นิพนธ์ต้นฉบับ

การผลิตและศึกษาคุณสมบัติของแอนติไอดิโอไทปิกแอนติบอดี ชนิดโพลิโคลนอล ต่อมาลาเรียเมอโรซอยท์แอนติเจน

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บทคัดย่อ

วัตถุประสงค์ : โปรตีนแอนติเจนขนาด 19 กิโลดาลตัน บนผิวเชื้อมาลาเรียในระยะเมอร์โรซอยท์ ($MSP1_{19}$) มีลำดับกรดอะมิโนค่อนข้างคงที่ในระหว่างสายพันธุ์ของมาลาเรีย สามารถนำมาใช้เป็นเป้าหมายในการผลิตวัคซีนได้ สำหรับ MAb302 ซึ่งเป็นโมโนโคลนอลแอนติบอดีต่อ $MSP1_{19}$ ชนิด IgG3 นั้นสามารถป้องกันหนูจากการติดเชื้อมาลาเรียสายพันธุ์ *P. yoelii* ได้ งานวิจัยนี้มีวัตถุประสงค์เพื่อผลิต anti-idiotypic antibody (anti-Id) ของ $MSP1_{19}$ ซึ่งจะเป็นแอนติบอดีที่สามารถเป็นตัวแทนของ $MSP1_{19}$ ได้

วิธีการ : นำ MAb302 (Ab1) ฉีดกระตุ้นให้กระต่ายทดลองเพื่อผลิต anti-Id (Ab2) แล้วนำซีรั่มไปคัดแยกบริสุทธิ์ให้ได้เฉพาะ IgG ทดสอบคุณสมบัติความเป็นแอนติเจนตัวแทนของ $MSP1_{19}$ ด้วย competitive ELISA โดยเปรียบเทียบความจำเพาะต่อ Ab1 กับ $MSP1_{19}$ จากนั้นนำ Ab2 ที่แยกส่วนFc ออกด้วยเอนไซม์ papain ฉีดกระตุ้นหนูทดลองเพื่อผลิต Ab3 แล้วนำกลับมาทดสอบกับ $MSP1_{19}$

ผลการทดลอง: Anti-Id ต่อ $MSP1_{19}$ (Ab2) (>50 μg/ml) สามารถยับยั้งการจับของ MAb302 ต่อ $MSP1_{19}$ ได้ (100% inhibition) รูปแบบทั้ง Fab และ IgG ของ Ab2 สามารถกระตุ้นการสร้าง Ab3 ที่สามารถตอบสนองต่อทั้ง Ab2 และ $MSP1_{19}$ แม้ไม่ได้ฉีดกระตุ้นด้วย $MSP1_{19}$ มาก่อน

สรุป: การศึกษานี้แสดงให้เห็นว่า สามารถผลิตแอนติไอดิโอไทปิก ต่อ $MSP1_{19}$ ชนิด โพลิโคลนอล ที่จับกับส่วนแอนติเจนของ MAb302 และมีส่วนเหมือนกับ $MSP1_{19}$ ในด้านโครงสร้าง โดยคุณสมบัติด้านภูมิคุ้มกันจะเป็นการทดลองต่อไป ทั้งนี้มีความเป็นไปได้ที่จะนำไปผลิตเป็นแอนติเจนและพัฒนามาลาเรียวัคซีนต่อไป

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คำหัส: Anti-Id antibody, $MSP1_{19}$, Mab302

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Abstract : Production and Characterization of Polyclonal Anti-idiotypic Antibody to Malaria Merozoite Antigen

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Objective: The 19-kDa carboxyl terminal fragment of merozoite surface protein 1 ($MSP1_{19}$) is a highly conserved region within malaria species. MAb302, which defines a highly conserved neutralization epitope of $MSP1_{19}$, showed protective activity against *P. yoelii*. The aims of this reseach were to produce polyclonal anti-Id mimic to $MSP1_{19}$, used as surrogate antigens for active specific immunity.

Method: MAb302 (Ab1) was used as antigen to immunize rabbit to produce anti-Id (Ab2). The binding of $MSP1_{19}$ to Ab1 was competed by Ab2 using competitive ELISA. Ab2, which was isolated Fc fragments by papain and affinity chromatography, was immunized to BALB/c mice to produce Ab3. Finally, its binding activity to $MSP1_{19}$ was performed.

Result: Anti-Id (> 50μg/ml) could block binding of $MSP1_{19}$ to MAb302 in a dose-dependent manner with 100% inhibition. Either whole molecule or Fab fragment of anti-Id were able to induce the production of both anti-Id and $MSP1_{19}$ neutralizing antibodies in BALB/c mice.

Conclusion: The binding of polyclonal anti-idiotype antibodies to antigenic determinant of MAb302 mimiced the three-dimensional structures of $MSP1_{19}$. The protective property against malaria infection should be studied. This study can be applied as strategies for developing malaria vaccine based on $MSP1_{19}$ by using anti-Id as another surrogate antigen for active specific immunity.

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Key words : Anti-Id antibody, $MSP1_{19}$, Mab302

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Introduction

Merozoite surface protein-1 (MSP1) is a protein synthesized and expressed on the surface membrane of merozoites during blood stage malarial infection and is recognized as a leading malaria vaccine candidate. The

protein undergoes proteolytic processing during schizogony and merozoite maturation giving rise to a number of fragments. One of these, the 19-kilodalton fragment at the carboxy terminus of MSP1(denoted $MSP1_{19}$) is carried to young ring form schizont in newly infected red blood cells [1]. $MSP1_{19}$ is highly conserved among *Plasmodium* species and is composed of two epidermal growth factor (EGF)-like domains which contain protective epitopes[2-3]. It is a potential vaccine candidate because it is directly exposed and interacts with the host during RBC invasion.

In the present study we attempted to present the epitopes of $MSP1_{19}$ by immunological regulation through the interactions between idiotype (Id) and anti-idiotype (anti-Id) antibodies [4]. Anti-Id antibodies represent internal images that was similar to the epitope of the original antigens and could serve as potential idiotypic vaccines for the induction of specific immune responses to the original antigens that they mimic without exposing the host to antigens of pathogenic origin [5]. It provided a reliable way to prepare vaccines against infectious diseases without potential hazard.

The antibodies (Ab1) produced not only react against the antigen, but they also served as antigen themselves. This triggers production of the second antibodies (Ab2). In turn, these antibodies reacted against Ab1 and served to induce the anti-Ab3 antibodies, and the process continues. Idiotypic vaccines composed of anti-Id antibodies have been applied in the control of many diseases caused by parasites such as *Trypanosoma rhodesiense* [6], *Schistosoma mansoni* [7], and *Babesia gibsoni* [8,12]. Thus, Jernes network theory [4] had opened a huge area of protection against pathogenic microbes by preparing anti-Id antibodies that mimic epitopes of their antigens. These successful investigations further encouraged the present study, in which the study prepared the anti-Id antibody showing the $MSP1_{19}$ internal image from the Fab (Id) of a monoclonal antibody (MAb) against $MSP1_{19}$ and examined its antigenicity in BALB/c mice.

Materials and methods

Monoclonal antibodies specific to $MSP1_{19}$ (MAb302) was a kind gift of Dr.Chakrit Hirunpetcharat (Hybridoma Technology of Queensland Institute of Medical Research Brisbane, Australia). Yeast-expressed $MSP1_{19}$ of *P. yoelii* produced as a His_6-tagged protein in *Saccharomyces cerevisiae* ($yMSP1_{19}$)[13] was obtained from Dr. David Kaslow (The Laboratory of Parasitic Disease, NIAID, National Institutes of Health, Bethesda, MD, USA)

Antigen preparation

$MSP1_{19}$ as a FLAG fusion protein (FLAG-$MSP1_{19}$) was produced from *Saccharomyces cerivisae*. The yeast strain was inoculated in YPEM medium (yeast-peptone extract medium) and incubated overnight at 250 rpm, 28 °C. The

yeast culture was transferred to YPHSM (yeast-peptone extract medium), and incubated at 28 °C, 250 rpm for 48 hr. The cell suspension was transferred, the supernatant was collected. The supernatant was resuspended in Tris-buffered saline (TBS), mixed with anti- FLAG $MSP1_{19}$ gel column for 1 hr at room temperature. The supernatant was discarded, the gel were washed twice as mentioned above [9].

Production of anti-Id IgG (Ab2)

New Zealand rabbits (weigh 1.2–1.5 kg Laboratory Animal Center, Faculty of Medicine, Chiangmai University) were immunized subcutaneously twice with 0.5 mg MAb302 (Ab1) in 0.5 ml PBS emulsified in Complete Freund's Adjuvant (CFA) for the first injection and in Incomplete Freund's Adjuvant (IFA) for the second injection 2 weeks later. Two weeks after the last immunization, rabbit anti-Id sera were collected and anti-Id IgG (Ab2) was purified by a protein G column. The binding activity of Ab1 and Ab2 was analyzed by Enzyme-Linked Immunosorbent Assay (ELISA). Ab1 was used for coating ELISA plates, and the wells were blocked with 1% BSA/PBS. The assay was developed with ALP-conjugated anti-rabbit IgG antibodies. Preimmunized rabbit serum was used as control.

Binding site assay

The microtitre plates (Maxi-sorb immunoplates, Nunc) were coated with 100 µl of 0.1 µg/ml $MSP1_{19}$ in carbonate-bicarbonate buffer pH 9.5 overnight at 4 °C. After three times of washings with 0.05% Tween/PBS, wells were blocked with 200 ml of 1% BSA/ PBS at 37 °C for 1h. Seriall dilution of Ab2 was co-incubated with fixed amount of Ab1 at 37 °C for 1h. The supernatant were discarded, and wells were washed 3 times with 0.05%Tween in PBS (PBST). Antibody mixture were added to each well and incubated for 1 h at 37 °C. After washing 3 time, 100 µl of 1:2000 HRP- conjugated anti-mouse/rabbit IgG antibodies were added. Following the incubation at 37 °C for 1h, wells were washed, substrate solution was added and incubated at room temperature for 30 min. The absorbance was measured at 405 nm, and the %inhibition was calculated by the following formula:

$$\%\text{inhibition} = 1 - \frac{(R_T - R_b) \times 100}{R_{max} - R_b}$$

Where R_T is the average OD of the experimental well with inhibitor, R_b is the average background OD, and R_{max} is the average maximum binding without inhibitor.

Fragmentation of anti-Id and murine model for determining the efficacy of anti-Id

The Fab (anti-Id) was obtained by digesting 5 mg/ml of anti-Id with 1 mg/ml of papain (Sigma) in digestion buffer containing 20 mM EDTA and 20 mM cysteine in phosphate-buffered saline (PBS) (137 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7

mM KCl, pH 7.2) for 16 h at 37°C[10]. Purification of Fab was subsequently performed using a protein G affinity column and analyzed by SDS-PAGE according to the previous procedure[10]. Female BALB/c mice aged 6–8 weeks (Laboratory Animal Center, Faculty of Medicine, Chiang Mai University) were used for immunization. Four groups of mice (4 mice in each group) were injected subcutaneously with two of different doses (2 g, 4 g, or 8 g) of anti-Id whole molecule or Fab fragment at 2-week intervals. Each dose of anti-Id-IgG used in each group was emulsified in CFA for the first injection and in IFA for the second injection, respectively. Another group of 4 mice injected with 0.2 ml saline in each injection was used as a non-immunized control group[11].

The binding assay

The 96-well ELISA plates were coated with 100 µl/well of $MSP1_{19}$ diluted in coating buffer (100 mM $NaHCO_3$, 34 mM Na_2CO_3, pH 9.5) and incubated overnight at 4°C. After blocking with 1% BSA/PBS 200 ml/well, 100 µl l/well of the mouse anti-rabbit serum from all conditions were added and incubated for 2 h at RT. The plates were washed with PBST and incubated with 100 µl/well of HRP-conjugated anti-mouse antibody (1:2000), for 60 min at RT. After washing with PBST, each well was incubated with 100 µl/well substrate solution for 30 min at RT in the dark. Reaction was stopped with 50 µl/well of 1 M H_2SO_4 and the absorbance was measured at 450 nm (OD_{450}).

Result

Immunization with MAb302 (Ab1) induces anti-Id antibody (Ab2) development

The MAb302 (Ab1) was purified with a protein G column, it was prepared for immunization of New Zealand white rabbit. The paratope (binding site) of Ab2 developed in rabbit after Ab1 immunization was analyzed by the inhibition of binding of Ab1 to MSP1. As described in Materials and methods, after immunization, sera collected from rabbit were purified with a protein G column pooled of each elute, diluted with PBS and used for analysis. As illustrated in figure 1, single immunization of Ab1 was sufficient to induce an Ab2 response that could significantly inhibit the binding of Ab1 to $MSP1_{19}$. Sera obtained from normal rabbit or pre-immune sera were used as control in this assay.

To demonstrate that anti-Id (Ab2) had a specific interaction against the antigen binding site of Ab1, the specific binding inhibition assay was conducted using MAb302 as an antigen for coating the plate and normal mouse IgG or MAb302 as a competitor. At the same concentration of inhibitor (MAb302 and normal mouse IgG), for example, at 100µg/ml, MAb302 inhibited the binding of anti-Id antibodies and plate coated MAb302 nearly 100% but normal mouse IgG also inhibited as high as 50% as shown in figure 2, suggesting that in the anti-Id antibody preparation some antibodies reacted to the binding site and other reacted to the constant part of the MAb302.

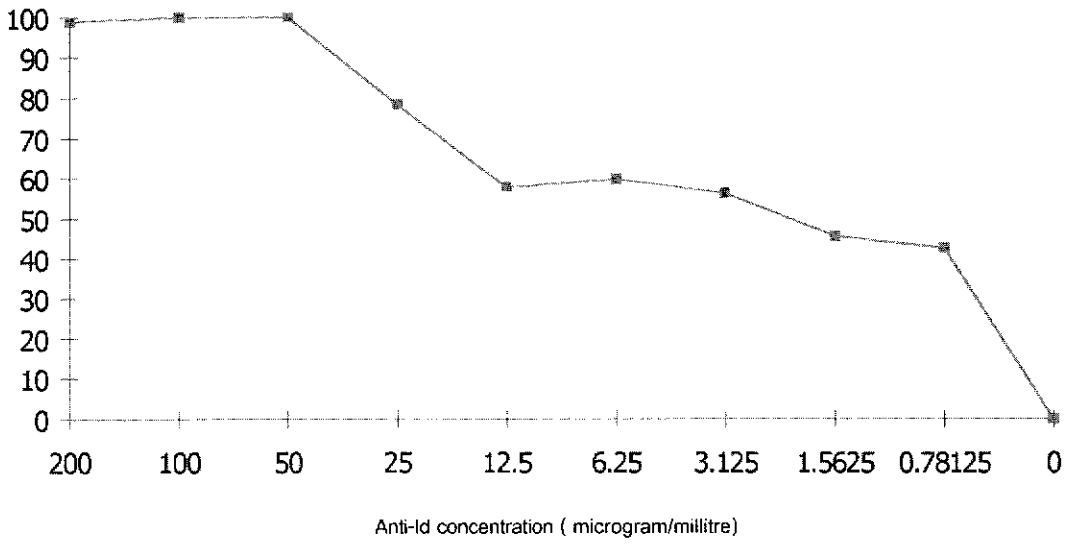

Figure 1 Enzyme-Linked Immunosorbent Inhibition Assay for anti-Id (Ab2) on the binding of Mab302 (Ab1) to MSP1-19. Microtiter plate was coated with MSP1-19, and % Inhibition was calculated as described. Anti-Id (Ab2) was used as competitive inhibitor.

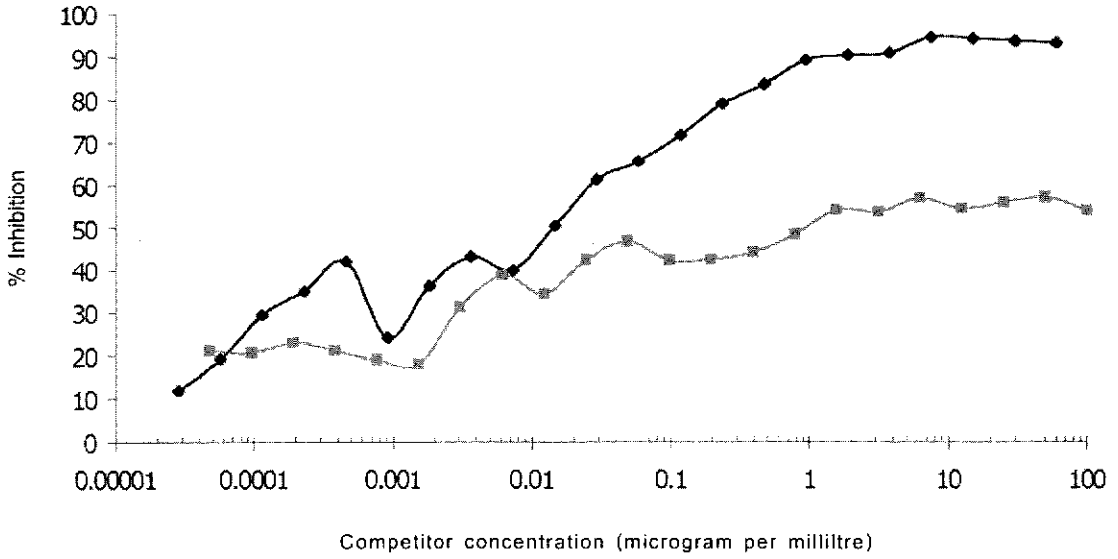

Figure 2 Assessment of specificity of anti-Id antibodies for the binding site of MAb302 by ELISA. A microtiter plate was coated with 0.5 μg/ml MAb302 and the mixture of 0.5 μg/ml anti-Id antibody and MAb302 at various concentrations were added. The reaction was detected as described in material and method.

Induction of anti-anti-Id (Ab3) by anti-Id (Ab2)

The anti-Id (Ab2) digested with papain was purified with a protein G column and analyzed by 12% SDS-PAGE. The preparation clearly consisted only of the 25 kDa protein, which was expected of the Fab (Figure 3, lane

3). The anti-Id IgG and neutralized rabbit sera were used to characterize the antigenicity of Ab2. The Fab Ab2 and neutralized mouse sera were used to confirm whether the internal image of Ab2 exhibits the $MSP1_{19}$-like conformation. Interestingly, both Fab and IgG of Ab2 induced anti-$MSP1_{19}$ antibody (Ab3)(Figure 4), which is expected to be similar to Ab1, while Fab of neutralized rabbit show no significantly effect in mouse anti-rabbit serum nor in IgG from rabbit. It appears that only polyclonal anti-Id showed the $MSP1_{19}$ internal image.

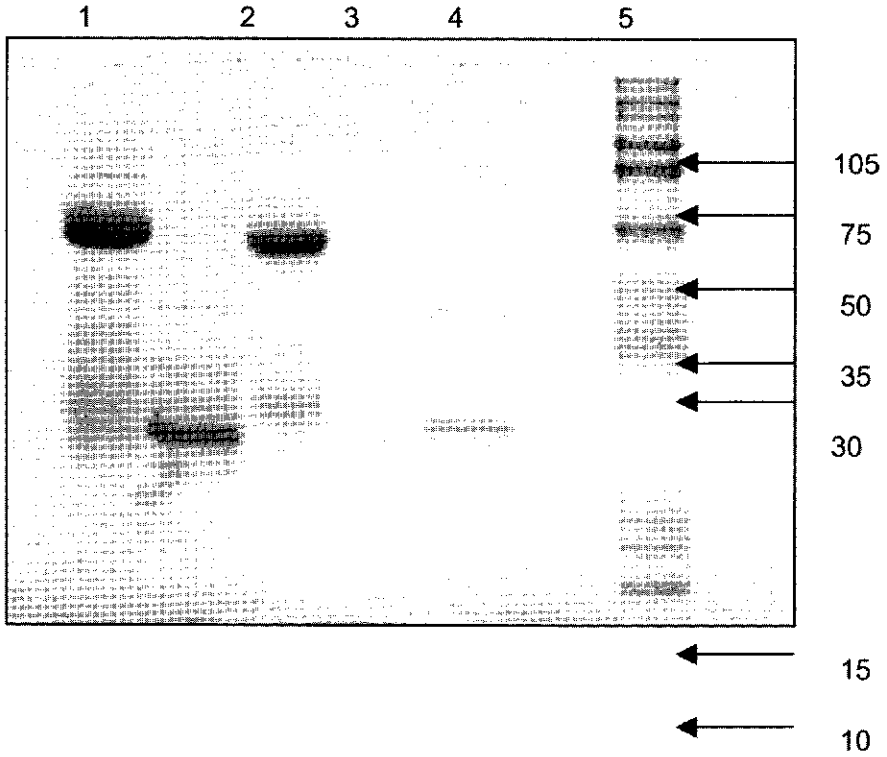

Figure 3 Assessment of antibody fragmentation by enzymatic degradation, papain. SDS-PAGE pattern of IgG before digestion (lane 1), IgG after digestion (lane2), unbound fraction from protein G column (lane3), bound fraction from protein G column (lane 4), protein marker (lane 5). Amount of antibodies used per Lane was 10 μg. All fragment of IgG was prepared by papain digestion

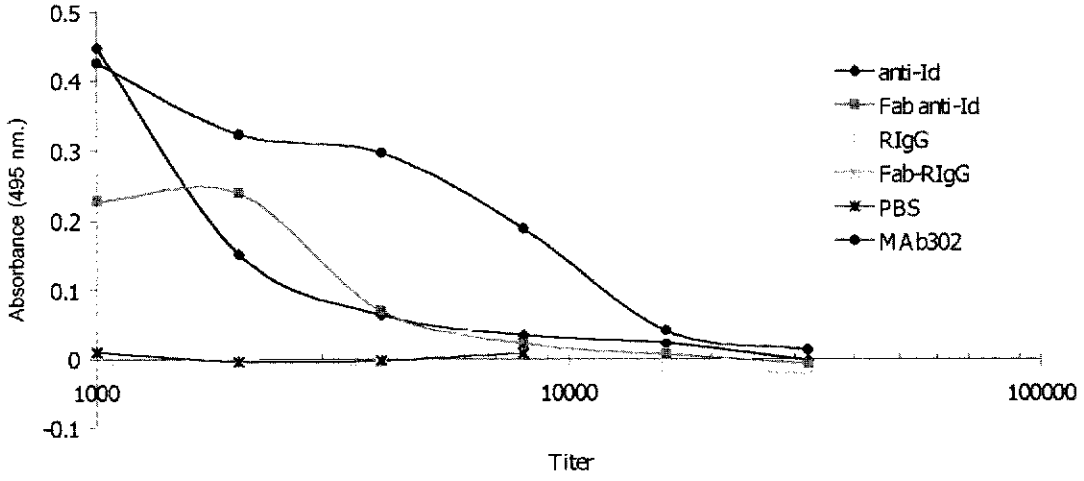

Figure 4 Binding of polyclonal mice Ab3 sera to MSP1-19 by ELISA. The plate was coated with MSP1-19 (100ng/well) and reacted with Ab3 sera (1/1000 dilution) from mice immunized with Ab2 (anti-Id) and unrelated IgG of the same animal. Immunization with PBS was used as control. The result are presented as the mean OD at 495 nm ; n = 4 for all groups.

Discussion

The anti-Id antibody with the internal image of $MSP1_{19}$, was used as an immunogen to immunize mice in the present study. A murine monoclonal antibodies against $MSP1_{19}$ (MAb302) was used for induction of polyclonal anti-Id in rabbit. Anti-Id showed high specific activity to the binding site of MAb302 as proven by no reactivity to normal mouse IgG. Under a variety of circumstances antibodies can be elicited against the variable region of other antibody molecules. Some of the antibodies are directed against the binding site of the eliciting antibodies. Of particular interesting are the antibodies that recognize epitopes of the original antibody which are in contact with antigen. This study postulated that polyclonal anti-Id might contain some antibody with internal-image reactivity for MAb302. The anti-Id was able to compete with $MSP1_{19}$ for binding to MAb302 in a dose-dependent manner, in a competitive ELISA. Hence, the data indicated that the rabbit anti-Id reacted with or near the antigen binding site of MAb302.[14-17]

Either whole molecule or Fab fragment of anti-Id was able to induce the protection of both anti-Id and $MSP1_{19}$ neutralizing antibodies in BALB/c mice. Neither whole molecule nor the Fab fragment of rabbit IgG control could

react with $MSP1_{19}$. The failure of the Ab2 to induce antigen reactive Ab3 thus appears to be due to neither intrinsic affinity nor idiotope frequency, but arises instead from structural reasons, for example, the incomplete penetration of the Ab2 into the binding-site cleft of the Ab1[18]. Consideration of the arrangement of residues, disclosed a region within the framework of the anti-Id of the heavy chain variable domain with similarity to epitope recognized by MAb. This region, formed from antiparallel chains, contains amino acid residues arranged in a conformation similar to that assumed by amino acid residues comprising the epitope within $MSP1_{19}$ [19]. This study indicated the presence of apparent endogenous internal-image Ab2 beta in $MSP1_{19}$. It was possible that this internal image Ab2beta might contribute to malaria vaccine. Although these observation suggest a potential for anti-Id vaccine against malaria, further studies are required to increase the immunogenicity of the anti-Id preparation

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