Ultrastructural Analysis of *Plasmodium berghei* Sporozoite Antigens Prepared by Freeze Thawing and Heat Inactivation

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Ultrastructural Analysis of *Plasmodium berghei* Sporozoite Antigens Prepared by Freeze Thawing and Heat Inactivation*

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SYNOPSIS. Ultrastructural analysis was performed on freeze-thawed and heat inactivated *Plasmodium berghei* NK65 sporozoite preparations, which in parallel studies served as the immunogens against a malarial infection in A/J mice. Sporozoites in the freeze-thawed sample appeared severely damaged with most of their cytoplasmic contents lost. The heat inactivated sporozoites remained intact and closely resembled the untreated controls.

Index Key Words: *Plasmodium berghei*; sporozoite antigen; sporozoites, freeze-thawed and heat inactivated; electron microscopy.

SCATTERED reports appear in the literature suggesting the feasibility of using sporozoites as sources of immunizing agents in the protection against malarial infection. Most recently Nussenzweig et al. (4) demonstrated the successful protection of mice using *Plasmodium berghei* sporozoites attenuated by X-irradiation. Alger et al. (1) presented additional evidence of *P. berghei* NK65 sporozoite immunogenicity by exposing 4-5 week old A/J female mice to freeze-thawed and heat inactivated crude sporozoite preparations. The sporozoites were obtained from manually dissected, infected mosquito salivary glands.

Ultrastructural analysis of the freeze-thawed and heat inactivated sporozoite antigens was undertaken to determine their nature and composition. The term antigen as used here refers to materials such as whole or fragmented sporozoites used to stimulate an immune response. It is probable that the sporozoites contain numerous antigen moieties. It is hoped that the knowledge obtained from this study will facilitate development of further purification procedures of the crude immunogens. There exist, to the best of our knowledge, no published electron microscopic studies dealing with the analysis of sporozoite antigen preparations.

MATERIALS AND METHODS

Aliquots were taken for electron microscopic studies from freshly prepared sporozoites ordinarily used for immunization of mice in parallel immunologic investigations. *Plasmodium berghei* NK65 sporozoites were released from ~ 800 *Anopheles stephensi* salivary glands by manually grinding the glands in a glass container. The resulting mixture was washed several times by differential centrifugation in order to minimize the salivary gland cell debris. The final supernate was divided into 3 equal portions and treated in the following manner. One sample remained untreated for control purposes. The 2nd sample was alternately frozen in liquid nitrogen vapor for 25-30 min and thawed 4 times. The 3rd sample was heated for 1 hr at 40-42 C. After the above treatment, the antigens were pelleted by centrifugation to facilitate processing for electron microscopy. The pellets were fixed at room temperature for 4 hr in 6% (v/v) glutaraldehyde buffered with a phosphate buffer giving a final concentration of 0.1 M phosphate, pH 7.2, followed by overnight storage at 4 C. A solution of 0.2 M sucrose in 0.1 M phosphate buffer, pH 7.2, was used to wash out the unreacted glutaraldehyde. The samples were stored overnight at 4 C in a fresh change of the sucrose solution. After a brief rinse with phosphate buffer, the pellets were postfixed in 1% (w/v) OsO_4 containing 0.1 M phosphate buffer, pH 7.2. Dehydration was carried out in an ascending series of ethanols, the final step ending in several changes of propylene oxide. The samples were embedded in Epon 812 (3). Sections were cut on a Reichert Om U2 ultramicrotome using a diamond knife. After being placed on naked 300 mesh copper grids, the sections were stained for at least 2 hr in a saturated solution of uranyl nitrate (7) and subsequently stained in lead citrate (6). The specimens were observed and photographed with a Hitachi HU 11-C electron microscope using 75 kV as the accelerating voltage.

RESULTS

Subcellular analysis of the various *P. berghei* sporozoite crude antigens reveals that all the preparations contain copious amounts of mosquito salivary gland cell fragments. It also appears that the preparations are relatively free of bacterial contamination. Several obvious differences are evident as a result of the various physical treatments to which the samples were subjected.

In the control sample (Figs. 1, 2), although there is no evidence of any intact salivary gland cells, many of the mosquito cell organelles such as mitochondria, ribosomes and electron-dense pigment granules are relatively intact. Most important,

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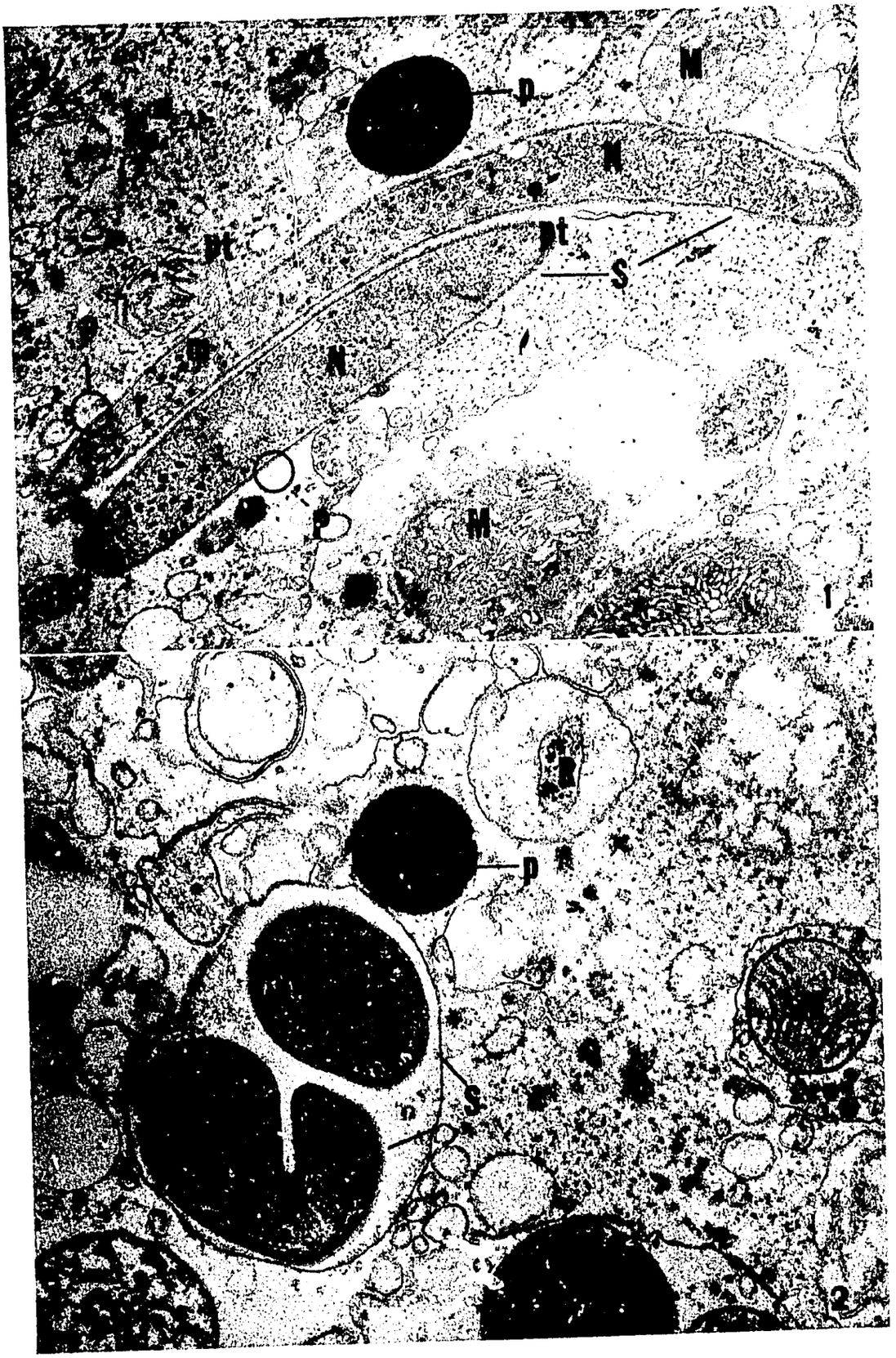
Figures 1-6 are electron micrographs of treated and untreated sporozoites of *Plasmodium berghei*.

Fig. 1. Partial longitudinal sections of 2 sporozoites (S) in an untreated control sample. The background cellular debris is of mosquito salivary gland origin. An intact electron-dense pigment granule (p) and mitochondria (M) are most evident. The pellicle (P) of the sporozoite contains a thin outer and a thick inner membrane and segments of peripheral tubules (pt). Also present are the micronemes (m), rhoptries (r), and nuclear area (N). × 18,000.

Fig. 2. Cross section of untreated control sample containing 2 (possibly a single doubled over) intact sporozoites (S). The electron-dense pigment granule (p), ribosomes (R), mitochondria (M) and various vesicles and membranes are of mosquito cell origin. × 30,000.


p
M
N
pt
pt
S
N
P
M
1
p
S
2

Figs. 3, 4. [Frozen-thawed sporozoites (S).] 3. In the center is a profile of a severely damaged sporozoite. × 58,000. 4. Cross section. Most of the cytoplasmic contents are lost and the only recognizable feature is its damaged pellicle (P). × 86,000.

however, is the fact that the integrity of the sporozoite is unaltered by the grinding technic used to release the sporozoites from the salivary gland cells. The pellicles of the sporozoites contain the proper number of layers, a thin outer membrane and a thick inner membrane and a row of microtubules. Also, depending on the level of the section taken through the sporozoites, various organelles such as the peripheral tubules, rhoptries, micronemes, ribosomes and nuclei can be seen.

In the freeze-thawed sample (Figs. 3, 4), on the other hand, intact salivary gland cell organelles rarely are found. The integrity of the sporozoite is destroyed. Sporozoites in the freeze-thawed preparation can be recognized at higher magnifications

Fig. 5. A longitudinal and a partial section of sporozoites (S). Several empty vacuoles (V) are seen. A nucleus (N), micronemes (m) and peripheral tubules (pt) also are clearly evident and the wall of the pellicle is intact. The organelles and profiles of various other structures are of mosquito origin. × 22,500.

Fig. 6. An example of cytoplasmic coagulation or clumping of a sporozoite (S) resulting from exposure of the sample to heat inactivation. × 21,000.

only. They are identified primarily on components of some of the partially remaining segments of the severely damaged pellicle. The cytoplasm, owing to its almost complete disruption, bears no resemblance to the untreated malarial organism. Only fragmentary pieces of agranular membrane, perhaps endoplasmic reticulum, remain. The electron lucidity of the matrix of the sporozoite suggests lysis of the organism resulting in a loss of its cytoplasmic substance.

The heat inactivated sporozoite antigen (Figs. 5, 6) resembles most closely the untreated control sample with some modifications. The cell debris of mosquito salivary gland origin contains membranous components of disrupted nuclei and numerous intact mitochondria. The pellicle of the heat inactivated sporozoites most often appears intact (Fig. 5) with the thin outer and the thick inner cell membranes clearly evident. The cytoplasm contains the usual components found in the controls: a nucleus, rhoptries, micronemes, peripheral tubules and ribosomes. In addition, the sporozoites in this preparation appear to have acquired large vacuoles. The vacuoles are believed to be artifacts caused by the heating process. In many, but not all, of the heat inactivated sporozoites, the cytoplasm gives the distinct impression of being coagulated (Fig. 6) as evidenced by the empty spaces adjacent to the compact cytoplasmic areas.

DISCUSSION

The results of this study attest the complexity of the crude sporozoite antigen. The immunogen contains not only sporo-

zoites, but also the entire range of *A. stephensi* salivary gland substances. It appears that mosquito salivary gland tissue cannot be eliminated by washing and differential centrifugation alone. The question that needs to be answered is whether elimination of the mosquito tissues is necessary, or for that matter, even desirable. Nussenzweig et al. (5) found no protection with normal salivary glands. Alger et al. (1), however, demonstrated that 7 injections of 70 normal mosquito salivary glands per injection provided some protection. Alger, on the other hand, found no protection with 7 injections of 2-6 normal salivary glands per injection (unpublished). At this time it is impossible to define the importance of the role of either the sporozoites or the mosquito cells in the induction of immunity since to date it has not been possible to obtain a pure sporozoite preparation. Even the use of more sophisticated technics such as sucrose density gradient centrifugation results in a sporozoite fraction containing contaminants of mosquito origin (2). It may well be that both substances contribute to protection. Remnants of the mosquito tissue may so entrap the sporozoites that in addition to itself inducing some as yet unexplained immune stimulation, it also may cause a slowing down of the release of the sporozoite antigen in the animal. In this manner the mosquito tissue would cause adjuvanting of the sporozoite material. Possibly this phenomenon might be explained on the same basis as that of the schistosome protection system of Smithers et al. (8).

The grinding technic employed in this study to release the sporozoites from mosquito salivary glands does not appear to alter the integrity of the parasite. The morphology of control sporozoites thus freed closely resembles that of sporozoites fixed in intact mature oocysts (10).

Our electron micrographs bear out the assertion of other investigators (9) that sporozoites are severely damaged by freeze-thawing. Furthermore, the heat inactivated sporozoites we observed, while more closely resembling the controls, also show some noticeable alterations in their physical make-up, unlike those seen by Spitalny & Nussenzweig (9). Such changes as we observed certainly would not be detectable by light microscopy.

As evidenced in the electron micrographs, the primary difference between the freeze-thawed and the heat inactivated sporozoites is a result of the physical nature of the inactivating process. The freeze-thawed sporozoites most likely were disrupted by ice crystal formation and their subsequent thawing. This type of disintegrating process causes not only fragmentation of the parasites, but also disruption beyond recognition of the cytoplasmic contents. Heat inactivation, on the other hand, induces coagulation of the cytoplasm and hence the sporozoites exposed to this method remained fairly well intact. That these dissimilar methods of sporozoite antigen preparation should yield immunogens capable of inducing similar levels of protection (1) suggests that the immunizing agent is of a rather stable molecular composition, perhaps of membrane origin.

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