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USE OF GLASS BEADS AND CF 11 CELLULOSE FOR REMOVAL OF LEUKOCYTES FROM MALARIA-INFECTED HUMAN BLOOD IN FIELD SETTINGS

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Passage of malaria-infected blood through a two-layered column composed of acid-washed glass beads and CF 11 cellulose removes white cells from parasitized blood. However, because use of glass beads and CF 11 cellulose requires filtration of infected blood separately through these two resins and the addition of ADP, the procedure is time-consuming and may be inappropriate for use in the field, especially when large numbers of blood samples are to be treated. Our modification of this process yields parasitized cells free of contaminating leukocytes, and because of its operational simplicity, large numbers of blood samples can be processed. Our procedure also compares well with those using expensive commercial Sepacell resins in its ability to separate leukocytes from whole blood. As a test of usefulness in molecular biologic investigations, the parasites obtained from the blood of malaria-infected patients using the modified procedure yield genomic DNA whose single copy gene, the circumsporozite gene, efficiently amplifies by polymerase chain reaction.

Key words: glass beads – CF 11 cellulose – leukocytes – malaria-infected human blood

Identification of candidate vaccine antigens and determination of their geographic variation are two key steps in the development of a subunit vaccine. In *Plasmodium falciparum* and *P. vivax* malaria parasite, several stage-specific proteins have been identified and tested for their ability to confer protective immunity against malaria infection (Miller et al., 1986; Nussenzweig & Nussenzweig, 1986). However, natural polymorphism of the immunodominant determinants in vaccine antigens may restrict the effectiveness of a subunit vaccine (de la Cruz et al., 1987; Good et al., 1988). For studies of antigenic variation to be predictive, studies of parasite proteins and the genes that encode them should be conducted on parasites from infected persons. The rationale for using field-derived parasites for antigenic variation studies is based on: (1) parasites grown in culture or in alternate hosts proliferate in the absence of host immune or biologic pressures; (2) certain lines of parasites proliferate preferentially, thus misrepresenting parasite types; and (3) culture-induced artifacts such as loss of determinants are known to occur in *in vitro* cultures (Fenton et al., 1985; Kemp et al., 1985, 1987; Corcoran et al., 1986, 1988; Pologe & Ravetch, 1986, 1988).

Recent advances in biotechnology, especially in recombinant DNA technology, have permitted investigations that employ small quantities of parasite material. However, in biochemical, some immunologic and in most genetic experiments, removal of host white cells is necessary because a small contribution of host-nucleated cells would result in parasite material largely contaminated with host proteins or nucleic acids. Three laboratory-based procedures effectively separate host nucleated cells from whole blood: those involving gradient centrifugation, where blood cells separate on the basis of density (Procell et al., 1986), those using columns of glass beads and CF 11 cellulose (Schmidt et al., 1982; Sinden & Hartley, 1985), where ADP is added to blood in order to activate platelets followed by passing blood separately through columns of glass beads and CF 11 cellulose, and more recent commercial Sepacell resins. Common disad-

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vantages of all three procedures are that they are time consuming, expensive, and require instrumentation unlikely to be found in many field settings. In view of these considerations, we undertook a study to modify the procedure that uses glass beads and CF 11 cellulose into a field-usable procedure. The modified procedure which uses a single step filtration of whole blood through column containing glass beads and CF 11 cellulose and eliminates use of ADP, not only works efficiently in a field setting but yields pure parasite material with larger volumes of blood in laboratory settings.

MATERIALS AND METHODS

Malaria parasites – The malaria parasites used in this study were *P. inui* O S strain, *P. simium* and *P. knowlesi* from monkeys, and *P. vivax* and *P. malariae* Uganda I from infected chimpanzees. The human malaria parasites used in the study, *P. falciparum* and *P. vivax,* were from Brazil. Parasites were diagnosed microscopically by thin and thick smears.

*Purification of parasites by Sepacell leukocyte removal filter** – Infected blood was passed through a Sepacell leukocyte removal filter (Baxter Healthcare Corporation, Deerfield, IL 60015, USA) to remove the white cells according to the instructions of the manufacturer. The red blood cells in the flow- through were collected by centrifugation at 251 g at room temperature, a blood smear was made, and the pellet was frozen at –85 °C.

*Purification of parasites on a glass bead/CF 11 cellulose column** – Depending upon the volume of infected blood to be processed, either a 1X 20 cms BioRad Econo column (for 10 ml blood volume) or a 6 ml disposable plastic syringe (for 1-2 ml blood volume) was used. In both cases, the columns were plugged with glass wool and filled to within one inch of the top with equal amounts of CF 11 cellulose (bottom layer) and acid washed glass beads (top layer). The CF 11 cellulose was obtained from Whatman Biosystems Ltd, Maidstone, Kent, England and the 150-212 micron glass beads were obtained from Sigma Chemical co., St. Louis, MO, USA. Before use the glass beads were incubated with nitric acid for 15 min, followed by extensive distilled water washes till neutral pH was obtained. The beads were then allowed to dry in oven. The resins (glass beads and CF 11 cellulose) were extensively washed with PBS before passing blood through the columns. After blood samples were applied, the columns were again washed with PBS, and the flow through was centrifuged as above.

Purification of genomic DNA and amplification of the CS gene – The genomic DNA from laboratory-induced parasites, *P. vivax* and *P. simium,* and from the field parasites, *P. falciparum* and *P. vivax,* were isolated (Dame & McCutchan, 1983). In the case of field specimen, the frozen red cell pellet was directly used for DNA extraction. One hundred nanograms of the DNA was used in a PCR amplification reaction (Saiki et al., 1987). The amplification primers were GTC GGA ATT CAT GAT GAG AAA ATT AGC TATT and CAG CGG ATC CTA ATT AAG GAA CAA GAA GG for the *P. falciparum* CS protein gene amplification and GTC GGA ATT CAT GAA GAA CTT CAT TCT C and CAG CGG ATC CTT AAT TGA ATA ATG CTA GG for amplification of the *P. vivax* and *P. simium* CS genes (Dame et al., 1984; Arnot et al., 1988). These primers have been synthesized with restriction sites for the endonucleases BamH 1 and EcoR I to facilitate bidirectional cloning into plasmid vectors for sequence analyses. The amplification procedure was accomplished by 25 cycles each of denaturation, annealing, and extension for 1.5, 2.0, and 4.0 minutes respectively. After 25 cycles, results of DNA amplifications were checked on an agarose gel.

RESULTS

Consistency and effectiveness of separation of leukocytes from malaria infected blood was equally comparable when Sepacell, a commercial resin, or the two layered glass bead/CF 11 cellulose columns were used (Table I). We have consistently observed that chimpanzee leukocytes do not separate on Sepacell as efficiently as monkey leukocytes. *P. inui* and *P. knowlesi,* two primate malaria parasites used in this study from laboratory-infected *Rhesus macaque* monkeys, separated equally well on Sepacell and on the co-layered glass beads/CF 11 cellulose column (Table I). For *P. knowlesi* parasites, we also monitored the percentage of

*Use of trade name is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Service.

TABLE I

Removal of leukocytes from malaria infected blood; comparison of Sepacell filter and glass bead: CF 11 cellulose column

Parasite species	Source	Purification procedure[a]			
		Sepacell		Glass bead/ CF 11 Cellulose	
		Before	After	Before	After
1. *Plasmodium inui*	O S Monkey	18	0	19	0
2. *P. malariae*	Uganda I Chimpanzee	46	15	ND[b]	ND
3. *P. knowlesi*	Monkey	44	0	44	0
4. *P. vivax*	Chimpanzee	22	2	ND	ND

a: WBC counts are per 10,000 red blood cells.
b: ND = not done.

TABLE II

Differential parasitemia before and after leukocyte removal by Sepacell and glass bead/CF 11 cellulose procedures

Treatment	Parasitemia (%)	Rings (%)	Trophs (%)	Schizonts (%)
None	30.00	6.00	33.00	61.00
Sepacell	22.50	20.00	23.00	57.00
Glass bead/ CF 11 cellulose	21.0	25.00	18.00	57.00

parasitemia and the presence of different stages of the parasites before and after the removal of leukocytes by both procedures (Table II). The results suggest that neither of the two procedures has any effect on the recovery of malaria parasites. We did however observe an increase in the level of rings after passing blood through Sepacell or glass bead/CF 11 cellulose column. This difference could be in part due to the rupture of schizonts and subsequent reinvasion into red cells during the time period between drawing of blood from infected animals and time when white blood cells are removed.

In order to test leukocyte removal efficacy of the two-layered column separation procedure in field conditions, we undertook experiments to separate infected red blood cells from leukocytes from malaria infected persons in Brazil. A disposable 6 ml plastic syringe used as a column was filled with 2 ml equivalent each of glass beads and CF 11 cellulose. This syringe was then prewashed with PBS and freshly collected slide-positive blood was passed through it. The flow-through was collected and centrifuged in a tabletop clinical centrifuge to pellet red cells. Thin and thick smears were prepared and stained with Giemsa solution before and after passing blood through the column. The parasitemia and presence of leukocytes before and after filtration through column was determined independently by two microscopist in a blind fashion. Out of the 22 human malaria blood samples used in this study, leukocytes were detected after filtration in only 8 samples (Table III). In these samples, a leukocyte count of only 1 to 4 per 10,000 red blood cells was recorded, compared to a wide range of between 4-150 leukocytes per 10,000 red blood cells before filtration.

We extracted genomic DNA from field samples of *P. falciparum* and *P. vivax* parasites. The CS gene was amplified from *P. falciparum* and *P. vivax* field parasite-derived DNA and from laboratory induced infections of *P. simium* and *P. vivax*. In one group of field parasite-derived DNA, 17 out of 23 DNA samples yielded a 1.2-1.3 kb DNA fragment

TABLE III

Removal of leukocytes from *Plasmodium falciparum*- and *P. vivax*-infected human blood in Paragaminos, Brasil, using glass beads/CF 11 cellulose columns

Parasites	Species	Purification[a]	
		Before	After
IEC-08-90	Pf	45	0
IEC-10-90	Pf	16	1
IEC-11-90	Pf	22	0
IEC-12-90	Pf	31	0
IEC-14-90	Pf	9	0
IEC-15-90	Pf	14	0
IEC-31-90	Pf	15	0
IEC-37-90	Pf	4	0
IEC-38-90	Pf	14	2
IEC-102-90	Pv	36	1
IEC-103-90	Pv	14	1
IEC-104-90	Pv	16	0
IEC-105-90	Pv	18	0
IEC-112-90	Pv	48	0
IEC-133-90	Pv	5	0
IEC-134-90	Pv	12	4
IEC-136-90	Pv	5	0
IEC-137-90	Pv	150	1
IEC-138-90	Pv	6	0
IEC-140-90	Pv	12	1
IEC-141-90	Pv	4	0
IEC-142-90	Pv	49	3

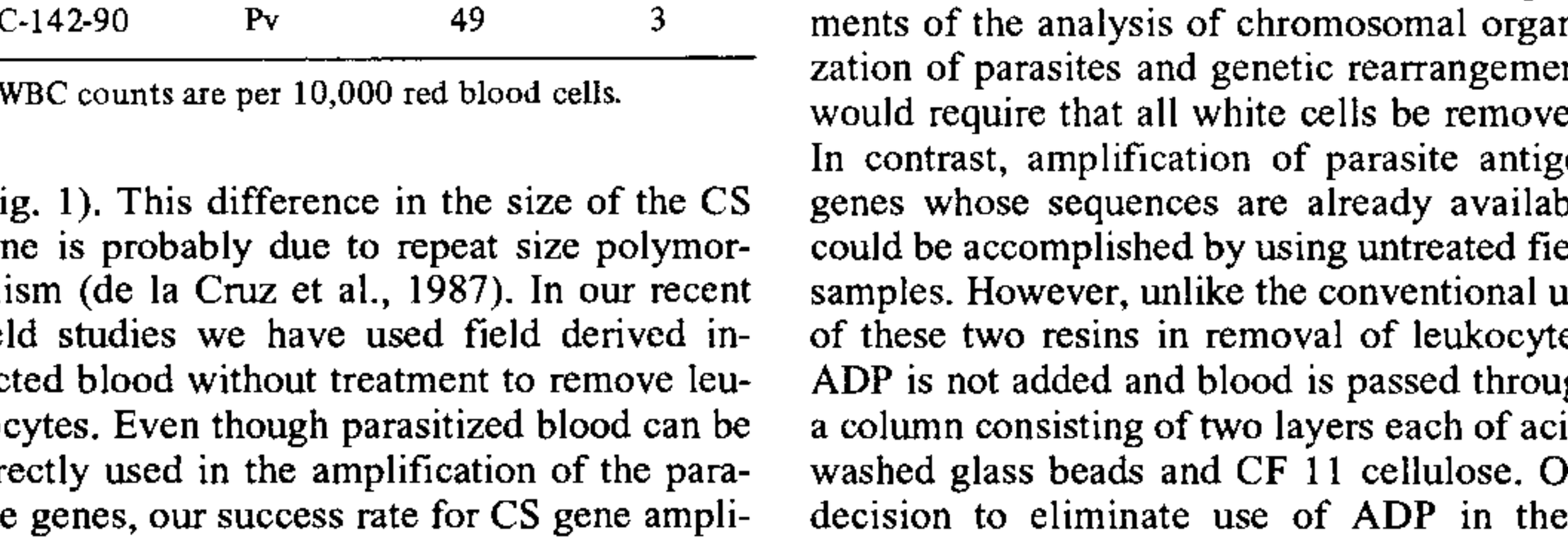
a: WBC counts are per 10,000 red blood cells.

(Fig. 1). This difference in the size of the CS gene is probably due to repeat size polymorphism (de la Cruz et al., 1987). In our recent field studies we have used field derived infected blood without treatment to remove leukocytes. Even though parasitized blood can be directly used in the amplification of the parasite genes, our success rate for CS gene amplification increases remarkably when white cells are depleted. Only 29% of the parasite DNA preparations obtained from leukocyte-free blood product failed to yield a CS gene fragment, compared with a 74% failure rate when whole infected blood-derived DNA was used in amplification. The failure rate in amplification reactions could be in part due to very low parasitemia or inadequate processing of parasitized cell.

Interestingly, *P. vivax* CS gene sequence-derived amplifying primers also work for the amplification of the CS gene of *P. simium* (data not shown). Our reason for using *P. vivax* primers to amplify the *P. simium* CS gene is derived from similar studies of *P. falciparum* and *P. reichenowi* antigens (Lal et al., 1990).

DISCUSSION

We have described a field-usable, column-based procedure that employs glass beads and CF 11 cellulose. The choice of using blood directly or first passing it through a column to remove white cells will depend on the field facility, numbers of blood samples, and the nature of investigation. For instance, experiments of the analysis of chromosomal organization of parasites and genetic rearrangement, would require that all white cells be removed. In contrast, amplification of parasite antigen genes whose sequences are already available could be accomplished by using untreated field samples. However, unlike the conventional use of these two resins in removal of leukocytes, ADP is not added and blood is passed through a column consisting of two layers each of acid-washed glass beads and CF 11 cellulose. Our decision to eliminate use of ADP in these



The CS gene amplification of *Plasmodium vivax* and *P. falciparum* parasite from Brazil. The CS gene amplification was carried out as described in materials and methods. Portions of the amplified fragments were on a 1.0% agarose gel at 50 volts for 5 hours. Lanes 1 and 26 are standard 1 kb DNA fragments, lanes 2-14 and 15-25 are amplified *P. vivax* and *P. falciparum* parasite CS gene fragments, respectively.

procedures was based on previous observations that platelets also bind to glass in absence of added ADP (Pachman et al., 1969; George, 1972). Even though we did not monitor for removal of platelets in procedures the results presented here show quantitative removal of leukocytes from blood.

Our modification of the procedure that uses glass beads and CF 11 cellulose is operationally simple, inexpensive, and most importantly, does not depend on sophisticated instrumentation. Taken together, these features should increase its use in molecular epidemiologic studies of malaria parasites as well as in laboratory procedures.

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