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X-ray sensitivity and DNA synthesis in synchronous culture of *Plasmodium falciparum*

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Abstract. The relationship between parasite development and sensitivity to irradiation with X-rays was investigated during a single synchronous cycle of *Plasmodium falciparum* in culture. The sensitivity of the parasites to irradiation was closely correlated with the phases of DNA synthesis. Their sensitivity was greatest at the ring stage in development, but decreased at the trophozoite stage when DNA synthesis begins. Lowest sensitivity was found when DNA synthesis was most rapid as the parasites were transforming from late trophozoite to schizont forms. These findings suggest that DNA is the target of the lethal radiation damage in the parasites.

Introduction

Our earlier work showed that "premunization" with an attenuated strain of *Plasmodium berghei* induced by irradiation can be considered as a means of immunoprophylaxis in rodent malaria (Waki et al. 1982). Later, the exposure of cultured *Plasmodium falciparum* parasites to irradiation at various stages of development showed that attenuation was possible also in human malaria (Waki et al. 1983).

Our aim is the isolation of stable attenuated parasites and, if this is achieved by mutation, the optimum conditions for the induction of mutants should be identified. It is known that exposure to ionizing radiation induces increased incidence of mutation in living mammalian cells, and that this occurs most readily at the time of DNA repair after sublethal damage.

In the present study, synchronous cultures were prepared by lytic treatment with sorbitol and the relationship between the parasite developmental stages and sensitivity to X-irradiation was investigated to obtain fundamental data on the radiobiology of malaria parasites.

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Materials and methods

Parasites. A strain of *Plasmodium falciparum* (designated GGG) used throughout the experiments was originally isolated in Geneva and has been maintained in culture in our laboratory since it was generously donated to us by Prof. P. Ambroise-Thomas in 1979. The parasites were cultured by the method of Trager and Jensen (1976) as previously described (Waki et al. 1983).

Synchronous culture. Synchronization of the culture at a stage of new ring forms was established by a modification of Lambros and Vanderberg's method (1979), as follows; cultures were treated with 5% D-sorbitol twice at intervals of 32 h. Twenty-eight hours after the second treatment, when the cultures were at a stage of transition from schizonts to ring forms, the parasites were again treated with sorbitol. Cultures were initiated (time zero), and the experiment was started 18 h afterwards to avoid the effect of sorbitol on the development of the parasites. Every 6 h from 18 h to 66 h thin blood films were made and at the same time, 0.5-ml aliquots of culture materials were transferred to either 24-well flat-bottomed culture plates (Linbro, New Haven, Conn.) for measurement of nucleic acids and protein synthesis, or into cryotubes (NUNC, Roskilde, Denmark) for irradiation. Parasitaemia and differential parasite counts were determined by counting the number of infected cells from a total of 10,000 RBCs or 200 parasitized erythrocytes in Giemsa-stained smears. In this study, the parasites were classified into the following three stages by morphological feature of the organisms; ring forms characterized by a clear central vacuole surrounded by blue cytoplasm with a single or double dots of chromatin and without pigment, trophozoites characterized by growing cytoplasm and nucleus, and schizonts defined by nuclear division of the parasites.

Measurement of DNA, RNA and protein synthesis. Incorporation of (^{3}H) hypoxanthine into DNA and RNA, and (^{3}H) isoleucine into protein was measured by adding 5 μCi (G-^{3}H) hypoxanthine (1 Ci/mM TRA 74, Amersham, Bucks, England) or 0.5 μCi L-(4,5-^{3}H) isoleucine (40 Ci/mM TRK 585, Amersham, Bucks, England) to each culture consisting of 5×10^8 erythrocytes with a 0.5% parasitaemia in 0.5 ml medium. Control cultures containing equivalent amounts of uninfected blood cells were incubated simultaneously, and incorporation of labelled compounds into DNA, RNA, or protein was determined at the same intervals as for the parasitized cultures. After incubation for 6 h, triplicate samples in each group were harvested. The red blood cells were washed with PBS and haemolyzed by suspending the cells in 5 mM phosphate buffer (pH 7.2). The parasites released were sedimented (5000 *g*, 10 min) and after removal of the supernatant, the pellet was suspended in 1 ml of PBS and the parasites disintegrated by freezing and thawing five times. For determination of DNA and RNA synthesis separately, each sample was divided into two parts; 0.5 ml of 2 *N* NaOH was added to one part for alkaline digestion of RNA, and 0.5 ml of distilled water to the other. Both were incubated overnight at room temperature by the method of Wilkinson and Pringle (1974). Alkaline-treated or control samples and non-treated samples for measurement of protein synthesis were added to 0.5 ml of 50% (w/v) trichloroacetic acid (TCA) to precipitate the cellular debris. After washing with 10% (w/v) TCA, the sediment was dissolved in 0.5 ml of solubilizer (NCS, Amersham, Bucks, England) and mixed in toluene containing 5.0 g 2,5-diphenyloxazole (PPO) and 0.1 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (Me_2POPOP) per liter and counted in a liquid scintillation counter (Aloka Liquid Scintilation Spectrometer LSC-673, Aloka, Japan).

Irradiation. Irradiation of parasites was carried out by X-ray deep therapy apparatus (180 KV, Toshiba) at varying doses; 20, 40, 60, 80, and 100 gray respectively by setting the dose rate at 8 gray/min (1 gray = 100 rad). After irradiation, each sample including controls was diluted with a suspension of fresh erythrocytes placed in culture dishes and the culture was continued.

Dose-response curves. The dose-response curves for the parasites against X-irradiation were obtained from the relationship between surviving fractions deduced by growth curves of irradiated parasites and X-ray doses as shown in Fig. 1. Multiplication of irradiated parasites was monitored daily and plotted on a logarithmic scale against time on a linear scale. Growth curves which showed straight lines were extrapolated on the vertical axis. The extrapolation

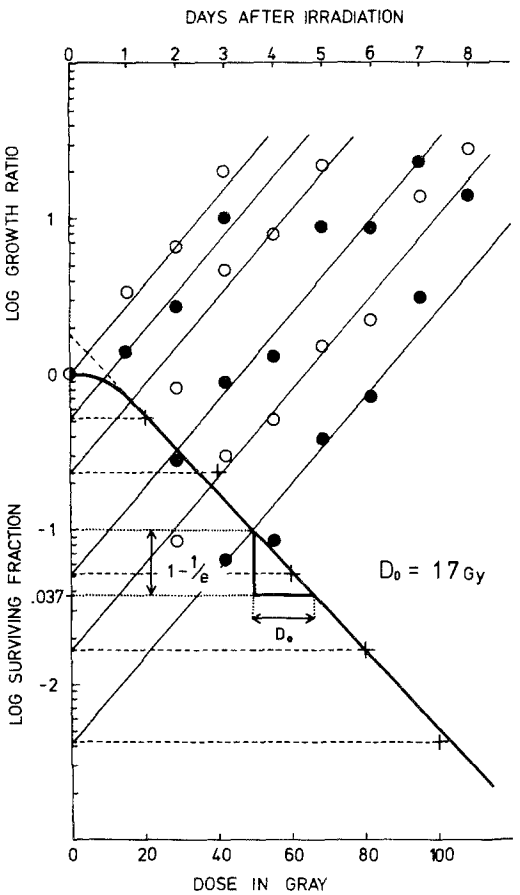

Fig. 1. Dose-response curve for *Plasmodium falciparum* exposed to X-irradiation. 10^6 parasites were irradiated at the dose levels shown and then cultured. The numbers of parasites were monitored daily and results were plotted as a ratio of the initial number. The dose-response curve was then obtained by extrapolation of growth curves of the surviving fractions on the vertical axis and comparison with irradiation doses. Sensitivity of the parasites to X-irradiation was expressed by the 37%-dose slope (D_0) which was determined from the straight portion of the dose-response relationship as the dose required to reduce the number of surviving parasites to 37%

value gave the survival rate after exposure to each dose of X-ray. The sensitivity of the parasites to X-irradiation was expressed as the 37%-dose slope (D_0 dose) which is required to reduce the number of surviving parasites to 37% ($1/e$).

Results

The asexual development and multiplication of parasites in synchronous cultures are shown in Fig. 2A. At the beginning of the experiment, 18-h culture after the last sorbitol treatment, the stages were in transition from ring forms to trophozoites, with 5% parasitaemia. The parasitaemia increased in the following 22 h (40 h). At 48 h all parasites were in the ring form and the parasitaemia showed a 4.4-fold increase. Ring stages persisted for the following 12 h, and at 66 h most parasites had transformed to trophozoites.

Figure 2B shows the course of DNA, RNA, and protein synthesis by parasites during one growth cycle. DNA synthesis as measured by ^{3}H-hypoxanthine incorporation into alkali-resistant parasite nucleic acids, was marked from 24 h to 42 h. This interval correlates with maturation of tro-

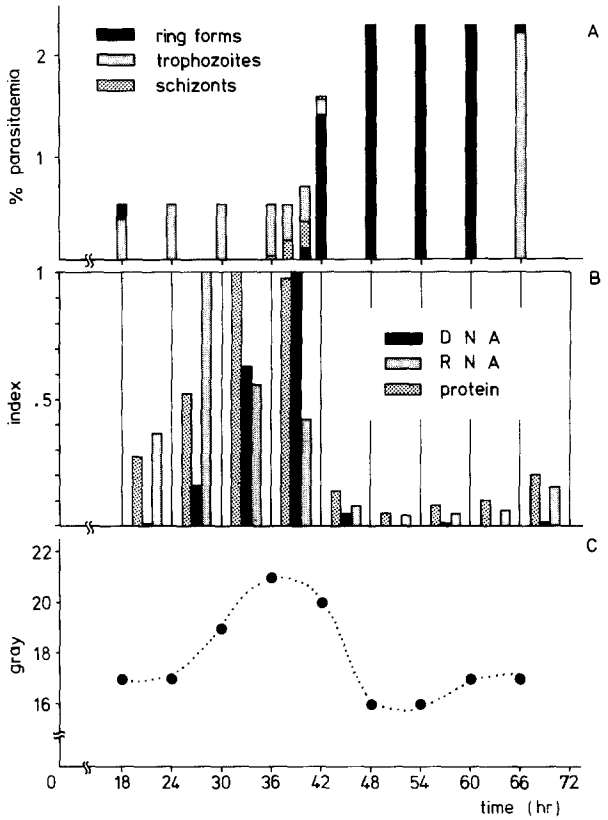

Fig. 2A–C. Development of stages DNA; RNA; protein synthesis and X-ray sensitivity during one growth cycle of *Plasmodium falciparum*. **A** Differential parasite counts in culture of *P. falciparum* following new ring forms at time zero. **B** Incorporation of ^{3}H-hypoxanthine into DNA and RNA and ^{3}H-isoleucine into protein of *P. falciparum* during a series of 6-h pulse-labelled culture. Each column represents the index of maximum value of radioactivity incorporated in each 6-h pulse labelling of DNA, RNA, and protein respectively. Maximum value which is shown by index in the orginate is equivalent to DNA; 3603 dpm, RNA; 68500 dpm and protein; 1507 dpm per 10^{7} parasitized RBCs respectively. Incorporation into DNA, RNA, and protein of uninfected control culture was less than 5% of the test groups. **C** Changes in sensitivity to irradiation expressed by D_0 dose which is required to reduce the number of surviving parasites to 37% during a cycle of *P. falciparum* development in culture

phozoites and development of schizonts. DNA synthesis showed maximum levels during the interval from 36 h to 42 h when schizogony was evident by microscopy. RNA synthesis, expressed by subtraction of ^{3}H-hypoxanthine incorporation into DNA from total value, increased from the beginning of trophozoite stages (at 18 h), reached a maximum level from 24 h to 30 h, and then decreased as the parasites matured into schizont stages. Incorporation of ^{3}H-isoleucine into parasite proteins increased in parallel with RNA synthesis. The maximum incorporation of isotope into protein occurred at 30 h to 42 h when parasites were in maturation stages.

Figure 2C shows the changes in sensitivity of the parasites to irradiation during one growth cycle. Dose-response curves were prepared every 6 h and the sensitivity expressed as the D_0 dose. From 24 h, the beginning of DNA synthesis, the parasites became progressively more resistant towards the latter part of the synthesis period. At 36 h, the parasites showed the highest resistance to irradiation with 21 gray D_0 dose. After transformation into ring forms, the sensitivity of the parasites was increased.

Discussion

In the present study the use of synchronous cultures and the measurement of DNA synthesis enabled us to examine the relationship between asexual parasite development and radiosensitivity more accurately than in our previous study (Waki et al. 1983).

DNA synthesis is continuous as parasites develop from trophozoites to schizonts and is prominent at the late stages of the development. It seems that, at least during the early stages of schizogony, DNA is still actively synthesized. It can be concluded that the first gap (G1) phase of DNA synthesis persists for nearly 30 h during the developmental stages from merozoites via ring to early trophozoites. Inselburg and Banyal (1984) recently showed that DNA synthesis of *P. falciparum* in culture is initiated at the early trophozoite stage of development, that is 30 h following the merozoite stage, and that it increased logarithmically until maturation. Our findings confirm their observations and correlate morphological developments of the parasite with phases of DNA synthesis in a cycle.

In the present experiment, it seems that the second gap (G2) phase, that is observed as an interval between S and mitosis (M) phases of mammalian cells, is not present or if it occurs, is of very short duration. The synthesis of RNA and protein paralleled one another and showed linear courses depending on development of the parasites.

The parasites showed an increased resistance to irradiation as the parasites moved to the late S phase. When the parasites moved out of the S phase into next G1 phase, their sensitivity increased. This pattern of response is similar to that obtained for HeLa cells (Terashima and Tolmach 1963) and Chinese hamster cells (Sinclair and Morton 1966). It seems that the lethal damage of radiation occurs mainly in DNA rather than in the membrane of the erythrocytes which harbor the parasites. The survival rate of normal mammalian cells and frequency of mutation are inversely proportional to each other with increasing dose (Thacker and Cox 1975). If the radiobiological characteristics of the parasites resemble those of mammalian cells, we should be able to induce mutant parasites readily in the most sensitive stage on the same basis. In our previous study, a few parasites which had survived a high dose of irradiation showed attenuated growth for several cycles in culture (Waki et al. 1983). Playfair and DeSouza have isolated partially attenuated parasites from a virulent *P. berghei* ANKA strain by repetitively cloning the growing parasites from mice inoculated with irradiated parasitized erythrocytes (personal communication). These

findings suggest that ring forms which are the most sensitive stage should be isolated from other stages before irradiation in order to obtain stable attenuated *P. falciparum* parasites from culture.

In vitro screening systems which could distinguish attenuated parasites from original organisms would be invaluable, although the virulence of isolates should be finally evaluated by in vivo studies using monkeys such as *Aotus trivirgatus*. Efforts to develop such systems are presently under way with the use of information from comparative studies with a radiation-induced attenuated strain of *P. berghei* (Waki et al. 1982) and the original virulent form.

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