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FINAL REPORT

PROJECT TITLE: EFFECT OF CHEMOTHERAPEUTIC AGENTS ON MALARIA SPOROZOITES IN VITRO

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SCIENTIFIC REPORT

1. General Scientific Aims:

Attempts were made to devise a system for *in vitro* development of exoerythrocytic (EE) stages of malarial parasites. Because sporozoite numbers and the availability of permissive host cells are frequently limiting, it was our aim to develop a micro assay similar to that used with the erythrocytic stages (Rieckmann, *et al.* 1978. Lancet 22). The research involved three parts: a) The in vitro production of gametocytes and their infectivity for mosquitoes. b) The preparation of sporozoites suitable for inoculation into cell cultures and their infectivity for cells *in vitro*. c) Detention, characterization, and quantitation of sporozoite receptors and EE stages in host cells.

2. Results:

a) The *in vitro* production of gametocytes and their infectivity for mosquitoes.

A clone of the Honduras I/CDC strain of the human malaria agent *Plasmodium falciparum* (Bhasin and Trager, 1983. J. Protozool. 30:7A) was maintained in a static culture system with red blood cells (Ifediba and Vandenberg. 1981.

Nature 294:364) where it formed stage V gametocytes. A membrane feeding program was established and nonsterile mosquitoes were allowed to engorge on cultures rich in stage V gametocytes. The gametocytes were not infectious and there was no evidence of even partial sporogonic development. The ratio of male to female gametocytes was approximately 1 to 100 and exflagellation was rarely observed. In addition, after 36 biweekly transfers and approximately 144 parasite doublings in continuous culture (4 months) the rate of gametocyte production declined and it was necessary to re-initiate gametocyte forming cultures using stocks stored in liquid nitrogen at lower passage levels.

Another gametocyte-forming clone from the Honduras I/CDC strain was grown in the continuous flow system. Stage V gametocytes were obtained but these were not infective for mosquitoes. The reason why this clone failed to generate mosquito infective gametocytes in either the stationary or the continuous flow system, is unknown, but this may be a result of frequent in vitro subculturing.

Because of these disappointing results, we spent considerable effort and time evaluating modifications of the static culture system that would improve gametocyte formation and mosquito transmission.

We evaluated the effects of supplementing the medium of the static culture of the parasites with the red blood cells, by adding glutamine, hypoxanthine, antibiotics, cholesterol, glucose, organic buffers, various types and concentrations of serum, and crude extracts prepared from cells and tissues of A. stephensi. The effects of a variety of culture manipulations (e.g., frequency of medium changes, and the timing of the subdivision of the gametocyte forming culture) were also tested. In summary, all of the parameters that we tried did neither improve gametocytogenesis nor promote mosquito transmission.

Clones exhibit differing capabilities of forming functional gametocytes that infect mosquitoes (Burkot, Williams and Schneider, 1984. Trans. Roy. Soc.

Trop. Med. Hyg. 78: 339-341). For this reason, we obtained the uncloned parental Honduras I strain from Dr. Trager for comparison and possibly the isolation of new clones. In the static culture system, the parasites are subjected to considerable shifts in temperature, gas tension, and pH during the routine culture handlings. These shifts may interfere with gametocytogenesis. We have observed that a temperature drop of 3 to 4 C over a period of several (6 to 8) hours leads to the collapse of a culture in the process of gametocytogenesis. We tried to evaluate the behavior of the clone in a continuous flow system (Trager, 1979. J. Protozool. 26: 125-129) in which environmental conditions are more stable.

In a later experiment we cultured in a static system the clone (7G8) of a Brazilian isolate (ITM-22) of P. falciparum (Burkot et al. 1984. Trans. Roy. Soc. Trop. Med. Hyg. 78, 339) (generously supplied by Dr. I. Schneider of the WRAIR). It formed gametocytes infective for Anopheles stephensi. The infection rates were, however, low with 15 to 20% of the females harboring 1 to 3 oocysts. The sporozoites invaded the salivary glands by day 14 after the blood meal, and approximately 3000 sporozoites were harvested from each infected mosquito. The clone was expanded and stocks of low passage number (12) were stored in liquid nitrogen.

b) The preparation of sporozoites suitable for inoculation into cell cultures and their infectivity for cells in vitro.

Sporozoites for the inoculation into cell cultures are usually obtained from salivary glands dissected from surface disinfected non-aseptic mosquitoes. In such studies microbial contamination limits the number of sporozoites that can be pooled. Though the production of aseptic sporozoite inocula in axenically reared mosquitoes is a laborious and time consuming endeavor, it represents the only means of procuring sporozoites sufficiently clean so that they can be used at the concentration necessary for the experiments outlined in

our original proposal (e.g. the effect of high sporozoite numbers on the rate of infection).

We evaluated whether the methods described for the mass isolation of salivary glands and sporozoites could be modified to obtain sporozoite preparations suitable for inoculation into cell cultures. The procedures involved the squeezing of infected salivary glands from decapitated mosquitoes (Bosworth et al. 1975. J. Parasitol. 61, 769) and/or the separation of sporozoites from contaminants using sterile ion exchange columns (Schmatz et al. 1984. J. Protozool. 31, 81). Two buffers were compared for their ability to elute a clean suspension of sporozoites from a column of DE 52. In order to keep microbial contaminants to a level that could be controlled by antibiotics, it was necessary to collect and use only the first fraction of sporozoites that came off the column. This limited the harvest to only 20 to 30% of the sporozoites placed onto the column. Continued elution gave improved sporozoite yields but these were obviated by an increase in microbial contamination and mosquito debris. Similar results were obtained with P. falciparum. An undesirable side effect was the loss of infectivity by approximately 50% of the sporozoites collected. Contamination was generally controlled using a cocktail of 3 antibiotics (penicillin, streptomycin and gentamycin), but this required daily medium changes and sometimes the use of these antibiotics in concentrations known to be detrimental to the erythrocytic stages of P. falciparum (Geary and Jensen. 1983. Am. J. Trop. Med. Hyg. 32, 221). This problem of contamination control is further exacerbated in the case of P. falciparum in which the EE stage requires 7 days for completion. Nevertheless, it has become apparent that such procedures do hold some promise and, with further modifications, may be suitable for some of our experiments.

We therefore tried to produce axenically reared mosquitoes in order to

obtain sterile invertebrate malaria stages, mainly sporozoites, and to devise methods to improve the sporozoite yield. Using A. stephensi, we developed an aseptic membrane feeding procedure. Non-sterile mosquitoes readily became infected when they were fed P. berghei gametocyte-infected hamster blood via a membrane feeder. The infection rates were improved when protease inhibitors, such as those present in chicken serum or lima beans, were added to the blood. This is in contrast to the results obtained from axenic mosquitoes where poor infection rates were obtained using gametocytes from hamsters. Proteolytic enzymes present in the gut of the mosquito destroy a large proportion of the malarial parasites ingested during the blood meal and they are among the factors responsible for the discrepancy between the number of gametocytes ingested and the number of oocysts, and, consequently, sporozoites obtained (Gass and Yeates. 1979. Acta Trop. 36, 243). This problem is further exacerbated by the use of heparinized blood in which the formation of the protective clot is prevented. Our results indicate that, compared with nonsterile mosquitoes, the gut of the axenically reared A. stephensi is a less suitable environment for the development of the early sporogonic stages. Parasite yields were markedly improved by infecting the axenic mosquitoes with in vitro formed ookinetes. A reliable and repeatable method for obtaining and purifying ookinetes of P. berghei was developed. The infection rate was related to the number of ookinetes ingested during the blood meal. Though the efficiency of infection (i.e., the percentage of ookinetes that develop in oocysts) was low at 0.1 to 0.5%, a blood meal with 5000 ookinetes per microliter resulted in infection rates of over 90% of the female mosquitoes, and yielded an average of 40,000 salivary gland sporozoites per infected mosquito. When mosquitoes were fed cultured gametocytes of P. falciparum, low infection rates were common. Our findings with P. berghei were especially germane to this problem, and we attempted to apply these concepts to the production of sporozoites of P.

falciparum in mosquitoes.

A fibroblast cell line isolated from embryonic human lung (MRC-5) was challenged with sporozoites of P. falciparum. Cell cultures were inoculated with infected salivary glands or with sporozoites separated using ion exchange chromatography. The cultures were fixed 3 and 7 days post inoculation but no microscopic evidence of EE development was observed.

Multi-well plates offer a convenient and economical in vitro microtechnique to do the assays we have proposed. The culture environment of the tissue culture plate differs from that of a closed flask or culture tube and we obtained incomplete development of the EE stages of P. berghei in MRC-5 cells grown in the tissue culture wells. This was an unanticipated observation and indicates that more research is needed to evaluate the influence of the gas composition and the culture vessel on development of the EE stages.

c) Detection, characterization and quantitation of sporozoite receptors and EE stages in host cells.

The use of multi-well culture systems and the low rates of host cell infection warrant that a method other than the Giemsa stain be used to detect the EE stages in a cell layer. Consequently an immunochemical approach was selected. We began raising polyclonal and monoclonal antisera against the blood stages as these have been demonstrated to cross react with the EE stages as well (Hollingdale et al. 1983. Am. Soc. Trop. Med. Hyg. 32, 682). These antisera could be used in an enzyme linked immune assay.

A novel enzyme linked lectin binding assay for the quantitative detection of carbohydrate receptors on cell membranes was developed. A hepatoma cell line HepG2, was exposed to biotinylated concanavalin A, Pisum sativum agglutinin and Lens culinaris agglutinin. The concentrations of cells and lectins were crucial with the assay. Receptors with mannose moieties were detected in the cells.

3. Conclusions:

Several important findings were made. A clone of P. falciparum which produces gametocytes was used to infect A. stephensi and to obtain sporozoites. The sporozoites were used to challenge cultures of human fibroblast cells (MRC-5) but no evidence of parasite growth or development was obtained. Further tests are, of course, needed but other cell types, especially hepatocytes, need to be evaluated as substrates for the growth of the EE stages. We evaluated various methods for the preparation of sporozoite inocula for cell culture systems. A method for the mass isolation of infected salivary glands was tried. Ion exchange chromatography was effective in removing mosquito debris and most of the microbial contaminants but modifications (e.g., cold column elution) are needed to reduce the concomitant loss of sporozoite numbers and infectivity.

A method for rearing axenic mosquitoes was developed (Munderloh, Kurtti, and Maramorosch. 1982. J. Parasitol. 68: 1085). A great advantage of preparing sporozoite inocula from infected gnotobiotic mosquitoes is that extensive manipulations and use of antibiotics are not needed to reduce microbial contaminants. A major need is the preparation of inocula with the numbers of sporozoites necessary to challenge replicated cell cultures. This is an important factor in the case of harvesting sporozoites of P. falciparum from mosquitoes in which the infection rates and yield of sporozoites are usually low. With P. berghei we found that mosquito infection rates and yields of sporozoites were augmented by the use of ookinetes and protease inhibitors in the infective blood meal. These techniques need now to be applied to P. falciparum infected mosquitoes. The EE stages of P. berghei, which complete development in closed culture systems such as tissue culture flasks, did not develop normally in human fibroblasts grown on coverslips in tissue culture wells. This indicates that the EE parasites, like the erythrocytic stages, are

sensitive to the composition of the culture atmosphere. Studies on the influence of gas composition of the culture atmosphere on EE development are needed. Finally, a novel enzyme linked lectin binding assay for analyzing the receptors for sporozoite invasion of cultured cells was developed.

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