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Ovalocytic erythrocytes from Melanesians are resistant to invasion by malaria parasites in culture

(*Plasmodium falciparum*/erythrocyte membranes/cytoskeletal structure)

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ABSTRACT **Ovalocytic erythrocytes from Melanesians in Papua New Guinea have been demonstrated to be resistant to infection by malaria parasites (*Plasmodium falciparum*) in culture by using a double-label fluorescence assay of merozoite invasion. That merozoites do not bind irreversibly to ovalocytes has been demonstrated by an assay that measures competition between ovalocytes and normocytes. Analysis of behavior on thermal deformation has demonstrated that ovalocytes are more more thermostable than normocytes, suggesting that there is a major difference in cytoskeletal structure. These findings with *P. falciparum* and epidemiological data demonstrating clinical resistance to *P. vivax* and *P. malariae* suggest that the membrane alterations(s) in these ovalocytes affect(s) invasion step(s) common to all three species of malaria parasite.**

The relationship between human erythrocyte variants and susceptibility to malaria infection has been the subject of intensive clinical and epidemiological study over several decades (1). The development of *in vitro* culture methods for *Plasmodium falciparum* (2) has recently permitted direct examination of the capacity of some of these variants to support parasite growth. Thus, under certain conditions, hemoglobin S (3), hemoglobin C (4), thalassemia, fetal hemoglobin, and glucose-6-phosphate dehydrogenase deficiency (5) limit the growth of the intraerythrocytic stages of *P. falciparum*.

Invasion of erythrocytes by malaria parasites involves interaction with the host cell membrane (6, 7), and elucidation of the molecular events concerned is central to some approaches to development of a malaria vaccine. In the case of *P. knowlesi* and *P. vivax*, one component of erythrocyte specificity is represented by Duffy blood group determinants (8) which are evidently involved in the formation of tight junctions (9). However, the susceptibility of Old World monkey erythrocytes, which are Duffy (fy^b) positive (10), to *P. knowlesi* and their resistance to *P. vivax* implies that the binding requirements of the two species are not identical and that Duffy determinants alone do not fulfill these requirements. These determinants do not appear to be essential for recognition and penetration by other malaria species in man.

The availability of human erythrocyte variants resistant to penetration by merozoites of several malaria species could be of help in identifying membrane components common to the process of erythrocyte recognition and entry by these species. In this context the observation, derived from population surveys among Melanesians in coastal Papua New Guinea hyperendemic for malaria, that persons with ovalocytic erythrocytes have decreased rates of infection with *P. vivax* and *P. malariae* and apparently lower *P. falciparum* parasite densities (11) is of great interest. The biochemical basis of Melanesian ovalocytosis is not known but membrane differences in these erythrocytes are inferred from the altered expression of blood group antigens (12). Further studies are also required to elucidate fully the genetic basis. Currently, *in vitro* studies can be carried out satisfactorily only with *P. falciparum*.

We report here an analysis of invasion by *P. falciparum* in culture which demonstrates that ovalocytic erythrocytes from Melanesians are very resistant to merozoite penetration.

MATERIALS AND METHODS

Parasite Strains. The strain of *P. falciparum* used in the majority of experiments was FCQ-2/PNG, isolated in Madang Province, Papua New Guinea, and maintained in continuous culture by the method of Trager and Jensen (2). In some experiments, strains FCQ-22/PNG, FCQ-27/PNG, FCQ-30/PNG, FCQ-31/PNG, FCQ-33/PNG, and FCQ-41/PNG (13) also were used.

Erythrocytes. Ovalocytic individuals were identified by population screening using the erythrocyte morphological criteria of Amato and Booth (14). Blood samples from 10 unrelated ovalocytic individuals and 5 normocytic Melanesian controls in Papua New Guinea were collected into acid/citrate/dextrose (ACD). There was no evidence of any other biochemical abnormality in these erythrocytes, and no malaria parasites were seen. The collections were made on three separate occasions, repeat samples being taken from given individuals to permit comparison between sample batches. Normocytic Caucasian controls from the Red Cross Blood Bank (Brisbane) were included in each study. The proportion of ovalocytes in each sample was determined from wet preparations.

Scanning Electron Microscopy. Blood was collected into 1% glutaraldehyde/0.1 M cacodylate, pH 7.4, and incubated at 4°C for 1.5 hr. Cells were centrifuged at 170 × *g* for 5 min, resuspended in cacodylate buffer, dehydrated in increasing concentrations of ethanol, dispersed on coverslips, airdried, and coated with gold. The cells were examined with a Cambridge Stereoscan 600 electron microscope.

Assay of Parasite Invasion. All erythrocyte samples were assayed quantitatively for *P. falciparum* merozoite invasion by using FCQ-2/PNG, or other parasite strains where specified. By using a schizont-enriched (15) parasite preparation, quantitation of merozoite invasion was carried out by a method described in detail elsewhere (16). The erythrocytes to be tested were labeled with fluorescein isothiocyanate (FITC) which allows identification of the labeled cells without affecting merozoite entry (16). The schizont-enriched parasitized donor erythrocytes and the FITC labeled recipient erythrocytes were mixed in appropriate proportions in replicate cultures and incubated for 24 hr in a candle jar at 37°C. Samples were taken,

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Abbreviation: FITC, fluorescein isothiocyanate.

smears were made on glass slides and fixed with methanol, and parasite DNA was stained with ethidium bromide. The smears were examined for ring forms of the parasite in FITC-labeled erythrocytes by fluorescence microscopy.

Relative Susceptibility to Invasion. Competition between normocytic and ovalocytic erythrocytes for invasion by *P. falciparum* was examined by mixing a standard number of FITC-labeled indicator normocytes with a varying ratio, but constant total number, of unlabeled normocytes and ovalocytes. These experiments were designed to give a direct measurement of the relative susceptibility of the two cell classes to invasion.

Erythrocyte Membrane Proteins. The discontinuous $NaDodSO_4$ system devised by Laemmli (17) was used for electrophoresis of erythrocyte membrane proteins; it was modified for use with a slab gel (130 mm long, 140 mm wide, 1.2 mm thick) with an 8.5% separating gel and a 3% stacking gel. Membranes were prepared by hypotonic lysis of fresh erythrocytes in 10 vol of cold 10 mM phosphate buffer (pH 7.4) followed by two washings, each with 10 vol of the same buffer. At each resuspension, care was taken to separate the erythrocyte ghosts from a tight pellet at the bottom of the tube. The membrane proteins were dissolved in $NaDodSO_4$ (10 mg/ml) and 2-mercaptoethanol (10 mg/ml).

Table 1. Invasion of normocytic and ovalocytic erythrocytes by *P. falciparum* in culture

Sample	% ovalocytes	Invasion index (Mean ± SEM)
Controls (n = 8)	3.4 ± 0.9	100 ± 4.72*
Ovalocytes		
Ov1	79	3.31 ± 0.83
Ov2	80	3.87 ± 1.32
Ov3	78	3.23 ± 0.31
Ov4	80	1.09 ± 0.42
Ov5	86	2.25 ± 0.89
Ov6	80	5.33 ± 1.76
Ov7	51	3.07 ± 0.92
Ov8	64	30.78 ± 9.16
Ov9	96	2.87 ± 2.96
Ov10	82	2.25 ± 0.90
Mean†		2.65 ± 0.34

P. falciparum was cultured by the method of Trager and Jensen (2) in RPMI-1640 medium (Commonwealth Serum Laboratories, Melbourne, Australia) containing 24 mM Hepes (Calbiochem), gentamicin sulfate (Sigma) at 20 μg/ml, and 10% human serum type AB (Red Cross Blood Bank, Brisbane, Australia). The pH was adjusted to 7.4 with $NaHCO_3$. The parasites were maintained in type AB cells. Schizonts were concentrated 10- to 20-fold from infected blood by sedimentation (10) in 0.75% swine skin gelatin (type II, Sigma) dissolved in medium without serum. Prior to their use in culture, recipient erythrocytes were labeled with fluorescein isothiocyanate (Sigma) to distinguish them from the donor cell population. Cells were washed once with isotonic saline; then, 0.5 ml of packed cells was resuspended in 1 ml of FITC (4 mg/ml in phosphate-buffered saline, pH 7.4) for 10 min at 20°C. Unreacted FITC was removed by washing the cells five times in buffered saline and once in medium containing serum. Approximately 10^6 schizont-enriched donor cells ($\approx 3 \times 10^5$ schizonts) were mixed with 10^7 FITC-labeled recipient cells in a final volume of 150 μl in triplicate wells in a microtiter tray. After incubation for 24 hr at 37°C in a candle jar, smears were made on glass slides and fixed with methanol. Parasite DNA was stained with ethidium bromide (2 μg/ml in water) for 15 min at 20°C, washed with water, and dried in air. The numbers of parasites in FITC-labeled erythrocytes were determined by using a vertical fluorescence microscope (Leitz Orthoplan) equipped with a 200-watt superpressure mercury lamp. The appropriate filter combination for fluorescein was used.

* The mean ± SD of parasitemia in controls from the series of experiments was 3.10% ± 1.62%. No significant difference was found, within one experiment, between Caucasian controls collected in Brisbane and Melanesian controls collected concomitantly with ovalocytes in Papua New Guinea. Three Brisbane Caucasian controls and five Melanesian controls were used.

† Weighted mean (see text).

Thermal Deformation of Erythrocytes. Washed erythrocytes were incubated in medium with 10% human serum at 10% hematocrit, at 37°C, in equilibrium with a gas phase of 90% N_2/5% O_2/5% CO_2(vol/vol) overnight. The suspension was then cooled to 0°C. Aliquots (10 μl) were put in microhematocrit tubes and one end was sealed. The tubes were placed in a water bath at the required temperature for 5 min, rapidly cooled in ice, and opened, and the contents were mixed with 10 μl of ice-cold 1% glutaraldehyde/1% paraformaldehyde/0.1 M sodium cacodylate, pH 7.4. The cells were left on ice for 30 min before being mounted and then examined by using a phase-contrast, ×100 oil-immersion objective.

Statistical Methods. The invasion index for ovalocytes is defined as

$$\frac{\text{Fraction parasitized ovalocytes}}{\text{Mean fraction parasitized normocytes}} \times 100$$

in which both ovalocytic and normocytic erythrocyte samples are infected in parallel with the same batch of schizonts. Throughout this paper, weighted means have been used with weights equal to the reciprocal of the estimated variance. Differences between means have been compared by using Student's t test; differences between invasion indices of different ovalocyte samples have been compared by using an analysis of variance.

RESULTS

Invasion of Ovalocytes. In a series of experiments (Table 1) ovalocytes had a significantly ($P < 0.001$) lower mean invasion

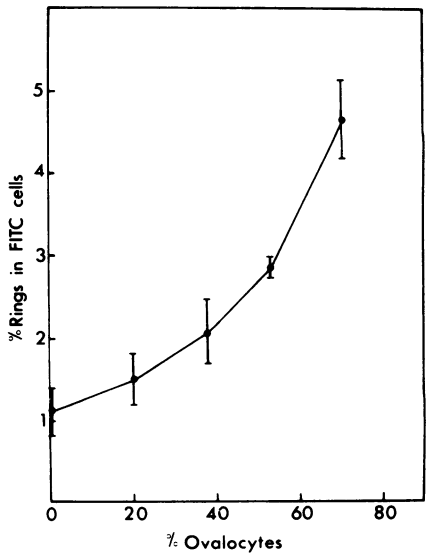

FIG. 1. Competition between normocytic and ovalocytic erythrocytes for invasion by *P. falciparum*. Indicator normocytes (1.7×10^7) were labeled with FITC as detailed in Table 1. Unlabeled normocytes and ovalocytes were added in different proportions to give a constant total number (6.5×10^7) of unlabeled erythrocytes. To the mixture was added a known number (5×10^6) of schizont-enriched (3.5×10^5 schizonts), parasitized, donor normocytic erythrocytes. The number of unparasitized erythrocytes in the donor population was taken into account in calculating the proportions of ovalocytes and normocytes. The final volume was 150 μl; each measurement was made in triplicate. Incubation was for 24 hr at 37°C in a candle jar. Smears were stained and examined as detailed in Table 1. The average parasitemia (in total cells, ovalocytes plus normocytes) did not significantly vary over the range 0–70% ovalocytes. Because the absolute parasitemia in the FITC-labeled cells varied according to the level of parasitemia in the donor cell population, a representative rather than an average curve of several experiments is shown. The ovalocytic sample contained 20% apparent normocytes. Error Bars = 1 SEM.

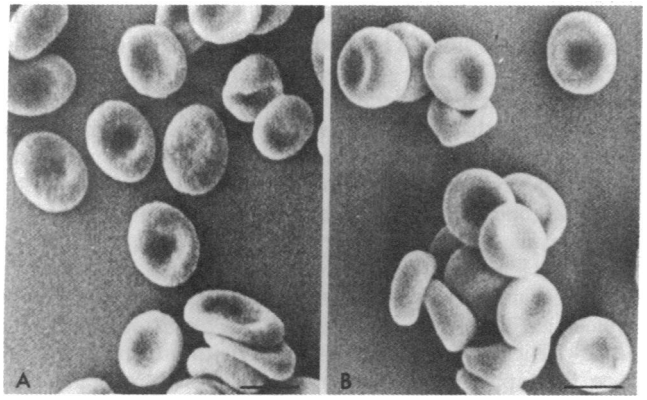

FIG. 2. Scanning electron micrographs of ovalocytes (*A*) and normocytes (*B*). (Scale = 5 μm.)

index compared with controls. The small proportion of cells invaded in ovalocytic samples appeared to be normocytic rather than ovalocytic as judged by fluorescence microscopy immediately after invasion, although by this criterion it is not possible to exclude the occurrence of parasite penetration of some ovalocytes.

Variation Among Ovalocytes. Within the one experiment using a single batch of schizonts, the invasion indices for Ov4, Ov6, Ov7, Ov8, Ov9, and Ov10 were compared by analysis of variance. On this basis, real differences between some ovalocyte samples from different donors are probable ($P < 0.001$). The invasion index of Ov8 was significantly greater than that of the next ranking sample, Ov6 ($P < 0.001$).

Variation with *P. falciparum* Strain. The susceptibility of ovalocytes to different parasite samples was tested by comparing infection of a single ovalocyte type, Ov4, by seven different *P. falciparum* strains listed above in the same set of experiments. The mean (±SEM) invasion index of Ov4 for these seven strains was 2.04 ± 0.26. No significant differences were detectable among the parasite strains ($P > 0.05$).

Competitive Invasion. By using the FITC-labeling technique it is possible to ascertain the relative invasion of ovalocytes and normocytes by competition assay. In such an experiment a standard number of FITC-labeled indicator normocytes was mixed with different proportions of unlabeled normocytes and ovalocytes, the total number of erythrocytes being kept constant. After incubation with a standard number of schizonts the percentage of parasitized indicator cells was recorded. When invasion of ovalocytes is negligible compared with that of normocytes, the parasitemia in normocytes must double as the number of available susceptible cells is halved, leading to an exponential relationship similar to that determined experimentally, as shown in Fig. 1 for representative data. If ovalocytes and normocytes were equally susceptible to invasion, the proportion of parasitized indicator cells should remain constant. This should also be true if merozoites were to bind irreversibly to ovalocytes without invasion. Thus, ovalocytes do not compete favorably with normocytes with respect to merozoite invasion, confirming the data from the direct infection experiments.

Ovalocyte Structure. These ovalocytic erythrocytes were elongated but retained a degree of biconcavity (Fig. 2). We have observed them to be more resistant than normocytes to lysis on storage (unpublished data). Electrophoresis of ovalocyte and normocyte membrane proteins showed no significant differences in distribution.

On thermal deformation, all 10 ovalocyte samples tested were substantially more heat resistant than normocytes as judged by the difference in temperature at which distortion of cell shape occurred (Fig. 3). The temperature at which half the

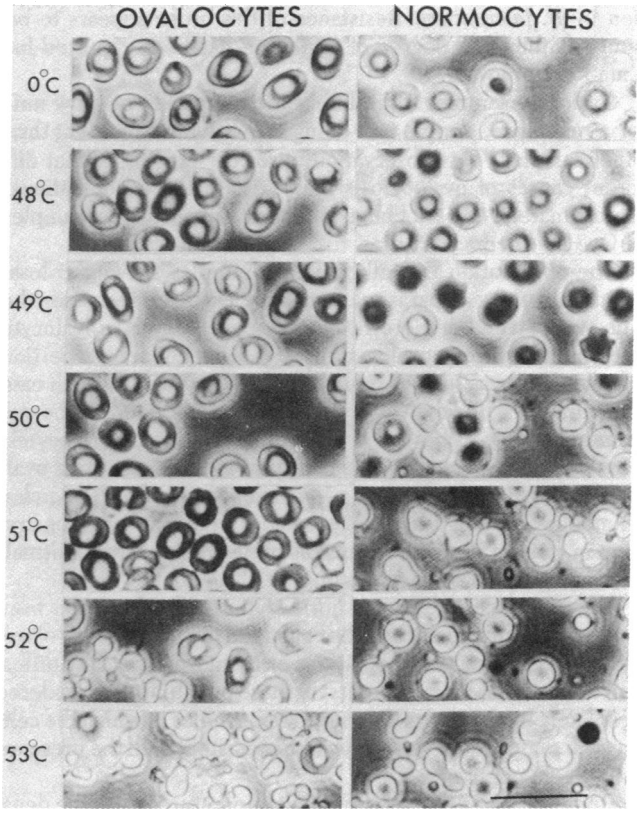

FIG. 3. Thermal deformation of ovalocytes and normocytes. (Scale = 20 μm.)

cells were deformed was approximately 49°C for normocytes and 51°C for ovalocytes. The normocytes became irregular and then rounded, with budding off of small spheroidal particles as reported (18). With ovalocytes, fewer spheroidal particles budded per cell and the final cell shape often was nonspherical with the cell body breaking into a few large fragments. Regardless of the number of apparent normocytes present, on thermal deformation all ovalocyte samples tested behaved as though a single cell population was present (Fig. 4).

DISCUSSION

By direct assay of invasion in culture, ovalocytic erythrocytes from Melanesians in Papua New Guinea are resistant to infec-

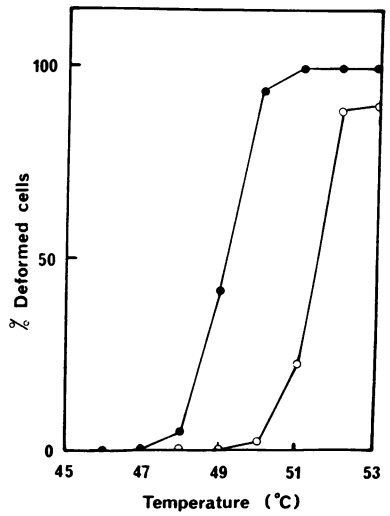

FIG. 4. Thermal deformation of ovalocytes (○) and normocytes (●).

tion by *P. falciparum*. Resistance of this order appears to be unique although a degree of inhibition has been reported for En(a-) erythrocytes (19).

Ovalocyte samples from different donors did not show uniform resistance, sample Ov8 being markedly less resistant than other samples. This observation raises the possibility that different causes of ovalocytosis may exist in these populations. Unfortunately, it has not been possible to obtain further samples of Ov8 for chemical studies.

The observation that all ovalocyte samples showed at least occasional entry of parasites into some erythrocytes raises the question of whether merozoites can indeed penetrate ovalocyte membranes. Every ovalocytic sample contained a proportion of normocytes as judged by light microscopy, and in each case the numbers of labeled erythrocytes invaded could be accounted for theoretically by the presence of these normocytes. However, the possibility that entry of a parasite into an ovalocyte alters its appearance [as has been shown for *P. knowlesi* in monkey erythrocytes (20)] precludes a definitive demonstration in stained films that parasites prefer the apparent normalocytes within an ovalocytic sample.

The presence of apparent normocytes in blood samples from ovalocytic individuals could be explained by the existence of two different erythrocyte populations or by morphologically different maturation stages. The first of these possibilities is rendered less likely by the thermal denaturation studies in which the cells in each sample behaved uniformly, consistent with the presence of a single population.

In normocytes the deformation temperature is also the denaturation temperature of the major cytoskeletal protein, spectrin (21). This close correlation between the deformation temperature of the cell and the denaturation temperature of its spectrin is also found in two conditions in which the erythrocytes show increased thermal sensitivity: hereditary pyropoikilocytosis, and certain cases of hereditary elliptocytosis (21, 22). It therefore is likely that for ovalocytes the increased thermal stability results from an altered cytoskeletal structure.

Several mechanisms can be postulated by which an altered cytoskeleton could inhibit merozoite invasion. A rigid skeleton may physically prevent merozoite penetration from occurring. Because the distribution (23) and lateral mobility (24, 25) of erythrocyte surface proteins are dependent upon the status of the cytoskeleton, it is also possible that an altered cytoskeleton may interfere with the recognition of particular sites on the erythrocyte surface by a merozoite. Such an explanation would be consistent with our data, derived from competition experiments, which show that merozoites do not bind tightly to ovalocytes. The observed depressed reactivity of some blood group determinants on ovalocytes (12) is consistent with an altered position in the membrane.

The data presented here may provide a mechanistic basis for the trend, in population surveys (11), toward lower *P. falciparum* densities in erythrocytes from ovalocytic compared with those from normocytic persons. This experimentally quantified resistance to *P. falciparum* and the resistance to *P. vivax* and *P. malariae* inferred from population survey data (11) imply that the altered membrane structure of these ovalocytes involves a change that is common to one or more steps in the process of recognition and penetration by all three species. Although the alteration or absence of specific receptors *per se* cannot be excluded, it seems more likely that ovalocyte resistance is secondary to the underlying cytoskeletal changes. Nevertheless, these ovalocytes provide a valuable tool for analyzing further molecular details of the process of invasion of erythrocytes by the major species of malarial parasites that cause disease in man.

From family studies it has been inferred (12) that ovalocytosis in Papua New Guinea is genetically determined. Therefore, the high geographical correlation of ovalocytosis and endemic malaria suggests that ovalocytosis confers a selective advantage with respect to malarial infection (11). From the observed pattern of ovalocytosis in a study of 96 Melanesian families, Booth *et al.* (12) concluded that the data were consistent with a high-frequency, autosomal recessive mode of inheritance. However, in three of four families involving the marriage of a Melanesian ovalocytic and a Caucasian normocytic person we have found ovalocytic children, in keeping with the report of Harrison *et al.* (26). These findings are consistent with dominant inheritance. The application of the criteria of *in vitro* resistance and thermal stability in addition to appearance may help to clarify the genetic basis of ovalocytosis. Indeed, in one Melanesian/Caucasian family we have been able to use all three criteria to demonstrate ovalocytosis in a newborn child of an ovalocytic mother. Full details of these genetic studies will be published elsewhere.

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