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Erythrocytic Abnormalities in Experimental Malaria.* (31931)

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In malarial infections, the osmotic fragility of both parasitized and nonparasitized red blood cells is significantly increased(1,2). We believed that measurements of the erythrocytic size, surface area, hemoglobin concentration and volume changes in hypotonic saline solutions would provide information about the effect of malaria upon red blood cells. This study reports the erythrocytic changes observed in hamsters infected with *Plasmodium berghei.*

Methods. Adult Walter Reed strain hamsters were used in these studies. For malarial experiments, hamsters were infected by intraperitoneal inoculation with 2×10^7 *P. berghei*-infected mouse erythrocytes (New York University gametocyte strain). In experiments in which acetylphenylhydrazine (APH) was used, 20 mg/kg of a saline solution of the drug was injected intraperitoneally each day for 13 days. Blood was collected from the axillary vein. Leishman-Giemsa stained blood films were prepared from fresh blood to determine the percentage of parasitized and polychromatophilic cells. Erythrocytic diameters were measured on these films with a calibrated micrometer eye piece using a simplified Price-Jones technique(3). For other studies the blood was anticoagulated with heparin and washed 3 times with normal saline. After the initial centrifugation heavily infected blood formed 2 distinct layers. In certain experiments these fractions were separated and tested individually. Mean corpuscular hemoglobin concentrations (MCHC) were calculated from microhematocrit and hemoglobin determinations.

Determinations of erythrocytic volumes were performed with the Coulter counter with a 50 μ siliconized aperture and a 25 window plotter (Coulter Electronics, Hialeah, Fla.). We used the method of Brecher *et al*(4) as applied by Weed and Bowdler to determine the critical hemolytic volume of erythrocytes in hypotonic saline(5).

Red blood cells were diluted 1:25,000, resulting in a final concentration of approximately 200,000 cells per ml. Various concentrations of phosphate buffered sodium chloride solutions (pH 7.4) were used and the cellular volumes were expressed as the fraction V/V^o, in which V^o equalled the volume of the red blood cell in a 1.0 percent buffered sodium chloride solution. Previous studies have demonstrated that alterations in the ionic concentration of the conducting medium do not alter measurements of the particle volume(6). Likewise, Weed and Bowdler showed that the volume of red blood cells in 1.0% saline solutions was similar to cells suspended in filtered plasma(6). The reason red blood cells require hyperosmotic concentrations of sodium chloride to maintain their volume is obscure(4,6). Since only relative changes in cellular volumes were important for interpretation of our results, explanations for this phenomenon are irrelevant to these experiments. Cellular suspensions were maintained in test solutions for 45 minutes before sizing. Preliminary experiments showed that

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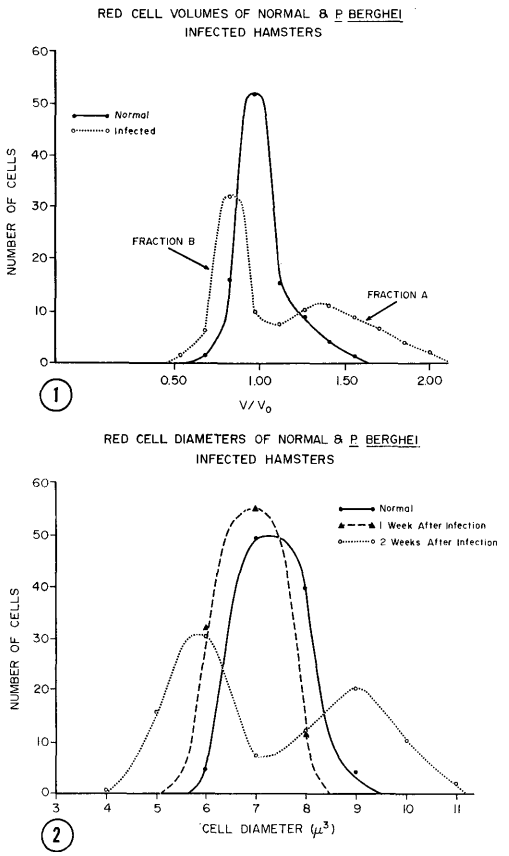

FIG. 1. Representative erythrocytic volume distributions in 1.0% buffered sodium chloride of normal hamsters and hamsters 2 weeks after infection with *P. berghei*. The mean cell volume of normal hamsters was set equal to 1.00 (V_0). Erythrocytes from infected hamsters were divided into 2 populations (A and B) by extrapolation. The number of cells on the ordinate represents the percentage frequency of various sized cells.

FIG. 2. Erythrocytic diameters in normal and *P. berghei*-infected hamsters. The normal curve is the mean of 5 normal hamsters, measuring 100 to 200 cells per slide. With infected animals, 500 cells per slide were measured. Individual hamsters 7 days (3% parasitemia, 1.6% reticulocytes) and 14 days (12% parasitemia, 11.3% reticulocytes) after infection are presented. The number of cells on the ordinate represents the percentage in frequency of various sized cells.

normal erythrocytes suspended in 1.0% saline solutions did not change their volume between 1 and 120 minutes. In 0.5% saline solutions erythrocytes swelled within 1 minute and maintained their new expanded volume for 2 hours. In 0.3% saline solutions a stable volume is achieved within 15 minutes and maintained for 2 hours; red blood cells shrink following hemolysis, and then the ghosts swell to the size achieved at their critical hemolytic volume(5). Washing erythrocytes in 3 changes of normal saline had no significant effect on cell volume determinations.

Results. A graphic representation of the volumes of normal hamster erythrocytes measured with the Coulter counter yielded a single-peaked distribution with a slight skew to the right, similar to the pattern for normal human red cells(4). Contrariwise, the blood of *P. berghei*-infected hamsters contained 2 populations of erythrocytes (Fig. 1), one with a significantly greater volume than normal cells (A) and the other with erythrocytes that were slightly smaller than normal (B). Confirmatory results were obtained when erythrocytic diameters were measured (Fig. 2). One week after infection, while parasite and reticulocyte counts were still low, erythrocytic size began to decrease. At 2 weeks, infection was severe and 2 distinct erythrocytic populations were present, one larger and one smaller than normal cells. Many of the smaller, nonparasitized erythrocytes appeared spherocytic on both stained and wet preparations.

When the blood from infected hamsters was centrifuged, 2 distinct layers of red blood cells were observed. The lower fraction contained erythrocytes with small volumes (B fraction in Fig. 1). The upper fraction was a mixture of large (A fraction) and small (B) erythrocytes, and contained almost all of the young and parasitized cells. Table I demonstrates the correlation between the percentage of cells in population A and the percentage of reticulocytes, polychromatophilic and parasitized cells.

The mean corpuscular hemoglobin concentration (MCHC) of the lower fraction of mature, nonparasitized erythrocytes from 11 hamsters, 7 to 15 days after infection, ranged from 29.0 to 36.5% with a mean of 31.7%. This was slightly greater than the MCHC of the lower fraction of centrifuged erythrocytes from normal hamsters (29.2 $\pm$ 2.3 (S.D.) %, n = 15, $P<0.01$). The MCHC of the upper fraction of centrifuged cells from malarious animals became markedly reduced

TABLE I. Identification of Red Blood Cell Populations of *P. berghei*-Infected Hamsters. Blood was obtained 2 weeks after infection and separated into top and bottom fractions by centrifugation. In each sample the relative frequency of large and small cells (populations A and B as represented in Fig. 1) was correlated with the concentration of various cell types.

Sample	Cells in population "A"	Cells in population "B"	Parasitized cells	Reticulocytes	Polychromatophilic red blood cells
	%				
1. Top	60.2	39.8	59.0	46.2	62.0
Bottom	.0	100.0	.0	.0	.2
2. Top	49.5	50.5	41.0	53.0	47.0
Bottom	.0	100.0	1.0	.9	1.0
3. Whole blood	24.5	75.5	12.3	16.1	24.0
Top	65.8	34.2	50.7	60.4	64.0
Bottom	.0	100.0	1.0	1.3	.0

when large, parasitized, polychromatophilic cells appeared.

The volume responses of both fractions of blood from *P. berghei*-infected hamsters were measured in solutions containing various concentrations of saline and the values were found to differ significantly from those observed with erythrocytes from normal animals. The mature, nonparasitized cells (B) from the lower centrifuged fraction were smaller than normal in the 1.0% saline and swelled much less than normal cells in hypotonic solutions (Fig. 3). The maximal volume attained was only 1.75 times the isotonic volume (1.40/0.80), compared to the maximal volume of 2.19 attained by normal cells. The large cells (population A, contained in the upper fraction) on the other hand, not only achieved twice their isotonic volume but swelled markedly in only slightly hypotonic solutions (0.85% and 0.70% sodium chloride).

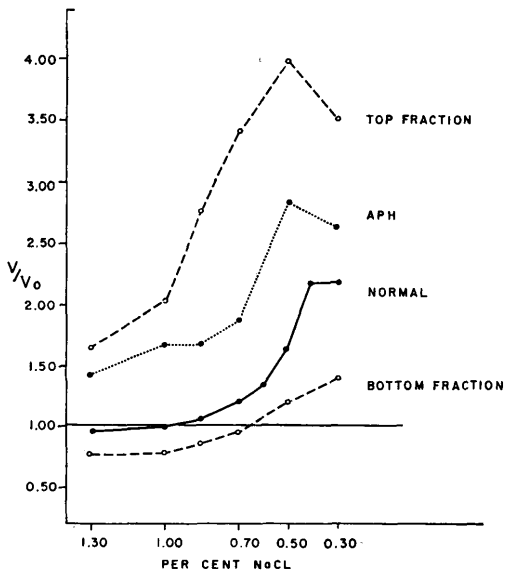

FIG. 3. Osmotic volume response of erythrocytes from normal hamsters, hamsters 2 weeks after infection with *P. berghei* whose blood was separated into top and bottom fractions, and hamsters treated with APH for 2 weeks. The osmotic volume response of bottom fractions of normal hamster blood was no different from the response of whole blood represented here.

To study the effects of hemolysis without the presence of intracellular parasitism, a group of hamsters was treated with daily doses of acetylphenylhydrazine (APH). Two weeks after initiating treatment there was a single population of large erythrocytes. Although these cells were similar in size to the reticulocytes from hamsters with malarial infections, their osmotic volume response showed less variation from normal cells (Fig. 3). In an additional experiment in which erythrocytes of 3 hamsters were studied at the time of maximal reticulocytosis (7 days) after a single large dose of APH (100 mg/kg), the results were similar to those of daily APH administration.

Discussion. Brecher and Stohlman(7) demonstrated that rats with APH-induced hemolysis preferentially destroyed older erythrocytes and with sufficient dosage produced a single population of reticulocytes. During the hemolysis induced by malaria, 2 populations of erythrocytes developed and were demonstrated by both volume determinations with the Coulter counter and measurement of the

diameter of circulating cells. This occurs because *P. berghei* preferentially parasitizes reticulocytes(8), thereby allowing mature erythrocytes to form a population distinct from that of the larger young cells. However, these mature, nonparasitized cells were not spared from the effects of the malarial infection. We previously reported that the osmotic fragility of the nonparasitized erythrocytes was increased(2). In the present study these cells were shown to be abnormally small and spherocytic. The increased MCHC and the diminished capacity of these cells to swell in hypotonic systems indicated that spherocytosis and a diminished cellular surface area occurred. Although the etiology of the spherocytosis is unknown, two possibilities are suggested by previous observations. Soluble antigens have been demonstrated in the sera of malarial-infected animals(9) and it has been postulated that circulating antigen-antibody complexes may become adsorbed to the erythrocytic membrane, fix complement, and provide an immunologic component to the hemolysis(10). Spherocytes are frequently seen in immune hemolytic anemias(11). Acquired spherocytes have also been observed in humans with hypersplenism resulting from nonmalarial infections in which autoantibodies were not demonstrated(12). In these patients increased activity of the reticuloendothelial system was thought to initiate the production of spherocytes and increase the sequestration of erythrocytes by the spleen. Thus the abnormalities of the nonparasitized erythrocytes may be induced by the hypersplenism accompanying malarial infections(13).

Reticulocytes from heavily infected animals were qualitatively different from both normal cells and APH-stimulated reticulocytes, swelling much more in only slightly hypotonic solutions. This indicated that an altered cation permeability defect occurred in the parasitized cells which permitted an increased influx of water into the cell. A membrane cation permeability defect during malarial infections was previously suggested by a report of abnormal sodium and potassium concentrations in erythrocytes from *P. knowlesi*-infected monkeys(14). Erythropoiesis during "marrow stress" without malarial infection has also been demonstrated to produce abnormal reticulocytes. These cells have a shortened survival(15) and retained "i" antigen upon their membrane(16). Although increased "marrow stress" may account for some abnormalities of the parasitized reticulocytes, comparison of the osmotic response of parasitized cells and APH-induced reticulocytes suggested that the intracellular parasitism probably caused additional membrane abnormalities.

Summary. Studies of erythrocytic indices and osmotic response revealed the presence of 2 abnormal populations of cells in hamsters infected with *Plasmodium berghei.* One population consisted of mature nonparasitized cells which became abnormally small and spherocytic with a diminished cellular surface area. This cell shrinkage could result from the hypersplenism of malaria or possibly from immunologic membrane damage. The other population was composed of parasitized reticulocytes which exhibited an increased rate of swelling in hypotonic saline, indicating impairment of the normal restricted cation permeability.

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Effect of Hyperbaric Oxygen on Aerobic Bacteria *in vitro* and *in vivo*.*† (31932)

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Hyperbaric oxygen has been used for therapy of osteomyelitis(1) and has been suggested as possible therapy for other infections due to aerobic bacteria(2,3). There are no controlled studies demonstrating efficacy of hyperbaric oxygen in therapy of aerobic infections, and in fact it is possible that oxygen therapy may have a deleterious effect in some instances. In addition, the possibility exists that hyperbaric oxygen used for treatment of conditions other than infection may increase susceptibility to infection by altering the delicate host-parasite relationship and allowing bacteria which are a part of the normal flora to produce infection.

The purpose of this research was to study by quantitative techniques the effect of oxygen under pressure on aerobic microorganisms *in vitro* and to investigate the influence of oxygen therapy on aerobic infections in animals.

Materials and methods. Clinical isolates of one strain each of *Escherichia coli, Aerobacter aerogenes, Pseudomonas aeruginosa, Salmonella typhimurium, Proteus mirabilis, Diplococcus pneumoniae* Type 6, *Staphylococcus aureus* (coagulase positive) and *Streptococcus pyogenes* were studied. Stock cultures were maintained by storing aliquots of a 24-hour broth culture at —20°C. The broth for *D. pneumoniae* was beef heart infusion broth with 5% sheep blood; trypticase soy broth without blood was used for the other bacteria. For each experiment an aliquot of stock culture was subcultured to broth and incubated at 37°C for 24 hours (24-hour culture). Four-hour cultures were prepared by diluting a 24-hour culture 1:10,000 in broth and incubating for 4 hours at 37°C.

The number of *D. pneumoniae* in broth was determined by serial dilution in beef heart infusion broth and streaking 0.1 ml aliquots on the surfaces of sheep blood agar plates (trypticase soy agar with 5% sheep blood). Numbers of bacteria other than *D. pneumoniae* were determined by serially diluting in trypticase soy broth and making pour plates with trypticase soy agar. All plates were read after incubation for 48 hours at 37°C.

In vitro *experiments. In vitro* experiments were performed in Torbal-B.T.L. Anaerobic Jars (Torsion Balance Co.) which were fitted with pressure gauges and could be pressurized to 2 atmospheres absolute or 30 lb per square inch absolute (30 p.s.i.a.). Air was eliminated from the jars by alternately evacuating with negative pressure and filling with oxygen under pressure. Suspensions of bacteria in broth in covered petri dishes were incubated in air at 15 p.s.i.a. or oxygen at 30 p.s.i.a. The broth was only 1.5-2.5 mm deep to allow diffusion of gas. Trypticase soy broth was used for all bacteria except *D. pneumoniae* for which beef heart infusion broth with 5% sheep blood was used. Ex-

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