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A monoclonal antibody directed against the sporozoite stage of *Plasmodium vivax* binds to liver parenchymal cells

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1. Summary

The circumsporozoite (CS) protein of malaria parasites is a major surface protein of the sporozoite stage. In the process of investigating the immunogenicity of this protein in the *Plasmodium vivax* complex, we found that a monoclonal antibody (mAb) directed against the CS protein of isolates of *P. vivax* recognizes New World monkey hepatocytes and human hepatoma cells HepG2A16 in Western blot and by immunoelectron microscopy. The mAb NVS3 binds to the amino acid sequence AGDR, which is also shared with the α3 domain of the human and primate major histocompatibility complex class I. In addition, *in vitro* experiments suggest that the binding of the mAb NVS3 to hepatocytes from *Saimiri* monkey enhances the invasion or development of malaria sporozoites. These results form the basis for investigating the relationships between parasite surface proteins and host-cell receptors.

Key words: Plasmodium vivax; Major histocompatibility complex Class I; Hepatocyte; Monoclonal antibody

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2. Introduction

The circumsporozoite (CS) protein of the sporozoite stage of malaria parasites is a target for malaria vaccine development [1]. The central third of all CS protein consists of repetitive amino acid sequences that contain immunodominant B cell epitopes [2]. The repeat region of the CS protein of most of the isolates of *P. vivax*, including Salvador I (Sal I), Chesson, and Belem strains, is characterized by the repeated sequences DRADGQPAG or DRAAGQPAG [3]. Passive transfer to *Saimiri* monkeys of NVS3, an IgG3 monoclonal antibody (mAb) directed against the AGDR sequence contained within the nine-amino acid repeated sequences, protects against sporozoite-induced malaria [4]. Moreover, sera obtained from these animals just prior to challenge inhibit the in vitro development of *P. vivax* liver-stage parasites in primary culture of *Saimiri* monkey hepatocytes [4].

These findings have led to efforts to construct synthetic peptide vaccines designed to produce antibodies to AGDR. However, a recent study has shown that the repeat region of the CS protein of the VK247 strain of *P. vivax* from Thailand does not include the sequence AGDR [5]. If antibodies to AGDR do not inhibit invasion of hepatocytes by VK247 sporozoites, a multi-epitope vaccine may be necessary. Here we report an enhancing effect of NVS3 on the development

of *P. vivax*, VK247 strain, sporozoites in primary culture of *Saimiri* monkey hepatocytes, and demonstrate that the MAb NVS3 interferes between the parasite and the hepatocyte by binding to hepatocyte molecules.

3. Materials and Methods

3.1. *In vitro culture of Siamiri monkey hepatocytes and mAbs*

The liver-stage parasite assay was performed using previously developed techniques [6]. Sporozoites were obtained from dissected salivary glands of infected *Anopheles dirus* mosquitoes previously fed on blood from chimpanzees (*Pan troglodytes*) infected with gametocytes of *P. vivax*, Sal I or VK247 strains. Sporozoites were counted and suspended in culture medium (Gibco, Grand Island, New York; formula #85-0227AK) supplemented with 10% fetal bovine serum, 2 g/l bovine serum albumin, 10 mg/l bovine insulin, 0.1 mM nonessential amino acids, 292 mg/l L-glutamine, 150 U/ml penicillin, and 150 μg/ml streptomycin. 30 μl of sporozoite suspension containing approximately 60 000 sporozoites was incubated with 30 μl of the following mAbs diluted in phosphate-buffered saline (PBS) for 15 min at room temperature: NVS3 (IgG3), directed against the repeat region of the CS protein of *P. vivax*, Salvador I strain [4]; 3G6 (IgM), directed against the repeat region of the CS protein of *P. vivax*, VK247 strain [5]; 2A10 (IgG2a), recognizing the sequence NANP on *P. falciparum* sporozoites [6]; and W6/32 (IgG2a), reactive with a monomorphic determinant on the Major Histocompatibility Complex (MHC), HLA-A, -B, and -C molecules [7]. The sporozoites were then overlaid on hepatocyte monolayers (30 000 sporozoites per monolayer containing 50 000 hepatocytes). Hepatocytes were obtained from a liver biopsy from an uninfected *Saimiri* monkey. After 2 h, sporozoites and plasma were removed, and medium was added to culture dishes. This medium was replaced 1 h later and then changed daily. After incubation for 7 days at 37°C in 5% CO_2, the cultures were fixed with methanol and Giemsa-stained. Schizonts were counted with the identity of each plate concealed.

3.2. *Western blot*

Western blotting [8] was performed as follows. Freshly dissociated hepatocytes were solubilized in reducing sample buffer (0.06 M Tris-HCl, 5% sodium dodecyl sulfate (SDS), 0.7 M 2-mercaptoethanol) and then incubated for 15 min at 75°C. After vertical electrophoresis in SDS gel (3.3% – 20%), the polypeptides were electrophoretically transferred to nitrocellulose paper. Strips of nitrocellulose paper were reacted overnight at 4°C with mAb NVS3 (A.D) or with two unrelated mAbs from other malarial parasites: 2A10 (*P. falciparum*) directed against the sequence $(NANP)_n$ (B,E), and 12D6 (*P. cynomolgi*, B strain) directed against the sequence $(NAGG)_n$ (C,F), diluted at 1:100 in PBS containing 0.3% Tween-20. After being incubated for 1 h at room temperature with peroxidase-conjugated goat antibodies to mouse IgG diluted in PBS/Tween, the strips were washed, and the antibodies bound to the strips were detected by staining with diaminobenzidine and hydrogen peroxide. Molecular weights were estimated from control-labeled proteins (Bethesda Research Laboratory, Gaithersburg, MD).

3.3. *Immunoelectron microscopy*

Immunoelectron microscopy [9] was carried out as follows. HepG2/A16 cells and *Aotus* hepatocytes were fixed with 1% paraformaldehyde and 0.2% glutaraldehyde in PBS for 40 min at 4°C. After dehydration, samples were embedded in LR Gold resin (Polyscience, NJ) at −20°C. Sections mounted on nickel grids were incubated for 30 min with PBS containing 5% non-fat dry milk (Carnation) and 0.01% Tween before being immunostained, first with NVS3 (500 μg/ml) overnight at 4°C and then with a goat anti-mouse IgG conjugated with 15 nm gold particles as a secondary antibody. Grids were examined with a Zeiss CEM902 electron microscope after being double stained with uranyl acetate and lead citrate.

Sequence similarity searches with the amino acid sequence AGDR were done using the Fasta program [10] to scan the SwissProt data bank [11], release 17.0 as of March 1991.

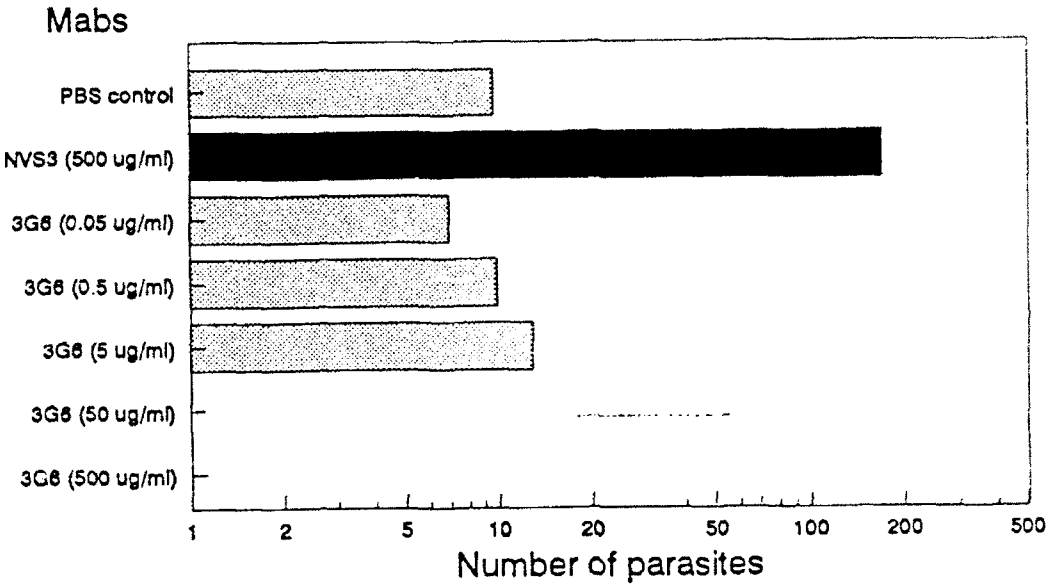

Fig. 1. Enhancements of *P. vivax*, VK247 strain, liver-stage development by incubation of sporozoites with MAb NVS3, compared with mAb 3G6 directed against the repeat region of the CS protein of *P. vivax*, VK247, and with control plate in which sporozoites were incubated with PBS only.

4. Results

MAb 3G6 inhibited 100% the development of VK247 parasites in primary cultures of *Saimiri* monkey hepatocytes at 500 μg/ml and 50 μg/ml dilution PBS (Fig. 1). In contrast, mAb NVS3, at 500 μg/ml, enhanced the number of liver-stage parasites to 17 times more than the number found in cultures exposed to PBS as a control, or to low concentrations (0.05 to 0.5 μg/ml) of mAb 3G6.

To determine whether mAb NVS3 was able to recognize hepatocytes from New World monkeys susceptible to human malaria parasite infections, we analyzed hepatocyte extracts from *Saimiri* and *Aotus* monkeys by Western blot (Fig. 2). The mAb NVS3 recognized a major band from both *Aotus* and *Saimiri* monkeys with a relative molecular mass of 45 KiloDalton (kDa). Two minor bands of 60 and 75 kDa were also detected.

Immunoelectron microscopy of *Aotus* monkey hepatocytes and of the human hepatoma cell line HepG2/A16 demonstrated that gold particles were associated with the microvilli and the cell surface as well as with the cytoplasm of both hepatocytes and hepatoma cell lines (Fig. 3).

To help identify the protein recognized by Western blot and immunoelectron microscopy, we conducted a sequence similarity searche with the amino acid sequence AGDR. Earlier investigations have been conducted with the repeat region of the CS protein of *P. vivax*, but these did not include the sequence AGDR [12]. In our search, this amino acid sequence was found to occur in 92 entries in the database. Thirteen of these sequences were primate (11 human and 2 chimpanzee) HLA-B class I α chains showing 100% homology to a highly conserved region of the extracellular α3 domain. When the sequence PAGDR, which is also present in the CS protein of *P. vivax*, Sal I strain, was used as a query in the search, more specific results were obtained (Fig. 4). This sequence was found in all 13 HLA sequences mentioned above (position numbers 255 to 280 in sequence) and in seven other proteins (two collagens, two potato virus X proteins, the APT synthetase ε subunit, the protease I precursor, and an estrogen precursor).

To determine if the interaction of antibodies with additional epitopes on class I MHC molecules could enhance sporozoite development in hepatocytes, sporozoites of *P. vivax*, Sal I strain

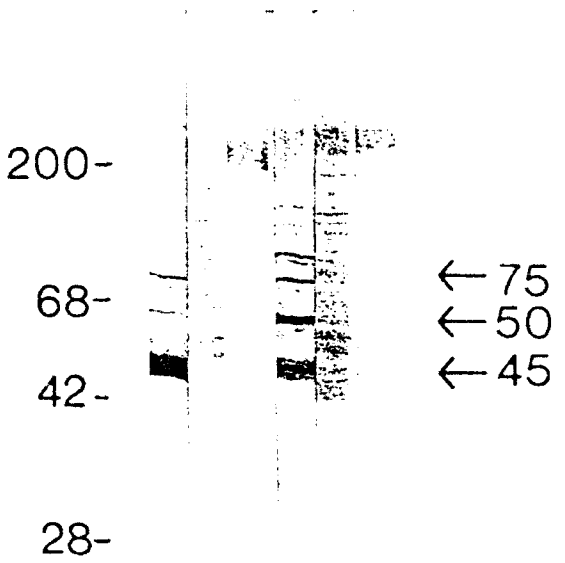

Fig. 2. Western blot analysis of hepatocyte extracts from *Saimiri* (A,B,C) and *Aotus* (D,E,F) monkeys.

Fig. 3. Immunoelectron micrograph of HepG2.A16 cells reacted with the mAb NVS3. The surface of HepG2.A16 cells shows many microvilli. Gold particles (arrows) are associated with the microvilli and the surface as well as the cytoplasm of the cells. (HC) × 70 000.

(containing the sequence AGDR) were incubated with an anti-MHC class I mAb W6/32. This antibody did not recognize the $(AGDR)_6$ peptide in an ELISA (data not shown). In the first experiment, mAb W6/32 and a control mAb 2A10 were mixed with sporozoites and then added to hepatocyte monolayers. In a second experiment, the mAbs were added to hepatocyte cultures 2 h before the cultures were infected with sporozoites and removed before the sporozoites were added. Identical results were obtained in the two experiments. The mAb W6/32 at 50 μg/ml enhanced liver-stage development 10 times more than both the control IgG2a mAb at equivalent concentration and control with culture medium only (Fig. 5).

5. Discussion

These results demonstrate that the mAb NVS3 directed against the CS protein of *P. vivax* sporo-

SwissProt database ID	Sequence	Position in sequence
HA1L$HUMAN	DTELVETRPAGDRTFQKWAAVVVPSGEEQR	280
HA1M$HUMAN	DTELVETRPAGDRTFQKWAAVVVPSGEEQR	280
HA1N$HUMAN	DTELVETRPAGDRTFQKWAAVVVPSGEEQR	280
HA1O$HUMAN	DTELVETRPAGDRTFQKWAAVVVPSGEEQR	280
HA1P$HUMAN	DTELVETRPAGDRTFQKWAAVVVPSGEEQR	280
HA1Q$HUMAN	DTELVETRPAGDRTFQKWAAVVVPSGEEQR	280
HA1R$HUMAN	DTELVETRPAGDRTFQKWAAVVVPSGEEQR	255
HA1S$HUMAN	DTELVETRPAGDRTFQKWAAVVVPSGEEQR	277
HA1T$HUMAN	DTELVETRPAGDRTFQKWAAVVVPSGEEQR	280
HA1U$HUMAN	DTELVETRPAGDRTFQKWAAVVVPSGEEQR	280
HA1W$HUMAN	DTELVETRPAGDRTFQKWAAVVVPSGEEQR	280
HA1D$PANTR	DTELVETRPAGDRTFQKWAAVVVPSGEEQR	280
HA1N$PANTR	DTELVETRPAGDRTFQKWAAVVVPSGEEQR	280

Fig. 4. SwissProt (Release 17.0) MHC entries, showing 100% homology to the *Plasmodium vivax* strain Sal I circumsporozoite pentapeptide PAGDR (highlighted).

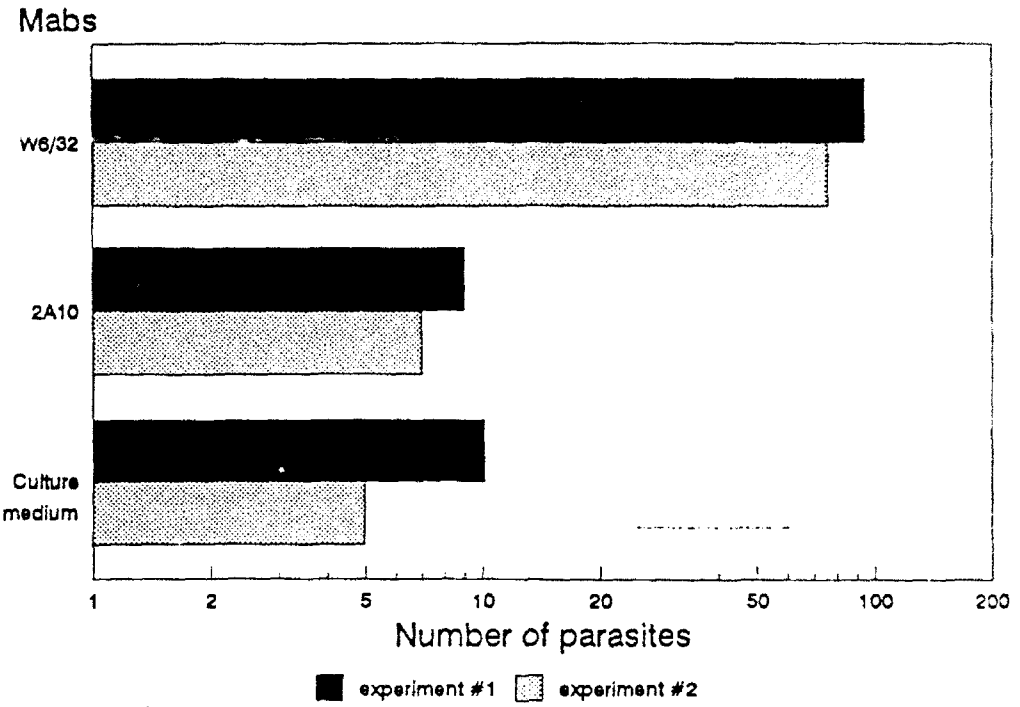

Fig. 5. Enhancement of *P. vivax*, Sal I strain, liver-stage development by incubation of sporozoites with mAb W6/32 directed against a monomorphic determinant on HLA A,B and C molecules. MAb 2A10 and culture medium were used as controls. Expt. 1, mAbs were mixed with sporozoites. Expt. 2, mAbs were in contact with hepatocytes for 2 h and removed before parasites were added.

zoites is able to recognize host-cell proteins. This mAb bound to monkey hepatocytes in Western blot, and to the cell surface and cytoplasmic molecules of human hepatoma cells in immunoelectron microscopy.

Sequence similarity searches with the amino-acid sequence AGDR, supported by other studies [13,14], showed that the sequence PAGDR recognized by NVS3, or its variant PAGDG, occurs in all primate or rodent MHC class I α proteins. In addition, we found that the variant sequence PAGDG is also a repetitive amino acid sequence of the CS protein of the monkey malaria parasite *P. cynomolgi*, Berok strain [15]. The *P. cynomolgi* complex has a close phylogenetic relationship with *P. vivax*, but we were surprised to find that the amino acid variations in the repetitive region of the CS protein were identical to those observed for the similar part of the α chains of the MHC class I network.

Interestingly, the mAb NVS3 reacted with a 45-kDa band in hepatocyte extracts (Fig. 2), and the size of MHC class I molecules is also 45 kDa [7]. However, attempts to immunoprecipitate the IgG3 mAb NVS3 to MHC class I molecules in parallel with anti-MHC class I mAbs (W6/32 or BB7) were unsuccessful. In addition, our results cannot exclude the relationship between the mAb NVS3 and other hepatocyte molecules, although an anti-MHC class I mAb was able to enhance the in vitro development of *P. vivax* sporozoites. Our in vitro results demonstrate that an anti-MHC class I mAb added before or during infection was able to enhance the development of malaria parasites in liver cultures, strongly suggesting that the interaction of the mAb with hepatocytes, not with sporozoites, was critical for mediating enhancement.

Amino acid sequence homology between isolates of *P. vivax* and a human protein might result from selection due to immune pressure. Indeed, individuals from a *P. vivax* endemic area did not recognize the sequence AGDR in ELISA (unpublished information). However, anti-AGDR

antibodies have been elicited by mice, leading to the production of the mAb NVS3. The lack of antibody response from humans confirms that this epitope is included in a human protein and is therefore protected from any immune response that could result in autoimmune complications. We are conducting additional investigations to confirm the relationships between sporozoites and the MHC class I molecule and to define its interaction with the liver-stage pathway of malaria parasites.

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