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A 175 kDa Plasmodium falciparum blood stage antigen which binds to erythrocytes and to merozoites, designated EBA-175, appears to be involved in the initial attachment of the merozoite to the erythrocyte, and it has been postulated that antibodies to EBA-175 could inhibit merozoite reinvasion (Camus & Hadley, Science, 1985;230:553). We have characterized and sequenced a 1.8 kilobase EBA-175 gene fragment cloned in lambda gt11. The clone was identified by monospecific antibodies selected from malaria immune monkey serum by affinity purification with EBA-175 adsorbed to nitrocellulose. Monospecific antibodies were then selected from malaria immune monkey serum by affinity purification with the expressed protein of the gene fragment and identified authentic EBA-175 in immunoblots. Southern analyses of genomic DNA digested with restriction enzymes Hind III or Xba I and probed with the cloned EBA-175 gene fragment showed no restriction fragment length polymorphism in 5 of 6 geographically distinct isolates of P. falciparum. Based on the deduced amino acid sequence of this EBA-175 gene gragment, several peptides were selected, synthesized,

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coupled to carrier molecules, and used for immunization studies. This work will enable evaluation of EBA-175 as a vaccine candidate.

FOREWORD

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____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

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INTRODUCTION

Malaria infects 100-200 million people a year and is considered by many to be the most important infectious disease in the world. The mosquito vectors of malaria have become increasingly resistant to insecticides, and Plasmodium falciparum, the most important human malaria parasite, has become increasingly restistant to antimalarial drugs. For these reasons there have been major efforts to develop malaria vaccines. The development of vaccines for malaria is complicated by the fact that potentially protective antigens are specific for each of the main developmental stages of this parasite. An effective vaccine against malaria is envisaged to contain a mixture of different stage antigens. Major interest has been directed against the blood stage of the parasite; the stage responsible for the pathology and morbidity of the disease.

Efforts include the identification of blood stage parasite molecules which are important for parasite survival. In the blood, host erythrocytes are invaded by the merozoite stage of the parasite. This invasion process is dependent on the capability of merozoites to recognise ligands on the erythrocyte membrane.(1,2). A recent study reported the isolation of a Plasmodium falciparum blood stage antigen from the CAMP strain which binds to host erythrocytes and to merozoites (3). It was postulated that this antigen designated EBA-175 is a receptor acting as a bridge between erythrocyte and merozoite. Since EBA-175 appears to be important in the initial attachment of the parasite for the erythrocyte, it has obvious value as a target antigen for vaccine development.

The objective of this project has been to isolate and characterise the gene encoding EBA-175, select portions of the protein encoded by this gene for immunogenicity studies, and to determine if the antibodies elicited by such antigens inhibit the invasion of merozoites into erythrocytes.

The technical approach used was to screen a genomic expression library with an affinity purified antibody to EBA-175, select appropriate clones, sequence the clones, analyze the deduced amino acid sequence for potentially immungenic domains, synthesize peptides from these domains, immunize mice and rabbits with these peptides, and determine if the antibodies induced by such immunizations recognized the peptides, blood stage parasites, EBA-175 on Western blots, and inhibited merozoite invasion of erythrocytes.

BODY:

i) Reagent to screen library with:

Preparations of EBA-175 were obtained from P. falciparum culture supernatants which had been incubated with human erythrocytes for 1 hour at room temperature and eluted off the erythrocytes with1.5M sodium chloride. EBA-175 preparations were fractionated by SDS-polyacrylamide gel electrophoresis and the gel was electroblotted onto nitrocellulose in Tris/Glycine buffer. Blots were blocked with TBS-0.3% Tween 20 for 40 minutes on a rocker platform, rinsed once with TBS-0.05% Tween 20 and air-dried. Using pre-stained molecular weight markers and a sample strip cut off from one end of the blot which is processed in the usual fashion of Western blots, a strip at the region of 175 kd is excised. This strip is then rehydrated in TBS-0.05% Tween 20 for 30 minutes. The strip is then incubated for 90 minutes with 1/100 dilution of immune monkey serum which contains antibodies to EBA-175. The EBA-175 strip is washed withTBS-0.05% Tween 20. Bound antibody was eluted from the strip with 2 ml aliquotes of 200 mM glycine, pH 2.8/ 150 mM NaCl/ 0.3% Tween 20, the eluants pooled and neutralized with Tris. The reagent obtained is affinity purified monospecific antibodies to EBA-175.

ii) Genomic Library:

A gt11 genomic expression library was constructed as follows. Genomic DNA from parasites in in vitro culture was obtained by standard procedures (4), and digested with the restriction enzyme mung bean nuclease. Ends of DNA fragments were repaired and EcoRI linkers were attached, before the DNA fragments were

cloned into the EcoRI site of the phage vector gt11, by standard techniques (5).

iii) Screening the Library

The library was plated out at $3x10^4$ pfu per large plate. Duplicate nitrocellulose filter lifts were made after the plaques were induced with IPTG and X-Gal (5), blocked in 0.5% Tween 20 before incubating overnight in the affinity purified antibody to EBA-175. Filter lifts were developed with alkaline phosphatase conjugated anti-monkey antibodies. $3x10^5$ pfu were screened and 7 plaques were selected for secondary screening. One plaque remained positive after tertiary screen.

This plaque designated gt11/EBA9 was plaque purified, amplified and the DNA obtained digested with restriction enzyme EcoRI to determine the insert size. The clone gt11/EBA9 contained an insert of 1.8 kb.

iv) Confirmatory Studies

To confirm that the 1.8 kb insert actually coded for a portion of EBA-175, several experiments were performed. The clone gt11/EBA9 was made a lysogen by transfecting lysogenic host Y1089 with the phage of gt11/EBA9, by standard procedures (6). The lysogen obtained was induced with IPTG. One ml of expressed protein was obtained from the pellets of 1 litre of induced lysogen by standard procedures (6). The expressed protein was fractionated on an SDS-Polyacrylamide gel and a standard Western analysis was performed using the affinity purified monospecific antibodies as a probe, and as a control, a duplicate lane was probed with anti beta-galactosidase. A major 65 kd and a minor 120 kd band was obtained which is reproducible even with the addition of a cocktail of protease inhibitors (TPCK, PMSF). The proposed interpretation of this is that the protein is expressed fused to beta-galactosidase and a clip occurs early after expression of the protein resulting in 65 kd of pure expressed protein and 4 kd fused to the 116 kd of beta-galactosidase.

Affinity purified antibodies to the 65 kd band as well as to an induced confluent plate of clone gt11/EBA9 was prepared off nitrocellulose by the method described in detail in section i). Affinity purified antibodies prepared to both the 65 kd band as well as the confluent plate identified the authentic EBA-175 in standard Westerns.

v) Sequencing the clone EBA9.

The 1.8 kb insert was cloned into the EcoRI site of the double stranded sequencing vector pGEM 4B (Promega) and transformed into the competant host cell XL-1Blue (Strategene), by standard procedures (6). The resultant clone was designated EBA9. Deletion clones made by the Henikoff procedure (7) were generated to facilitate sequencing the clone. Sequencing was essentially by the "dideoxy" procedure (8). The 1.8 kb fragment was completely sequenced and was found to have a long open reading frame that terminates with multiple stop codons in its highly AT-rich 3' end. The deduced amino acid sequence was 576 residues, with a molecular weight of 66028 Daltons.

vi) Characterization of the 1.8 kb insert.

To investigate whether this 1.8 kb fragment was conserved among strains of P. falciparum, genomic DNA was isolated by standard procedures (4) from 6 different geographical isolates. The DNA obtained were digested by restriction enzymes EcoRI, XbaI, HpaI, DdeI and HinDIII and then fractionated on a 0.8% agarose gel. The gel was subsequently transferred by method of Southern (9) and probed with the 1.8 kb fragment which had been labeled by nick-translation (10). There was strong hybridization indicative of strong homology of the 1.8 kb fragment to all the strains tested (FCR3 from Gambia, 7G8 from Brazil, Honduras, Santa Lucia, IMTM22 from Brazil). The restriction patterns generated on the Southern analyses were identical in all strains except the FCR3 strain which had restriction site differences.

vii) Synthetic peptides predictive of antigenic determinants

The deduced amino acid sequence obtained from the 1.8 kb fragment had an average PI of 4.3 indicating that this was an acidic and highly negatively charged

section of the protein. Hydrophilicity plots and beta turn charts of amino acid sequences derived show several regions predictive of antigenic determinants.

The peptides designated KIM2 and KIM4 were synthesized by a modification of the method of Merrifield (11) with an Applied Biosystems synthesizer, Model 430-A, using the manufacturers software on a 0.1mMole scale.

These peptides were conjugated to the carrier KLH either with glutaraldehyde or with the divalent cross-linkers SPDP or MBS. An average of 10 molecules of peptide to one of KLH was obtained.

viii) Immunization with synthetic peptides.

These conjugated peptides were emulsified in Freund's complete adjuvant seperately, and used to immunise 5 Balb/c mice each with 100ug of peptide. These mice were boosted with 100ug of the respective peptide emulsified in Freund's incomplete adjuvant at 3 weekly intervals. A control group of mice were immunized with an equivalent amount of KLH alone emulsified with Fruend's adjuvant given in the same schedule.

The different groups of mice were bleed 1 week after their second boost, and the sera from each individual group were pooled. ELISA were run according to standard protocols(12) using peptide alone as capture antigen at concentrations of 10ug/ml. Titres obtained for both the peptides KIM2 and KIM4 were significant at dilutions of 1: 50,000 and above.

ix) Isolating other regions of the EBA-175 gene.

To isolate other regions of the EBA-175 gene, a suitable restriction enzyme which would give a restriction fragment of a clonable size was needed. Genomic DNA of the CAMP strain of P. falciparum was digested with several restriction enzymes individually. The digested DNA was fractionated on an agarose gel and analysed by method of Southern (9). The probe used was the 1.8 kb fragment, radiolabeled by nick-translation (10). The genomic Southern revealed a single 5.5 kb Xba I fragment which included the entire 1.8 kb fragment. Since this meant that there were possibly as much as 3.3 kb of upstream sequences in this 5.5 kb Xba I fragment, efforts were made to isolate this particular fragment. To this end, an Xba I genomic library was constructed in the double stranded sequencing vector PGEM-4B (Promega). The genomic DNA used to construct the library was size selected over a 15%-30% continuous gradient, to select for DNA fragments of the sizes between 4.4 kb-6.5 kb.

CONCLUSIONS

An affinity purified monospecific antibody to EBA-175 was prepared and used to screen a CAMP strain gt11 genomic expression library. A reactive clone containing a 1.8 kb insert was identified, isolated characterized and completely sequenced. The 1.8 kb gene fragment contained a long open reading frame translating to a minimum of 576 amino acid residues.

This 1.8 kb gene fragment cross-hybridized strongly to 5 other isolates of P. falciparum from various geographical locations. Restriction patterns on genomic Southerns were identical in all strains tested except for the strain FCR3 which showed restriction site differences. From the deduced amino acid sequence of the 1.8 kb gene fragment, synthetic peptides predictive of antigenic determinants were synthesized. Synthetic peptides KIM2 and KIM4 were conjugated to carrier molecules and used to immunise mice. The mice made extremely high titres of antibody to the immunizing peptide. At the same time, efforts were directed at isolating other regions of the EBA-175 gene.

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