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## Original Article

### Mini-Column for Cytoadherence: A New Method for Measuring the Relative Size of Binding Subpopulations in *Plasmodium falciparum* Isolates

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#### ABSTRACT

**Background:** Cytoadherence of *Plasmodium falciparum*-infected red blood cells to endothelial cells is an important mechanism for parasite survival and a major trigger for diseases pathology. Here, we describe a new adhesion assay in which different cell types (CHO, CHO/CD36 and CHO/ICAM-1) are attached to Cytodex beads in a mini-column format to measure the relative sizes of various binding subpopulations as a percentage of the total population.

**Methods:** Relative size of CD36 and ICAM-1-binding subpopulations of erythrocytes infected with *P. falciparum* were measured by amount of parasitemia before and after passing the infected erythrocytes through a particular column.

**Results:** The mini-column adhesion assay was a suitable method as parasitemia always reduced after passing through a particular column in independent experiments. For example, in a typical experiment using *P. falciparum* ITG line, 75% of the parasites are retained on a CHO/ICAM-1 while 0% of clone 3D7 is retained.

**Conclusion:** This work introduced and validated a method for measuring the relative size of parasite binding subpopulations and the selection of them. Also, the mini-column method is of value for assessments of cytoadherence and can be used as tool for different applications.

**Keywords:** Cytoadherence, CHO cells, *P. falciparum*

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## Introduction

**D**uring falciparum malaria, infected erythrocytes with late stages of parasite (late trophozoites and schizonts) do not appear in the peripheral blood, but are sequestered in the post-capillary venules, especially in deep tissues (1). This sequestration is responsible for the pathology and leads to severe diseases particularly cerebral malaria (2, 3). It has been studied in detail and is mediated by interaction of various host endothelial receptors and *P. falciparum* antigens expressed on the surface of infected erythrocytes. It is mainly processed through the interaction of *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) with various receptors such as cell differentiation 36 (CD36) (4), intercellular cell adhesion molecule-1 (ICAM-1)(5, 6) and chondroitin sulfate A (CSA) (7) which are expressed on the surface of vascular endothelial cells, infected and uninfected erythrocytes and platelet.

Since 1980, different adhesion assays have been developed to modeling the process. At the outset, human umbilical vein endothelial cells (HUVECs) were used in an in vitro cytoadherence model by Udeinya et al., 1981 (8). In subsequent assay models, different cells (9) and purified proteins (3, 10) attached to a solid substrate are used. These adhesion assays have been performed under static or flow conditions and both techniques reveal great deal about the molecular mechanisms by *P. falciparum*-infected erythrocytes adhered to the vascular endothelium. But they suffer from common problems such as intensive labouring and complexity of the techniques. These methods are unable to measure relative size of different binding subpopulations within maternal population (11). On the other hand, any parasite population, whether taken directly from a patient or adapted for long-term in vitro cultivation, represents a mixture of different sub-popula-

tions, each with different binding characteristics (12). Therefore, accurate quantification of particular binding subpopulation of erythrocytes infected with *P. falciparum* remains challenging in performing classical adhesion assays. Here, we describe a new adhesion assay in which different cell types (CHO, CHO/CD36 and CHO/ICAM-1) are attached to Cytodex beads in a mini-column format to measure the relative sizes of various binding subpopulations as a percentage of the total population.

## Materials and Methods

### *Parasites and cells*

Three *P. falciparum* lines A4 (13, 14), 3D7 (from NF54 from Netherland received from D. Walliker), ITG (5,14) were used. All parasites were cultured in human blood group O<sup>+</sup> using RPMI-1640 containing AB<sup>+</sup> human serum (RPMI-HS) mostly described by Mphande et al., 2008 (15). Chinese Hamster Ovary cells (CHO) or CHO transfected with CD36 or with ICAM-1 cells were cultivated as described by Anna Vogt, 2008 (16). These cells were kindly prepared by Dr. Russell Howard.

### *Micro-carrier culture*

Cytodex-1 beads (Sigma-Aldrich Corporation, USA) were suspended and hydrated for two hours in phosphate buffer saline (PBS) and then sterilized by autoclaving for 15 min at 121°C as recommended by manufacturer. Micro-carrier cell culture was carried out in a Silicone (Sigmacote, Sigma) treated 1,000 ml spinner flask containing a suspended magnetic rod. 250 mg of Cytodex-1 beads were added to 200 ml of RPMI-1640 containing Foetal calf serum (RPMI-FBS). Then, about  $3.6 \times 10^6$  CHO/ICAM-1, CHO/CD36 or CHO cells were added to the flask. The culture process was initiated by

hand agitation for 5 min, kept static for 25 min and then the stirring speed was kept at about 60 rpm over night (17). If the beads were coated by CHO-cells, the beads were used for the on-column cytoadherence technique.

#### ***Cytoadherence on mini-column***

A column was made by suspending the beads in a 1 ml pipette tip fitted with a polyethylene disc to retain the beads in the column. The column was then washed once with RPMI-FCS followed by the addition of 1 ml of *P. falciparum* culture at 2% hematocrit (all isolates used after about 30-36 hours retrieval at any parasitemia). The column was washed three times with RPMI-HS to remove unbound infected erythrocytes.

Parasitemia was measured before and after passing the *P. falciparum*-infected erythrocytes through the column by counting at least 2,000 cells and the percent of retained infected erythrocytes was obtained as explained in Fig. 1. Each experiment was repeated at least two times. The bound cells were eluted from the Cytodex beads by transferring them to a clean tube and shaking them gently to suspend the cells in RPMI-HS. After the beads settled, the supernatant was collected and centrifuged. A thin smear was made from the pellet and stained with Giemsa.

In order to examine the efficacy of the mini-column binding assay for the detection of subpopulations of parasites within a test sample three independent experiments were performed which are explained below.

#### ***1) Mixing of parasites***

The min-column adhesion assay was carried out on samples containing various proportions of an ICAM-1/binding line (ITG) and an ICAM-1/ non-binding isolate (3D7). The two *P. falciparum* isolates, of known adherence phenotypes, mixed in different proportions and allowed to bind to cultured CHO

cells expressing ICAM-1 in the mini-column format. Prior to the assays, two cultures were grown to late stages and accurate counts of the parasitaemia were made for both cultures. Then, two parasite lines were mixed in seven different proportions of 100:0, 90:10, 75:25, 50:50, 25:75, 10:90 and 0:100 in order to artificially create heterogeneous parasite.

#### ***2) Selection of non-binding parasites to ICAM-1***

The mini-column binding assay was performed on CHO/ICAM-1, using *P. falciparum* line A4 as described before. After washing the column, the washed through cells (not retained by the column) were collected and centrifuged. The pellet was grown to the mature stage of the next generation and called "non-binding to ICAM-1 subpopulation". Then, mini-column cytoadherence method was applied with CHO/ICAM-1 or CD36 using the non-binding to ICAM-1 subpopulation.

#### ***3) Selection of clones binding to ICAM-1 and CD36***

Selection of binding subpopulations was carried out in the static situation, using a standard technique that has been previously described by Marsh et al., 1988 (18). The selection was repeated three times. The ring stages of the fifth generation of the selected parasites were cryopreserved and retrieved. Late trophozoites/ schizonts of the parasites were used for performing adhesion assay with CHO/ICAM-1 or CD36 in the mini-column format.

## **Results**

#### ***Mini-column cytoadherence***

In the first series of experiments, infected erythrocytes with late stages of *P. falciparum* line A4 was passed through a column made with CHO/ICAM-1 or CHO/CD36 cells. Results obtained from these experi-

ments demonstrated that parasitemia was always reduced after passing through the mini-column in various independent assays. For example, the parasitemia of the sample added to the columns was 1.4% and was reduced to 1% and 0.55% (a retention ratio of 30% and 60.1%) after passing through columns made with CHO/CD36 and CHO/ICAM-1, respectively. These results can also be evaluated if parasitemia of the red cells retained on the column is measured directly. This was achieved by washing the column with medium, detaching of the bound cells and stained with Giemsa. Results obtained here showed that parasitemia of retained red blood cells was 24.4% and 41.4% in the CHO/CD36 and CHO/ICAM-1 columns, respectively (at least 500 red blood cells were counted). Figure 2 shows bound infected erythrocytes to CHO/ICAM-1 cells which detached and stained with Giemsa. In addition, in order to make sure the column was not saturated the experiment was repeated with the parasite at different hematocrit. This experiment indicated that suitable hematocrit is two percent compared with one and four percent (data are not shown).

#### ***Mixing of parasites***

When mixed samples containing different proportions of ITG clone which bind to ICAM-1 and 3D7 a non-binding isolate exposed to mini-column with CHO/ICAM-1 cells, a fall in the percentage of adhered infected erythrocytes with ITG line to the column was seen as the proportion of infected erythrocytes with 3D7 within the sample increases. It has also showed that the highest binding rate (75%) and no binding (0%) is seen when 100 % of proportion of parasitized cells are ITG isolate and 3D7, respectively (Fig. 3).

#### ***Selection of unbound parasites to ICAM-1***

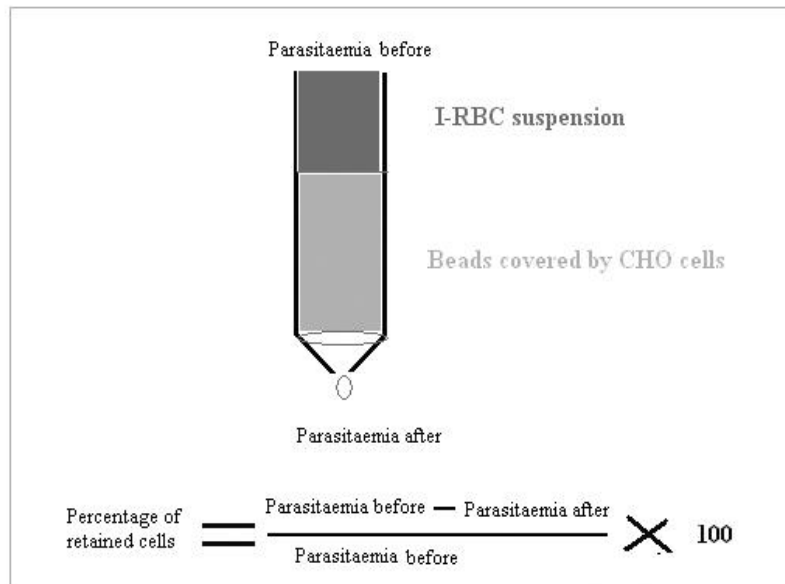
Results obtained from previous experiments showed that the percentage of retained A4-infected erythrocytes is 51.3% (data are not

shown). It could conclude that approximately 48.7% of infected erythrocytes are not able to bind to CHO/ICAM-1 cells. In the next experiment, an ICAM-1-unbound subpopulation of A4 isolate was selected and used (after one generation in culture) to perform mini-column adhesion method with CHO/ICAM-1 or CHO/CD36 cells.

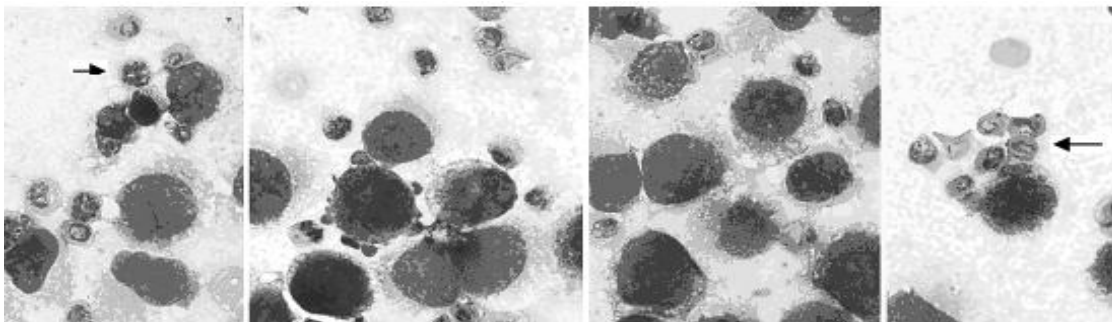
When the selected parasites were used, the percentage of infected erythrocytes bound to column with CHO/ICAM-1 was reduced from 51.3% to 12.5% compared with non-selected parasites. The percentage of the selected parasites bound to CD36 in comparison with non-selected parasites was increased from 31.5% to 45%. This experiment demonstrated a major difference between percentage of cells bound to ICAM-1 and CD36 using a non-bound to ICAM-1 subpopulation of *P. falciparum* A4 (12% Vs 45%) (Fig. 4). It also revealed that the selected parasite (unbound to ICAM-1) show a major difference in binding characteristic.

#### ***Selection of clone binding to ICAM-1 or CD36***

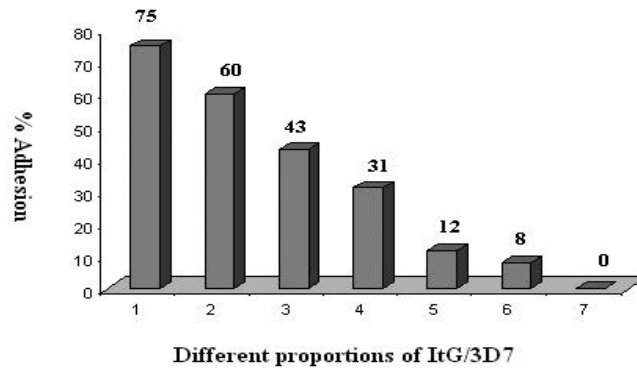
When ICAM-1-binding subpopulation of *P. falciparum* line A4 (selected under static situation) used for performing mini-column adhesion assay, proportion of infected cells bound to CHO/ICAM-1 column increased from 51.3% to 56%. The percentage of bound infected erythrocytes to CHO/CD36 increased from 31.5% to 50.7% when the CD36/selected clone was used. A considerable difference was seen between the percentages of infected erythrocytes bound to CHO/ICAM-1 (56%) in comparison with infected cells bound to CHO/CD36 (35.3%) when the ICAM-1/selected subpopulation was used. Also, a major difference was seen between the percentage of parasite infected cells bound to CHO/CD36 (50.7%) and infected cells bound to CHO/ICAM-1 (30.3%), when the CD36/selected subpopulation was used.



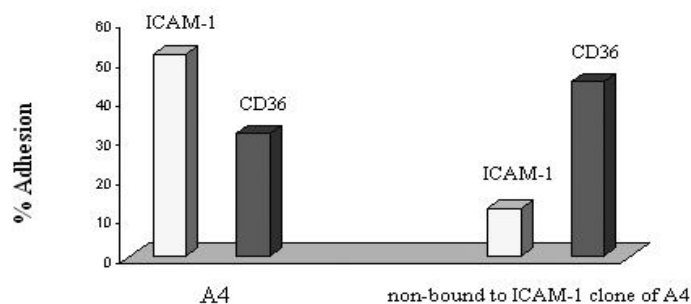
**Fig. 1:** Shows a schematic diagram which describes procedure of the mini-column adhesion assay. It also explains calculation of the percentage of retained infected erythrocytes with *P. falciparum* in the column



**Fig. 2:** *P. falciparum*-infected erythrocytes A4 bound to CHO/ICAM-1, using mini-column binding assay. Arrows denote retained and bound infected erythrocytes (Giemsa stain) on the column in different microscope fields. It shows a good binding of parasitized cells, at least one infected erythrocytes per one CHO cell. The 1000 X magnification with oil was used



**Fig. 3:** Adhesion rate of mixing various proportions of two different *P. falciparum* isolates, ITG (ICAM-1/binding clone) with 3D7 (non- ICAM-1 binding clone), to CHO cells expressing ICAM-1 using mini-column binding assay. Data shows the percentage of adhesion of two parasite lines which were mixed in seven different proportions of ITG and 3D7. The proportions of ITG:3D7 are 100:0, 90:10, 75:25, 50:50, 25:75, 10:90, 0:100



**Fig. 4:** Percentage of adhesion to CHO/ICAM-1 and CD36 cells on the mini-column format using non-binding to ICAM-1 clone of *P. falciparum* A4

## Discussion

This study describes a method capable to quantitating a particular binding subpopulation as a percentage of the total population. In the main, all studies on cytoadherence of *P. falciparum*-infected erythrocytes to different endothelial receptors involve adding a suspension of infected erythrocytes to target cells or purified proteins immobilized on a glass or plastic. These methods are able to find out whether an isolate of *P. falciparum* can bind to particular ligand or whether there is a high or low binding but they can not

show what percentage of the isolate has capacity to bind to a specific receptor (11). The first series of experiments were designed to find out the best condition for attachment and growth of CHO cells on the beads using micro-carrier system and set-up of the mini-columns to perform cytoadherence assays. The micro-carrier culture technique used by other investigators suggested  $10^5$  cells/ 0.4 g beads as a suitable amount of cells to cover the beads after 4-5 days cultivation (17). In the current study, we find

that  $10^6$  CHO cells per 0.25 g beads are reasonable amount of cells and beads to do the assay easier and over a short time. This avoided having to grow the cells on the beads over long period in order to have enough cells and mature stage of parasite at the same time. This also reduced the risk of bacterial contamination especially when selection of a parasite binding subpopulation had been planned.

These experiments showed that the mini-column binding assay was a suitable method as parasitemia was always reduced after passing through a particular column. The results obtained were highly repeatable when the same experiment was performed many times. This work demonstrated many advantages of the mini-column binding assay in comparison to other methods. It uses simple equipments, easy to do, enable the selection of binding subpopulations in a short time and easy to repeat the experiment with the first generation of the selected parasites. The major advantage of using mini-columns is that it enables the quantitative study of subpopulations of *P. falciparum* within an infection. This is confirmed by three different experiments. These are: 1) mixing two parasite lines in known qualities; one that exhibits high binding to ICAM-1 and one, which does not display ICAM-1 binding at all (19). The percentage of retained cells was reduced when the proportion of the adherent population was decreased (Fig. 3).

2) using a non- ICAM-1/binding population for mini-column cytoadherence with CHO/ICAM-1 and CHO/CD36 cells. The findings show the percentage of bound cells to ICAM-1 was greatly reduced and adhesion to CD36 was increased. These findings showed that the most infected red blood cells which are able to bind to ICAM-1 receptor were removed. It also indicated that removing one binding subpopulation can increase the binding to other ligands. These findings are in good agreement with results obtained

from a study which indicated that *P. falciparum* variants are different in efficiency of adhesion and compete for binding to endothelial receptors (20). Doing the assays under situation like to flow conditions for short time periods leads to select a unique binding subpopulation of the parasite which has surface antigens with configurations which could better match with existing receptors.

3) Binding assays using the mini-column technique with ICAM-1 or CD36 binding parasites previously selected under static condition. The adhesion rate of any particular selected parasite to the same receptor using mini-column technique was increased in comparison with unselected parasite. It has also showed that the percentage of adhesion to CHO/CD36 (using CD36-selected clone) increased more in comparison with CHO/ICAM-1 cells (using ICAM-1-selected clone). These results indicated that the ICAM-1 selected subpopulation was able to bind to the CD36 receptor and the CD36 selected parasite were able to bind to ICAM-1. Possible explanations are: 1) the selection of parasites over long incubation periods in a static situation, gives an opportunity to all parasite subpopulations to bind to the particular receptor. This could be due to presence of different domains on parasite binding ligand (PfEMP-1) (21) which make it possible for various binding subpopulations to bind to particular receptors. Another possibility is that parasites may bind to molecules on the surface of CHO cells that might be similar to human endothelial receptors. 2) Antigenic variation (13) and switching in binding feature of parasite (22), it is well established that the switching rate of *var* gene family is about 2% or even faster per generation *in vitro*. Therefore, the adhesive ligand of selected parasite could change while the parasites are grown to achieve a suitable amount of infected cells for cryopreservation.

In summary, this work demonstrated that many advantages of the mini-column binding assay in comparison with other methods. Firstly, it enables the quantitative study of subpopulations of *P. falciparum* within an infection. Secondly, it enables to select various binding subpopulations of the parasite in a short time and perform further experiments on the selected parasites. Finally, the mini-column is not only simple in design but also avoids the need for formaldehyde pre-treatment which would seem to contribute to erroneous results.

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