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SYNTHETIC PEPTIDE-BASED DIAGNOSIS OF *PLASMODIUM FALCIPARUM* INFECTION IN HAITI

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Executive Summary

The purpose of this project was to develop practical, field-applicable diagnostic tools to aid in the monitoring of malaria endemicity in Haiti. Synthetic peptide-based immunoassays were developed from peptide sequences derived from a 70 kDa *Plasmodium falciparum* exoantigen; peptides were synthesized and then constructed as a copolymer (SPf70) for diagnostic purposes. Studies on the characterization of SPf70 resulted in a publication (Parasitol. Res., 79: 501-507, 1993) and a US Patent (No. 5,456,911 issued October 10, 1995) which covered the development of this technology. An advantage for international development is that this reagent can also be used to detect *P. vivax* malaria. Peptide ELISAs for antibody and antigen detection were standardized in a microplate format, and a rapid latex agglutination test was optimized with SP70 peptides for the detection of anti-*P. falciparum* antibodies. Antibody detection assays could serve as useful tools in the epidemiologic surveillance of malaria transmission in Haiti, facilitating and streamlining resources allocated for the malaria control program. Internal political strife in Haiti impeded the progress of the project at several points in its duration; longitudinal field studies could not be carried out as a result. Nonetheless, the project was successful in achieving its scientific goals, and technology was appropriately transferred as the Haitian counterparts and laboratory personnel at the Hopital Sainte Croix, Leogane, Haiti, were trained in methods and procedures developed herein.

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A. Research Objectives

1. Develop a synthetic peptide-based enzyme immunoassay for the detection and quantitation of *Plasmodium* blood-stage antibodies and antigens in sera of infected individuals. Serologic reactivity to synthetic and native *P. falciparum* peptides will be correlated with the immune status/clinical characteristics of individuals residing in Leogane, Haiti and the surrounding communities.

Synthetic peptide-based immunoassays using the peptide complex SPf70 were successfully developed in this project for the detection of antibodies and antigens [using anti-SPf70 antibodies] for either *P. falciparum* or *P. vivax*. The high immunogenicity and antigenic reactivity of SPf70 indicate the potential of this synthetic peptide polymer as an immunogen and a diagnostic reagent (US Patent No. 5,456,911 "Synthetic Malarial Antigens and Uses Thereof" issued on October 10, 1995). These assays are presently carried out in the microplate ELISA format. Studies on the characterization of SPf70 were published in Parasitology Research (79: 501-507, 1993) and a copy of this paper is attached as Appendix 1. Antigen detection assays are designed to complement the classical microscopic diagnosis of malaria to facilitate the implementation of treatment regimen. A logical extension of this work would be to adapt the standard immunoassay for Pf70 antigen to a rapid format, e.g., dipstick or dry-strip technology. Antibody detection assays can aid in the epidemiologic surveillance of malaria transmission in mesoendemic areas. The SPf70 peptide-ELISA is particularly suited for this purpose as 85-90% of all persons with antimalarial antibodies (indirect fluorescent antibody test-positive) have antibodies to PF70 (Ferreira da Cruz et al., 1995; Tshefu and James, 1995). This ELISA assay could be readily available for use by national malaria control programs in Latin America and the Caribbean region.

2. Develop and standardize rapid immunoassays, e.g., dot-ELISA or latex agglutination tests, for detection of antibodies and antigens in the diagnosis of *P. falciparum* infections.

A simple latex agglutination test was developed using SPf70 for the detection of antimalarial antibodies. This rapid assay could be used by technical personnel in malarial control programs. Although a similar test was not developed for antigen detection, discussions are underway with commercial organizations who have expressed interest in the potential licensing and development of such a product. One advantage of such a SPf70-based assay as compared with the commercially available ParaSight-F™ test is the ability of the former to detect *P. vivax* infections.

3. Train Haitian scientists in novel procedures for malaria diagnosis.

Dr. Marise McNeeley, director of the Clinical Pathology Laboratory at Hopital Sainte Croix, Leogane, Haiti, trained at Tulane in November 1992 in the immunodiagnosis of malaria. She was able to carry out any activity that was needed on-site in Haiti. A fluorescence-equipped microscope and an automated microplate reader were purchased for the project at Hopital Sainte Croix. During the course of the project, several other persons (medical technologists trained in microscopy) in the diagnostic laboratory of Hopital Sainte Croix were introduced to the synthetic peptide-based immunoassays. They all demonstrated a willingness to learn. They agreed that deficiencies exist in the present state of microscopic diagnosis of malaria. This was attributed to various problems with old equipment, Giemsa stain stock solutions, or inconsistencies in blood smear preparation and staining. All personnel were able to carry out the essential assay procedures. Although microscopists and lab technicians required some supervision to perform the peptide-ELISA in the conventional microplate format, a rapid dot-ELISA was easily managed by all trainees. Laboratory staff recognized the advantage of the microplate ELISA for testing large numbers of samples and found it to be less physically demanding than microscopy. However in its present format, the peptide-ELISA is probably too time-consuming for individual diagnosis.

B. Methods and Results.

1. Characterization of a synthetic peptide complex (SPf70) derived from a 70 kDa *Plasmodium falciparum* exoantigen.

A 70 kDa *Plasmodium falciparum* exoantigen was purified from supernatant fluids of continuous in vitro *P. falciparum* cultures. The purified protein was then digested with chymotrypsin and amino-acid sequences were determined for the resulting fragments. Four peptides (termed C2, C3, C5, C10) were subsequently selected for synthesis based on their predictability for antigenic sites. The peptides were effectively used as a synthetic immunogen (SPf70) when they had been copolymerized with glutaraldehyde in the absence of a carrier. When given with Freund's adjuvant, the synthetic peptide complex was found to be highly immunogenic in rabbits. Serologic reactivity to the peptide complex and peptides C2 and C5 was uniformly high, followed by the responses to peptides C3 and C10. Peptide antigenicity was also assessed with human anti-*P. falciparum* sera from malaria-endemic regions. ELISA data showed that anti-*P. falciparum* antibodies were specific for and reactive to the peptides. The specificity of the rabbit anti-SPf70 antibodies for *P. falciparum* antigen was shown by immunoprecipitation of metabolically labeled proteins and by immunoblotting. The high immunogenicity and antigenic reactivity of SPf70 indicate the potential use of this synthetic peptide

polymer as an immunogen and a diagnostic reagent (patent pending). This work was published in Parasitology Research (79: 501-507, 1993) and a copy of the paper is attached as Appendix 1.

2. Production of rabbit anti-SPf70 antibodies: preparation and titration of enzyme-antibody cojugates for antigen-detection ELISA.

Four rabbits were immunized with SPf70 in a regimen of 3 subcutaneous doses with Freund's adjuvant at 3-week intervals. Sera were collected 10 days after the third immunization, and screened for reactivity to native antigen and the SPf70 complex by immunoblots and ELISA, respectively. Two rabbits, in particular, produced anti-peptide antibodies which were strongly reactive with native P. falciparum antigen in immunoblots. IgG was isolated by sequential fractionation of sera collected 7, 10 and 14 days after the third immunization with caprylic acid and ammonium sulfate. Enzyme-antibody conjugates were prepared using both alkaline phosphatase (glutaraldehyde method) and horseradish peroxidase (sodium periodate method). The respective conjugates were titrated for optimal reactivity with native Pf70 found circulating in peripheral blood.

3. Microplate ELISA for detection and quantitation of antigen and antibody.

A. Antigen detection assay.

Direct peptide-ELISA. One approach to the immunodiagnosis of malaria lies in the detection of circulating *Plasmodium* antigens. Such techniques can provide sensitive methods for detecting active, and possibly recent, infections. Rapid, simple assays that provide information on the status of infection in large populations should be helpful tools in mass examination for the control of malaria. With this goal in mind, we developed the direct ELISA for antigen detection, using polyclonal anti-peptide antibodies. Tests are conducted in a standard microplate format with 96-well polystyrene ELISA plates (Immulon 2; Dynatech Laboratories, Inc., Chantilly, VA). Serum/plasma samples are diluted 1:100 in PBS pH 7.4 and applied directly to the plate. After a blocking step (5% nonfat dry milk/0.5% Tween 20/PBS pH 7.4), antigen is detected in a single reaction with AP-conjugated anti-peptide antibody. Following addition of the enzyme substrate, color development is measured with an automated ELISA reader. A special feature of the assay is its rapidity. Once samples have been added to the plates, approximately 320 sera (assayed in duplicate) can be tested in a 3-hour period.

B. Antibody detection assay.

Microplate peptide-ELISA. The serological reactivity (anti-peptide IgG) of sera and filter paper bloodspot eluates can be tested to determine the presence of antimalarial (asexual blood-stage) antibodies. Briefly, microplates (NUNC MaxiSorp) are coated with the synthetic peptide polymer SPf70 at a concentration of 10 μg/ml. Alkaline phosphatase-conjugated goat anti-human IgG is used

as the enzyme conjugate. The test can be easily adapted for the detection of IgM with the use of a specific anti-IgM conjugate. Sigma p-NPP tablets in diethanolamine buffer are used as the substrate. Absorbance (405 nm) values are measured after 30 min using an automated ELISA reader. Samples are considered positive when absorbance values are greater than the mean optical density plus 3 standard deviations obtained with normal control samples from the USA and endemic area (endemic normals). The antibody test can supplement epidemiologic surveillance activities. The utility of antibody detection is particularly evident in asymptomatic individuals who actually exist as carriers and a source of transmission and/or potential outbreaks.

Peptide-ELISAs for antigen and antibody detection were performed on sera (n=100) collected from presumptive malarious patients presenting at the Hopital Sainte Croix. Prevalence rates for circulating malarial Pf70 antigen and anti-Pf70 antibodies were 30.0%, 39.4% (IgM), and 44.6% (IgG), respectively. The slide positivity rate for this group was 20.7%. Upon interview, 17% of these subjects claimed automedication with antimalarial drugs. This fact may contribute to a possible under-estimation of the actual incidence of malaria cases.

Because of the political situation in Haiti during the project, we were unable to carry out studies to validate the sensitivity, specificity and predictive value of these assays under field conditions. However, previous evaluation of the peptide-ELISA in Honduras for detection of Pf70 antigen (2,041 samples) demonstrated that the assay was highly sensitive (89.3%) and specific (96.7%) with positive and negative predictive values of 95% and 93%, respectively.

4. Two-sited antigen-capture enzyme immunoassay in dot-ELISA format.

A dot-ELISA test was developed, but remained to be standardized and optimized for the rapid diagnosis of circulating antigen in P. falciparum-infected individuals. The test employed rabbit anti-SPf70 IgG as an antigen-capture reagent, and the same antibody cojugated to enzyme for detection of the antigen. Conditions that have thus far proved satisfactory for antigen detection are as follows: capture antibody concentration = 0.1 mg/ml or 100 ng IgG per dot (1 ul volume); blocking of nonspecific sites carried out with PBS/0.5% Tween 20 for 16 h at 4C; conjugate dilution = 1:600 in PBS/0.1% Tween 20.

5. Synthetic peptide-based latex agglutination test for antibody detection.

A latex agglutination test designed to detect antimalarial antibodies in serum was developed by adsorbing SPf70 peptide onto polystyrene microparticles (PS-MP; 0.813 μm diameter) in glycine-buffered saline pH 8.6. Briefly, 100 μl PS-MP was mixed with 1 ml of glycine-buffered saline (0.15 M NaCl, 0.01 M glycine, 0.1%

sodium azide), pH 8.6, and then 100 μl SPf70 peptide (10 mg/ml PBS pH 7.4) was added. The suspension was mixed thoroughly with a pipettor, and then allowed to mix gently on an orbital rotator for 90 min at room temp (RT). Next, the latex particles were pelleted by spinning at 10,000 x g for 20 min at RT. The supernatant was removed and the beads washed twice in 1 ml of the same buffer. Finally, the particles were resuspended in a 1 ml-volume of glycine-buffered saline pH 8.6 for use. To test serum samples for the presence of antibody, 50 μl of sera was pipetted onto a clean glass slide (dark background to aid in visualization) and an equal volume of latex suspension was added. The reactants were mixed with a wooden applicator stick and then the slide was hand-rotated for 2 min. Evidence of agglutination in the presence of antibody was observed under indirect light. Negative reactions resulted in a homogeneous suspension without agglutination of latex particles.

Reference samples (n=54) known to be positive for antibodies to *P. falciparum* by the IFA assay were tested with the SPf70 latex reagent. All (100%) produced an agglutination reaction which varied in intensity. Seven negative control sera (determined negative by the IFA test) did not show agglutinating activity. This test still needs to be evaluated in terms of its relative sensitivity and specificity under field conditions.

5. Comparison of antigen slides prepared for indirect immunofluorescence from in vitro cultures of various P. falciparum isolates.

With the development of the synthetic peptide-based SPf70 ELISA for detection of specific antibodies to Pf70, one needs to compare this reactivity with the indirect fluorescent antibody (IFA) test for detection of total antimalarial antibodies to asexual blood-stage parasite antigens. Therefore, IFA antigen slides derived from different culture-adapted P. falciparum isolates (ITG-2, Haiti, Indochina I/CDC, Geneve/SGE-1) were compared for reactivity with sera collected from presumptive malarious subjects (n=19) presenting at the Hopital Sainte Croix for diagnosis and treatment. Results are as follows:

	ITG-2	Haiti	Indo.	Geneve
Reciprocal IFA Titer:				
Range =	<20-2560	40-2560	80-2560	<20-5120
Mean =	514	574	857	257
% pos =	94.7	100	100	78.9

Comparison of inter-strain reactivity is important because the SPf70 peptides are derived from Pf70 of the Indochina I strain.

6. Seroprevalence of anti-SPf70 and total antimalarial (IFA) antibodies in sera collected at the Malaria Outpatient Clinic, Hopital Sainte Croix, Leogane, Haiti (Oct. 1993).

Sera from 85 subjects were tested for antibodies to Pf70 and polyspecific asexual blood-stage antigens by the SPf70-ELISA and IFA test (Indochina I/CDC antigen), respectively. The respective seroprevalence rates for anti-Pf70 antibodies (IgG or IgM) and total antimalarial antibodies were 34.1% and 90.6%. The mean reciprocal IFA titer of sera positive for antibodies to Pf70 was 458; the titer of sera negative for anti-Pf70 antibodies was 272. Future studies will address the role of naturally-acquired anti-Pf70 antibodies in the protection against malaria. Moreover, it is believed that the SPf70-ELISA for antibody detection would be a useful tool in the epidemiologic surveillance of malaria transmission.

7. Use of a nested PCR system for diagnosis of malaria.

In an effort to compare the sensitivity of our newly developed synthetic peptide-based ELISA with other diagnostic tests with inherently different sensitivities (including conventional microscopy), we adapted a nested PCR system for malaria diagnosis. Detection of parasites on thick blood smears is still considered the gold standard for diagnosis of malaria. Identification of parasites and differentiation of *Plasmodium* species in smears can be difficult. In this study a nested PCR technique was used to diagnose malaria in 230 filter paper blood samples of malarious individuals. Parasite DNA was extracted using the QIAGEN Tissue Kit System. PCR was optimized to use a volume of 10 ul PCR reaction mix/sample. Malaria diagnostic primers were based on *Plasmodium* small-subunit RNA genes: outer primers amplified a conserved genus-specific segment and species-specific primers distinguished *P. falciparum, P. vivax* and *P. malariae* infections (Snounou, 1995). In Haiti 100% of malaria cases are caused by *P. falciparum*. Analytical sensitivity for the assay was 8 parasites/ul blood, equivalent to 0.0002% parasitemia. Although this type of assay may have limited applications under field conditions, it could be used to monitor the quality of diagnosis being performed by laboratory personnel using classical microscopic methods. In other studies unrelated to the present project, we are using a combined nested PCR system for the simultaneous diagnosis and determination of drug-resistant malaria.

C. <u>Impact, Relevance and Technology Transfer</u>.

Dr. Marise B. McNeeley was instrumental in the first two years of the project for daily supervision of activities at the Hopital Sainte Croix. Activities included: sample collection and processing; training of laboratory personnel; assembly of equipment; and ordering and cataloging of materials and supplies purchased for the project. Public health workers were trained in the collection of census data which will be of value if and when

these technologies are used in field studies in the Leogane community. Thus, the present staff in the Clincal Pathology Laboratory has the training and equipment (fluorescence-equipped microscope and automated microplate reader) to implement the immunodiagnostic procedures developed in this project.

It is anticipated that the results from the project will be used to improve the diagnosis of malaria cases at the Hopital Sainte Croix by offering an alternative technique for rapid screening and for the quality control of existing microscopic methods. The SPf70 peptide-ELISA for antibody detection would benefit the government agencies responsible for epidemiologic surveillance and assessment of malaria endemicity.

D. Project Activities/Outputs.

Training:
Dr. Marise B. McNeeley, Haitian co-investigator, November 1992, Tulane University, New Orleans, LA USA. Trained in immunodiagnostic methods--indirect immunofluorescence, ELISA, immunoblotting.

Laboratory personnel, Hopital Sainte Croix, Leogane, Haiti. Dr. James conducted a one-week training session in April 1993 on immunodiagnostic applications in the diagnosis of malaria.

Seminar:
Dr. James presented a seminar entitled "Immunodiagnosis of Malaria" to medical staff at the Hopital Sainte Croix in Leogane, Haiti, June 23, 1995.

Publication:
James MA, Montenegro-James S, and Fajfar-Whetstone CJ. 1993. Immunogenicity and antigenic reactivity of a carrier-free synthetic peptide complex derived from a 70 kDa *Plasmodium falciparum* exoantigen. Parasitol. Res., 79: 501-507.

Patent:
A US patent (filed by Tulane University on January 15, 1993 for inventors Mark A. James and Sonia Montenegro-James) entitled "Synthetic Malarial Antigens and Uses Thereof" was issued on October 10, 1995 (Patent No. 5,456,911). The standard non-exclusive license to the United States Government is attached as Appendix 3.

E. Project Productivity.

The project was productive in that synthetic peptide-based immunoassays were developed that can be used for the sensitive detection of either *Plasmodium* antibodies or antigen. This technology was patented has part of this project. A rapid latex

agglutination test was developed for detection of antimalarial antibodies that could be immediately adopted for epidemiologic surveillance purposes. A major impediment to progress at field sites in Haiti was caused by the precarious political situation for most of the project duration. These interruptions prevented travel by the Tulane PI to carry out research on the field evaluation of newly developed immunoassays, and longitudinal studies designed to assess the functional immunity to malaria in the Leogane community, i.e., the naturally-acquired antibody response to Pf70 and its role in protection against malaria.

F. Future Work.

It is hoped that future studies can be carried out in Haiti and at the Hopital Sainte Croix, in particular. The hospital is fortunate to have a dynamic medical director, Jack G. Lafontant, MD, who has a genuine interest in research and continued medical education. The community health program of Hopital Sainte Croix is an excellent resource and provides an appropriate infrastructure for the technical support needed to conduct field-oriented studies. The program has 16 years of field experience in the community and provides comprehensive preventive and curative services for the more than 108,000 inhabitants throughout the Commune of Leogane. Village health workers have surveyed the entire community, and thus a significant amount of background information is available concerning the socio-economic status and health of the residents.

In addition, it is envisioned that the technology developed herein could provide assistance to national governments in their malaria control strategies. Furthermore, commercial development of the SPf70-based antigen detection assay in a rapid format would be especially beneficial for use by community health workers in malaria-endemic regions.

G. Literature Cited.

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Tshefu K and James MA. 1995. Relationship of antibodies to soluble *Plasmodium falciparum* antigen (Pf70) and protection against malaria in a human population living under intense transmission in Kinshasa, Zaire. Trop. Med. Parasitol., 46: 72-76.

Appendix 1

Publication

Parasitol Res (1993) 79:501-507

Parasitology Research
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Immunogenicity and antigenic reactivity of a carrier-free synthetic peptide complex derived from a 70-kDa *Plasmodium falciparum* exoantigen

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Abstract. A 70-kDa *Plasmodium falciparum* exoantigen was purified from supernatant fluids of continuous in vitro *P. falciparum* cultures using sequential cation-exchange and high performance liquid chromatographic procedures. The purified protein was then digested with chymotrypsin and amino-acid sequences were determined for the resulting fragments. Four peptides (termed C2, C3, C5, and C10) were subsequently selected for synthesis based on their predictability for antigenic sites. The peptides were effectively used as a synthetic immunogen (SPf70) when they had been copolymerized with glutaraldehyde in the absence of a carrier. When given with Freund's adjuvant, the synthetic peptide complex was found to be highly immunogenic in rabbits. Serologic reactivity to the peptide complex and peptides C2 and C5 was uniformly high, followed by the responses to peptides C3 and C10. Peptide antigenicity was also assessed with human anti-*P. falciparum* sera from malaria-endemic regions of Uganda and Venezuela. Enzyme-linked immunosorbent assay (ELISA) data showed that anti-*P. falciparum* antibodies were specific for and reactive to the peptides. The specificity of the rabbit anti-SPf70 antibodies for *P. falciparum* antigen was shown by immunoprecipitation of metabolically labeled proteins and by immunoblotting. Herein we describe the peptide sequences of a 70-kDa *P. falciparum* exoantigen (Pf70) that, when synthesized and constructed as a copolymer (SPf70), are capable of inducing the formation of antibodies that are reactive with the native malarial protein. The high immunogenicity and antigenic reactivity of SPf70 indicate the potential use of this synthetic peptide polymer as an immunogen and a diagnostic reagent.

Many of the malarial asexual blood-stage antigens thus far identified are associated with cytoplasmic inclusions and/or accumulate within the parasitophorous vacuole of the infected erythrocyte. Such antigens may be shed or secreted into the plasma of infected individuals or into the supernatant medium of in vitro cultures, especially upon schizont rupture. These soluble antigens have been historically referred to as exoantigens (Wilson et al. 1969). Since exoantigens are known to occur in the circulation of patients with malaria, they may be responsible for excess production of tumor necrosis factor (TNF), a mediator that is thought to play a central role in the pathogenesis of the disease (Scuderi et al. 1986; Kwiatkowski et al. 1989, 1990; Taverne et al. 1990a, b; Playfair et al. 1991; Mendis 1992). TNF may promote cytoadherence and sequestration of *Plasmodium falciparum*-infected erythrocytes (Grau et al. 1989; Molyneux 1990) and, when produced in excessive levels, may predispose to cerebral malaria and a fatal outcome (Kwiatkowski et al. 1990). In addition, malarial fevers resulting from *P. falciparum* and *P. vivax* infections may be mediated, at least in part, through paroxysmal TNF release associated with schizont rupture (Kwiatkowski et al. 1989; Karunaweera et al. 1992; Mendis 1992). It has been suggested that immunization with an exoantigen(s) might provide a means of protection against the clinical effects of malaria and of generating immunity to the disease by reducing cytokine production (Bate et al. 1990; Playfair et al. 1990). Such an exoantigen-based, antidisease vaccine would minimize the clinical manifestations of *P. falciparum* malaria, thereby extending life until the development of solid, naturally acquired immunity.

The present study was designed to test the immunogenicity and antigenicity of a unique synthetic peptide hybrid (SPf70) derived from internal chymotryptic fragments of a major 70-kDa *P. falciparum* (Indochina I/CDC) schizont protein (Shamansky et al. 1985; Braun-Breton et al. 1986). It has been suggested that the 70-kDa polypeptide is a degradation product of a 120-kDa schizont membrane protein, with the 70-kDa protein increasing in amount at the time of merozoite release/reinvasion (Braun-Breton et al. 1986). This antigen was selected for study because of its immunogenicity and efficacy in inducing partial protective immunity in sus-

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(2.5 mg) were given at 4-week intervals. The first two injections were given with Freund's complete adjuvant, and the third immunization was given with Freund's incomplete adjuvant. Rabbits were bled at 7 and 14 days after each immunization and the sera, processed for analysis.

Enzyme-linked immunosorbent assay

The immunogenicity and antigenicity of the synthetic peptide complex was analyzed in standard microplate ELISAs. First, sera collected from three rabbits at 14 days after the third immunization were pooled, serially diluted in PBS/0.05% Tween 20 from 1:50 to 1:6,400, and reacted with the individual peptides and the peptide complex in a microplate ELISA. NUNC MaxiSorp (Denmark) polystyrene 96-well plates were coated with either individual peptide-bovine serum albumin (BSA) conjugates, the peptide copolymer (without BSA), or BSA-glutaraldehyde alone at a protein concentration of 10 μg/ml PBS (pH 7.4). Individual peptides were previously conjugated to BSA at a molar ratio of 25:1 (peptide to BSA) with glutaraldehyde in PBS. The preparations were then purified over a G-25 Sephadex column. After a blocking/wash step with PBS/0.5% Tween 20, the sera were placed in triplicate wells and allowed to react for 2 h at 37° C. The plates were then washed three times with PBS/0.1% Tween 20 and then incubated with alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (H and L chains; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) for 1 h at 37° C. After a final series of washes, anti-peptide IgG reactivity (mean absorbance values read at 405 nm and corrected for the negative BSA control) was determined in an automated ELISA reader after a 30-min reaction period with *p*-nitrophenyl phosphate (*p*-NPP) substrate.

In another experiment, the serum pool was diluted 1:50 and preincubated with the respective peptides and the peptide complex at a final peptide concentration of 250, 25, and 2.5 μg/ml, and no peptide. The final serum dilution was 1:100. Serum/peptides were incubated for 16 h at 4° C, and immunoprecipitates were removed by centrifuging at 13,000 rpm (microcentrifuge) for 20 min. Supernatants containing antibodies were reacted in triplicate in a standard peptide ELISA with microplates coated with the homologous peptides (10 μg/ml). Antigenic reactivity was measured as described above. Finally, in a similar competitive-inhibition ELISA experiment, the antigenicity of the peptides was determined after preincubation with hyperimmune human serum pools containing anti-*P. falciparum* antibodies from malaria-endemic Uganda and Venezuela (respective IFA titers = 1:10,240) and with a normal human serum control. These sera were kindly provided by Dr. I. Kakoma, University of Illinois, and Dr. O. Noya, Central University of Venezuela, respectively. The final serum dilution was 1:100. Antigenic reactivity was measured after incubation with AP-conjugated goat anti-human IgG (H and L chains, Kirkegaard & Perry) and *p*-NPP substrate as described above.

Biosynthetic labeling/immunoprecipitation and immunoblotting of P. falciparum antigens

In vitro cultures of the Geneve/SGE-1 strain of *P. falciparum* were synchronized by sorbitol lysis, and late-stage-parasitized erythrocytes were concentrated using Percoll. Stage-specific labeling of schizont proteins (approx. 70% parasitemia) was carried out using [^{35}S]-methionine in RPMI 1640 plus 10% human serum (1 mCi/5 ml) for 5 h according to standard procedures. Schizonts were removed by centrifugation, lysed in cold 10 m*M* TRIS buffer (pH 8), and solubilized in 1% Nonidet P-40 in PBS. IgG fractions (rabbit anti-peptide complex and normal rabbit control) were coupled to Protein A-Sepharose CL-4B, washed, and subsequently incubated with 200 μl of labeled parasite extract. After a final washing, 100 μl of sodium dodecyl sulfate (SDS)/dithiothreitol (DTT) sample buffer was added to each immunoprecipitate (IP), boiled for 1 min, and centrifuged. Then, 60 μl of each IP supernatant was loaded onto a 10% SDS-polyacrylamide gel and electrophoresed at 70 V for 20–24 h. The gels were enhanced for fluorography, and films were developed by standard procedures.

P. falciparum (Indochina I/CDC strain) antigens (infected erythrocyte lysate and affinity-purified exoantigens) from asynchronous in vitro cultures were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting. Exoantigens were affinity-purified from culture supernatant fluids using monoclonal anti-peptide C3 and human anti-*P. falciparum* antibodies (generously provided by Dr. I. Kakoma) on Reacti-Gel columns (Pierce Chemical, Rockford, Ill.). Electrophoresis was performed on 10% acrylamide gels under nonreducing conditions. After transfer, antigens were reacted with 1:100 dilutions of sequentially collected rabbit antisera (day 0, days 7 and 14 after the first immunization, and day 7 after the second immunization) to the SPf70 copolymer. The antigens were subsequently developed after incubation with horseradish peroxidase (HRPO)-conjugated goat anti-rabbit immunoglobulins (Kirkegaard & Perry) and diamine benzidine substrate.

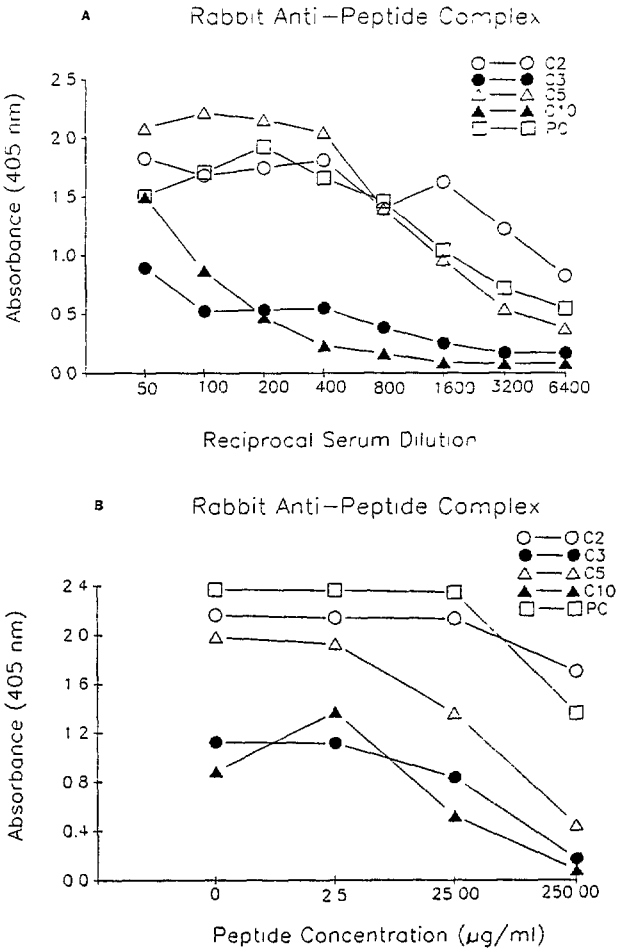

Fig. 1 A, B. Immunogenicity of the synthetic peptide complex SPf70. Rabbit antibody responses to the peptide complex were measured by ELISA. **A** Reactivity (mean absorbance values of triplicate wells) of individual peptides (*C2, C3, C5, C10*) and the peptide complex (*PC*) to antibodies in serially diluted pooled antisera. **B** Antibody reactivity in a competitive-inhibition ELISA after preincubation with homologous peptides

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ceptible Bolivian *Saimiri* monkeys (James et al. 1985). Partially purified (enriched for the 70-kDa antigen) supernatant fluids of *P. falciparum* Indochina I and Geneve/SGE-1 strains conferred significant clinical protection of squirrel monkeys against challenge with the homologous Indochina I strain and a moderate degree of heterologous strain immunity. Subsequently, monospecific rabbit antibodies to the 70-kDa polypeptide were shown to have schizont specificity by immunofluorescence and caused approximately 50% inhibition of *P. falciparum* growth after 72 h of in vitro culture (C. Fajfar-Whetstone, unpublished observations).

Materials and methods

Antigen purification

The 70-kDa exoantigen was purified from supernatant fluids of continuous in vitro *Plasmodium falciparum* cultures using sequential chromatographic procedures previously described elsewhere (Shamansky et al. 1985; Shamansky 1986). *P. falciparum* parasites (Indochina I/CDC strain) were cultured in vitro using human A+ erythrocytes and 10% human A+ serum (Trager and Jensen 1976). Supernatants were collected daily from asynchronous cultures at an average parasitemia of 2%–3%. Supernatants were stored at −70° C before their use in purification procedures.

Exoantigens were partially purified from crude culture supernatants by sulfopropyl (SP)-trisacryl (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) cation-exchange chromatography (Thelu et al. 1985). Briefly, supernatant fluids were thawed and pooled, concentrated 10 times by pervaporation at 4° C, and then dialyzed against 60 vol. of 0.01 *M* sodium acetate buffer (pH 3.7) for 48 h at 4° C. Subsequently, this material was placed on an SP-trisacryl column (50 × 200 mm), and proteins were eluted in 0.01 *M* sodium acetate buffer (pH 3.7) with an increasing stepwise salt gradient to 0.3 *M* NaCl at a flow rate of 300 ml/h. Antigenic fractions (250 ml/fraction) were monitored by an enzyme-linked immunosorbent assay (ELISA) using an immune human reference serum pool [African origin; indirect fluorescent antibody (IFA) titer = 1:5,120]. Positive fractions were pooled, concentrated by lyophilization, and dialyzed against 0.01 *M* phosphate-buffered saline (PBS, pH 7.2) for 48 h at 4° C. These fractions, enriched for the 70-kDA exoantigen as determined by immunoblotting, were subsequently purified by a sequential high-performance liquid chromatography (HPLC) series. A three-step HPLC scheme employing gel filtration (Dupont Zorbax GF-250, 9.4 × 250 mm), diethylaminoethyl (DEAE) anion-exchange (Waters Protein Pak DEAE-5 PW, 7.5 × 75 mm), and phenyl hydrophobic interaction (Beckman TSK-phenyl-5PW, 7.5 × 75 mm) columns was used. Chromatographic conditions were carried out according to previously described procedures (Shamansky et al. 1985; Shamansky 1986). Fractions were monitored by ELISA and immunoblots. Fractions containing the 70-kDa protein were pooled, concentrated by vacuum dialysis, and then stored at −20° C for further purification. With the use of the multi step purification scheme (conventional cation-exchange chromatography plus sequential HPLC), the 70-kDA *P. falciparum* exoantigen could be routinely purified to homogeneity (confirmed by two-dimensional electrophoresis).

Enzymatic digestion and gas-phase sequencing of proteolytic fragments, analysis and synthesis of peptides

Our strategy for the determination of amino-acid sequences in the 70-kDa protein was to carry out a complete enzymatic digestion using chymotrypsin, followed by isolation of the resulting peptides with reverse-phase HPLC. Alpha-chymotrypsin (Sigma Chemical, St. Louis, Mo.) was used at an enzyme-to-protein ratio of 1:25 (w/w) to digest 500 pmol (approximately 35 μg/ml) of the purified antigen according to the method of Stone et al. (1990b). Individual peptides were isolated by analytical reverse-phase HPLC (Delta Pak C-18 column, 3.9 mm × 15 cm) according to procedures described previously (Stone et al. 1990a). Ten peptide fragments were submitted to the Genetic Engineering Facility at the University of Illinois (Urbana-Champaign, Ill.) for gas-phase sequencing. Peptides were sequenced by automated Edman degradation on an Applied Biosystems Model 470A protein sequencer.

All sequences were analyzed for similarities to known protein sequences, both nonmalarial and malarial in origin, using the Swiss Protein Database Version 2.0 and the Malaria Protein Sequence Database (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia), respectively. No significant homology to published sequences was found. Next, sequences were analyzed by the IBM DNASTAR software package that provided structural predictions based on computer algorithms. These included the Hopp and Woods (1981) and Kyte and Doolittle (1982) algorithms for prediction of B-cell antigenic sites (based on hydrophilicity) and the Chou and Fasman (1974) prediction of protein conformation (associated with the α-helical propensity of T-cell epitopes). With this information, four peptides (termed C2, C3, C5, and C10) were selected for synthesis based on the following criteria: (1) peptide length; (2) hydrophilicity; (3) secondary structure, i.e., α-helices; and (4) content of proline residues. The respective peptide sequences (patent pending) are detailed in Table 1. Peptides C2, C3, C5, and C10 of the 70-kDa *P. falciparum* antigen were synthesized by the Merrifield (1963) method in an Applied Biosystems peptide synthesizer at the Genetic Engineering Facility of the University of Illinois. Peptides were lyophilized and stored in a dessicator at −20° C until used.

The individual peptides were finally tested by ELISA for reactivity with anti-*P. falciparum* antibodies in sera previously collected from patients ($n = 126$) with acute malaria from Bolivar State, Venezuela (Toebe et al. 1993). Overall seropositivity (IgG) rates for peptides C2, C3, C5, and C10 were 20%, 40%, 20%, and 58%, respectively. IgM reactivity to the respective peptides was 53%, 30%, 83%, and 70%. We also found that immune sera from Ugandan subjects had high reactivity with the peptides (IgG seropositivity rates of 90% for C2, C3, and C5 and 70% for C10; S. Montenegro-James, unpublished observations). To improve on the antigenicity and to overcome the restricted (variable) immune responsiveness to individual epitopes, we thus targeted the construction of a synthetic peptide hybrid.

Preparation of the synthetic peptide complex and immunization of rabbits

Our laboratory chose to follow a novel approach for the formulation of the synthetic peptide immunogen – the copolymerization of multiple peptides in the absence of carrier molecules. This results in a product unmodified by carrier conjugation or the potential effects of epitopic suppression (Leclerc et al. 1987). Peptides C2, C3, C5, and C10 were copolymerized as follows. Equal molar amounts of each peptide were mixed in PBS (pH 7.4) to obtain a final protein concentration of 3 mg/ml. Peptides were polymerized with 0.6% (v/v) glutaraldehyde (final concentration), added dropwise under stirring at 25° C. Three successive additions of 0.2% glutaraldehyde were carried out, the first two steps lasting 10 min each and the final reaction step, 30 min. Finally, a sodium borohydride reduction step (10 mg/ml peptide solution) was carried out for 1 h at 4° C to restore the charge to the derivatized amino groups, in effect increasing the solubility of the peptide complex (Briand et al. 1985). Following copolymerization, the peptide solution was dialyzed against PBS for 48 h at 4° C to remove free peptides and glutaraldehyde.

Three rabbits were given a series of three subcutaneous immunizations with the synthetic peptide complex SPf70. Peptide doses

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Table 1. Synthetic peptides[a] constructed from internal chymotryptic digests of the 70-kDa *Plasmodium falciparum* (Indochina I/CDC) protein

Peptide fragment	Sequence[b] (number of AA)	Secondary structure (%)[c]		
		α-Helices	β-Extended	β-Turn
1. C2	GQDEGEENEG (10)	60	30	70
2. C10	GRNGLGANTDQDDQLEDE (18)	33	28	72
3. C5	DQFFDANPNLFQLLEPVEFDED (22)	64	64	41
4. C3	LVFLVQQPFLFVLWDQNEKFPVFM-GVYDP (29)	31	69	21

[a] Peptides ranked according to hydrophilicity
[b] Single-letter code for amino-acid (AA) residues
[c] Secondary structure (% amino acids) as predicted by the Chou-Fasman algorithm in the DNASTAR program

Results

The present study showed that a unique set of peptides (termed C2, C3, C5, and C10) derived from a 70-kDa *Plasmodium falciparum* exoantigen could be effectively used as a synthetic immunogen (SPf70) when they had been copolymerized with glutaraldehyde in the absence of a carrier. When given with Freund's adjuvant, the synthetic peptide complex was found to be highly immunogenic in rabbits. Specific anti-peptide responses were analyzed by conventional peptide ELISA (Fig. 1). Serologic reactivity to the peptide complex and peptides C2 and C5 was uniformly high, followed by the responses to peptides C3 and C10. Normal rabbit serum gave negligible background reactivity (data not shown). These data suggest that the most immunogenic epitopes are components of peptides C2 and C5. The apparent efficacy of C2 and C5 for eliciting good IgG antibody levels may be correlated with the presence of putative T-cell epitopes as demonstrated by their respective content of alpha-helices (Table 1). Moreover, antibodies to the peptide complex are reactive with *P. falciparum* schizonts as demonstrated by indirect immunofluorescence assays, with titers ranging from 1:320 to 1:640 after the third immunization (data not shown).

Peptide antigenicity was assessed with human anti-*P. falciparum* sera from malaria-endemic regions of Uganda and Venezuela. ELISA data showed that anti-*P. falciparum* antibodies were specific for and reactive to the peptides (Fig. 2). With the exception of peptide C10, the peptides demonstrated varying degrees of antigenic reactivity; peptides C3 and C5 were the most antigenic. Normal human serum gave minimal background reactivity (mean absorbance value = 0.11, data not shown). The greatest reactivitiy in terms of absolute values was seen with the Ugandan sera. The lesser reactivity with antibodies in Venezuelan immune sera may be attributable to strain differences or may indicate a lower level of naturally acquired anti-70-kDa (and anti-peptide) antibodies in the sera. Inhibition data indicated that peptide C5 completely inhibited the binding of Venezuelan sera and reduced the binding of Ugandan sera by approximately 50%, confirming the serological specificity of these antibodies from antimalarial sera. The serological activity of the other peptides was considerably lower.

The specificity of the rabbit anti-SPf70 antibodies for *P. falciparum* antigen was shown by immunoprecipitation of metabolically labeled proteins (Fig. 3) and by immunoblotting (Fig. 4). The major protein precipitated

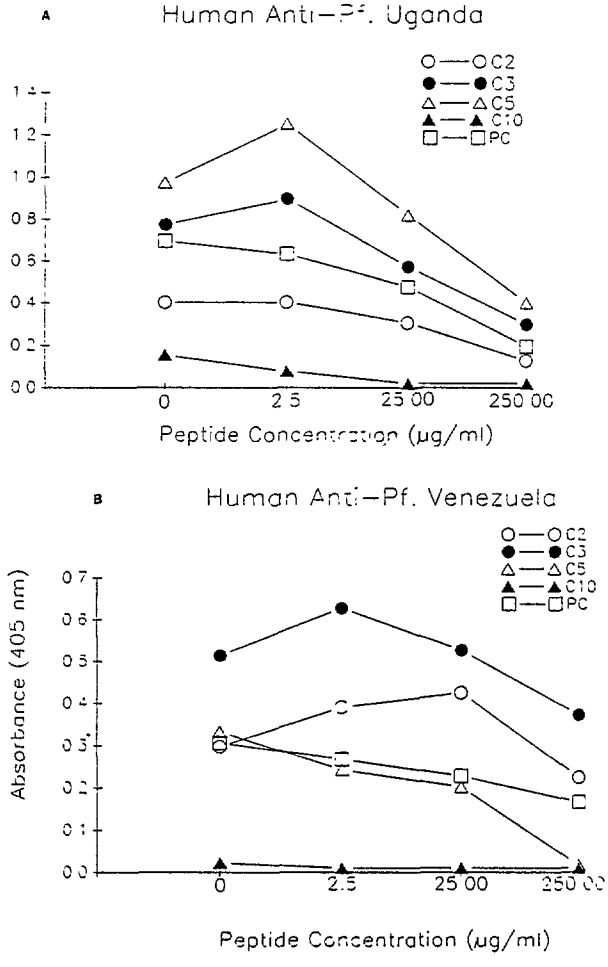

Fig. 2A, B. Antigenicity of synthetic peptides derived from the 70-kDa *Plasmodium falciparum* exoantigen. Hyperimmune human serum pools containing anti-*P. falciparum* antibodies from malaria-endemic Uganda (A) and Venezuela (B) were preincubated with peptides *C2*, *C3*, *C5*, and *C10* and the peptide complex (*PC*) prior to measurement of the antigenic reactivity (mean absorbance values of triplicate wells) of homologous peptides in a standard peptide ELISA

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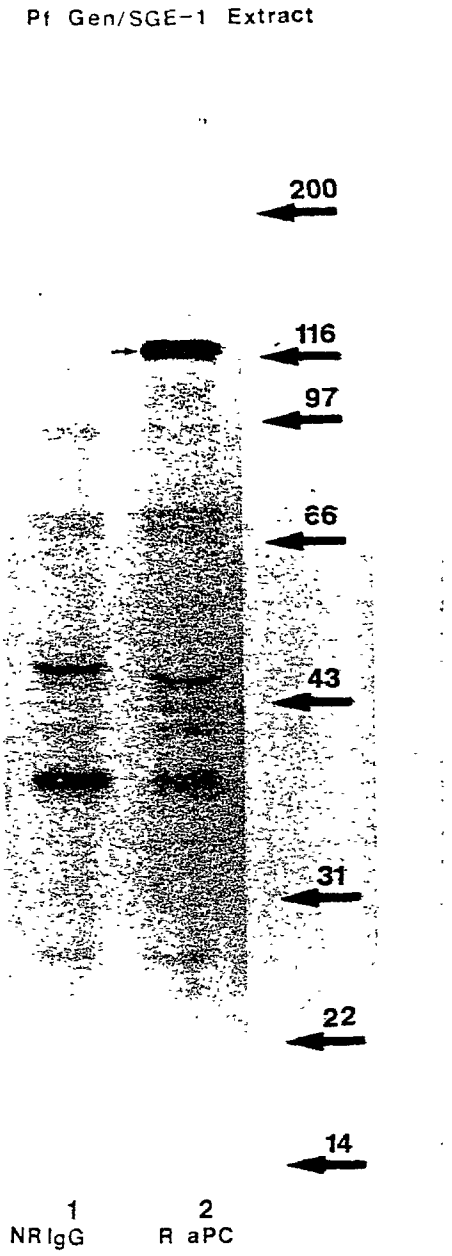

Fig. 3. Biosynthetic labeling and immunoprecipitation of *P. falciparum* antigen [^{35}S]-Methionine-labeled *P. falciparum* (*Pf*) Geneve/SGE-1 schizonts were solubilized and immunoprecipitated with rabbit anti-peptide complex (*R aPC*) IgG (*lane 2*). Normal rabbit IgG (*NR IgG*) was used as a control (*lane 1*). A major 120-kDa *P. falciparum* antigen precipitated by immune IgG is indicated with an *arrow*

by anti-peptide complex (PC) IgG was a 120-kDa schizont antigen. Several other minor proteins were also precipitated nonspecifically by normal rabbit IgG. It appears that the 120-kDa membrane protein is degraded in mature schizonts to the 70-kDa exoantigen, which increases in amount at the time of merozoite release/reinvasion (Braun-Breton et al. 1986). At this point, a substantial amount of 70-kDa antigen is released into the surrounding medium. Hyperimmune sera collected sequentially from rabbits immunized with the synthetic copolymer reacted in immunoblots (Fig. 4) with the 70-kDA antigen from (1) *P. falciparum* Indochina I-infected erythrocyte extracts; (2) supernatant fluid, i.e., exoantigens, from Indochina I cultures affinity-purified with monoclonal antibody to peptide C3; and (3) Indochina I exoantigens affinity-purified with *P. falciparum*-immune IgG of Ugandan origin.

Discussion

Herein we describe the peptide sequences of a 70-kDa *Plasmodium falciparum* exoantigen (Pf70) that, when synthesized and constructed as a copolymer (SPf70), are capable of inducing the formation of antibodies that are reactive with the native malarial protein. The knowledge of peptide sequences provided the potential for construction of synthetic peptides representing immunodominant and, hopefully, immunorelevant epitopes of the native protein. If Pf70 is involved in the induction of TNF production in clinical malaria, our approach would be to design and construct a synthetic immunogen that could theoretically be used to improve upon nature. With the careful selection of parasite-specific epitopes, one could enhance the immunogenicity and induce a solid immunity against the disease (Good and Miller 1990; Berzofsky 1991).

In this initial phase of our studies, the construction of the synthetic peptide hybrid was carried out by glutaraldehyde copolymerization (Leclerc et al. 1987). Although glutaraldehyde copolymerization results in a relatively randomly linked product, it is conceivable that this randomized relationship of multiple parasite-specific B-cell and T-cell epitopes would be adequate in meeting the requirements for induction of potent immune (antibody- and T-cell-mediated) responses. Immunogenic and antigenic epitopes must now be experimentally defined and mapped. Nevertheless, the high antigenic reactivity of SPf70 and the individual component peptides demonstrates the induction of T-cell-dependent antimalarial antibody (IgG) responses and indicates the potential use of synthetic peptide polymers as immunogens and diagnostic reagents (together with anti-peptide antibodies for the detection of antigen).

One of the important indicators of functional immunity to malaria is the recognition of malarial antigens or synthetic derivatives by defined sera from human populations immunized by natural exposure to *P. falciparum*. Recent studies in epidemiologically defined populations from Indonesia, Brazil, and Africa (Burkina Faso) have shown anti-SPf70 reactivity to correlate with naturally acquired protective immunity. The first study was conducted by J.K. Baird et al. (unpublished data). A total of 218 serum samples from Irian Jaya were analyzed for antibody to SPf70 by ELISA. Sera were collected from individuals during a malaria epidemiology study by Baird et al. (1991). Subjects were grouped according to age and immune status (recent versus lifelong residents). Antibody reactivity to SPf70 was found to increase with age among migrant people with only 2 years'

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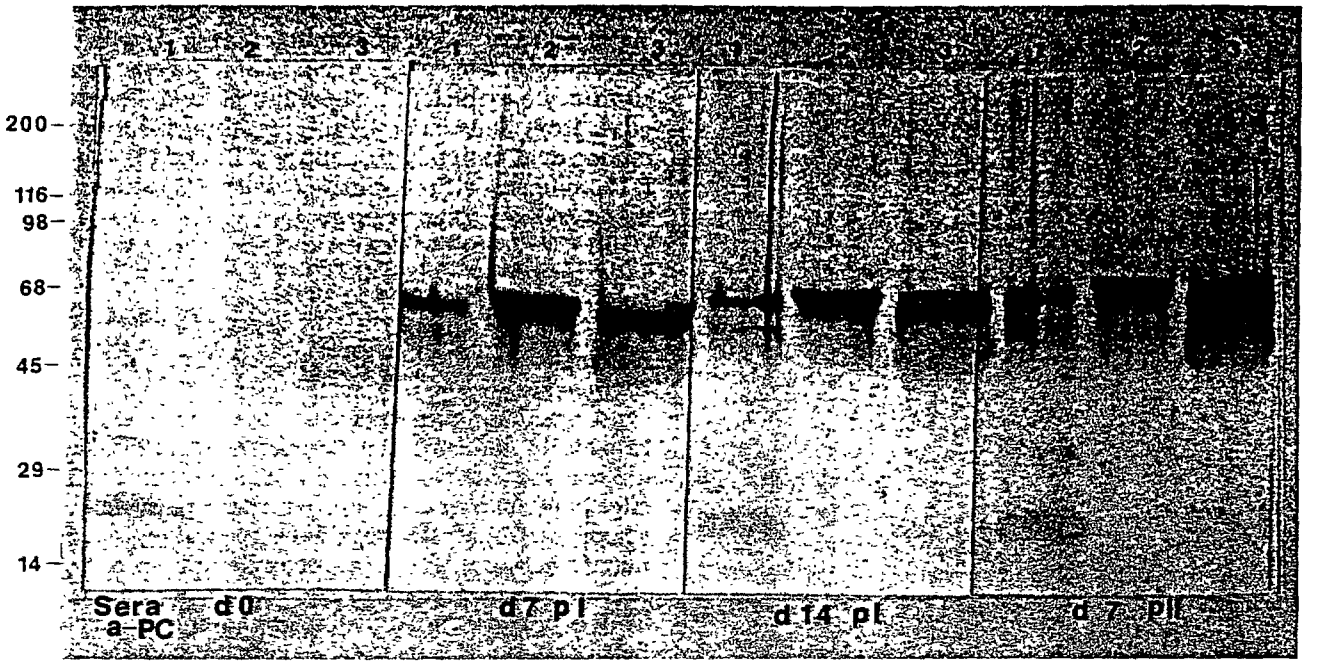

Fig. 4. SDS-PAGE/immunoblotting of the 70-kDa *P. falciparum* protein. Antigens from *P. falciparum* Indochina I/CDC in vitro cultures (infected erythrocyte lysate, *lane 1*; exoantigen affinity-purified with monoclonal antibody to peptide C3, *lane 2*; exoantigens affinity-purified with immune IgG from Ugandan subjects, *lane 3*) were reacted in immunoblots with antisera (pooled) collected sequentially from rabbits immunized with the synthetic peptide complex (*PC*). Sera collected 7 and 14 days (*d7*, *d14*) after the first (*pI*) and second (*d7 pII*) immunizations were compared with baseline (*dO*) sera

exposure to hyperendemic malaria. The increased frequency and level of anti-SPf70 antibody observed among 110 migrants was quantitatively parallel to that noted among 100 lifelong residents of the hyperendemic area. Overall, 83% of the people had antibodies reactive with SPf70. The proportion of people positive for anti-SPf70 antibody increased from 45% among 2- to 5-year-olds to 95%–100% among adults (>15 years of age).

The second study was conducted by Ribeiro et al. (1991). In a seroepidemiology study designed to assess the natural humoral response to various *P. falciparum* antigens, including Pf70, the humoral immune recognition of SPf70 was analyzed by ELISA. Sera were collected from diverse human populations, e.g., Amazonian Indians and hyperimmune Africans from Burkina Faso. Seropositivity rates for antibodies reactive with SPf70 were 58% for the Amazonian subjects and 85% for the population from Burkina Faso as compared with rates of 51% and 20%, respectively, for antibodies to an $NANP_4$ peptide derived from the central repeat region of the circumsporozoite protein.

The above studies indicate that the target Pf70 peptides are immunogenic in nature, as 85% of hyperimmune West African adults and 95%–100% of immune Irian Jayan adults possess antibodies to Pf70. Moreover, the present results confirm the immunogenicity and antigenic reactivity of the synthetic peptide complex SPf70. Future studies should provide information as to the cellular and humoral mechanisms involved in the development of immunity to the 70-kDa *P. falciparum* protein, expecially the correlation of epitope- or peptide-specific responses to infection.

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