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Plasmodium falciparum Parasites Expressing Pregnancy-Specific Variant Surface Antigens Adhere Strongly to the Choriocarcinoma Cell Line BeWo

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Placenta-sequestering *Plasmodium falciparum* parasites causing pregnancy-associated malaria express pregnancy-specific variant surface antigens (VSA_{PAM}). We report here that VSA_{PAM}-expressing patient isolates adhere strongly to the choriocarcinoma cell line BeWo and that the BeWo line can be used to efficiently select for VSA_{PAM} expression in vitro.

Women living in areas of stable *Plasmodium falciparum* transmission become highly susceptible to infection during their first pregnancy, regardless of previously acquired protective immunity (reviewed in reference 7). The resulting pregnancy-associated malaria (PAM), which is caused by parasites selectively accumulating in the placenta, is an important cause of poor mother-child health in areas where *P. falciparum* is endemic (18). Placenta-sequestering infected erythrocytes (IE) express particular parasite-encoded variant surface antigens (VSA) on the IE surface that are functionally and antigenically distinct from VSA expressed by nonplacental IE (1, 6, 13). Functionally, the PAM-specific VSA (VSA_{PAM}) are unique in mediating IE adhesion to placental host receptors such as the glycosaminoglycan chondroitin sulfate A (CSA) that are not receptors for VSA expressed by IE in nonpregnant individuals. The fact that *P. falciparum*-exposed males and females who have never been pregnant do not possess VSA_{PAM}-specific antibodies, despite high levels of antibodies specific for other VSA, is evidence of the antigenic distinctiveness of VSA_{PAM}. These findings, and the evidence that links VSA_{PAM}-specific immunoglobulin G (IgG) to acquired immunological protection from the adverse clinical consequences of PAM (5, 17), suggest that VSA_{PAM} can be used in a syndrome-specific vaccine against PAM. Consequently, functional and molecular characterization of VSA_{PAM} and, not least, determination of the intraclonal and interclonal diversity of these antigens are a current high-priority research area (14).

Some *P. falciparum* lines can acquire the VSA_{PAM} phenotype following in vitro selection for IE adhesion to CSA (13, 16), but this type of selection is inefficient with other lines (our unpublished data). Furthermore, many placental *P. falciparum* isolates have relatively low affinity for CSA (6), while some nonplacental isolates have been reported to adhere to CSA (3). Finally, recent studies have shown that IE can acquire the ability to adhere to the placental choriocarcinoma cell line BeWo following selection in vitro and that this adhesion can be partially inhibited by soluble CSA and chondroitinase treatment (9, 20). These studies suggest that IE adhesion to BeWo cells may be a valuable tool in the characterization of the adhesion specificity of VSA_{PAM}-expressing parasites isolated from PAM patients and that this cell line can be used for efficient selection of VSA_{PAM}-expressing IE in vitro. However, no patient isolates were included in the earlier studies (9, 20), and other than line 3D7 only long-term in vitro adapted laboratory lines that can be readily selected for IE binding to CSA were used. Finally, acquisition of VSA_{PAM} expression in response to selection of IE for adhesion to BeWo cells in vitro has not previously been documented.

To further validate the role of BeWo cells in PAM research, we first examined the VSA expression of 16 *P. falciparum* patient isolates obtained from the peripheral blood of pregnant women living in an area of stable parasite transmission. The isolates were collected, and their VSA expression was characterized as previously described (12). Fifteen of the isolates clearly expressed VSA_{PAM} when tested in a flow cytometry assay (13, 15). Thus, for each of these isolates the levels of VSA-specific IgG in the plasma of 27 *P. falciparum*-exposed multiparous women were much higher than levels in plasma from 30 sympatric men (*t* test, $P < 1 \times 10^{-7}$ for each isolate) (Fig. 1). This strongly sex-specific recognition implies a placental infection focus (12). Plasma levels of IgG with specificity for the VSA expressed by isolate DP168 were less sex specific (*t* test, $P > 5 \times 10^{-3}$) (Fig. 1). Thus, the VSA expressed by this isolate appeared to be a mixture of VSA_{PAM} and non-PAM VSA (12).

We next measured the ability of IE from the 16 patient isolates to adhere to CSA and to four cell lines purchased from American Type Culture Collection (http://www.lgcpromochem-atcc.com). The lines used were the Chinese hamster ovary (CHO) line K1 (ATCC CCL-61) that expresses CSA, a CHO glycosylation mutant line (A-745; ATCC CRL-2242) that does not express CSA, a CHO cell line transfected with human CD36 (CHO-CD36; ATCC CRL-2092), and the choriocarcinoma cell line BeWo

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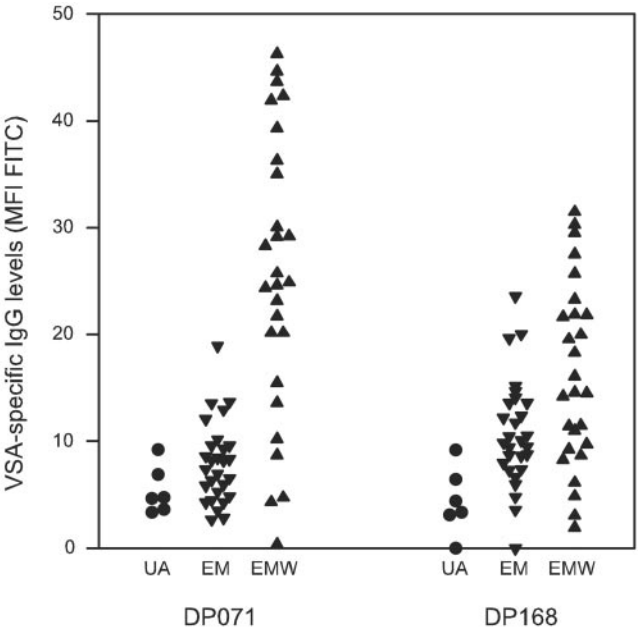

FIG. 1. Plasma levels of IgG with specificity for the VSA expressed by *P. falciparum* parasites isolated from the peripheral blood of pregnant women exposed to malaria. VSA-specific IgG levels in individual plasma samples from 6 unexposed control adults (●, UA), 30 *P. falciparum*-exposed men (▼, EM) and 27 sympatric, multiparous women (▲, EMW) are shown for isolate DP071 having typical VSA_{PAM} expression (clear difference between levels in exposed men and exposed multiparous women) and for isolate DP168 with "partial" VSA_{PAM} expression (probably a mixture of VSA_{PAM} and non-PAM-type VSA). VSA-specific IgG levels were measured by a flow cytometry assay as described in detail elsewhere (15).

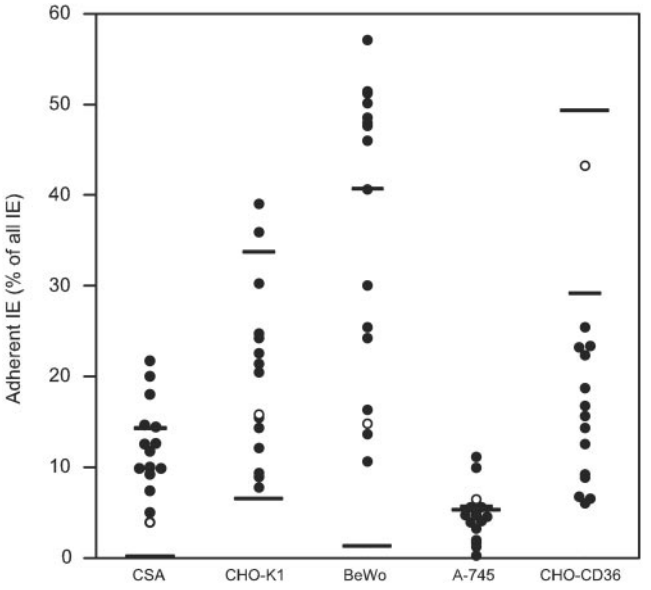

FIG. 2. Adhesion of *P. falciparum* isolates obtained from malaria-exposed pregnant women to CSA (50 mg/ml) and to monolayers of four cell lines in vitro. The percentage of adherent IE for each of 15 individual isolates expressing VSA_{PAM} (●) and a single isolate not expressing VSA_{PAM} (○) are shown. Adhesion of sublines of laboratory line FCR3 selected for adhesion to CSA by panning on CHO-K1 cells (FCR3-CHO, short horizontal line) or for adhesion to CD36 by panning on CD36-transfected CHO cells (FCR3CD36, long horizontal line) are shown for comparison. Parasites were [^{3}H]hypoxanthine labeled for 18 to 22 h and synchronized by gelatin sedimentation before IE were allowed to adhere for 1 h to CSA or cell layers in 96-well flat-bottomed microtiter plates. Nonadhering IE were washed away, and the percentage of adherent IE was calculated after measuring the remaining [^{3}H]hypoxanthine activity by liquid scintillation spectrometry.

(ATCC CCL-98). All cell lines were maintained as suggested by the American Type Culture Collection. In some preliminary experiments, we pretreated the BeWo cells with forskolin to induce multinucleated syncytia (23). However, forskolin treatment did not markedly affect selection efficiency, and forskolin was not used in the results presented in this paper.

Compared to the CSA-deficient cell line (A-745), the 15 VSA_{PAM}-expressing *P. falciparum* patient isolates bound significantly better to CSA and to each of the other cell lines (Kruskal-Wallis test, $P < 0.001$, and Dunn's post hoc test, $P < 0.05$ in all cases) (Fig. 2). Adhesion of these isolates to CSA and to the CSA-expressing CHO cell lines (CHO-K1 and CHO-CD36) was similar, whereas adhesion to the BeWo line was significantly higher (Kruskal-Wallis test, $P < 0.001$, and Dunn's post hoc test, $P < 0.05$ CSA versus BeWo) (Fig. 2). DP168 did not bind well to CSA but bound strongly to CHO-CD36 (Fig. 2). A subline of the long-term in vitro-adapted *P. falciparum* line FCR3 (FCR3-CHO) that expressed VSA_{PAM} (*t* test as above, $P < 1 \times 10^{-10}$) following selection for binding to K1 and included as a control for CSA-mediated adhesion (10) bound well to CSA and to all the CSA-expressing lines (Fig. 2). In contrast, a CD36-adhering FCR3 subline (FCR3-CD36) expressing non-PAM VSA (*t* test as above, $P = 0.16$) and included as a control line for CD36-mediated binding (10) only bound to the CD36-transfected CHO cell line (Fig. 2). Specifically, FRC3-CD36 did not bind well to the BeWo cell line, which does not express CD36 (20). These results show that VSA_{PAM}-expressing patient isolates bind strongly to BeWo cells, suggesting that this line is a useful tool in selecting for VSA_{PAM}-expression in vitro.

To test this hypothesis, we subjected six long-term in vitro adapted laboratory lines (3D7, Dd2, FCR3, HB-3, K1, and NF54) to selection for adhesion to BeWo cells (Table 1). The lines were

TABLE 1. Acquisition of VSA_{PAM} expression following in vitro selection for IE adhesion to various cell lines and receptors

Parasite name	Reference(s)	Selection protocol (no. of rounds of selection)[a]		
		BeWo (3)[b]	CHO-K1 (10)[c]	CSA (10)[d]
3D7	21	+/−	−	−
Dd2	11, 22	−	−	−
FCR3	8	+	+	+
HB-3	2	+	+	−
K1	19	−	−	−
NF54	4	+	ND	+
Busua	17	+	ND	+
N0031		+	ND	ND
N0045		−	ND	ND

[a] Complete (+), incomplete (+/−), or no (−) acquisition of VSA_{PAM} expression following selection protocol indicated. ND, not determined.
[b] Selection protocol performed as in Megnekou et al. (10), except for the cell line used (BeWo instead of CHO).
[c] Selection protocol performed as in Megnekou et al. (10).
[d] Selection protocol performed as in Ricke et al. (13).

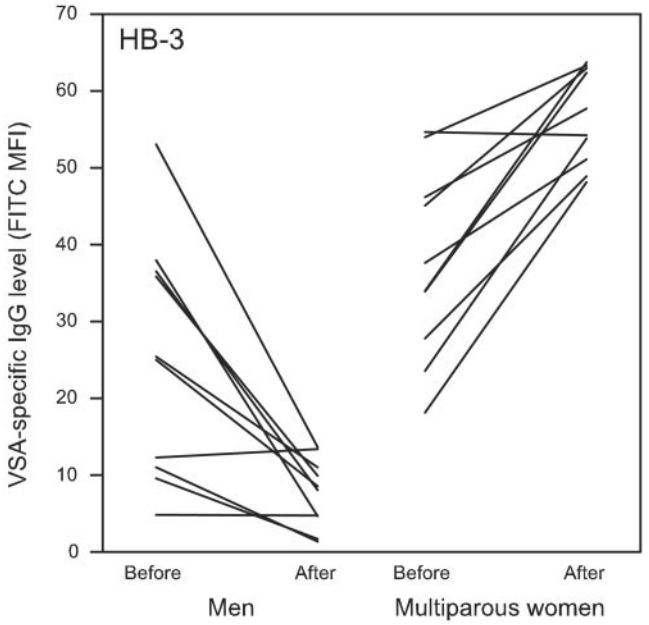

FIG. 3. Plasma levels of IgG with specificity for the VSA expressed by *P. falciparum* line HB-3 before and after three rounds of selection for adhesion to BeWo cells. Levels of VSA-specific IgG in plasma from 10 *P. falciparum*-exposed men and 10 sympatric, multiparous women are shown. Levels of IgG with specificity for the VSA expressed by HB-3 following selection were significantly lower in male plasma (paired *t* test, $P = 0.002$) and significantly higher in female plasma ($P = 0.0002$) than levels of IgG with specificity for the VSA expressed prior to selection. This is consistent with acquisition of VSA_{PAM} expression in HB-3 in response to selection. VSA-specific IgG levels were measured by a flow cytometry assay as described in detail elsewhere (15).

chosen because none of them spontaneously expressed VSA_{PAM} and because our attempts to select for VSA_{PAM} expression by 10 rounds of panning on CSA (13) had been unsuccessful for four of them (Table 1). For three of five lines tested, selection for VSA_{PAM} expression by selection for adhesion to the K1 cell line (10) had been equally ineffective (Table 1). After only three rounds of selection for adhesion to BeWo cells, three of the lines had clearly acquired VSA_{PAM} expression (Table 1 and Fig. 3). With respect to 3D7, plasma levels of VSA-specific IgG were lower (paired *t* test, $P = 0.01$) in males and higher in multiparous women ($P = 0.0001$) after three rounds of selection, but substantial male reactivity remained, indicating that acquisition of VSA_{PAM} was incomplete for this clone.

Finally, we subjected three *P. falciparum* isolates (Busua, N0031, and N0045) recently obtained from nonpregnant malaria patients to selection for IE adhesion to BeWo cells. Two of these isolates rapidly acquired VSA_{PAM} expression (Table 1). Although the VSA expressed by the third isolate clearly changed in response to selection, it did not become VSA_{PAM}-like, and the IE from this isolate appeared to have affinity for the plastic surface of culture plates rather than for receptors on the BeWo cells (data not shown).

In conclusion, we have shown that VSA_{PAM}-expressing *P. falciparum* patient isolates implicated in the pathogenesis of PAM adhere more strongly to the placental choriocarcinoma cell line BeWo than to CSA and cell lines often used in studies of *P. falciparum* involved in PAM. Furthermore, we demonstrate that the BeWo cell line is an efficient tool to select for VSA_{PAM} expression in vitro. These findings establish the BeWo cell line as an important tool for research on the VSA_{PAM} that are centrally involved in the pathogenesis of and protective immunity to PAM. Studies on the ability of VSA_{PAM}-specific IgG to interfere with adhesion of VSA_{PAM}-expressing IE are currently under way.

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