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THE ISOLATION OF MALARIA VACCINE ANTIGENS: A REVIEW *

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ABSTRACT

A review and evaluation is made of *Plasmodium berghei* vaccine fractions isolated from infected blood. *Plasmodium berghei* were released from infected blood cells by passage through a French Pressure Cell (FPC) at low pressure. The released parasite material was separated from the disintegrated blood by centrifugation, washed and disintegrated in the FPC at high pressure. The undisintegrated portion was separated from the disintegrated parasite components by centrifugation. The disintegrated parasite material was chromatographed on a Sephadex G-200 column. The void volume eluate acted as a vaccine against *Plasmodium berghei* infection in mice. The vaccine fraction has been found to fix complement in the presence of specific antibody and to be serologically free of erythrocyte stromal contamination. The isolated plasmodia and large plasmodial fragments from which the partially purified vaccine fraction originated also protected mice against *P. berghei* challenge. Large parasite fragments not disintegrated by high pressures in the FPC similarly contained vaccine antigens. From this it is suggested that the partially purified vaccine antigens are in some way associated with the large parasite fragments and may be of membrane origin.

Parasites isolated from their associated erythrocytes at FPC pressures between 800 psi and 1000 psi appeared morphologically intact by light microscopy. These, along with large parasite fragments, were readily separated from the disintegrated erythrocytes by high gravity centrifugation. Sedimentation and washing of the isolated free parasites greatly disrupted them, though their immunogenic properties remained unchanged.

Index Descriptors. Malaria vaccine antigens, isolation, partially purified, *Plasmodium berghei*.

INTRODUCTION

The isolation of malaria vaccine antigens has only recently been reported (D'Antonio et al. 1966a, 1969c 1970). Though a number of crude plasmodial preparations have been utilized as vaccines in the past (Brown 1969, D'Antonio 1971 , D'Antonio et al. 1969a), no attempts to identify the origin or character of the active antigens have been reported. In 1966, D'Antonio et al. described a new method for the separation of plasmodia from erythrocyte stroma and the isolation of a partially purified plasmodial fraction. This fraction was found to fix complement in the presence of specific plasmodial antibody and to be serologically free of host stromal contamination (D'Antonio 1966a, 1966b). A fraction of Plasmodium berghei prepared in the same manner was subsequently found to vaccinate mice against P. berghei malaria (D'Antonio et al.1969b,1970).More recently a partially purified fraction of Plasmodium knowlesi was similarly found to protect Rhesus monkeys against P. knowlesi infection (D'Antonio et al., 1971).

The following report is a review and evaluation of the various isolated plasmodial materials obtained during the step-wise preparation of the partially purified plasmodial antigens. The possible origins and character of the final isolated antigens is discussed.

MATERIALS

A/J[1] mice were used as the vaccination model (D'Antonio et al. 1969c) Plasmodium berghei strain NK65, Line D., served as the source of antigen and infective material (D'Antonio et al.1969c,1970).The French Pressure Cell[2] (FPC) with automatic hydraulic press was used to process parasitized blood and plasmodial materials.

1. Obtained from Jackson Laboratories, Bar Harbor, Maine.
2. American Instrument Co., Silver Springs, Maryland.

Parasitized blood and isolated plasmodial materials were processed according to the following basic plan (Fig. 1) (D'Antonio et al. 1966a). Variations in the processing procedure for different lots of material were made as described. The processing diluent was either isotonic phosphate buffered saline (pH 7.0) or isotonic saline throughout.

Pooled, washed, P. berghei infected erythrocytes were suspended in diluent to 20%. The 20% suspended blood was passed through an FPC at low pressure to release the parasite from the red cell. The low pressures varied from a low of 800 pounds per square inch (psi) for some lots to a high of 2500 psi for others. Any remaining intact erythrocytes were separated from the FPC effluent by low gravity centrifugation at between 50g maximum and 1100g maximum for 10 minutes. The supernatant contained free parasites, large parasite fragments and a mixture of disintegrated parasites and erythrocytes. The supernatant was centrifuged at high gravity forces which varied from 7000g maximum to 12000g maximum for 30 minutes at 4°C. The high gravity supernatant (B) was chromatographed through a Sephadex G-200 column. The high gravity sediment (A) containing free parasites and parasite fragments was washed three times and finally resuspended in up to 10 times its volume of diluent. Resuspended washed sediment (A) was passed through the FPC at high pressure. The pressure differed for individual lots from a low of 3000 psi to a high of 20,000 psi. The high pressure FPC effluent contained disintegrated parasites and large undisintegrated parasite fragments (E).

The undisintegrated parasite fragments (E) were separated from the disintegrated parasite components by centrifuging the high pressure FPC effluent at 12000g maximum for 30 minutes at 4°C. The resulting supernatant (F) was chromatographed through Sephadex G-200 at 4°C.

Evaluation of the Immunogenicity of the Parasite Materials

Immunogenicity of the various parasite fractions resulting from the above procedure was tested in A/J mice as reported (D'Antonio, et al. 1969c, 1970).

Various groups of A/J mice were injected one time intraperitoneally (IP) with their respective parasite fractions. Starting at 8 weeks after treatment the injected mice were challenged IP with 10×10^6 virulent P. berghei NK65D.

The parasite fractions tested (Fig. 1) were as follows:

Preparation	Description
A	The FPC isolated free parasites and associated large parasite fragments.
E	The large parasite fragments remaining following high pressure disintegration of isolated parasite Material A.
G	The Sephadex G-200 void volume eluate of disintegrated parasite material A.
C	The Sephadex G-200 volume eluate of the mixture (B) containing disintegrated erythrocytes and parasites.
DSH.	The Sephadex G-200 retarded fractions for (B) and (G).

RESULTS

Low Pressure FPC Passed Infected Blood

Light microscopy of the FPC effluent obtained by low pressure FPC passage of infected blood revealed the presence of plasmodia apparently free of encircling red cell stroma (Fig.3). A broad distribution of parasites ranging in size from the smallest merozoites to large segmenter forms were observed in the effluents obtained at FPC pressures below 1000 psi.

The number of larger parasite forms progressively decreased at pressures above 1000 psi. Only a few merozoite sized parasites were observed at 2500 psi.

Some undisintegrated erythrocytes were present in the low pressure FPC effluents. These were effectively removed by low gravity centrifugation. The most effective removal occurred at 1100g maximum for 10 minutes.

Most of the smaller parasite forms remained behind in the supernatant following the low gravity centrifugation. The number of large segmenter forms removed along with the intact erythrocytes increased with increase in the force of centrifugation.

Following the sedimentation and washing procedures the free plasmodia appeared disrupted, matted, and morphologically indistinct (Fig. 4).

Sephadex G-200 Fractionation

Fractionation of the disintegrated isolated plasmodial material (F) resulted in an initial brownish, finely turbid void volume peak, followed by a smaller clear second peak (D'Antonio et al., 1970). Fractionation of the mixture (B) of disintegrated erythrocytes and parasites resulted in an initial brownish-red turbid void volume peak followed by a clear hemoglobin containing peak and a clear colorless terminal peak (D'Antonio et al., 1970).

Vaccination

Vaccinated mice responded to virulent *P. berghei* challenge with transient low parasitemias, mild to moderate anemias and high survival rates (see D'Antonio et al., 1969c, 1970). Protection was associated with the free parasites (A), the parasite fragments (E) and the Sephadex void volume eluates G and C.

There was no protection associated with any of the Sephadex G-200 retarded fractions (D and H).

Of the mice in the groups receiving the above preparations; 31 out of 34 receiving A or E were protected; 16 out of 20 receiving G, and 11 out of 15 receiving C were protected. None out of 55 mice receiving retarded fractions D or H were protected (see D'Antonio et al., 1969c, 1970 and Fig. 1).

DISCUSSION

It is apparent that the active parasite antigens were distributed throughout each of the parasite products obtained during the step-wise preparation of the final partially purified plasmodial vaccine fraction. The FPC released parasites (A) as well as the large parasite fragments (E) derived from them were protective. The disintegrated parasite fraction (G) originating from the FPC released parasite material (A) and appearing in the void volume eluate of Sephadex G-200 likewise protected. This distribution of the parasite vaccine antigens suggests that they are somehow intimately related with the parasite fragments. The quantity of parasite antigens released by FPC disintegration of intact and fragmented parasites has been found to increase with increasing disintegration pressures. At 20,000 psi, in contrast to lesser pressures, only a small residual of weakly immunogenic undisintegrated parasite material (E) remains. The immunogenicity of the Sephadex G-200 void volume eluate (C) suggests that the protective antigens released as a result of disintegration of infected red blood cells are the same as those obtained from the parasites first separated from their associated erythrocytes.

A number of observations suggest that the partially purified vaccine antigens are extremely large or even particulate in nature. The Sephadex G-200 void volume eluates containing them is turbid. A similar appearing immunogenic fraction results following chromatography through Sepharose 4B (D'Antonio et al., 1969b). Sepharose 4B has an exclusion limit above molecular weight three million. Finally, centrifugation of the parasite void volume eluate at 100,000g maximum for several hours results in a dark opaque immunogenic pellet (D'Antonio and Silverman, 1970).

One suggestion is that the vaccine antigens are portions of parasite membranes. The most finely disintegrated membrane material would remain suspended following centrifugation at 12000g maximum while the larger undisintegrated parasite fragments containing the remainder would be sedimented.

The suggestion that the vaccine antigen may be of membrane origin would be consistent with the proposal that the blood stage of malaria infection is finally aborted by host antibody contact with free merozoites (Cohen, 1969).

The parasite antigens (G) appearing in the void volume eluate of Sephadex G-200 are the most highly purified obtained to date. They fix complement in the presence of specific antibody and have been found serologically free of host stromal contaminants (D'Antonio et al., 1966a). The free parasites from which they originated have been found to contain only trace amounts of stroma by serologic test (D'Antonio et al., 1966a). This is in contrast the high level of stromal contamination found present in plasmodial materials prepared by other methods (D'Antonio et al., 1966a).

Killby and Silverman (1969) ultrastructurally examined a large number of free parasites in a series of washed FPC preparations. They concluded that the FPC released the largest number of parasites generally free of host stromal membranes. They also found, as did Cook et al. (1969),that parasites prepared by other methods still retained a large amount of the original red cell stroma.

In studies to date, the best release of intact free parasites occurred at the lowest FPC pressures studied (i.e. 800 to 1000 psi). At these pressures, a large number of intact free parasites resulted while only a few intact red blood cells remained in the FPC effluents. In experiments with either normal blood or infected blood processed through the FPC at these

pressures, better than 99% of the original erythrocytes were disintegrated.

The FPC freed plasmodia and plasmodial fragments were readily separated from the disintegrated host erythrocytes by centrifugation at gravity forces between 7000g maximum and 12000g maximum. Centrifugation of FPC disintegrated (1000 psi) normal blood at 12000g maximum for 30 minutes resulted in no more than a thin film sediment (Cook, Aikawa, 1971 and D'Antonio, Silverman, 1971). In one experiment, the sediment obtained from 10 ml of packed normal erythrocytes was found to be less than 0.15 ml. This erythrocyte sediment would constitute a very small fraction of the bulky parasite sediment which results from similar processing of infected blood.

As noted in Figure 3, the FPC released parasites appeared morphologically intact. Once free of the erythrocyte stroma, however, they were readily disrupted by centrifuging and washing (Fig. 5). Similar findings have been made upon examining washed parasite sediments by electron microscopy (Killby and Silverman, 1969; Cook et al., 1969). Despite this disruption the immunogenic properties of the parasite material remained unimpaired.

The partially purified plasmodial vaccine fraction described here has been obtained from other species of malaria (D'Antonio et al., 1966a). A similar fraction of P. knowlesi has recently been found to vaccinate Rhesus monkeys against P. knowlesi (D'Antonio et al., 1971).

Additional vaccination studies, as well as further investigation into the origin and character of the vaccine antigens themselves, are planned. It is anticipated that with the emergence of more basic knowledge, more effective vaccination procedures will be found and new, more direct, methods for isolation of the vaccine antigens will be devised.

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Fig. 1. Schematic diagram depicting the French Pressure Cell isolation of plasmodial materials and the preparation of a final partially purified plasmodial fraction.

Low pressure refers to FPC pressures between 800 psi and 2500 psi; high pressure refers to FPC pressures between 3000 psi and 20,000 psi.

Low gravity refers to centrifugation forces between 50g maximum and 1100g maximum; high gravity refers to centrifugation forces between 7000g maximum and 12000g maximum.

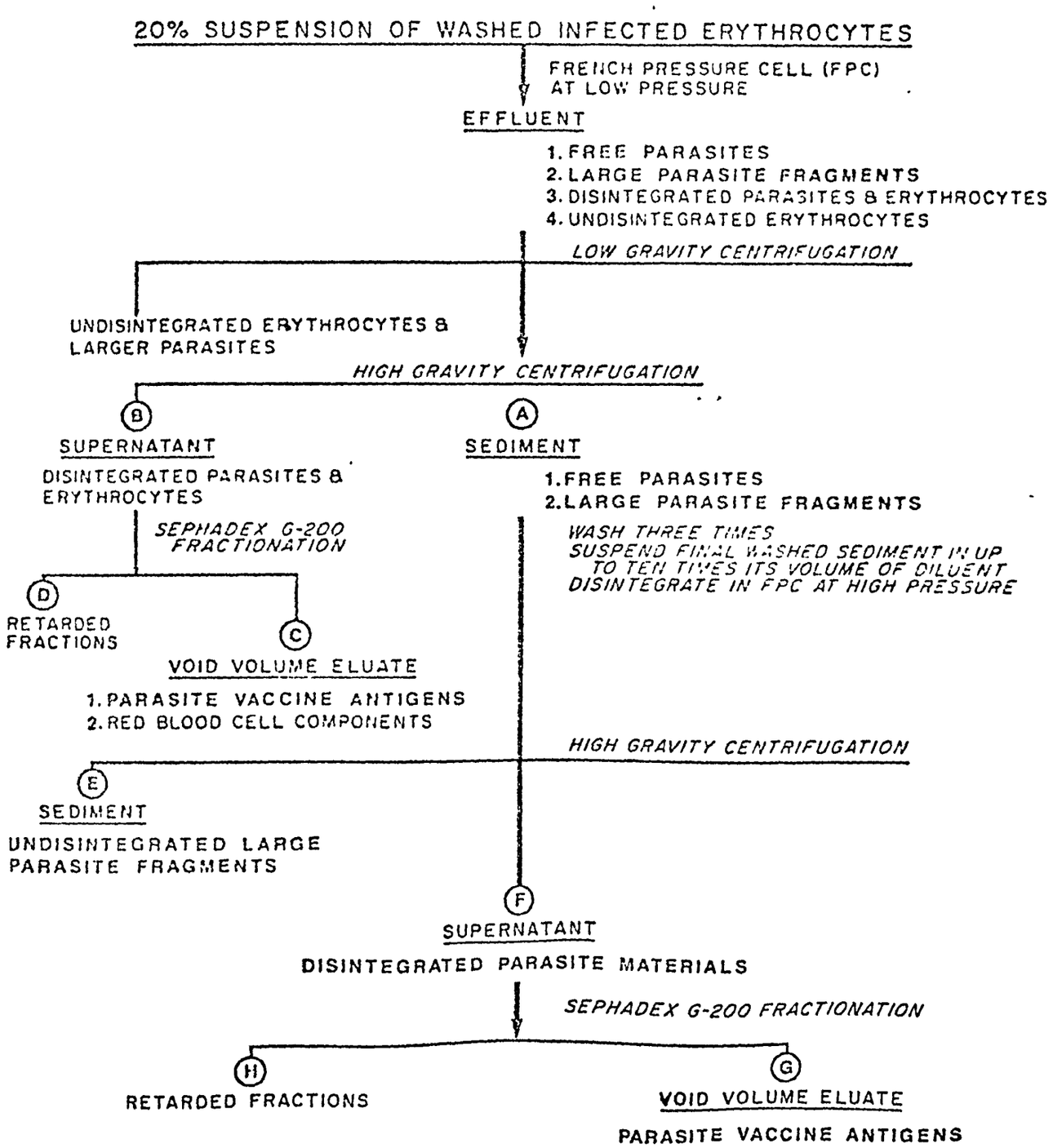
20% SUSPENSION OF WASHED INFECTED ERYTHROCYTES
FRENCH PRESSURE CELL (FPC) AT LOW PRESSURE
EFFLUENT
1. FREE PARASITES
2. LARGE PARASITE FRAGMENTS
3. DISINTEGRATED PARASITES & ERYTHROCYTES
4. UNDISINTEGRATED ERYTHROCYTES
LOW GRAVITY CENTRIFUGATION
UNDISINTEGRATED ERYTHROCYTES & LARGER PARASITES
HIGH GRAVITY CENTRIFUGATION
B
SUPERNATANT
DISINTEGRATED PARASITES & ERYTHROCYTES
A
SEDIMENT
1. FREE PARASITES
2. LARGE PARASITE FRAGMENTS
SEPHADEX G-200 FRACTIONATION
WASH THREE TIMES
SUSPEND FINAL WASHED SEDIMENT IN UP TO TEN TIMES ITS VOLUME OF DILUENT
DISINTEGRATE IN FPC AT HIGH PRESSURE
D
RETARDED FRACTIONS
C
VOID VOLUME ELUATE
1. PARASITE VACCINE ANTIGENS
2. RED BLOOD CELL COMPONENTS
HIGH GRAVITY CENTRIFUGATION
E
SEDIMENT
UNDISINTEGRATED LARGE PARASITE FRAGMENTS
F
SUPERNATANT
DISINTEGRATED PARASITE MATERIALS
SEPHADEX G-200 FRACTIONATION
H
RETARDED FRACTIONS
G
VOID VOLUME ELUATE
PARASITE VACCINE ANTIGENS

Fig. 2. Photomicrograph of *P. berghei* infected mouse blood before release of parasites by the FPC. X1000.

Fig. 3. Photomicrograph of *P. berghei* following release from red blood cells. A number of parasite forms are present ranging from small merozoites to large segmenters. X1000.

Fig. 4. Photomicrograph of washed *P. berghei*. Note the disrupted, matted appearance. X1000.