte receptors. Activate B lymphocytes differentiate into antibody secreting plasma cell clones, and the resulting antibody response is usually polyclonal. In contrast, monoclonal antibodies (mAbs) are produced by a single B lymphocyte clone. These were first recognized in sera of patients with multiple myeloma in which clonal expansion of malignant plasma cells produced high levels of an identical antibody resulting in a monoclonal gammopathy. In 1975, Köhler and Milstein devised a technique for generating mAbs *in vitro*[1].

The decision whether to use polyclonal antibodies or mAbs as laboratory reagent depends on a number of factors, the most important of which is its intended use. Polyclonal antibodies can be generated much more rapidly at less expense and technical skill that is required to produce mAbs. However, with mAbs it is possible to target unknown molecules, undetectable with polyclonal antibodies. Moreover, mAbs are homogeneous and available in a limitless supply, and their monospecificity is useful in evaluating changes in molecular conformation, protein-protein interactions and phosporylation states, as well as in identifying single members of protein families. Also, mAbs are acquiring more and more importance as therapeutic agents. There are more than twelve mAbs licensed for therapeutic use, including two that are labelled with radionuclides to deliver tumoricidal radiation (Ibritumomab-tiuxetan-^{90}Y, and Tositumomab-^{131}I, anti-lymphoma). The other commercial mAbs are used in the treatment of asthma, autoimmune diseases, cancer, respiratory syncytial virus, and preventing organ rejection[2].

More than one hundred thousand scientific papers are available on mAbs, clearly showing that these antibodies (as a reagent) have contributed directly or indirectly to many

scientific discoveries. In research, they are employed for analysis, purification, enrichment, and to mediate or modulate physiological responses. Basic techniques such as immunoblots, enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunospot assay (ELISPOT), immunofluorescence analysis (IFA) and immunoprecipitation are examples in which mAbs are used to establish whether an antigen or related molecule is present in a biological samples (i.e., body fluid, cell suspension or tissue lysate) (Chapter 2-6). Combinations of these analytic techniques and mAbs has been successfully used in diagnosis of diseases such as hematological malignancies[3,4], also in the monitoring of the disease[5], and the detection of many cancers[6]. MAbs are also tools to identify and purify target antigens for serodiagnosis and vaccine candidates[7-10] (Chapter 4). They have been used, at the same time, as fundamental tools for both functional (neutralize activity and activate signaling) and molecular biology studies of target proteins.

Within the framework of the present thesis, mAbs were generated to study *M. ulcerans* and *P. falciparum* antigens, allowing the identification and characterization of protein targets as candidates for serological tests, molecular fingerprinting and/or vaccine design.

7.2 Buruli ulcer

In contrast with the existing knowledge on tuberculosis and leprosy, and despite that research on *M. ulcerans* has intensified in the last years, many aspects of Buruli ulcer are still incompletely understood. The identification and characterization of immunodominant antigens of *M. ulcerans* may contribute to our knowledge in its transmission, micro-epidemiology, and immune protection.

7.2.1. Immune protection and vaccine design

It is thought that antibodies play no significant role in protection against intra-cellular mycobacterial infections such as tuberculosis and leprosy. Therefore humoral responses have drawn little attention in the field of vaccine design against these diseases. Traditionally, most of our knowledge on antibody responses during mycobacterial infections has been derived from serodiagnostic studies.

The advances in recombinant DNA technology have facilitated the identification of several major antigens of *M. tuberculosis* and *M. leprae*. Young *et al.* reported the first breakthrough in this field by using a lambda gt11 phage expression system for efficient expression of mycobacterial DNA as fusion proteins in *E. coli*. They screened the mycobacterial libraries with antibody probes and succeeded in identifying several recombinant mycobacterial antigens[11]. Testing these antigens with T-cell lines and clones showed that human T-cell recognized most of them as well[9,12-20]. Several studies suggest that a number of these *M. tuberculosis* immunodominant antigens have potential as candidate antigens for a new vaccine against tuberculosis[21]. The most extensively studied antigens used in developing a new vaccine against tuberculosis are Heparin-Binding Hemagglutinin[8], hsp65, Ag85[22], ESAT-6[23], MPT64 and 38-kDa antigen. Recombinant antigens, synthetic peptides, recombinant live vaccines and recombinant plasmids for DNA vaccines have all been tried out to protect against tuberculosis in experimental animals.

Unlike other mycobacteria, *M. ulcerans* remains extracellular throughout the infection[24] and stimulates antibody responses[25-29], therefore the potential of antibodies in controlling the infection is higher. An important question about the pathogenesis of the disease concerns T-cell down-regulation. Buruli ulcer is characterized by a down-regulation of Th-1 responses, potentially associated with the cytopathic activity of mycolactone[26-28,30]. Spontaneous healing is frequently observed in late stages of *M. ulcerans* diseases. It is currently not clear, how the host eventually overcomes immune suppressed and develops a protective immune response? The explanation could be that the mycobacterium is neutralized by an antibody response that develops slowly over the course of the disease. However, antibody responses against mycolactone have not been observed in Buruli ulcer patients. Future studies in humoral response against recombinant immunodominant antigens identified in the present work may help to understand the role of antibodies in the control of the disease. Passive immune protection experiments in a mouse model with the mAbs generated in this thesis may help to give insight into the potential role of antibody responses against mycobacteria. If antibodies play a major role, it may in the end be easier to develop a vaccine against *M. ulcerans* disease than against leprosy or tuberculosis.

7.2.2. Identification of polymorphic immunodominant proteins and molecular fingerprinting of M. ulcerans isolates

The antigenic overlap among mycobacterial species has limited the studies of antibody responses against mycobacterial pathogens, of the immunopathogenesis of mycobacterial diseases, and the development of serological tests (diagnosis, mycobacteria exposure and evaluation of new vaccine). All humans are exposed to water- and air-borne environmental mycobacteria, which frequently come into contact with the skin and mucous membranes (digestive and respiratory epithelia). In addition, many children, particularly in developing countries, are inoculated with live BCG vaccine and frequently exposed to *M. tuberculosis*. Antigen overlap is also revealed for *M. ulcerans*, since most immunodominant antigens characterized in this thesis by mAbs contained epitopes present in many mycobacterial species (chapter 4), resulting in serological cross-reactivity. The use of these antigens in serological tests for *M. ulcerans* exposure of disease has limitations in endemic areas where BCG vaccination has been implemented and tuberculosis is also endemic. However, cross-reactive antigens could be used as polymorphic genetic markers, and also as vaccine candidates. *M. tuberculosis* immunodominant antigens such as hsp65 showed more divergence between mycobacterial species than the 16S rRNA gene did[31]. Other polymorphic *loci* of *M. tuberculosis* included a phospholipase C, a membrane lipoprotein, members of an adenylate cyclase gene family, and members of the PE/PPE gene family, some of which have been implicated in virulence or the host immune response. Several gene families, including the PE/PPE gene family, also had significantly higher synonymous and non-synonymous substitution frequencies compared to the genome as a whole. A number of these polymorphisms appear to have occurred multiple times as independent events, suggesting that these changes could be under selective pressure[32].

The studies of *M. ulcerans* diversity performed with different typing techniques have all revealed a clonal population structure within specific geographical regions[33-38] and a low level of genetic variation, as also observed in *M. tuberculosis*. Nevertheless, sequence analysis of immunodominant antigens may reveal genetic diversification. In this thesis, gene sequencing of only one immunodominant antigen (Laminin-Binding protein) allowed the description of eight different alleles, four of them were found in Australian isolates.

7.2.3. Identification of target antigens for serological analysis

The clinical diagnosis of *M. ulcerans* can be confirmed by Ziehl-Neelsen (ZN) staining, the culture of *M. ulcerans*, the detection of characteristic histopathological changes in excised tissue or the detection of *M. ulcerans* DNA by PCR. Since the vast majority of mycobacterial diseases (tuberculosis, leprosy and Buruli ulcer) occur in developing countries with limited resources, rapid and inexpensive diagnostic tests would help limit the spread of the disease in the community.

Assays based on the detection of immunological responses to *M. ulcerans* are attractive alternatives to current methods. In particular for pre-ulcerative disease where the collection of punch biopsies may enhance the spreading of the pathogen, a test requiring only a peripheral blood would be highly preferable. One of the aims of the present thesis was to identify *M. ulcerans* specific antigens as targets for a serodiagnostic markers or T-cell stimulation based assay. Unfortunately, the most immunodominant antigens characterized contained epitopes present in other mycobacteria. In contrast, *M. ulcerans* 18 kDa shsp, which has no homologue in *M. tuberculosis* and *M. bovis*, turned out to be a suitable marker for exposure to *M. ulcerans* (chapter 3). The publication of the fully annotated *M. ulcerans* genome will open new possibilities to identify specific immunodominant antigens for serodiagnostic tests or T-cell based tests differentiating the *M. ulcerans* exposure and disease.

In the case of *M. tuberculosis,* the availability of the genome sequence has accelerated identification of antigens for serodiagnosis of tuberculosis, and a number of new antigens are being tested in various combinations to produce cocktails with high sensitivity and specificity. In tuberculosis, assays based on the serological immunodominant antigen 38-kDa antigen[22] alone or in combination with other proteins have achieved a high sensitivity (~80%)[39,40]. T-cell based assays using ESAT-6 and CFP10 are already used for diagnosis of active tuberculosis by the detection of interferon (IFN)-γ production by ESAT-6/CFP10-specific CD4 T cells. Released IFN-γ can be measured by assessment of the supernatant of the stimulated cells[41] or by using the enzyme-linked immunospot (ELISPOT) method[42].

The development of PCR-based assays to detect *M. ulcerans* has provided a tool to study its epidemiology and its mode of transmission[43]. Nevertheless, the epidemiology of *M. ulcerans* is only incompletely understood and the irregular distribution of cases and considerable underreporting make it hard to draw an overall picture of the prevalence of Buruli ulcer disease. In tuberculosis, exposure has been measured through use of a delayed hypersensitivity (DTH) skin-test reaction using a purified protein derivate (PPD). However, the highly cross-reactive nature of the PPD confounds the interpretation of skin-test positive for individuals who either have a history of vaccination with *M. bovis* BCG or who live in areas with a high environmental load of non-tuberculous bacteria[44]. A Buruli test is not suitable to measure exposure to *M. ulcerans*, because early Buruli ulcer patients are predominantly negative[45], and the first stage of the disease is characterized by a down-regulation of Th-1 responses[26-28,30]. The detection of *M. ulcerans* exposure could be based on antibody responses, since household contacts and Buruli ulcer patients produce antibody responses against *M. ulcerans* antigens[26-28]. One of the main contributions of this thesis was the identification of a specific immunodominant protein (18 kDa protein; chapter 3), which could be used as a serological exposure target in Buruli ulcer endemic regions. Previous studies on tuberculosis suggest that for the development of a serological test, a cocktail of specific antigens is required to increase the sensitivity of the test. Serological studies using multiple purified protein antigens have shown that the antibody response during tuberculosis is directed against many mycobacterial antigens. Moreover, the antigen recognition is highly heterogeneous -no two single antigens or common set of antigens respond alike[46]. The identification and characterization of other *M. ulcerans* specific immunodominant antigens may help to increase the sensitivity of the *M. ulcerans* exposure test further.

7.2.4. Antigen detection in environmental samples

While it is clear from many epidemiological studies that the proximity to swamps and wetlands is a risk factor for *M. ulcerans* infection, the exact mode of transmission remains unknown[47]. Farming activities close to rivers in endemic areas, wading and swimming in rivers while not wearing protective clothing may represent risk factors[48,49]. The predominance of Buruli ulcer lesions on the extremities and, in males, on the trunk involves passive exposure of exposed body parts, such as by bites of insect vectors. Since

M. ulcerans has been detected in the salivary glands of carnivorous aquatic insects, it has been hypothesized that it may be transmitted by water bug bites[50-52]. *M. ulcerans* DNA has also been detected in aquatic organisms and biofilms of aquatic plants[37,52-54], but the contribution of these environmental elements in transmission remains largely unknown. The discovery of IS2404 and IS2606 sequences in *M. liflandii*[55] complicates the interpretation of past environmental studies. Immunoassays based on the sensitive detection of *M. ulcerans* specific antigens may in the future complement PCR-based analyses. In this thesis, it is shown that *M. ulcerans* antigens can be detected in Buruli ulcer lesions by western-blotting (chapters 2 and 3). Future studies using magnetic beads coated with mAbs against specific *M. ulcerans* immunodominant antigens may help to selectively recover and concentrate *M. ulcerans* from environmental samples to discover their ecological niche.

7.3. Malaria

Malaria, together with HIV and tuberculosis, is one of the major causes of disease in tropical countries. The accurate estimation of how many people die from malaria per year is difficult to achieve. Most estimates suggest that malaria directly causes about 300-660 million clinical cases and between one million deaths per year[56]. Malaria has become a priority for the international health community and is now the focus of several new initiatives. The emergence and/or resurgence of malaria in many parts of the world have resulted from the spread of the parasite's drug-resistant to various drugs, which has to rising malaria-associated mortality, especially in Africa[57]. An antimalarial vaccine has long been a public health priority but, despite extensive research, there is still no effective vaccine available[58]. Several studies have indicated that malaria vaccines may be feasible. First, immunization with irradiated sporozoites protects or partially protects rodents[59], monkeys[60] and humans[61,62] from being infected by sporozoites. Second, people infected repeatedly by malaria develop "natural acquired non-sterile immunity"[63]. Passive transfer experiments showed that immunoglobulins of such semi-immune individuals can protect against clinical disease[64].

7.3.1. Characterization of potentially novel vaccine candidate by mAbs

Plasmodia are complex organisms. Each infection launches thousands of antigens against the human immune system, which differ with each stage of the parasite's life cycle. The main difficulty in finding a successful vaccine is *i*) due to the fact that *P. falciparum* can adapt to the human immune system and misdirect or suppress it[65], and *ii*) our poor understanding of the natural immune response to malaria. Conserved epitopes of the leading vaccine candidates are often not particularly immunogenic[66]. While many other implicated epitopes are highly polymorphic (vary between strains) or change with time within strains[67].

Vaccine candidates targeting the erythrocytic stages of the parasite have been the most intensively studied[68]. Antibody mediated neutralisation of the merozoites, preventing their entry into erythrocytes[69], or clearance of infected erythrocytes expressing merozoites surface antigens, represent potential mechanisms of immune protection[70]. Recent, access to

the sequence of the entire genome of *P. falciparum* has provided opportunity to deduce the function of the predicted proteins through the identification of orthologue genes and motifs in other organisms[71]. The drive to identify novel vaccine candidates has in part focussed on identifying genes coding for transmembrane or secreted proteins of *P. falciparum*[71,72]. Furthermore, these antigens should be accessible to the immune system, inducing protective immune responses in animal models. They should either lack antigenic diversity or have at least limited diversity, this allowing to focus the immunoresponse on conserved functional domains[73]. Monoclonal antibodies represent an important tool to analyse new candidate vaccine antigens (localization, purification, enrichment and functional activity).
In the framework of these strategies for malaria subunit vaccine design, using together computational models, expression tools and mAbs, the characterization of a novel antigen designated D13 was accomplished. As shown by Western blot analysis with anti-D13 mAbs (chapter 5), D13 is stage-specific, expressed during schizogony and is localized on one distinct pole of individual merozoites. The D13 N'-terminal region is a highly conserved gene product, which seems to have a functionally essential role in parasite biology, since anti-D13 mAbs have shown parasite growth inhibitory activity. In particular, the highly conserved N-terminal domain may represent a suitable target for malaria vaccine design.

7.3.2. Characterization of potentially drug target by mAbs

Spreading of resistance to chloroquine in the early 1960's has had a dramatic impact on malaria treatment worldwide, making the development of new antimalarial drugs necessary. The identification and characterization of novel protein targets for drug development has been a very active area of research in the past 30 years that crosses many disciplinary boundaries such as biochemistry and molecular biology of malaria parasites, focusing on specific parasite molecules, which are key to the parasite's life-cycle or the induction of its pathogenesis. In the case of the asexual blood stage, the parasite resides in erythrocytes: There are multiple membranes that must be traversed to access most intraparasitic targets, including the host cell-membrane, the parasitophorous vacuolar membrane, the parasite plasma membrane, and in some cases, a further organelle membrane. Therefore, the parasite trafficking function and specificity offers huge

potential for the future of parasite chemotherapy. In this thesis, using the anti-pfGAPDH mAbs by immunofluorescence and confocal microscopic analyses, we demonstrated that GAPDH is segregated in the late stages of parasite development from the cytosol, enriched in the apical region of merozoites. Furthermore, Western blot analyses showed that pfGAPDH is in both the membrane-containing pellet and the supernatant fraction of parasite lysate. In addition, a quantitative microsomal recruitment assay confirmed that pfGAPDH and the N'-terminal domain thereof were recruited to HeLa cell-derived microsomes in a Rab2-dependent manner. These data together suggest that pfGAPDH exerts non-glycolytic function(s) in *P. falciparum*, including possibly a role in vesicular transport and biogenesis of apical organelles (chapter 6). The obligate dependence of *P. falciparum* on glycolysis for ATP production combined with the possible involvement of pfGAPDH in other biological functions makes it an attractive target for antimalarial drug development. This is supported by the development of anti-trypanosomatid parasite drugs. The trypanosomatid glycolysis is compartmentalized, and many of its enzymes display unique structural and kinetic features. Structure- and catalytic mechanism-based approaches have been applied to design compounds which inhibit the glycolytic enzymes like adenosine and GAPDH[74,75]. The limited amino acid sequence identity with human GAPDH, and the unicity of sequence features at the active site of pfGAPDH, suggests that potent and selective inhibitors could affect only the growth of *P. falciparum* without affecting the corresponding proteins of the human host. Screening of a large library of compounds for pfGAPDH inhibitory activity in a high-through put screen has recently identified several structures with potential as lead compounds for a drug development process (unpublished results).

7.4. Concluding Remarks

This PhD thesis identifies and characterizes immunodominant antigens of *M. ulcerans* and characterizes of two *P. falciparum* proteins. These antigens were characterised by mAbs, which were generated from mice immunized with either *M. ulcerans* antigens or *P. falciparum* recombinant proteins. We can conclude:

1. The study of *M. ulcerans* antigens contributed: i) to the development of a serological test which may allow the assessment of exposure to *M. ulcerans* in endemic areas and ii) the identification of a polymorphic marker for strain typing.

2. Characterisation of the D13 protein and GAPDH of *P. falciparum* revealed their potential as vaccine and drug targets, respectively.

3. Besides the availability of parasite genome sequences and the development of new bioinformatics models, mAbs will continue to provide scientists with a powerful and important research tool. Working together, both technologies could improve and speed-up the advance in the development of serological tests (diagnosis, exposure test) and/or the identification of a potential vaccine candidates and potential drug targets, needed in developing countries.

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MEETINGS AND SEMINARS ATTENDED

Medicine and Health in the Tropics. Immunodominant antigens of *M. ulcerans*: From monoclonal antibodies via the protein to the genes (Poster). Marseille, France. September 11-15, 2005

XVIITH Meeting of the Swiss Immunology PhD Students**.** Immunodominant antigens of *M. ulcerans*: 18 kDa protein (Poster). Wolfsberg, Switzerland. March 30-April 01, 2005

International Mini-symposium on Buruli ulcer**.** Immunodominant Antigens of *M. ulcerans.* Swiss Tropical Institute (Oral presentation). Basel, Switzerland. March 12-13, 2004

Swiss Meeting for Doctoral Students in Parasitology and Tropical Medicine. Immunodominant Antigens of *M. ulcerans* (Poster). Müncheswiler, Switzerland. October 14-15, 2004

Tropenkongress 2004. Diversity of the laminin-binding protein (LBM) of *M. ulcerans* (Poster). Wuerzburg, Germany. September 23-25, 2004

Swiss Meeting for Doctoral Students in Parasitology and Tropical Medicine. Production of species specific and cross-reactive Monoclonal antibodies against *M. ulcerans* antigens (Oral presentation)**.** Müncheswiler, Switzerland. October 16-17, 2003.

The 15th International Congress of Tropical Medicine and Malaria. Characterization of a Crossreactive Peptide Domain Involved on Invasion of Erythrocytes by *Plasmodium falciparum.* Cartagena, Colombia, August 20-25, 2000**.**

AQUILA-WHO-Instituto de Inmunología. Seminar on the immunization protocol for the antimalarial vaccine SPf-66: QS 21: phase-1 study. Bogotá, Colombia. May 15-25, 1997.

WHO-Instituto de Inmunología. Validation of Three batches of the Antimalarial Vaccine SPf-66. Bogotá, Colombia, May 1996.

WHO-Instituto de Inmunología. Implementation of a New Adjuvant for the Antimalarial Vaccine SPf-66. Bogotá, Colombia, October 1996.

Participant in the Semester Seminars of the Faculty of Sciences-Department of Chemistry-Universidad Nacional de Colombia, Faculty of Medicine-Universidad Militar Nueva Granada, and the Masters Program in Microbiology of Universidad Javeriana (Oral presentation). Bogotá, Colombia. 1995-2000

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Inmunocompetentes III Congreso de la Asociación Latinoamericana de Inmunologia. Santiago de Chile, Chile. April 15-17 1993**.**

III Congreso de la Asociación Latinoamericana de Inmunología. Further Characterization of the MTP-40 Species-specific Protein of *Mycobacterium tuberculosis* by Peptide-induced Monoclonal Antibodies (Oral Presentation). Santiago de Chile, Chile. April 15-17 1993.

UNDERGRADUATE THESIS ADVISING

Characterization of specific Monoclonal Antibodies against CMV. Clinical usage in terminal patients with renal insufficiency. Yenny Fernanda Castro, Diana Mildred Cedeño y Luz Alba Colorado. Faculty of Health Sciences, Bacteriology Program, Universidad Colegio Mayor de Cundinamarca. Bogotá, Colombia. November, 1996.

Synthesis of the Monomeric Peptides for the production of Monoclonal Antibodies against the SPf66 Vaccine**.** Iván Gómez P. Department of Chemistry. Faculty of Sciences, Universidad Nacional de Colombia. Bogotá, Colombia, 1995.

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