Applicant Organization

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Title of Proposal

Plasmodial Induction of Epstein-Barr Virus

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October 1983 to September 30, 1984

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Relevance of Proposed Work to the Agency for International Development

Malaria, schistosomiasis, onchocerciasis, and other parasitic diseases are common in tropical Africa. These diseases increase infant mortality, decrease productivity of affected individuals, and strain the economic resources of developing countries.

Research in tropical diseases is directed at the control and eradication of the diseases that affect the quality of life in developing countries. Though individual investigators limit their studies primarily to one parasite, residents of tropical Africa are infected with a host of parasites, bacteria, and viruses. The infections and diseases caused by these microorganisms have varying physiologic consequences for the host; therefore, the influence of one infection on the outcome of another requires study. One such host-multiple parasite interaction is that of *Plasmodium falciparum*, the cause of severe malaria in tropical Africa, and the Epstein-Barr virus (EBV) which causes infectious mononucleosis and is associated with Burkitt's lymphoma and nasopharyngeal carcinoma.

Epstein-Barr virus infection in Africa occurs within one to two years after the disappearance of transplacentally acquired maternal antibodies; however, infectious mononucleosis is virtually unknown in developing countries. Infectious mononucleosis does not result from EBV infection very early in life. In developed countries, EBV infection occurs later in life and results in infectious mononucleosis.

EBV infects only the antibody-producing cells (B lymphocytes) of the lymphoid systems of humans and higher primates. The virus causes rapid proliferation of infected cells. Destruction of the infected cells by viral multiplication may also occur. This destruction of lymphocytes may eliminate cells that are capable of responding to various substances, and immunosuppression characterized by weak immune responses to viral and bacterial vaccines may result in children infected at a very young age. After primary infection with EBV, the virus may remain latent in lymphocytes indefinitely.

Plasmodium infection in Africa occurs at an early age also. *Plasmodium* also causes lymphocytes to multiply rapidly and is capable of suppressing the host's immune response. Though infection with EBV and *P. falciparum* occur within the same time frame in the young of developing countries, the interaction of these two organisms in disease manifestation, refractoriness to childhood vaccinations, and in the development of childhood malignancies has not been investigated.

Presently, intensive research efforts in tropical medicine are directed toward the development of a vaccine contra malaria. The successful employment of a vaccine depends upon the host's immunologic capabilities at the time of vaccine administration. If children are the primary targets of vaccine campaigns, then the study of the effects of early EBV infection on the efficacy of the vaccine should be studied. The following questions necessitate investigation of the *P. falciparum*-EBV-host interaction:

1. Does P. falciparum induce (activate) the EBV in latently infected lymphocytes? If it does, what are the consequences of administering a vaccine to a young EBV-infected population?

2. Does EBV infection increase or decrease the efficacy of vaccination because it is an immunostimulating and an immunosuppressive agent?

3. Is chronic lymphoid stimulation such as occurs with malaria and EBV infection a factor in the development of childhood tropical malignancies?

To begin to address the significance of the P. falciparum-EBV interaction in disease, preliminary studies are designed to assess the effect of P. falciparum on the latent EBV genome in Burkitt's lymphoma cell lines. The effects of EBV infection on the efficacy of a P. falciparum vaccination will be determined in future studies with New World primates which do not harbor EBV. These studies will be the subject of a subsequent proposal.

Scientific Aspects of the Proposed Work

Objectives

The specific objective of these studies is to measure the effect of P. falciparum on the expression of the genes of the Epstein-Barr virus in Burkitt's lymphoma cell lines which carry multiple copies of the EBV genome.

Rationale

Malaria is holoendemic or stable in some regions of tropical Africa and in New Guinea, i.e., transmission occurs throughout the year. Burkitt's lymphoma is endemic in these areas also. Burkitt's lymphoma from tropical Africa is associated with the Epstein-Barr virus, and the virus is present in virtually every cell of African Burkitt's. The virus is rarely associated with sporadic cases of Burkitt's lymphoma that occur in other parts of the world.

The geographic occurrence of malaria and Burkitt's lymphoma in Africa and in New Guinea has led to speculation of a cofactor role for malaria in the development of Burkitt's lymphoma. The proliferation of the cells of the lymporeticular system and the immunosuppression caused by persistent Plasmodium infection are believed to be important factors in the development of Burkitt's lymphoma. Though P. falciparum can cause T and B lymphocytes to divide rapidly, the effect of this mitosis-inducing property on the EBV has not been determined.

Since infection with Plasmodium and EBV occur in infants in tropical Africa, it is possible that infection with one of these parasites influences the outcome of infection with the other. Plasmodium and EBV are capable of causing lymphocytes to divide. Dividing lymphocytes are

more susceptible to virus infection than are resting cells. Substances that stimulate division in lymphocytes may also activate latent viruses residing in these cells. Therefore, the studies proposed here are designed to determine if P. falciparum can activate the latent EBV genome in Burkitt's lymphoma cell lines. The cells of these lines contain many copies of the EBV genome; however, the genes for mature virus production are not expressed. Therefore, these cell lines can be used to assay for virus gene activation. When the viral genome is activated, various proteins associated with the production of mature virus are produced. These proteins can be detected in the infected cells with fluorescent antibody techniques.

Literature Review

Human malaria is caused by Plasmodium falciparum, P. vivax, P. ovale, and P. malariae. Plasmodium falciparum malaria occurs in the tropics and subtropics and is the most common form of malaria in sub-Saharan Africa. Malaria caused by P. falciparum is the most severe of the malarias and is responsible for most of the malaria mortality. Two hundred million people yearly are either chronically infected or suffer from acute attacks of Plasmodium (UNDP/Work Band, WHO Fourth Annual Report, 1980).

Because malaria is transmitted by mosquitoes, eradication and control of the disease in many countries has been achieved by the application of insecticide; however, malaria still occurs at a constant rate, i.e., is endemic, in tropical Africa (Wyler, 1983).

Malaria is characterized by chill and fever cycles which occur when the red blood cells that are infected with Plasmodium burst open after the parasites complete a cycle of growth. The spleen and the lvier enlarge, and there is excessive lymphoreticular proliferation. Patients have increased levels of parasite-nonspecific immunoglobulins in the blood (Greenwood, 1974). The occurrence of parasite nonspecific globulins during Plasmodium infection led investigators to speculate that substances capable of causing nonspecific activation and multiplication of lymphocytes (mitogens) were associated with Plasmodium (Greenwood, 1974). Plasmodium falciparum preparations have been shown to be mitogenic for thymus-derived human lymphocytes (T cells) and for B (immunoglobulin producing) lymphocytes (Wyler et al., 1979 and Greenwood et al., 1979, respectively). Furthermore, Gabrielsen and Jensen (1982) showed that P. falciparum from continuous cultures causes normal human lymphocytes to proliferate.

Burkitt's lymphoma is a malignancy of the B lymphocytes of the immune system in humans. This tumor was first described in Ugandan children by Dennis Burkitt in 1961. Tumors are found in the bones of the jaw and in the kidneys and ovaries (Ziegler, 1981).

Though malignant lymphoma is a rare disease in children, Burkitt's lymphoma occurs with significant frequency in children in countries in tropical Africa and in New Guinea (O'Connor, 1970). Burkitt's lymphoma is rarely found outside of these areas. The rarity of the disease outside of these geographical areas provides strong evidence for the participation of environmental factos in the development of the malignancy (Burkitt, 1969; O'Connor, 1970 and Ziegler, 1981). Malaria has been suggested to be an important environmental factor which influences the geographic occurrence

of Burkitt's lymphoma, because the malignancy is common in tropical Africa and in New Guinea, areas of the world where the incidence of malaria is stable (endemic). In holoendemic malarious areas, there is year-round transmission of malaria and the parasitemia rate and the spleen rates (enlarged spleen) in infants and in young children is greater than 75% (O'Connor, 1970). Further strengthening the connection between holoendemic malaria and the occurrence of Burkitt's lymphoma is the observation that cases of the lymphoma in older children and in young adults are most often seen in immigrants coming from malaria-free and Burkitt's lymphoma-free areas into areas where malaria is holoendemic (Burkitt, 1966).

In 1964, Epstein et al. described a herpes-like virus in electron micrographs of a Burkitt's lymphoma from Africa. This virus is now known as the Epstein-Barr virus (EBV). The virus infectes only the B lymphocytes of humans and primates (Jondal and Klein, 1973). Primary infection by EBV usually leads to immunity; however, the virus may remain latent in B lymphocytes for many years following the primary infection (Thorley-Lawson, 1980). Primary infection with EBV may be manifested as infectious mononucleosis.

EBV is present in virtually every cell of African Burkitt's tumors (Kieff et al., 1982). The cells contain nearly all the same viral DNA as is found in mature virus particles (Sugder, 1977). However, the genes coding for mature virus formation are not expressed (Lindahl et al., 1974).

Though EBV has not been shown to cause cancer, there is epidemiologic, clinical and experimental evidence which suggests a causal role for EBV in the genesis of Burkitt's lymphoma. Epidemiologic data indicate that EBV is present in virtually all cells of Burkitt's lymphoma; however, the virus is rarely found in the sporadic cases of the lymphoma that occur outside of endemic areas (Kieff et al., 1982). Seroepidemiologic studies have shown that African children with high antibody titers to EBV capsid (coat) antigen run a high risk of developing Burkitt's lymphoma (de The et al., 1978). Because infection with EBV in Africa occurs in infants within the first year of life, children that develop Burkitt's lymphoma may have chronic EBV infections (de The et al., 1978) and consequently higher levels of antibody that is specific for EBV capsid antigen.

Clinical studies point to a role for EBV in the genesis of lymphomas. Children with certain immunodeficiencies develop lymphomas after primary infection with EBV which presents as infectious mononucleosis (Purtilo et al., 1980). Robinson et al. (1980) described the development of a malignant lymphoma in a 4-year old girl who was initially hospitalized for an infectious mononucleosis-like syndrome. The B lymphocytes of the malignancy were infected with EBV.

The study of the biologic properties of the EBV has also implicated EBV as a cancer causing agent. Malignant lymphoma can be established in marmosets after the injection of the virus (Shope et al., 1973). In the laboratory, suspensions of the virus will cause B lymphocytes to proliferate indefinitely, i.e., to transform (Henle, 1979).

Several models have been suggested to explain the role of EBV infections

in Burkitt's lymphoma. EBV causes B lymphocytes to proliferate rapidly under laboratory conditions, i.e., EBV is mitogenic for B cells. Klein (1979) suggested that EBV may play the role of initiator in tumor formation by establishing a pool of precancerous B lymphocytes. Klein suggested that a genetic change in these precancerous cells might result in the expression of genes that favor continuous growth over those that counteract cell multiplication.

According to the target cell conditioning model proposed by Zur Hausen (1981), primary infection by EBV in infancy results in heightened lymphocyte proliferation at a time when the immune system is not fully mature. This early proliferation provides a pool of conditioned cells that become susceptible to other agents which may have super transforming properties. Lymphocytes activated by mitogen are more susceptible to virus infection than are resting lymphocytes (Greaves et al., 1973). Zur Hausen and Gissmon (1979) isolated a new virus from dividing monkey lymphocytes which is dependent upon lymphocytes that are dividing for its replication. In Zur Hausen's model, the lymphocyte that is stimulated to divide would become more susceptible to agents that might cause cancer.

The geographic paradox of the occurrence of EBV-associated endemic Burkitt's lymphoma with holoendemic and hyperendemic malaria has led several investigators to postulate that malaria is an important factor in the outcome of the EBV-host interaction (Ziegler, 1981). Burkitt (1969) and O'Connor (1970) suggested that stimulation of the immune system by chronic Plasmodium infection is a cofactor in the development of Burkitt's lymphoma. Plasmodium falciparum is mitogenic for thymus-derived human lymphocytes (Wyler et al., 1979) and for B lymphocytes (Greenwood et al., 1979). Recently, Gabrielsen and Jensen (1982) showed that P. falciparum from continuous cultures is mitogenic for human lymphocytes.

Children in malaria/Burkitt's lymphoma-endemic areas are exposed to Plasmodium and to EBV at a very early age (O'Connor, 1970; de The 1980). Both of these parasites are capable of causing a high degree of lymphocyte proliferation (Gabrielsen and Jensen, 1982; Adams, 1980). Both of these parasites may suppress host immune responses (Wyler, 1983 and Tsukuda et al., 1981). If P. falciparum activates the latent EBV genome in infected cells, and if mature virus production results, a larger precancerous pool of cells would be created. Such a postulate is feasible because of the observation of increased levels of antibody to EBV capsid antigen that have been observed in children that later develop Burkitt's lymphoma. Activation of latent virus which results in mature virus formation may cause destruction of lymphocytes. The destruction of B lymphocytes following viral infection may result in depletion of lymphocytes which are responsive to a variety of antigens, among them Plasmodium antigens. The fact that infection with Plasmodium and with EBV occur at a very early age in African children indicates that the interaction of these two lymphocyte-stimulating agents in the genesis of Burkitt's lymphoma, in the observed immunosuppression in malaria and in the refractoriness of African children to childhood vaccinations (Wyler, 1983) require further study.

Experimental Design with Time-Phased Plan-of-Work

Establishment of Petri dish cultures of P. falciparum; months 1-3. Petri dish cultures of P. falciparum (FCR-3/Gambia) will be established as described by Jensen and Trager (1977). Outdated human type O red blood cells (RBC) from the Howard University Blood Bank will be used to establish cultures from a P. falciparum-infected RBC inoculum (Dr. Susan Langreth, Uniformed Services University). The cultures will be incubated at 37°C as described by Jensen and Trager (1977). The growth of the parasites will be monitored daily by light microscope examination of Giemsa-stained thin blood films. Subcultures will be started at 3-day intervals with the addition of fresh RBC (Trager and Jensen, 1976).

Plasmodium falciparum cultures will be synchronized after sorbitol treatment (Lambros and Vanderberg, 1979). Unsynchronized cultures contain parasites in various stages of development. Sorbitol destroys the RBC that are infected with mature parasites; therefore, a population of RBC infected with young forms is obtained.

Preparation and characterization of P. falciparum schizonts and merozoites; months 4-7. The dividing forms (schizonts) and the infectious forms (merozoites) resulting from this division will be isolated according to established methods. Schizont-infected RBC will be separated from other RBC in the cultures after incubation at 37°C for 30 minutes with Plasmagel (Reese et al., 1979). Schizont-infected cells are less dense than are uninfected RBC and RBC with immature ring forms. Consequently, the schizont-infected cells do not sediment and can be removed from the upper phase of the Plasmagel-infected RBC suspension. The schizonts will be washed in phosphate buffered saline (PBS), resuspended in PBS and stored in a freezer at -20°C.

Merozoites may be harvested from isolated schizont-infected cells in the following manner. Schizont-infected cells recovered after Plasmagel treatment will be washed in culture medium (RPMI 1640) and resuspended in this medium. These schizonts will be returned to culture in Petri dishes (Jensen and Trager, 1977). The parasites will mature and the release of merozoites will occur. Merozoites will be isolated from the medium after centrifugation. The merozoites will be washed in PBS, resuspended in PBS, and stored at -20°C.

Normal human type O RBC will also be prepared for controls. Normal type O RBC will be cultured in Petri dishes as described above for infected RBC. After a 48-hour incubation period, the cells will be washed twice in PBS and resuspended in cold water to effect lysis of the cells. (Membrane damage occurs when RBC are mixed with water without physiologic saline.) The hemoglobin of the cells will leak out, and after high-speed centrifugation (25,000 g), the RBC membranes can be isolated. The RBC membranes will be washed twice in PBS, resuspended in PBS and stored at -20°C.

The effectiveness of the methods used to prepare schizonts and merozoites will be evaluated by light and electron microscope. Each preparation will be examined by phase contrast microscopy before being stored. A small aliquot of each sample will be fixed and embedded for electron microscopy.

Immunofluorescence assays for the induction of the EBV genome in Burkitt's lymphoma cell lines after exposure to P. falciparum; months 8-12. The Burkitt's lymphoma cell lines, Raji, P3HR-1, and NC37 are being maintained in suspension cultures in RPMI 1640 medium. (These cell lines were supplied by Dr. D.V. Ablashi of the National Cancer Institute.) The cells of these lymphoblastoid lines harbor the EBV. P3HR-1 cells produce the virus; however, the virus is not a transforming strain of EBV.

To assay for virus genome activation in these cell lines by P. falciparum, schizont-infected cells, merozoites, and RBC membranes will each be solubilized after three cycles of freezing in a dry-ice-acetone bath followed by rapid thawing in 37°C water. The material will be further solubilized after a short exposure to ultrasound. Material that is insoluble after this treatment will be recovered after high speed centrifugation at 25,000 g at 4°C. The insoluble sediment will be returned to storage at -20°C. The solubilized material will be filtered sterile, assayed for protein (Bensadoun and Weinstein, 1976), and for endotoxin (Felton et al., 1980). (High endotoxin levels may be indicative of bacterial contamination in the preparations. Endotoxin or lipopolysaccharide is a very potent activator of B lymphocytes. Preparations containing high levels of endotoxin will be discarded.)

Various microgram doses of P. falciparum schizonts or merozoites or of normal RBC membranes will be incubated with each Burkitt's lymphoma line in sterile tissue culture dishes. All cultures will be set up in triplicate and incubated at 37°C. At 24-hour intervals for 5 days, a small sample of each culture will be removed. The cells will be washed in PBS and resuspended in PBS. A drop of each suspension will be placed in each well of microscope slides designed especially for fluorescence microscopy. The buffer in each well will be allowed to evaporate and the cells on the slide will be fixed in cold acetone and stored at -20°C until needed.

For immunofluorescence tests, slides will be removed from the freezer, and various concentrations of human serum specific for EBV early antigen (EA) and for virus capsid antigen (VCA) will be incubated with the fixed cells at 37°C for 30 minutes. (Serum samples which contain antibodies that are specific for EBV EA and VCA were provided by Dr. W. Henle, Children's Hospital of Philadelphia.) After the 30-minute incubation, the slides will be washed three times in PBS and then incubated for 30 minutes at 37°C with serum from goats which contains antibodies that react with human antibodies. The goat antiserum will be labeled with fluorescein, a substance which emits a yellow-green light when it is exposed to ultraviolet light. The fluorescent-labeled antiserum will be obtained from Cappell Laboratories. After this incubation, the cells on the slides will be washed in PBS and covered with PBS-glycerin solution and a coverslip. The cells will be examined for fluorescence with a Leitz fluorescence microscope. The number of cells fluorescing per 500 cells will be determined. Significance will be assessed with the student's t test.

Positive controls for these studies will be Raji, NC37, or P3HR-1 cells incubated with 12-O-tetradecanoylphorbol-13-acetate (TPA) as described by Ablashi et al., 1980. TPA activates the EBV genome in Burkitt's lymphoma cells. Cells incubated with medium or with RBC membranes will be negative controls for all studies.

Future studies. In future studies, the effect of P. falciparum on the transformation of human lymphocytes by EBV will be determined. Lymphocytes taken from the umbilical cord blood of delivered human infants will be incubated with various amounts (doses) of P. falciparum schizonts or merozoites and with a measured dose of transforming EBV. The lymphocytes will be incubated under the conditions previously described for Burkitt's lymphoma cell lines except that radioactive thymidine, a substance that is used in DNA syntheisis will be added to the cultures. DNA synthesis in the cultures will be determined by counting the radioactivity of each culture in a scintillation counter. Transformation of the cells will be determined as described by Thorley Lawson (1980). The presence of EBV nuclear antigen in the treated cells will be detected with fluorescent antibody techniques described by Reedman and Klein (1973). The EBV nuclear antigen is the earliest and possibly the only viral product made following the infection of lymphocytes by transforming virus (Pearson, 1980).

If P. falciparum is capable of inducing expression of the EBV genome, the specific component responsible for the induction will be isolated and characterized.

New World primates do not harbor EBV, yet they can be infected in the laboratory with the virus. Though the squirrel monkey, the woolly monkey, and the marmoset can be infected with EBV, only the marmoset develops lymphoma following the injection of the virus (Adams, 1980). New World monkeys can also be infected with Plasmodium. Therefore, these primates offer the perfect non-human model system for the study of Plasmodium-EBV host interactions. However, studies with the higher primates are very expensive and greater resources than are presently available to me are required for such studies.

Rationale of proposed methodologies and alternatives. The cells of Burkitt's lymphoma have the characteristics of the lymphocytes that produce antibodies, i.e., they appear to be B cells. These lymphomas do not contain macrophages, cells which ingest substances and present these substances to lymphocytes. In many experimental systems, macrophages are required in order for a lymphocyte to respond to stimuli (antigens) in its environment. The use of B-cell lymphoma lines for studying activation of a viral genome inside the cell may be questioned, because it must be supposed that the B lymphocyte binds the stimulus (P. falciparum) to its membrane. This binding then may stimulate rapid cell division which may trigger latent virus gene expression. Tovey et al. (1979) used various mitogens (substances that stimulate a large number of lymphocytes to divide) to activate the EBV genome in Burkitt's lymphoma cell lines. Furthermore, the B cells from lymphoma cell lines are efficient at binding certain substances and presenting these substances to T lymphocytes (Chestnut et al., 1982). Normal B lymphoctytes are not as efficient. These results indicate that B cell lymphomas act in much the same way as do macrophages in antigen binding and presentation. Chestnut et al. (1982) theorized that B cell lymphomas might be capable of some critical step in antigen processing that resting B cells are incapable of performing. Therefore, the use of Burkitt's lymphoma cell lines in assaying for EBV genome induction after exposure to P. falciparum is feasible.

Merozoite preparations are unstable and deteriorate rapidly (D. Haynes and J.P. Kreier, personal communications). Substances that inhibit protein digestion by enzymes (protease inhibitors) can be added to merozoite preparations to stabilize them. Protease inhibitors cannot be added to the preparations to be used in the proposed studies, because the merozoite preparations will be added to the Burkitt's lymphoma cultures. It may be possible to use freshly prepared merozoites for the proposed studies instead of frozen suspensions.

The methods described for solubilizing the parasites will not release all components of the parasites into the liquid phase. Proteins in the membranes of the parasites may not be solubilized. Detergents can be used to solubilize membranes; however, the detergent is required to keep the proteins in the soltuion after solubilization (Strominger et al., 1981). Therefore, detergents cannot be used as membrane solubilizing agents in the studies proposed here.

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Gravely, S.M., J. Hamburger, and J.P. Kreier. 1975. T and B cell population changes in young and in adult rats infected with Plasmodium berghei. Annual Meeting of the American Society of Parasitologists. Abstract 234, p. 999.

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Budget Information and Estimates

1. Estimated total funding requested	$34,560
2. Cost-sharing arrangements	0
3. Detailed budget	
a. Salaries*	
Principal investigator, Sally M. Gravely, Ph.D. 20% time and effort	5,820
Fringe benefits, 28% of salary	1,630
Total salary and fringe benefits	$ 7,450
b. Travel	
Annual meeting of the American Society of Tropical Medicine and Hygiene	750
c. Expendable supplies and equipment+	10,000
(Tissue culture reagents and supplies, general chemicals, glassware, immunological reagents, and electron microscopy supplies)	
d. Other direct costs	1,000
(Library literature retrieval, photocopying, publication page charges)	
e. Total direct costs	19,200
f. Indirect costs	
(80% of direct costs)	15,360
g. Total direct and indirect costs	$34,560

*Though I am requesting 20% of my university's salary, I expect to spe 55% of my time completing the proposed studies.

The following equipment is available in the Department of Microbiology for the proposed studies: CO_2 incubators, dry-air incubators, RC-2B refrigerated centrifuge, walk-in cold room, Zeiss inverted microscope, Leitz fluorescence microscope, Zeiss EM 10-B transmission electron microscope, and a Heat Systems Ultrasonics sonicator cell disruptor. A virology suite with six cubicles for tissue culture work and an electron microscopy suite are available. The principal investigator has a laboratory with an adjoining office in the Department of Microbiology.