PN-AAX-575

62

FINAL REPORT

PLASMODIAL INDUCTION OF EPSTEIN-BARR VIRUS

DAN-1460-G-SS-4003-00

by

Sally M. Grovely, Ph.D.

Howard University

Washington, D.C. 20059

001361

004783

S

RECEIVED
JUN 11 1985
STI/RUR
hilp

DESCRIPTION of the PROBLEM

Despite mosquito control campaigns, malaria remains endemic in tropical Africa. Infection with Plasmodium falciparum results in high mortality in infants and in young children (World Health Organization, 1980). Plasmodium infection is debilitating and immunosuppressive. The humoral immune response to murine tumor viruses (Salaman et al., 1969; Bomford and Wedderburn, 1973), to sheep red blood cells (Greenwood et al., 1971), and to bovine serum albumin (Strambachova-McBride and Micklem, 1977, 1978) is suppressed during malaria infection. Plasmodium yoelii infection in mice facilitated the growth of a transplanted, syngeneic, virus-induced lymphoma (Wedderburn et al., 1981). Thymic involution, depletion of T lymphocytes from the spleen and lymph nodes, and disturbed responsiveness of lymphocytes to mitogens characterize Plasmodium infection (Krettli and Nussenzweig, 1974; Gravely et al., 1976; Taylor and Siddiqui, 1978). The debilitating and immunosuppressive effects of Plasmodium infection may predispose children in malaria endemic areas to other diseases. High incidence rates of Burkitt's lymphoma (BL) have been recorded in malaria endemic areas (tropical Africa and New Guinea), and malaria has been suggested to be a precipitating factor in the development of this disease in children (Morrow et al., 1976; de The et al., 1978; Ziegler, 1981).

Though malaria is immunosuppressive, patients with malaria have increased levels of parasite nonspecific immunoglobulins in their sera (Greenwood, 1974). The occurrence of these parasite nonspecific immunoglobulins during Plasmodium infection led investigators to speculate that substances (mitogens) capable of causing nonspecific activation and multiplication of lymphocytes were associated with Plasmodium (Greenwood, 1974). Plasmodium falciparum preparations have been shown to be mitogenic for T and for B 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).

In 1964, Epstein et al. described a herpes-like virus in electron micrographs of a BL from Africa. This virus is now known as the Epstein-Barr virus (EBV). The virus infects 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).

EBV is present in virtually every every cell of African Burkitt's tumors; however, the virus is rarely found in the sporadic cases of the lymphoma that occur outside of endemic areas (Kieff et al., 1982). The cells contain nearly all the same viral DNA as is found in mature virus particles (Sugden, 1977).

Seroepidemiologic studies have shown that African children with high antibody titers to EBV capsid (coat) antigen run a high risk of developing BL (de The et al., 1978). Because infection with EBV in Africa occurs in infants within the first year of life, children that develop the lymphoma may have chronic EBV infections (de The et al., 1978) and consequently higher levels of antibody that is specific for EBV capsid antigen.

EBV is capable of transforming normal B lymphocytes (Henle, 1979), and malignant lymphomas can be established in marmosets after the injection of EBV (Shope et al., 1973). Though EBV is a transforming virus, its role in the genesis of BL is unknown. Klein (1979) suggested that EBV may play the role of initiator in tumor formation by establishing a pool of precancerous B lymphocytes. Klein (1979) also suggested that chronic holoendemic malaria may act as an environmental promoter of tumor formation by causing the precancerous EBV-infected cells to proliferate. Chronic proliferation induced by Plasmodium would increase the possibility of a genetic change occuring in a cell. Immunosuppression that occurs during Plasmodium infection would enhance the survival of a cell that had undergone a cytogenetic change (Klein, 1979). T cell immunity is impaired in humans infected with P. falciparum (Whittle et al., 1984), and Thorley-Lawson (1980) has shown that T cells prevent the outgrowth of EBV-infected cells.

Cytogenetic changes are found in BL. Burkitts lymphomas (EBV positive or negative) carry chromosomal translocations which involve chromosomes that encode the genes for immunoglobulins (Ohno et al., 1979). Seventy-five percent of BL carry t(8;14) translocations. The remaining 25% carry either t(8;22) or t(2;8) translocations (Croce et al., 1983).

The cellular myc (c-myc) gene maps to chromosome 8, band 24 (Taub et al., 1982). In lymphomas that carry t(8;14) translocations, c-myc is translocated to the region of chromosome 14 that encodes the mu heavy chain of IgM (Taub et al., 1982). In lymphomas that carry the t(8,22) or t(2;8) translocations, c-myc remains on chromosome 8, and the lambda light chain locus or the kappa light chain locus is translocated to chromosome 8. These translocations result in transcriptional deregulation of the c-myc gene and its increased expression, while untranslocated c-myc is transcriptionally silent. (Croce et al., 1983). The c-myc gene is activated by mitogens (Persson et al., 1984), and c-myc transcription in normal somatic cells is related to the rate of cell division (Stewart et al., 1984).

The cellular myc gene is also referred to as a proto-oncogene; i.e., a gene that is a potential cancer gene. Though the translocations observed in BL involve myc and fullfill the cytogenetic events in tumor formation as postulated by Klein (1979), there is no proof that activated proto-oncogenes are necessary for cancer formation (Duesberg, 1985). However, c-myc can immortalize rat embryo fibroblasts but cannot induce transformed foci. Transformed foci do form in c-myc immortalized rat embryo fibroblasts that are treated with the tumor promoter, 12-O-tetradecanoylphorbol 13-acetate (TPA) (Connan et al., 1985).

Because P. falciparum is mitogenic for T and for B lymphocytes and because EBV associated BL and malaria share endemicity in Africa and in New Guinea, the studies reported here were designed to determine whether P. falciparum induces EBV activation in BL cell lines and whether the parasite enhances the proliferation of rapidly dividing BL cells. These studies have become increasingly significant because of the possible role of proto-oncogenes and environmental promoters in tumor formation.

MATERIALS and METHODS

In vitro Cultivation of *Plasmodium falciparum*

Plasmodium falciparum (FCR-3) was cultured in 5 to 15-ml volumes in 25-cm^2 or in 75-cm^2 tissue culture flasks (Corning Glass Works, Corning, NY) as described by Haynes et al., 1976 For cultures, human type 0, Rh positive red blood cells (RBC) were collected aspectically into sterile acid-citrate-dextrose (ACD) solution, and the blood was stored at 4°C for 7 days prior to use. Blood (100 ml) was collected from the same healthy American male donor at 20 to 25-day intervals. The donor has no history of malaria nor has he ever resided in or traveled to an area where malaria is endemic.

Type A serum (Rh positive) was obtained from healthy volunteers in the College of Medicine at Howard University or from the Plasma Alliance in Knoxville, TN. The sera from all type A donors were clarified after centrifugation, heat inactivated at 56°C for 30 min and stored in 50-ml volumes at -20°C. All donors were negative for hepatitis B virus and had no history of malaria or residency in malaria-endemic areas. All donor sera contained antibodies specific for the virus capsid antigen (VCA) of EBV as determined in the indirect fluorescent antibody test (Litton Bionetics, Charleston, SC).

Normal human type 0 RBC were washed three times in RPMI 1640 medium (M.A. Bioproducts, Walkerville, MD), and the buffy coat which contained white blood cells (WBC) was removed after the first wash. A small amount of the upper portion of the packed cell volume (PCV) was removed after each of the final two washes to eliminate WBC contamination in cultures.

The PVC was measured after the final wash and adjusted to a 5% hematocrit by the addition of RPMI 1640 medium containing 10% heat-inactivated human type A serum, 25 mM hepes and 0.3 mg/ml glutamine (complete medium *P. falciparum*, CM-P).

Plasmodium falciparum (FCR-3) was obtained from the Walter Reed Army Institute of Research. Subcultures were made twice weekly by dilution of infected blood from an old culture (3 or 4 days old) with a 6% suspension of fresh normal type 0 RBC so that the percentage parasitemia in each culture was 0.12%. Each culture was gassed with a mixture of 5% CO_2, 5% O_2 and 90% N_2 (Air Products, Allentown, PA), tightly sealed and incubated at 37°C.

Cultures of normal human type 0 RBC were subcultured as described for *P. falciparum*-infected cultures. The inoculum for normal cultures was RBC from and old uninfected culture.

Plasmodium falciparum cultures were synchronized after sorbitol treatment of infected cultures as modified from Lambros and Vanderberg (1979) by Vernes et al., 1984. Synchronized cultures were fed and subcultured as described above.

Percentage parastiemias were determined by counting the number of parasitized RBC in 500 RBC on Giemsa-stained thin blood smears. Percentage parasitemias in cultures averaged 5% after 4 days *in vitro* when a calculated inoculum of old culture was added to a fresh suspension of normal RBC so that the starting parasitemia was 0.12% and the hematocrit was 6%. Lower starting parasitemias did not yield parasitemias that were high enough for use in the studies described below. Higher parasitemias (7.9 to 10%) were obtained after 6 to 7 days of *in vitro* culture with 24 or 48-hr feedings in the absence of subculturing; however, some of the mature parasites in such cultures were not typical parasites and resembled crisis forms.

Isolation of merozoites. Suspernatant medium from cultures was centrifuged at 5000 *g* in the IEC PR-J centrifuge for 15 min at 4°C. The pellet was resuspended in RPMI 1640 medium without serum and washed once more by centrifugation at 5000 *g* for 15 min at 4°C. Supernatant medium was removed and the pellet was resuspended in enough medium without serum to make a 1:10 dilution of the pellet. A drop of the preparation was smeared on a slide, fixed in methanol and stained with Giemsa stain. These preparations contained merozoites, mature schizonts in which merozoites had not dispersed and RBC. Each preparation was stored at -20°C until used. Protein concentration in each of these preparations was determined by Biuret.

Maintenance of Burkitt Lymphoma Cell Lines

The BL lines, Raji-3, NAB, P_3HR-1 and AG876 were obtained from Dr. D.V. Ablashi of the National Cancer Institute. These cell lines carry multiple copies of the EBV genome. Each cell line was maintained in suspension culture in 25-cm^2 or in 75-cm^2 tissue culture flasks (Corning Glass Works, Corning, NY). PRMI 1640 medium (Advanced Biotechnologies, Silver Spring, MD) was supplemented with 10% heat-inactivated fetal bovine serum, 0.30 mg/ml glutamine and antibiotics (penicillin-streptomycin-fungizone) for BL cell lines (CM-BL). The cells were passaged twice weekly and were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. BJAB, an EBV negative BL line, was maintained as described for EBV-positive cell lines.

Immunofluorescence Assay for the Induction of EBV by *Plasmodium falciparum* (FCR-3)

BJAB, Raji-3, P_3HR-1 and AG876 cells were cultured *in vitro* as described above. For assays, the cells of each culture were pelleted by centrifugation at room temperature for 10 min in an IEC desk top clinical centrifuge. The pelleted cells were resuspended in medium without serum and the centrifugation step was repeated. The cells were then resuspended in CM-BL. Each cell suspension was diluted in CM-BL so that each ml of suspension contained 1 X 10^6 viable cells. Viability was determined by trypan blue exclusion, and all cell counts were performed in a hemacytometer (Reichert, Bright-Line).

RBC from P. falciparum-infected or from normal cultures were washed twice in RPMI 1640 without serum by centrifugation at room temperature in the IEC clinical centrifuge. Each pellet was resuspended in enough CM-BL to yield a 6% hematocrit. Infected cultures were used when parasitemias were 5% or more and when 80 to 85% of the RBC contained schizonts.

One ml of each cell line (1 X 10^6 viables/ml was mixed with 0.1 ml of a 6% suspension of P. falciparum-infected RBC, with 0.1 ml of normal RBC (6%), with 0.1 ml of merozoite-RBC lysate or with 0.1 ml of CM-BL in 16 X 125-mm crew cap culture tubes (Corning Glass Works, Corning, NY). All mixtures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 72 hr. After incubation, the cells were centrifuged at room temperature in an IEC clinical centrifuge and the pelleted cells were washed two times in Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS). A drop of each suspension was placed on each well of an 8-well slide (Roboz Surgical Instrument Co., Washington, D.C.). Slides were air dired and fixed for 15 min at 4°C in acetone. Fixed slides were dried and stored at -20°C until used.

For immunofluorescence assays, slides were removed from the freezer and allowed to warm to room temperature. A 1:80 dilution of human anti-EBV (supplied by Dr. W. Henle) serum was prepared in PBS. A 1:10 dilution of normal human serum that was negative for antibody for EBV was also prepared in PBS. Five of the wells on each slide were incubated with the 1:80 dilution of human anti-EBV (VCA titer, 640; early antigen titer, EA, 40). Two of the wells contained the 1:10 dilution of normal serum and the last well was incubated with PBS alone. All slides were then incubated in petri dishes that contained moistened filter paper at 37°C for 40 min. After the incubation, the slides were washed two times in PBS (5 min/wash) and once in distilled water. The slides were air dired. Each well was then covered with a 1:15 dilution of affinity purified, fluorescein-labeled goat anti-human IgG (Cappel Laboratories, Cochranville, PA) or with fluorescein-labeled goat anti-human immunoglobulin containing Evans blue counterstain (Litton Bionetics, Charleston, SC). The slides were incubated at 37°C for 40 min, and after incubation, the slides were washed twice in PBS, once in distilled water and air dried. The slides were mounted with PBS-glycerin (1 part PBS, 9 parts glycerin) and coverslips. The percentage of cells that were positive for VCA was determined by counting the number of fluorescing cells in a total of 500 cells in a Leitz fluorescence microscope.

Stimulation of DNA synthesis in BL Cell lines by Plasmodium falciparum

Plasmodium falciparum, normal RBC, and BL cell lines were maintained in vitro as described above.

Normal RBC or P. falciparum-infected RBC were washed twice in RPMI 1640 medium without serum and resuspended to a 6% suspension in CM-BL.

BJAB, Raji-3, NAB, P_3HR-1, and AG876 cells were washed once in RPMI 1640 medium and resuspended in CM-BL. Viability was determined by trypan blue exclusion, and cell counts were done in a hemacytometer. Each cell suspension was adjusted to contain 1 X 10^6 viable cells/ml.

One ml (1 X 10^6) of each of the cell lines was mixed with 0.1 ml of one of the following: a 6% suspension of normal human type O RBC; a 6% suspension of P. falciparum-infected type O RBC (5% parasitemia or greater); merozoite-RBC mixture that was prepared from culture supernatants; CM-BL, or with 0.1 ml of PHA (2 µg/ml, Sigma, St. Louis, MO) in CM-BL.

Each test was set up in triplicate in 16 x 125-mm tissue culture tubes and incubated for 18 or for 48 hr at 37°C in an atmosphere of 5% CO_2 in air. After 18 or 48 hr of incubation, 1 µCi of ^{3}H-thymidine (specific activity, 6.7Ci/mmol, ICN Radiochemicals, Irvine, CA) in 0.1 ml of CM-BL was added to each culture tube, and the tubes were incubated for an additional 24 or 48 hr at 37°C in an atmosphere of 5% CO_2 in air.

After incubation with ^{3}H-thymidine, the cells were washed twice in PBS by centrifugation at 1000 g in the IEC PR-J centrifuge for 10 min at 4°C. The supernatants were discarded, and the pelleted cells were dissolved in 1N NaOH. Each suspension was then precipitated in trichloracetic (TCA) acid as described by Waithe and Hirschhorn, 1979. TCA precipitates were dissolved in Hyamine hydroxide (New England Nuclear, Boston, MA), and the amount of ^{3}H-thymidine incorporated in to TCA precipitable protein was measured as counts per minute (cpm) in a Beckman LS 3133 P liquid scintillation counter.

Coloration due to hemoglobin caused quenching in test cultures that contained RBC. A drop of H_2O_2 was added to hemoglobin containing cultures to reduce coloration and quenching. H_2O_2 had no effect on counts in test cultures that did not contain RBC.

Statistical methods. The mean of triplicate or of duplicate cultures was calculated for each stimulator tested. Significance was assessed by the two-tailed paired student t test.

RESULTS

Detection of VCA in Indirect Fluorescent Antibody Assays

VCA expression was slightly increased in AG876 cells that were incubated with P. falciparum-infected RBC; however these increases were not significant ($p < 0.1$). NRBC did not increase VCA expression above control levels in AG876 cells (Table I).

VCA expression was increased above control levels in P_3HR-1 cells that were incubated with P. falciparum ($p < 0.05$). This was a significant increase above control levels (Table I). NRBC did not significantly alter VCA expression in P_3HR-1 cells. Cells of the P_3HR-1 line were frequently observed to be damaged in fixed preparations. The significance of this finding cannot be assessed at present, because whether or not lytic virus production was increased in P_3HR-1 cells under the conditions employed was not determined in this study.

VCA was not detected with any stimulator in Raji-3 or in NAB cells.

Incorporation of ^{3}H-thymidine into BL Cell Lines Incubated with P. falciparum

NRBC and P. falciparum-infected RBC increased ^{3}H-thymidine incorporation in Raji-3, NAB, and in P_3HR-1 cells (Table II) when ^{3}H-thymidine was added after 18 hr of incubation. The increase in ^{3}H-thymidine incorporation above control levels was most dramatic in the Raji-3 cell line where stimulation indices were 2.69 and 3.25 for NRBC and P. falciparum-infected RBC, respectively (Figure 1). These increases are very significantly different from control levels with $p < 0.01$ for Raji cells incubated with NRBC when compared to the RPMI controls and $p < 0.001$ for Raji cells incubated with P. falciparum-infected RBC. The difference between the counts obtained for P. falciparum-infected cells incubated with Raji-3 and the counts obtained when NRBC were incubated with this cell line is also statistically significant ($p < 0.01$). Even the crude merozoite preparation which is contaminated with RBC increased ^{3}H-thymidine incorporation above control levels in Raji-3 cells (Table II and Figure 3).

^{3}H-thymidine incorporation was increased above control levels in NAB cells that were incubated with NRBC or with P. falciparum-infected RBC ($p \leq 0.01$ and $p < 0.05$, respectively). There were no significant differences in ^{3}H-thymidine incorporation between NRBC and P. falciparum stimulators in NAB cells (Table II).

Though ^{3}H-thymidine incorporation was increased above control levels in P_3HR-1 cells that were incubated with NRBC or with P. falciparum-infected RBC, there were no significant differences in ^{3}H-thymidine incorporation between NRBC and P. falciparum stimulators in P_3HR-1 cells.

Counts per minute recorded for AG876 and P. falciparum as stimulator did not differ significantly from cpm recorded for AG876 cells with RPMI alone.

Though cpm for AG876 cells and NRBC were lower than those recorded for AG876 cells and RPMI and for AG876 cells and P. falciparum, the differences were not significant ($p < 0.1$).

Incorporation of ^{3}H-thymidine was depressed in BJAB cells (EBV-negative BL) when these cells were incubated with NRBC, P. falciparum-infected cells, the crude merozoite preparation, or with PHA (Table II). The lowered counts in BJAB cells with P. falciparum as stimulator different significantly from cpm for BJAB and RPMI ($p < 0.01$). The stimulation index was 0.83 for P. falciparum-infected RBC and BJAB. Though all other test stimulators lowered cpm for BJAB cells, the differences between tests and controls were not significant.

Quenching due to coloration caused by hemoglobin was a problem in preliminary experiments that were designed to measure DNA synthesis in BL cell lines after ^{3}H-thymidine incorporation. Results from an experiment where BL cell lines were incubated with the various stimulators for 48 hr prior to the addition of ^{3}H-thymidine are shown in Table III. The results presented in this table indicate that quenching due to hemoglobin is a serious problem in assays where RBC or lysates of RBC are used. Standard deviations greater than 20% of the mean cpm were recorded for some cultures (Table III). Variations in the cpm greater than 15 to 20% of the mean are indicative of technical errors or contamination (Bradley, 1980). In these studies, these variations are indicative of hemoglobin induced quenching. The following cpm were recorded for triplicate cultures of Raji cells and RPMI: 10,555; 11,364 and 12,939. When Raji-3 cells were incubated with P. falciparum-infected cells, the following cpm were recorded: 7094; 10,022 and 14,660. The standard deviation of the first set of counts with RPMI alone is 1444, or 12.6% of the mean of 11,453, whereas the standard deviation for Raji-3 cells and P. falciparum is 3815 or 36% of the mean of 10,592. H_2O_2 was added to all hemoglobin containing cultures after preliminary experiments showed that it reduced quenching but did not significantly alter cpm (Table IV).

Infected RBC nor NRBC contributed significantly to the counts reported in these studies (Table II).

Rosettes were never observed when P. falciparum-infected RBC or NRBC were incubated at 37°C or at 4°C with any of the cell lines tested. Parasites did not survive the 72-hr incubation with BL cell lines as determined from Giemsa-stained thin blood films.

TABLE I

VCA Expression in BL Cell Lines Incubated for 72 hr with *Plasmodium falciparum*

Cell line	Mean % cells fluorescing and standard error of the mean with		
	RPMI	NRBC	IRBC
EJAB	0	0	0
Raji-3	0	0	0
NAB	0	0	0
P_3HR-1	0.646 ± 0.29	0.69 ± 0.36	2.47 ± 0.71[a]
AC876	6.50	6.05 ± 1.43	9.53 ± 0.87[b]

[a]The *t* statistic for two means was calculated. No significant differences were found between NRBC and RPMI in P_3HR-1; however, the difference between the mean percentage for P_3HR-1 incubated with *P. falciparum* was significantly different from the means with RPMI or with NRBC ($p < 0.05$).

[b]$p < 0.1$.

TABLE II

Incorporation of ^{3}H-Thymidine into BL Cell Lines Incubated with *Plasmodium falciparum*[a]

Cell line	Mean cpm and standard error of the mean				
	RPMI	NRBC	IRBC	Merozoites	PHA
BJAB	548,891 ± 6,943	516,167 ± 27,997	454,995 ± 39,376	457,202 ± 13,981	462,256 ± 1,231
Raji-3	7,531 ± 431	20,272 ± 343[b]	24,495 ± 298[b]	13,291 ± 428[b]	N.D.[c]
NAB	388,916 ± 18,318	580,410 ± 18,941	585,048 ± 10,675	N.D.	N.D.
P_3HR-1	231,364	347,743 ± 53,666	310,931 ± 9,620	N.D.	N.D.
AG876	211,018 ± 18,044	194,707 19,876	261,948 ± 31,080	N.D.	N.D.
NRBC	192 ± 60				
IRBC	150 ± 37				

[a]One x 10^6 viable cells of each cell line were mixed with 0.1 ml of a 6% suspension of NRBC, of *P. falciparum*-infected RBC (IRBC), merozoites, medium or with PHA and incubated at 37°C for 18 hr prior to the addition of 1 μCi of ^{3}H-thymidine.

[b]$p < 0.001$ for IRBC and Raji-3; $p < 0.01$ for NRBC and merozoites.

[c]Not done.

TABLE III

Incorporation of ^{3}H-Thymidine into BL Cell Lines Incubated with *Plasmodium falciparum* for 48 hr

Cell line	Mean cpm and standard error of the mean[b]				
	RPMI	NRBC	IRBC	Merozoites	PHA
BJAB	436,071 ± 56,959	253,077 ± 94,755	208,268 ± 42,390	308,961 ± 49,636	578,351 ± 48,931
Raji-3	11,453 ± 834	9,962 ± 1,378	10,592 ± 2,203	13,049 ± 2,633	6,604 ± 244
AG876	124,470 ± 32,765	172,007 ± 14,471	140,384 ± 39,740	131,609 ± 14,615	137,626 ± 15,856

[a]One x 10^6 viable cells of each line were mixed with 0.1 ml of a 6% suspension of NRBC, *P. falciparum* infected RBC, crude merozoites preparation, medium or with PHA and incubated at 37°C for 48 hr prior to the addition of 1 μCi of ^{3}H-thymidine.

[b]Results are expressed as mean cpm ± the standard error of the mean of triplicate cultures except for AG876 tests which were performed in duplicate.

TABLE IV

Effect of H_2O_2 in Reducing Quenching in Test Cultures

Test	cpm	
	H_2O_2	No H_2O_2
BAJB + NRBC	432,480	133,475
BJBA + RPMI	538,979	549,079

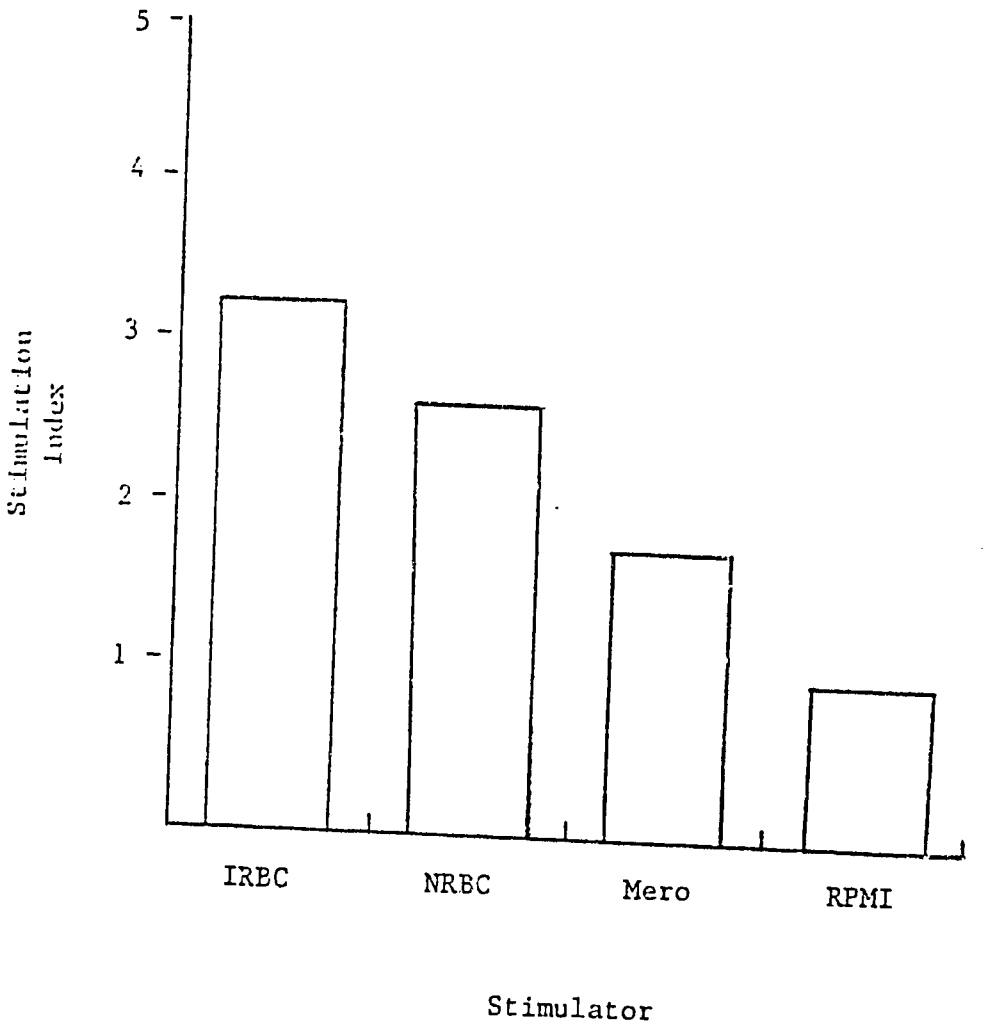

Figure 1. Stimulation indices for Raji cells incubated with NRBC, IRBC, or with merozoites. The stimulation index was calculated by dividing the cpm of Raji cells with a stimulator by the cpm of cells in RPMI alone.

DISCUSSION

Since BL was first described in Ugandan children by Burkitt in 1961, malaria has been postulated to be a factor in the development of this rare malignancy. Klein (1979) suggested that malaria may act as an environmental promoter of BL formation in malaria-endemic areas of Africa by promoting the proliferation of precancerous EBV-infected B lymphocytes. The experiments described in this report were designed to investigate *in vitro* the interactions between the human malaria parasite, *P. falciparum*, and the EBV in BL.

Plasmodium falciparum increased the expression of VCA in the lytic virus producer cell line, P_3HR-1, at 37°C. This cell line was not maintained at 34°C nor were the cells starved to enhance virus production. On the other hand, *P. falciparum* had no effect on VCA synthesis in the cell line that produces transforming virus, i.e., AG876.

The results obtained with P_3HR-1 and *P. falciparum* are indeed significant because EBV may remain latent indefinitely in the B lymphocytes of humans who have recovered from primary EBV infection (Thorley-Lawson, 1980). T lymphoctes prevent the malignant outgrowth of these infected cells (Thorley-Lawson, 1980; Whittle et al., 1984). Phytohemagglutinin increased EBV early antigen expression in Raji cells and other mitogens activate latent viruses (Tovey et al., 1979). The *P. falciparum*-induced increase in VCA synthesis in P_3HR-1 cells observed in this study may indicate increased EBV synthesis. Increased synthesis of EBV during *Plasmodium* infection may be the cause of increased anti-EBV capsid antigen antibody titers that have been recorded in children in Africa who later develop BL (de The et al., 1978). In Africa, development of BL coincides with the development of maximum antibody titers to *Plasmodium* (Morrow et al., 1976).

The most significant increases in DNA synthesis above control levels in BL cell lines as determined from ^{3}H-thymidine incorporation were in Raji-3 cells that were incubated with NRBC or with *P. falciparum*-infected RBC. Only in this cell line did *P. falciparum*-infected RBC significantly increase DNA synthesis above the level observed for NRBC. This result indicates that *P. falciparum* has a specific effect on Raji-3 cells; however, the effect of *P. falciparum* or of NRBC on the latent EBV genome in Raji cells cannot at this point be determined. The Raji cell line is an African BL line that contains latent EBV. These cells do not produce VCA; therefore, activation of virus by either of the two stimulators could not be determined in this study. EBV DNA in Raji cells replicates during the early S phase of the cell cycle when host cell DNA is also replicating (Gussander and Adams, 1984). Studies are now being designed to determine quantitatively whether or not EBV DNA replication accounts for some of the increased ^{3}H-thymidine incorporation observed in Raji-3 cells.

The highly significant increases in DNA synthesis observed in Raji-3 cells with P. falciparum and with NRBC may also indicate that Raji cells are more efficient antigen presenters than are the cells of the other BL cell lines. B cell tumors can function as antigen presenters (Chestnut et al., 1982).

All BL cell lines that contain the EBV genome except AG876 cells increased their DNA synthesis with NRBC and with P. falciparum-infected RBC. For NAB and P_3HR-1 cells, the differences between NRBC and P. falciparum stimulators were not significant. Only one type O positive donor of normal RBC was used for all of the studies described in this report. The responses observed may be to type O positive RBC antigens or to some other RBC constituent. White blood cells could not have caused the stimulation observed as RBC were aged before use and buffy coats were removed during the first and second RBC washes. Cpm for NRBC and for P. falciparum-infected RBC suspensions were well below background levels. Future studies with hemoglobin and with RBC ghosts should elucidate the significant phenomenon of RBC-induced proliferation in BL cell lines.

Though proto-onc genes have not been shown to cause cancer in humans (Duesberg, 1985), BL cell lines have been shown to contain translocations involving chromosome 8 and chromosomes 14, 2, and 22 which carry the genes for immunoglobulin synthesis (Taub et al., 1982). Chromosome 8 contains the myc gene whose transcription in normal cells is related to the rate of cell division (Stewart et al., 1984). In addition to the myc gene, BL also contains an activated Blym-1 gene. This gene shares sequence homology with transferin (Diamond et al., 1984). The Blym-1 gene from BL cells transforms NIH3T3 cells (Diamond et al., 1983).

The results presented in this report have clearly shown that P. falciparum influences EBV synthesis of capsid antigen. They also show that NRBC and P. falciparum can cause even more rapid proliferation in rapidly proliferating cells. Clearly, additional studies are needed to define the roles of RBC or RBC constituents and of P. falciparum in cell proliferation. The relationship of these factors to protooncogene expression and cell proliferation requires further study.

LITERATURE CITED

Bomford, R. and N. Wedderburn. 1973. Depression of immune response to Moloney leukemia virus by malarial infection. Nature 242, 471-472.

Bradley, L.N. 1980. Cell proliferation. In "Selected Methods in Cellular Immunology" (B. Mishell and S. M. Shiigi, eds.), pp. 153-172. W. H. Freeman and Company, San Francisco, CA.

Burkitt, D. and G.T. O'Connor. 1961. Malignant lymphoma in African children. I. A clinical syndrome. Cancer 14, 270-283.

Chestnut, R., S.M. Colon, and H.N. Grey. 1982. Antigen presentation by normal B cells, B cell tumors, and macrophages: Functional and biochemical comparison Journal of Immunology 128, 1764-1768.

Connan, G., M. Rassoulzadegan, and F. Cuzin. 1985. Focus formation in rat fibroblasts exposed to a tumour promoter after transfer of polyoma plt and myc oncogenes. Nature 314, 277-279.

Croce, C., J. Erikson, K. Huebner, and K. Nishikura. 1985. Coexpression of translocated and normal c-myc oncogene in hybrids between Daudi and lymphoblastoid cells. Science 227, 1235-1237.

Diamond, A., G. Cooper, J. Ritz, and M.A. Lane. 1983. Identification and molecular cloning of the human Blym transforming gene activated in Burkitt's lymphomas. Nature 305, 112-116.

Diamond, A., J. Devine, and G. Cooper. 1984. Nucleotide sequence of a human Blym transforming gene activated in a Burkitt's lymphoma. Science 225, 516-519.

Duesberg, P.H. 1985. Activated proto-onc genes: Sufficient or necessary for cancer. Science 228, 669-677.

Epstein, A.L., B.G. Achong, and Y.M. Barr. 1964. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. Lancet 1, 702-703.

Gabrielsen, A. and J. Jensen. 1982. Mitogenic activity of extracts from continuous cultures of Plasmodium falciparum. American Journal of Tropical Medicine and Hygiene 31, 441-448.

Gravely, S.M., J. Hamburger, and J.P. Kreier. 1976. T and B cell population changes in young and in adult rats infected with Plasmodium berghei. Infection and Immunity 14, 178-183.

Greenwood, B.M. 1974. Possible role of a B-cell mitogen in hypergammaglobulinaemia in malaria and trypanosomiasis. Lancet 1, 435-436.

Greenwood, B.M. A. Oduloju, and T. Platts-Mills. 1979. Partial characterization of a malaria mitogen. Transaction of the Royal Society of Tropical Medicine and Hygiene 73, 178-181.

Greenwood, B.M. J. Playfair, and G. Torrigiani. 1971. Immunosuppression murine malaria. I. General characteristics. Clinical and Experimental Immunology, 8, 467-478.

Gussander, E. and A. Adams. 1984. Electron microsopic evidence for replication of circular Epstein-Barr virus genomes in latently infected Raji cells. Journal of Virology 52, 549-556.

Haynes, J.D., C.L. Diggs, F.A. Hines, and R. Desjardins. 1976. Culture of human malaria parasites Plasmodium falciparum. Nature 263, 767-769.

Henle, W. 1979. The association of Epstein-Barr virus with Burkitt's lymphoma. In "Epstein-Barr Virus Oncogenesis" (T. Osato, ed.), pp. 27-39, Hokkaido University School of Medicine, Sapporo, Japan.

Jondal, M. and G. Klein. 1973. Surface markers on human B and T lymphocytes. II. Presence of Epstein-Barr virus receptors or B lymphocytes. Journal of Experimental Medicine 138, 1365-1378.

Kieff, E., T. Dambaugh, M. Heller, W. King, A. Cheung, V. Van Santen, M. Hummel, C. Beisel, S. Fennewald, K. Hennessy, and T. Heineman. 1982. The biology and chemistry of Epstein-Barr virus. Journal of Infectious Diseases 146, 506-517.

Klein, G. 1979. Lymphoma development in mice and humans: Diversity of initiation is followed by a convergent cytogenetic evolution. Proceedings National Academy of Science U.S.A. 76, 2442-2446.

Krettli, A. and R. Nussenzweig. 1974. Depletion of T and B lymphocytes during malarial infections. Cellular Immunology 3, 440-446.

Lambros, C. and J. Vanderberg. 1979. Synchronization of Plasmodium falciparum erythrocytic stages in culture. Journal of Parasitology 65, 418-420.

Morrow, R., N. Cutensohn, and P. Smith. 1976. Epstein-Barr virus-malaria interaction models for Burkitt's lymphoma: Implications for preventive trails. Cancer Research 36, 667-669.

Ohno, S., M. Bahovits, F. Wiener, J. Spira, G. Klein, and M. Potter. 1979. Nonrandom chromosome changes involving the Ig gene-carrying chromosomes 12 and 6 in pristane-induced mouse plasmacytomas. Cell 18, 1001-1007.

Person, H., L. Hennighausen, R. Taub, W. DeGrado, P. Leder. 1984. Antibodies to human c-myc oncogene product: Evidence of an evolutionarily conserved protein induced during cell proliferation. Science 225, 687-693.

Salaman, M.H., N. Wedderburn, and L.J. Bruce-Chwatt. 1969. The immunosuppressive effect of murine Plasmodium and its interaction with murine oncogenic viruses. Journal of General Microbiology 59, 383-391.

Shope, T., D. Dechairo, and G. Miller. 1973. Malignant lymphoma in cotton-top marmosets after inoculation with Epstein-Barr virus. Proceedings National Academy Science U.S.A. 70, 2487-2491.

Stewart, T.A., A.F. Bellve, and P. Leder. 1984. Transcription and promoter usage of the myc gene in normal somatic and spermatogenic cells. Science 226, 707-710.

Stambachova-McBride, J. and H.S. Micklem. 1978. Immunosuppression in murine malaria. II. The primary response to bovine serum albumin. Immunology 33, 253-259.

Strambachova-McBride, J. and H.S. Micklem. 1978. Immunosuppression in murine malaria. III. Induction of tolerance and immunological memory by soluble bovine serum albumin. Immunology 36, 607-614.

Sugden, B. 1977. Comparison of Epstein-Barr virus DNAs in Burkitt lymphoma biopsy cells and in cells clonally transformed in vitro. Proceedings of the National Academy of Sciences U.S.A. 74, 4651-4655.

Taub, R., I Kirsch, C. Morton, G. Lenoir, D. Swan, S. Tronick, S. Aaronson, and P. Leder. 1982. Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proceedings of the National Academy of Science U.S.A. 79, 7837-7841.

Taylor, D. and W. Siddiqui. 1978. Suppression of lymphocyte transformation by plasma from owl monkeys actuely infected with Plasmodium falciparum. Infection and Immunity 21, 147-150.

de The, G., A. Geser, N. Day, P. Tukei, E. Williams, D. Beri, P. Smith, A. Dean, G. Bornkamm, P. Toerino, and W. Henle. 1978. Epidemiological evidence for casual relationship between Epstein-Barr virus and Burkitt's lymphoma from Ugandan prospective study. Nature 274, 756-761.

Thorley-Lawson, D. 1980. The suppression of Epstein-Barr virus infection in vitro occurs after infection but before transformation of the cell. The Journal of Immunology 124, 745-751.

Tovey, M., G. Lenoir, J. Begon-Lours, H. Tapiero, and C. Rochette-Egly. 1979. The effect of mitogens on the expression of of Epstein-Barr virus antigens in human lymphoid cells lines. The Journal of Immunology 123, 138-142.

Vernes, A., J.D. Haynes, P. Tapchaisri, J.L. Williams, E. Dutoit, and C.L. Diggs. 1984. American Journal of Tropical Medicine and Hygiene 33, 197-203.

Waithe, W. I. and K. Hirschorn. 1979. Lymphocyte response to activators. In "Hanbook of Experimental Immunology" (D.N. Weir, ed.), pp. 26.1-26.10. Blackwell Scientific Publications, Oxford.

UNDP/World Bank WHO. 1980. Malaria. In "UNDP/World Bank WHO Fourth Annual Report, Special Programme for Research in Tropical Diseases," pp. 17-88.

Wedderburn, N., M. Compa, C.E. Tosta, and D.C. Henderson. 1981. The effect of malaria on the growth of two syngeneic transplantable murine tumors. Annals Tropical Medicine and Parasitology 75, 597-605.

Whittle, H.C., J. Brown, K. Marsh, B.M. Greenwood, P. Seidelin, H. Tighe, and L. Wedderburn. 1984. T-cell control of Epstein-Barr virus infected B cells is lost during P. falciparum malaria. Nature 312, 449-450.

Ziegler, J.L. 1981. Burkitt's lymphoma. The New England Journal of Medicine 305, 735-745.

ACKNOWLEDGEMENTS

The very fine technical assistance of LaMona Monteiro, Faye E. Phillips, and Kimberly Williams, all laboratory technicians who were employed part-time or full-time on Specific Support Grant No. DAN-1406-G-SS-4003-00, is acknowledged and sincerely appreciated.

The expert typing and editorial assistance of Ms. Alisia Cooper, Mrs. Maureen Crawford, and Mrs. Charlene Johnson, secretaries in The Department of Microbiology at Howard University, in the preparation of the research proposal and this report is also acknowledged and sincerely appreciated.