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STUDIES IN HUMAN MALARIA[1]

I. The Preparation of Vaccines and Suspensions Containing Plasmodia

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In spite of the vast amount of knowledge of the malarias that has accumulated through the years, little has come to light regarding the chemical and immunological properties of the plasmodia responsible for malaria in humans. This has been in part due to the difficulties, in normal times, of collecting large volumes of heavily parasitized human blood, lack of methods for the concentration of the plasmodia, and the difficulties inherent in the organization of controlled experiments on the immunization of humans.

Under wartime conditions many of these difficulties appeared less important, particularly in view of the urgency of the malaria problem, and the present studies were accordingly undertaken.

A primary requisite for the investigation of the antigens of human malaria was the preparation of concentrates of plasmodia from infected human blood. These were required for (1) an attempt to terminate chronic relapsing *vivax* malaria by specific vaccination of patients, (2) attempts to prevent mosquito-borne infection by *Pl. falciparum* and *Pl. vivax* and blood-borne infection by *Pl. vivax* by prior vaccination of subjects, and (3) study of the immunological properties of the parasites and the immunochemistry of their components. Owing to the exigencies of the war effort precedence was given to the more immediately practical aspects of these problems.

In the earlier stages of the work the samples of human blood used were infected with *Pl. falciparum*, *Pl. vivax*, or *Pl. malariae*. Those not obtained locally were sent in by air express (shipments from outside the United States under permit) in vacuum jars containing cracked ice. The writers are greatly indebted for such material to Lt. Col. G. R. Biskind, Letterman General Hospital, San Francisco; Dr. M. F. Boyd, Tallahassee, Fla.; Col. W. C. Cox, Quarry Heights, Canal Zone; Col. W. P. Davenport, San Juan, Puerto Rico; Dr. C. M. Johnson, Gorgas Memorial Inst., Panama; Drs. S. B. McLendon and M. D. Young, Columbia, S. C.; Dr. J. A. Shannon and staff, New York; Dr. L. H. Sophian, U. S. Marine Hospital, Staten Island, New York; Dr. R. B. Watson, Memphis, Tenn.; Capt. M. D. Willcuts, Naval Hospital, San Diego; and Dr. J. Zozaya, Mexico City.

[1] The work described in this paper was carried out under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University, Dec. 1942–Dec. 1945. The work was also carried out in part under the Harkness Research Fund of the Presbyterian Hospital in New York City.

At a later stage the study was facilitated by the cooperation of the Bureau of Medicine and Surgery of the U. S. Navy, and malaria patients at the St. Albans Naval Hospital were authorized to donate blood on a voluntary basis under the supervision of Comdr. W. A. Coates, MC, USNR. These samples were all infected with *Pl. vivax*.

The initially used method of preparation of vaccines involved lysis of washed erythrocytes in chilled water saturated with CO_2 and removal of hemoglobin from the stromata, leucocytes, platelets and parasites by repeated washing with chilled 0.2 per cent NaCl solution saturated with CO_2. About one-half of the vaccines used in the attempt to influence relapsing *vivax* malaria were of this type, as were also most of those used in the experiments on active immunization.

EXPERIMENTAL

1. Separation of formalinized Pl. vivax by differential centrifugation. The method of concentration which follows was gradually evolved out of the earlier attempts. It yields the purest concentrates with bloods containing large, pigmented forms of *Pl. vivax*, preferably at concentrations of 10,000 per mm^3, or above. Fifteen to 20 hours are required for processing 500 ml of blood, speed being desirable owing to the deterioration in appearance of parasites on standing for even a few days at temperatures near 0 C.

All operations, including centrifugation, are carried out in the cold and with aseptic precautions, and only water and saline especially prepared for infusions were used with material intended for use as vaccine. The citrated blood sample is at once chilled. The plasma is removed after centrifugation and the erythrocytes are washed twice with 2 to 3 volumes of chilled 0.9 per cent saline solution containing 0.2 per cent formalin. The plasma and washings are recentrifuged, and any sediment, usually rich in parasites, is combined with the main portion for lysis.

Meanwhile, 5 l of water (for 500 ml blood) are saturated in an ice-bath with CO_2 by passage of a carefully filtered stream of the gas for about 1 to 1.5 hrs. Ten ml of formalin are added and the washed, packed erythrocytes are poured into the chilled, carbonated water, with constant mixing until all masses of cells have disintegrated. When the stromata and parasites have settled (about 1 hr) the transparent red supernate is sucked off and the whitish deposit collected by centrifugation in four 250 ml bottles. The clear supernate is removed and the packed residues are suspended smoothly in 800 to 900 ml of chilled 0.9 per cent saline solution containing 0.2 per cent formalin. After about 20 min the suspension is centrifuged for 15 to 20 min at 2,000 rpm. The sediment (P_1) consists mainly of leucocytes with a brown, upper layer of parasites. The turbid supernate which contains most of the stromata and many parasites, especially if these are small or ring forms, is sucked off and recentrifuged for 1–2 hrs at 2,500 rpm. This time a brown sediment (P_2) is obtained, consisting mainly of parasites. The supernatant fluid, which still contains most of the stromata and small or ring forms if present, may be centrifuged once more for recovery of another small sediment (P_3), while the smaller parasites remain with the stromata.

Separation of leucocytes and parasites in Faction P_1. The leucocyte and parasite sediments are suspended in small portions of the formalinized saline, combined, and recentrifuged. After about 20 minutes at 2,000 rpm the turbid supernate, which contains mainly stromata, is removed and added to P_3 and the leucocyte-parasite deposit is transferred with formalin-saline to a 15 ml test tube. Since the leucocytes are only slightly heavier than the parasites, as many as six or seven runs may be required to effect the separation by differential centrifugation. The speed and time of centrifugation are chosen so as to deposit most of the leucocytes as well as a few parasites, leaving only parasites and stromata in the supernates. On repetition, progressively fewer parasites appear in the sediment, so that eventually a gray leucocyte deposit is formed, essentially free from parasites. To accomplish this the suspension of P_1 is centrifuted 3 to 5 min at 600 to 800 rpm. The somewhat

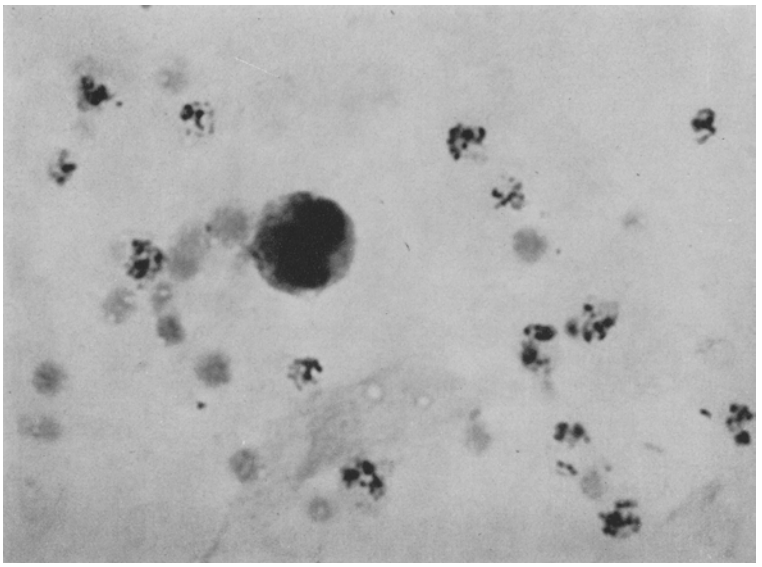

PLATE 1

TYPICAL P 1 FRACTION, × 1800

U. S. Navy Giemsa Stain. Nitrogen, 4.7 mg per billion parasites

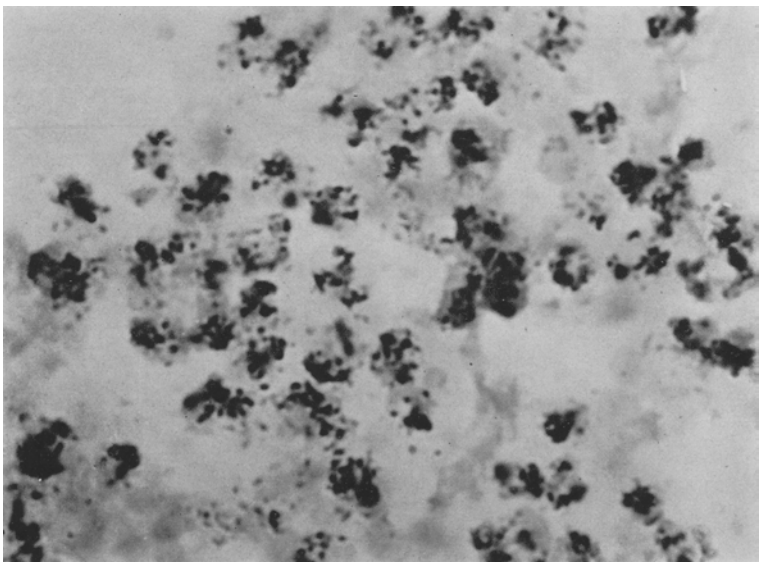

PLATE 2

TYPICAL P 2 FRACTION, × 1800

Nitrogen, 1.7 mg per billion parasites

turbid supernate, which contains parasites and stromata, is sucked off through a sterile syphon into a 250 ml centrifuge bottle. The dense bottom portion is mixed thoroughly with formalin-saline up to about 10 ml and centrifuged as before. The slightly turbid upper

portion is again transferred to the 250 ml bottle and the dense sediment again resuspended in formalin-saline up to about 10 ml. This washing process is repeated until the gray sediment no longer shows an upper layer of brown parasites. The combined supernates are then centrifuged 15–20 min at 2,000 rpm. The sediment is transferred to a 15 ml test tube with formalin-saline. At this stage there are still stromata present and these are removed by the procedure described below for P_2.

Separation of parasites in Fraction P_2. This fraction contains mainly parasites, stromata, and platelets. Removal of stromata is effected by differential centrifugation under conditions chosen to obtain complete sedimentation of the parasites and limited deposition of the stromata. Fraction P_2 is suspended in about 10 ml of formalin-saline in a 15 ml test tube and centrifuged for 5 to 10 minutes at 2,000 rpm. The supernate is sucked off and the process repeated 5 to 6 times with the residue, with progressive reduction of centrifugation time to about 4 to 5 min. The effectiveness of the separation of the almost white stromata from the brown parasites is easily noted. Supernates may be collected and combined with P_3.

TABLE 1

LOT NO.	PARASITE COUNT OF BLOOD	TOTAL PARASITES IN ENTIRE LOT	PREDOMINATING FORMS	FRACTION P_1		FRACTION P_2		YIELD $P_1 + P_2$
				Nitrogen per billion parasites	Total number	Nitrogen per billion parasites	Total number	
	millions per ml	*billions*		*mg*	*billions*	*mg*	*billions*	*per cent*
199	18	12	large trophs.	7.5	1.5	1	7	70
201	30	17	large trophs.	0.8	9.5	0.4	11*	130
202	6.7	4.4	large rings	1.9	0.1	1.9	0.1	5
203	6.5	4.4	large trophs.—some pigmented	1.4	0.5	0.48	1.2	39
204	7.8	4.8	large trophs.		0.8	0.74	0.4	25
205	2.1	1.3	large trophs.	2.1	0.2	2.1	0.1	23
206	3.1	0.5	med. trophs.		0.4†			80

* An additional billion parasites were recovered in fraction P_3; N, 0.8 mg per billion.

† With lot 206 centrifugation of the P_1 fraction was prolonged in order to concentrate most of the parasites into this fraction.

The removal of stromata from P_1 and P_3 is carried out in the same manner. Representative photomicrographs of fractions P_1 and P_2 are given in plates 1 and 2, respectively.

If the final product is desired free from formalin, the last three washings for removal of stromata should be carried out with saline solution containing 1:10,000 merthiolate.[2]

The data on lots which were processed as described are summarized in table 1.

The first two preparations in the table were concentrated as above from heavily parasitized bloods, while the remaining lots illustrate the somewhat less satisfactory results with bloods showing parasite counts in the usual range. Most of the parasites in lot 202 would probably have been found in the discarded layers of stromata.

2. Separation of Pl. vivax or Pl. malariae in a magnetic field. Some years ago, Rous and Beard (*J. Exp. Med.*, 1934, **59,** 577–) showed that Kupffer cells could be separated from accompanying tissue components by causing them to take up finely divided ferric oxide and exposing the mixed cells to a magnetic field. On this basis it appeared possible that hematin or related malarial pigments

[2] Manufactured by Eli Lilly and Co., Indianapolis, Ind.

might show a magnetic behavior different from that of hemoglobin in the uninfected red cell, and that pigment-containing malarial parasites might therefore be concentrated magnetically. Experiment showed that this could be accomplished.

Through the courtesy of Dr. David Rittenberg of Columbia University, the electromagnet described by Nier (*Rev. Sci. Instr.*, 1940, **11**, 212) was made available. This was originally used without modification and was calculated to have a flux density in the air-gap of about 5,000 gauss at the 3 amp. used. With this apparatus concentrates or malarial bloods containing pigmented parasites deposited thin columns of parasitic material in the most intense portions of the field. In order to make the magnetic field as unsymmetrical as possible one pole-piece was replaced with a wedge-shaped piece and used as indicated in fig. 1, preferably in a cold room.

With this arrangement 20 ml of citrated parasitized blood, diluted with 10 ml of saline solution containing 0.3 per cent merthiolate, may be exposed to the action of the unsym-

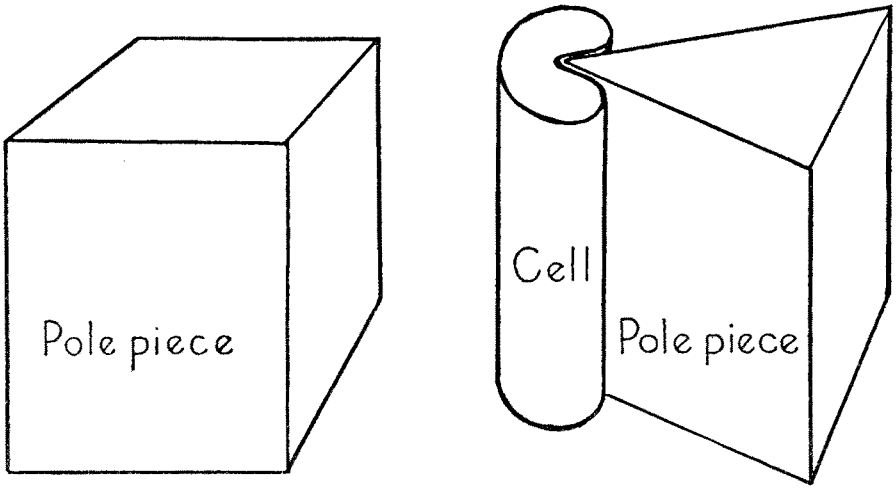

FIG. 1. ARRANGEMENT OF POLE PIECES AND CELL FOR MAGNETIC SEPARATION OF MALARIAL PARASITES

metrical magnetic field. The cell is a "lusteroid" tube, softened in boiling water and bent sharply around the edge of the wedge-shaped pole. After the sample is added the top is closed with Parafilm. Pigmented parasites are drawn toward the edge of the pole-piece and adhere to the wall of the cell, forming a brown streak. The blood sample is diluted so that the red cells settle in 6 to 12 hrs, and during this time a current of air from an electric fan is driven past the cell to dissipate the heat of the magnet so as to minimize convection currents in the blood. When the red blood cells have settled, the parasite streak is sucked off along with the supernatant plasma, and the parasites and accompanying cells are recovered by centrifugation. In a typical experiment with blood infected by *Pl. malariae*, 20 per cent of the parasites present were recovered along with unparasitized red cells. The counts showed a 140-fold concentration (from 1.7 parasites per 1,000 RBC in the original blood to 240 per 1,000 in the concentrate). A blood sample infected with *Pl. vivax* was extracted twice in succession with a total yield of about 25 per cent of the parasites and a 15-fold purification in terms of the ratio of infected to uninfected red cells (from 1 in 100 to 1 in 6 or 7).

The method is applicable only to small blood volumes, requires apparatus difficult of access, and is described solely for its scientific interest.

DISCUSSION AND SUMMARY

Brownish-black concentrates of *Pl. vivax* were readily obtainable from 500 ml samples of heavily infected blood by the method of lysis and differential centrifugation described above. In general, purity was highest and the yields best when the parasites consisted mainly of large, pigmented forms, and in one such instance the nitrogen content of the product was only 0.4 mg per billion parasites.

Large, pigmented forms of *Pl. vivax* and *Pl. malariae* are also slowly separable from unparasitized red cells in 5 to 25 ml blood samples in a strong, unsymmetrical magnetic field, but the method does not appear suited to the preparation of large quantities of plasmodia.