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Research Article

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Impairment of the Growth of Plasmodium Falciparum in HbEE Erythrocytes

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A B S T R A C T   HbE is a β-chain mutant frequently found among inhabitants of Southeast Asia and surrounding territories. We find that *Plasmodium falciparum* multiplies more slowly in erythrocytes from individuals homozygous for HbE than in cells from HbA individuals. In contrast, this parasite grows normally in erythrocytes heterozygous for HbE. This is the first direct evidence that suggests what has been suspected on the basis of circumstantial data, that HbE-containing erythrocytes might be advantageous to the carrier in regions with endemic malaria.

INTRODUCTION

Hemoglobin E is a β-chain mutant in which glutamic $\beta26$ is substituted by lysine (1). This mutant, although polymorphic and carried by an estimated 30 million individuals in Southeast Asia, is rare elsewhere. In a large area involving portions of Laos, Cambodia, Thailand, and their mutual borders, the gene for β^E exhibits its highest frequency and reaches an incidence of up to 55% among the Khmer people in Sarim and around the ancient temple of Angkor Wat (2). The gene frequency decreases concentrically from this area, with frequencies of 10–20% in Western Thailand, Burma, Southern Vietnam, and Sri Lanka. The frequency of β^E falls to between 2 and 10% in Bengal,

Northern Vietnam, and Malaysia (except for Sakai and Senoi where the frequency is higher). Frequencies below 2% are typical for South China, Philippines, Indonesia, and Nepal. In the rest of the world HbE is discovered occasionally and its frequency is probably exceedingly low.

A relation between HbE and malaria has been suggested (2) but no definitive evidence has been presented. Since Southeast Asia is a holoendemic area for *Plasmodium falciparum* and other types of malaria, it is reasonable to suspect that malaria might be acting as a selective factor for this gene. The recent description of an in vitro method for the culture of *P. falciparum* (3) has allowed us to examine this question directly, by studying the growth curve of the parasite in AA, AE, and EE erythrocytes.

METHODS

Blood samples were obtained in citrate phosphate dextrose or heparin and kept at 4°C until tested. The absence of α-thalassemia among the heterozygotes studied was deduced by the 40%/60% proportion of HbE/HbA (4). HbE was defined pH 8.6 starch gel electrophoresis and pH 6.4 agar electrophoresis. The absence of α-thalassemia among our homozygous EE patients was ascertained in one of the homozygotes by endonuclease mapping performed by Dr. Gregory Mears. One of the homozygous EE samples was shipped from Rochester, Minn., but the others were fresh samples from New York. Shipping controls of AA individuals of the same ethnic stock were included in the sample of AE individuals from France. The five homozygous individuals were of

Chinese, Laotian, and Thai origin. The heterozygous individuals were of Laotian background.

Culture conditions were those described by Trager and Jensen (3). The *P. falciparum* strain used (FCR-3) was a kind donation of Dr. William Trager. Erythrocytes from the donors were washed and infected as described (5) to obtain an initial concentration of 0.1% of parasitized cells. Infected cells with AA genotype and 10% parasitemia were used as infective sources but were diluted 100-fold with the cells to be studied. Petri dishes (15 × 22 mm) holding 1.5 cm³ of erythrocyte suspensions were used. Every 24 h the media was changed and a smear was taken and stained with Giemsa. Parasites were counted in a field of 1,000 cells, and the results expressed as percent parasitemia.

Percoll-Renografin gradients were prepared as described by Vettore et al. (6), using Percoll:Renografin-76:water:0.9% NaCl ratio of 3.5:1.7:4.3:0.7. The resulting osmolarity was 320 mosmol and the pH was 7.5. For anemic patients the hematocrit was adjusted to 40–50 before the cells and plasma were added to the gradient mixture. For analytic studies, 0.1–0.2 ml of whole heparinized blood was added to 5.9 or 5.8 ml of gradient mix. The tubes were spun for 20 min at 17,000 rpm (35,000 g) in a Sorvall SS-34 angle head rotor at 4°C.

RESULTS

When growth curves of *P. falciparum* in AA and AE cells were compared, no difference was observed (Fig. 1). The cells from AA individuals from Laos, used as control, sustained the growth of the parasite as well as cells from normal Caucasian AA individuals.

In contrast, *P. falciparum* multiplies more slowly in EE erythrocytes than in AA erythrocytes (Fig. 2). Samples from three other homozygous EE patients behaved in the same manner. This strongly suggests that these erythrocytes are less capable of sustaining the growth of *P. falciparum* than AA or AE erythrocytes. We consider this finding the first direct evidence that strongly suggests a connection between HbE and malaria.

DISCUSSION

These observations are a clear indication that homozygous EE individuals could be significantly protected from the disease; they do not, however, provide evidence for the selective pressure of malaria acting on the heterozygote as expected in a classical balanced polymorphism. On the other hand, the data does not exclude the possibility that the AE heterozygote may be protected from malaria by another mechanism. The benign clinical condition of the homozygote for HbE added to the apparent hindrance of parasitic growth in these cells might mean that the HbE gene is not at equilibrium in the region and could indeed be increasing in frequency. The available population data is not conclusive in this respect (2).

More work is needed to determine the mechanism of impairment of growth of *P. falciparum* in EE erythrocytes. HbEE erythrocytes are microcytic (low mean
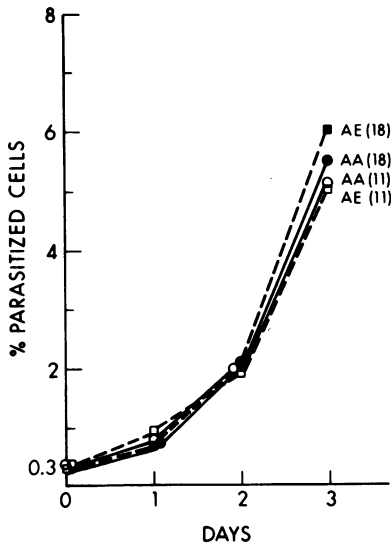

FIGURE 1 Parasite growth curves of *P. falciparum* in HbAA and HbAE erythrocytes. Between parentheses in the number of days of storage in citrate phosphate dextrose mixture (CPD) before infection. Each curve corresponds to a different donor. Each point is the average of three culture plates. In the ordinate, notice the concentration after initial inoculum (0.3). Culture became saturated for all samples at day 4. Identical results were obtained with other cases of AE erythrocytes.
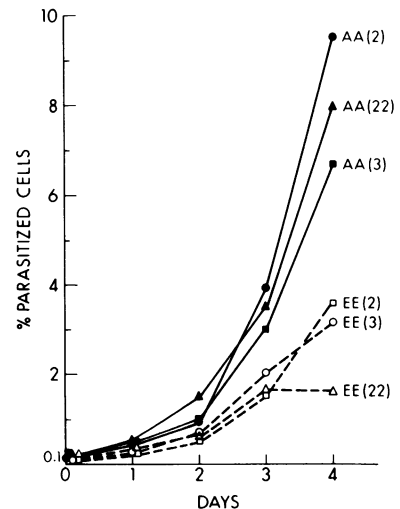

FIGURE 2 Parasite growth curves of *P. falciparum* in HbaA and HbEE erythrocytes. Between parentheses is the number of days of storage in CPD before infection. One set of donors of HbAA and HbEE were studied after 2 and 22 d of storage in CPD. Each point is the average of three culture plates. In the ordinate, notice concentration after initial inoculum (0.1): culture became saturated for AA cells on day 5, but not for EE cells. HbEE erythrocytes of other three independent cases behaved in the same manner.

corpuscular volume) but have a normal mean cellular concentration of Hb. As expected from these observations, the mean corpuscular hemoglobin (the total amount of Hb per cell) is low (7). Because the carriers of this genetic condition are not anemic and no hemolysis has been detected in the absence of other diseases, the number of erythrocytes is elevated.

That HbEE red erythrocytes have a normal (MCHC)[1] can be confirmed by isopycnic centrifugation. In the technique used the cells move in a spontaneously formed continuous density gradient until they reach their isopycnic density. The method has two advantages: (a) MCHC differences of as little as 0.5 g % can readily be detected and (b) the range of MCHCs present in the sample is immediately obvious. Thus, if a sample has a heterogeneous distribution of cells (as is the case for HbSS samples) the heterogeneity is readily detected. From Fig. 3 we can see that AA and EE cells have similar density distributions. This is in contrast to the CC cells, also shown, that are microcytic but have elevated MCHC.

Recently, Fairbanks (7) and Traeger et al. (8) have

presented convincing evidence that β^E synthesis is defective. Traeger et al. (8) have, in addition, provided evidence that a reduced level of β^E mRNA is the probable cause for this defect. The magnitude of the deficiency in β^E synthesis in HbE homozygotes is of the order of that found in β-thalassemia heterozygotes. Erythrocytes of individuals heterozygous for β-thalassemia, nevertheless, sustain the growth of P. falciparum normally (9). So we need to search further for the cause of the impairment. This finding also eliminates microcytosis, per se, as a cause for the inability of the parasite to grow efficiently in HbEE cells. A lead worth pursuing is the possibility that HbE may be mildly unstable to oxidative stress, as suggested (10), and that this instability can be elicited by the presence of the parasite.
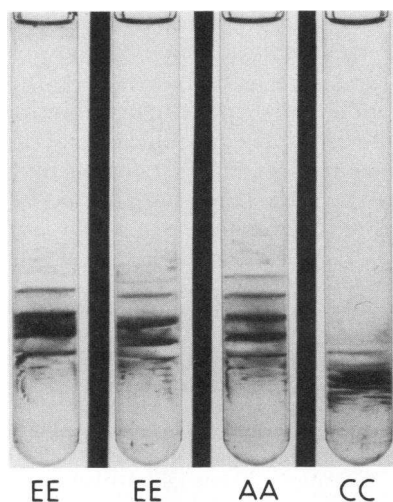
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FIGURE 3 Percoll-Renograffin continuous density gradients for two different EE patients, an AA subject, and a CC patient. The density of the medium in which the cells are suspended varies continuously, and increases as the depth in the tube increases. The density of erythrocytes can be shown to be directly proportional to their MCHC; therefore, the depth at which the cells are found in the tube reflects their MCHC. From these results we can deduce that, the mean corpuscular hemoglobin concentration of the AA and EE subjects is 33 g/dl and that of the CC is 39 g/dl. The result is comparable to that obtained by conventional MCHC measurements.

[1] Abbreviation used in this paper: MCHC, mean corpuscular hemoglobin.

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