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Malaria sporozoite penetration

A new approach by double staining

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To determine, whether a sporozoite is outside the hepatocyte membrane or internalized, a double staining test was carried out using, successively, antibody labeled with peroxidase and fluorescein. This test permits the quantification of sporozoite entry and outline sporozoite-hepatocyte interactions.

Key words: Malaria; Double staining test; Sporozoite; Hepatocyte; Culture

Introduction

During the life cycle of malaria parasites, the sporozoite invades the liver and gives rise to thousands of merozoites which are able to infect red blood cells. Invasion of liver cells is thus a critical step by which infection becomes established in the host and against which an antiparasitic attack might be effectively mounted. In contrast to merozoite-erythrocyte interactions which have been exhaustively studied at both morphological and molecular levels, understanding of sporozoite-hepatocyte interactions is still incomplete (Aley et al., 1986).

The recent development of hepatic culture systems has greatly improved the means of studying the events associated with sporozoite entry into target cells (Hollingdale et al., 1982; Mazier et al., 1986). These studies, however, are subjective because of the difficulty inherent in ascertaining whether or not a sporozoite is attached to the hepatocyte surface membrane or is inside the cell. To eliminate subjectivity, an immunoradiometric assay measuring the amount of internalized circumsporozoite (CS) protein, the major sporozoite surface protein, has been proposed by Zavala et al. (1985). This method claims to quantify sporozoite penetration. This approach, unlike microscopic observation, does not relate precisely the movement of the parasite. Here, we describe a new method, based on stepwise staining, using a single monoclonal antibody (Mab) specific for sporozoite surface, revealed by two secondary antibodies, the first coupled to an enzyme, and the second to a fluorochrome.

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Abbreviations: CS, circumsporozoite; IFAT, immunofluorescent antibody test; IPAT, immunoperoxidase antibody test.

Materials and methods

Hepatocyte culture

Cultures of metabolically active human or rodent hepatocytes were prepared as previously described (Mazier et al., 1982, 1985). Briefly, 10^5 hepatocytes were added into eight-chamber Lab-Tek plastic culture slides (Miles Labs., Elkhart, IN). The cells were incubated in supplemented minimal essential medium (MEM) at 37°C in 5% CO_2 in air, for 24 h. The medium was replaced by fresh medium, supplemented with 7×10^{-5} hydroxycortisone hemisuccinate. The cultures were incubated for 24 additional hours before being inoculated with infective sporozoites.

Sporozoites

Salivary glands of *Anopheles stephensi* infected with the 265 BY strain of *Plasmodium yoelii yoelii*, the NF 54 strain of *P. falciparum*, or the Sal I strain of *P. vivax* were aseptically dissected, pooled and disrupted by trituration in a glass tissue grinder. The sporozoites were counted in a Neubauer chamber, the count per unit volume adjusted and the appropriate inoculum (40 000 sporozoites per 10^5 hepatocytes) was added to each culture well.

Parasite staining

At appropriate times after inoculation (3 h for *P. yoelii*, 48 h for *P. falciparum* and *P. vivax*), cultures were rinsed three times in phosphate-buffered saline (PBS), pH 7.5, fixed for 5 min at room temperature in 4% paraformaldehyde in PBS, and again washed three times. In the first part of the assay, external sporozoites were detected by an immunoperoxidase antibody test (IPAT) using antibodies chosen for their capability to react only with sporozoite surface. The selected monoclonal antibodies (Mabs) were diluted with PBS, added to the appropriate culture, and incubated for 30 min at 37°C. After three rinses, a peroxidase-conjugated goat anti-mouse immunoglobulin (G,A, MIg) (Biosys) diluted 1 : 100 in PBS was applied as the revealing antibody. Peroxidase activity was detected with 3,3′-diaminobenzidine tetrahydrochloride (1 mg/ml) in Tris-HCl (0.1 M; pH 7.6), and 0.03% H_2O_2 in PBS. The cultures were washed three times in PBS, treated with ethanol (99.9%) for 10 min at 22°C and washed again three times. The cultures were then kept in PBS overnight at 37°C. The second part of the assay, the immunofluorescence test (IFAT), was performed on the following day. These same cultures were first rinsed three times in PBS and incubated for 30 min at 37°C, with the same dilution of monoclonal antibody as the day before. After three washes, a fluorescein-labeled anti-mouse immunoglobulin (Nordic) diluted 1 : 100 in 0.5% Evans Blue was applied. The cultures were viewed with an Olympus microscope, using transmitted and UV lights, alternatively.

Antisporozoite antibodies recognition pattern

Using the 'wet sporozoites' technique, slide wells were coated with 20 µl of poly-L-lysine (Sigma Chemical Co.) at a concentration of 25 mg/ml, and dried at 37°C. Thereafter, drops of freshly isolated sporozoites were deposited, and the preparations kept at 4°C for 24 h in a moist chamber to permit attachment. The slides were handled following the same protocol as for the parasite staining. A battery of murine monoclonal antibodies was tested for their ability to react only with the sporozoite surface. They were specific for *P. falciparum*: 3SP2 (Verhave et al., 1988), NFS1, NFS2 (Charoenvit et al., manuscript in preparation), B4 (Boulanger et al., 1988); for *P. vivax*: NVS3 (Wirtz et al., 1987); and *P. yoelii*: NYS1, NYS3 (Charoenvit et al., 1987).

Assessment of fixative treatment

Non-infected cultures, fixed either with paraformaldehyde or with paraformaldehyde followed by ethanol treatment, were incubated with a Mab reacting with a nucleus.

Results and discussion

Fixation of liver cell monolayers with paraformaldehyde was shown to impede penetration of antibodies through the hepatocyte membrane since no staining was observed with a Mab reacting with the cell nucleus. In contrast when paraformaldehyde fixation was followed by ethanol treatment, nuclei were stained.

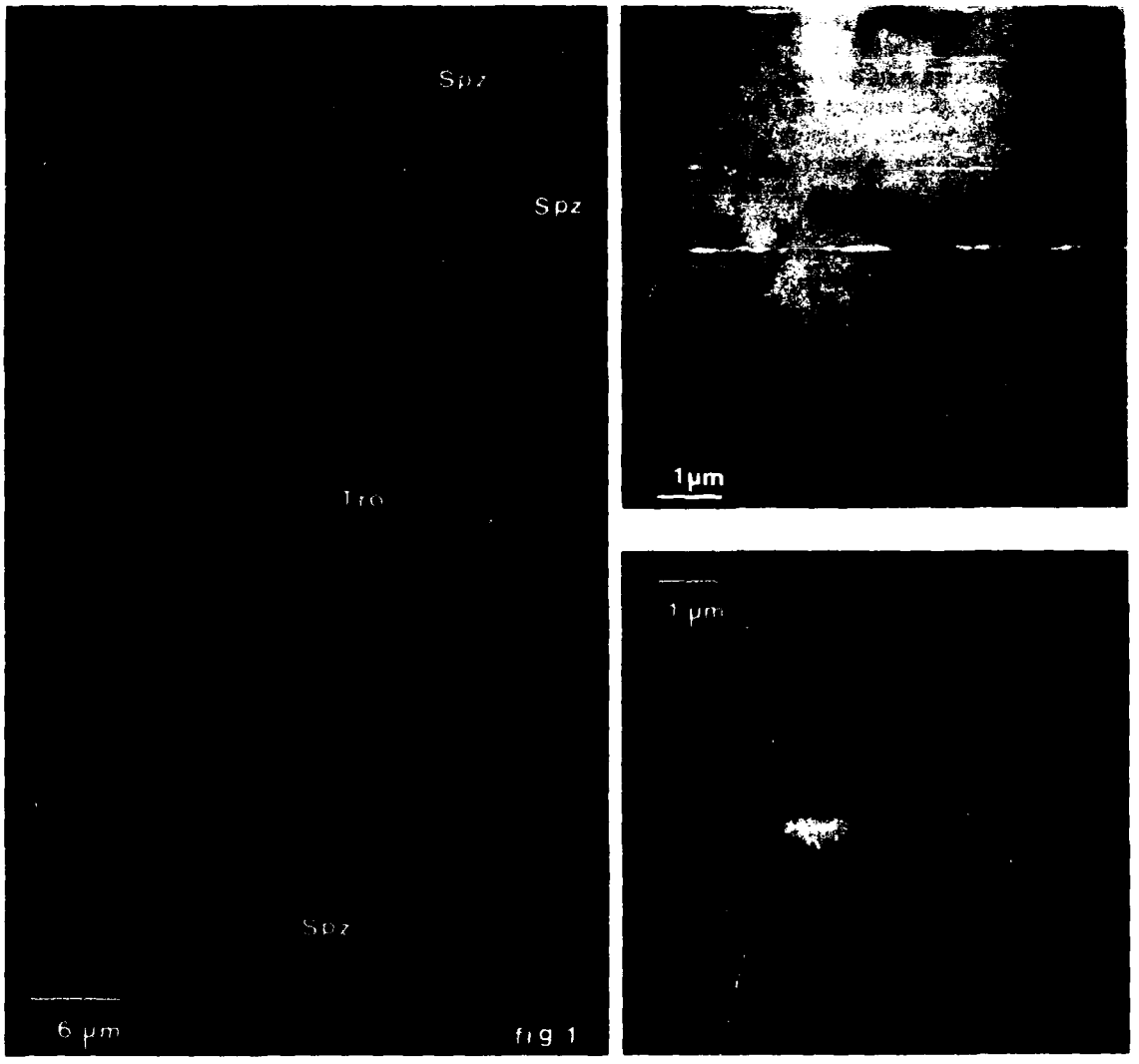

Fig. 1. Sporozoites external to the hepatocyte plasma membrane are stained brown by the peroxidase-labeled antibody (Spz). In contrast, a trophozoite (Tro), developing from an internalized sporozoite, is stained by fluorescein-labeled antibody.

Fig. 2. The external part of sporozoite which has not completed entry into the hepatocytes is stained brown by peroxidase-labeled antibody (Fig. 2*A*), while the inner part is stained by fluorescein-labeled antibody (Fig. 2*B*).

The next step, using freshly isolated sporozoites, demonstrated that epitope covered with a Mab revealed by a peroxidase-labeled antibody was no longer accessible, after ethanol treatment, to the same Mab revealed by a fluorescein-labeled antibody. This fact is the result of a saturation and not of a denaturation of the site recognized by the Mab. Moreover, we noticed that ethanol did not destroy the peroxidase staining. All the Mabs, under study, fulfilled these criteria. These initial results justified the use of this double staining test to discriminate in infested culture membrane-attached and internalized sporozoites. External sporozoites were stained by peroxidase and, after ethanol treatment which permeates the hepatocyte membrane, and thus allows interaction of Mab with surface epitope of internalized sporozoites, interiorized parasites were stained with fluorescein (Fig. 1). The method even allowed discrimination of the portion of a sporozoite which has not completed entry into the hepatocyte. The internal part of the parasite fluoresced, while the external part was stained brown from peroxidase staining (Figs. 2*A* and 2*B*). Identical results were observed with *P. yoelii, P. falciparum*, and *P. vivax* sporozoites and their corresponding Mabs. In earlier experiments, we noticed that, if the IFAT was done immediately after the ethanol fixation, the fluorescence was quenched by the presence of the oxygen, since peroxidases catalyze the transformation of H_2O_2 into H_2O and O_2. Oxygen inhibits fluorescence intensity by collision with the fluorophore (Lakowicz and Weber, 1973). To avoid this phenomenon, a minimum of 8 h at 37°C was found sufficient to allow total disappearance of reactive oxygen in the medium, before the fluorescein staining.

In the in vitro inhibition assay, Hollingdale et al. (1984) used a peroxidase staining after methanol fixation. In this assay, internalized sporozoites, surrounded by the parasitophorous vacuole, were revealed by a sausage shaped staining, in contrast to external sporozoites which were sharply outlined. Using these criteria, discriminating between external and internal sporozoites remains subject to error. With the present procedure of double staining, external sporozoites are invariably sharply outlined, whereas those having gained entry into the hepatocyte are revealed by a staining either limited to the parasite body or displaying a fluorescent halo surrounding the sporozoite.

The combination of both IPAT on paraformaldehyde fixed material and subsequent IFAT on the same material after re-fixing in ethanol, removes all doubt in the discrimination between extracellular and intracellular parasites and provides the means to understand the sequence of steps by which the sporozoite enters the host cell. This technique is now routinely used in our laboratory to assess the effects of antibodies or drugs on host-parasite interactions (Nüdelman et al., Goma et al., manuscripts in preparation).

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