Effectiveness of Gamma Rays in Attenuating Rodent Malaria Parasites of *Plasmodium berghei* in Blood of Mice

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ABSTRACT

Malaria is a major public health problem in Indonesia. Therefore, an effective vaccine against this disease is actively being sought by using gamma rays to attenuate the parasites. However, the safety and efficacy of the resulting vaccine are dependent on the precise irradiation dose. The aim of this research was to determine the exact time when the parasites are attenuated by gamma ray exposure. Mice blood containing *Plasmodium berghei* of 5,0 X 10^7 parasites/ml was irradiated with gamma rays at doses of 0, 150, 175 and 200 Gy (doses rate of 380 Gy/h) and then was injected intraperitoneally to mice at 0, 1, 2, 3, and 4 h post irradiation. The parasitemia (parasite density) in mouse blood was observed starting with day 2 and repeated every 2-4 days up to 28 days. The survival of the mice was also observed during the experiment. The results showed that the pre-patent period advanced with exposing infected blood to 150 and 175 Gy irradiations, suggesting some degree of attenuation. The amount of radiation required to render the parasites non-viable is about 175 Gy for an inoculum of a number of parasites, but a delay of 4 h resulted in the death of parasites. There was no difference in the infectivity of irradiated parasite injected 1 h and 2 h post irradiation in terms of parasitemia and the survival of mouse. For a dose of 200 Gy which was injected 2 h post irradiation, no parasitemia was found in the blood and animals which died after times varying from 1 to 4 weeks. We concluded that irradiated parasites should be injected into the host within 1 h after irradiation.

INTRODUCTION

Malaria kills more than one million people each year and is the leading cause of death of children in the developing world. Each year, there are around 500 million clinical episodes of this disease worldwide [1]. Malaria is a mosquito-borne disease and hence it can be controlled at the level of both human and mosquito. However, malaria control is a never-ending battle and requires long-term sustainability and commitment [2]. Malaria has significant impacts on health, social, and economic in countries where the disease is endemic [3]. Drug treatment is continually undermined by the development of drug-resistant parasite strains and insecticide resistance of the vector [4].

In Indonesia, the achievement of malaria control through elimination was done step by step from one island to another in order to gain a healthy community which is free from malaria transmission by 2030. In the Strategic Plan of the Ministry of Health 2010-2014 the malaria control is one target by reducing annual paining index from 2 to 1 per 1.000 people. However in 2009 the index was 1.85 per 1000 people, so it needs an effective effort such as vaccination [5]. In addition to other, existing measures, an effective vaccine will be needed to achieve eradication of malaria.

Among the practical applications of radiobiological techniques which may be of considerable interest for public health is the use of ionizing radiation in the preparation of vaccines. There has long been an interest at the use of attenuated parasite vaccines for malaria control [6], and so far the major efforts to develop such whole organism vaccines have focused on generating attenuated sporozoites by radiation [7,8]. More than four decades ago, Ruth Nussenzweig and co-workers [9] demonstrated that as a result of injection of mice with irradiation-attenuated sporozoites, the most infectious stage of the rodent malaria parasite *P. berghei* completely prevented onset of blood-stage parasitemia after challenge. This was a

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landmark finding which set the standards for immunological protection against malaria infection. Humans immunized with irradiated *P. falciparum* parasites have been effectively protected from subsequent challenge with homologous and heterologous infectious *P. falciparum* [7]. Irradiation introduces random mutations and breaks in the deoxyribonucleic acid (DNA) of parasites [10]. Immunity resulting from immunization with irradiated parasites is also multifactorial, includes reaction to malaria antigens on infected hepatocytes and has cell-mediated components whereas the synthetic vaccines do not induce such reactions.

The successful use of irradiated plasmodium as a vaccine depends on finding the radiation dose which significantly reduces the pathogenic effect of the microorganism without seriously impairing their immunogenic power [11]. Besides that, due to live-attenuated vaccine, the time of injection of irradiated parasites into host should be considered; thus, this paper presents the results of this study which shows that the first one hour post irradiation is the best time to inject parasites in terms of parasitemia and the survival of the mice.

EXPERIMENTAL METHOD

Parasites and mouse

Plasmodium berghei (strain Antwerpen-Kasapa, ANKA) infected mouse blood batches were received from Eijkman Institute for Molecular Biology, Indonesian Ministry of Research and Technology and National Institute of Health Research and Development, Indonesian Ministry of Health. Male Swiss-Webster mice *Mus musculus* ssp. (6–8 weeks old) were purchased from the National Institute of Health Research and Development, Indonesian Ministry of Health, and were housed at the animal facility at the Biomedical Laboratory of the Center for Technology of Radiation Safety and Metrology, National Nuclear Energy Agency of Indonesia (BATAN) and handled according to institutional guidelines. All procedures were reviewed and approved by the Animal Care and Use Committee at the National Institute of Health Research and Development, the Indonesian Ministry of Health.

Infection of mouse

The mice were divided into batches, each of which consisted of equal numbers of infected and control mice. The infected mice were intraperitoneally (IP) injected with about 5.0×10^7 *P. berghei* parasitized blood and control mice were sham injected with uninfected blood. Parasitaemia was monitored by Giemsa-stained blood smears using light microscopy. Parasitaemia and survival of mice were evaluated. Mice were sacrificed at days post-infection to harvest blood, liver and spleen for further analysis.

Irradiation of blood stage parasites

Infected batches of mouse blood with 10-25% parasitemia were irradiated *in vivo* inside a gamma irradiator (Cobalt-60 of the IRPASENA at the Center for Application of Isotope and Radiation, BATAN), with doses of 0, 150, 175 and 200 Gy at a dose rate of 380.0 Gy/h. The radiation dose was calculated from the machine specific estimate of 1505 Rads per minute. Afterward, the irradiated parasites were injected intraperitoneally to mouse at various times (0, 1, 2, 3, 4 h) post irradiation. During the extension times the irradiated parasites were kept in waterbath at 37^{o}C. Unirradiated and/or uninfected blood batches served as controls.

Parasitemia monitoring

Parasitemias in mice from each infected group were monitored started at day 2 and repeated every 2-4 days up to 30 days by conventional Giemsa staining. Thin blood films were prepared by tail bleeding; those blood samples were subsequently air dried and methanol fixed before staining. Staining was done with a 10% Giemsa solution for 10 minutes at room temperature. Slides were evaluated at a 1000× magnification (oil-immersion) using a Nikon E400 Eclipse microscope by reading 20 fields per slide. The total number of red blood cells counted was between 2500 to 6000 cells per treatment.

RESULTS AND DISCUSSION

The results of experiments at the 0 h injection (directly injected after irradiation) of irradiated parasites into mouse showed that the pre-patent period advanced with exposing infected blood to radiation, suggesting some degree of attenuation, except for 200 Gy where parasitemia was continuously increased. Higher parasites densities were found in mouse injected with 0 Gy irradiation where as high as 69.0% of parasitemia was observed on day 28 post injection. The amount of radiation

required to render the parasites non-viable is about 175 Gy for an inoculum of a number of parasites (Fig. 1). The infectivity of the parasites gradually decreased with increasing exposure to irradiation up to 175 Gy. For a dose of 200 Gy the parasitemia continued to rise until the animals died at times which varied from 2 to 5 weeks. To attenuate the parasites, the dose of irradiation is the most crucial factor. Additionally, another important information which is being investigated is the optimal number of irradiated parasites needed to obtain a maximal protection in mouse in term of parasitemia.

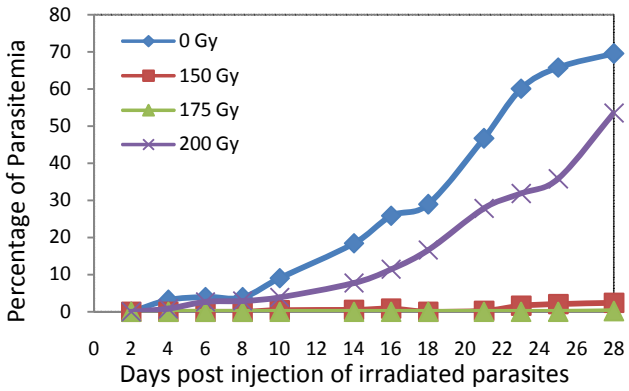

Fig. 1. The percentage of parasitemia in mouse blood at days post injection of irradiated parasited blood 0 h after irradiation. Notes that data for 0 and 200 Gy started on day 10 was obtained from only 2 surviving mice out of 3 in the initial experiment.

This experiment found that longer pre-patent period and lower parasitemia or day of its peak was seen on 150 and 175 Gy (Table 1). It is approved that live-attenuated parasites with irradiation are protective to the host where the contents of parasites in red blood cells are low. However there was no boost conducted to the primed mice, as done by other researchers, to gain the higher degree of acquired immunity which is directly proportional to the number of immunizing doses [12]. Challenges with live parasites is also required to test whether the injection of attenuated parasites as vaccine materials could elicit an antibody in the host; it is the most relevant approach to testing the efficacy of experimental malaria vaccines.

Table 1. Pre-patent period, peak of parasitemia and the survival time (days) of mouse post injection of irradiated *P. berghei* directly after irradiation.

Dose (Gy)	Pre-patent Period (day)	Day of peak of parasitemia (percentage)	Survival time (day)
0	2	28 (68.5%)	<30 (2 mice)
150	5	9 (2.2%)	>40
175	5	9 (2.4%)	>40
200	3	28 (54.2%)	<35 (2 mice)

Macroscopic observations, performed 2 weeks after injection, of the livers and spleens of mice directly after irradiation (0 h) showed that those organs exhibited fresh colors in mice injected with irradiated parasites and in uninfected control mice, whereas organs exhibiting much darker colors, containing hemoglobin digested by parasites, and larger than normal size were found in 0 Gy-irradiated mice (Fig. 2). These results seem to suggest that 175 Gy is the most effective dose for attenuating parasites, similar with the finding in the percentage of parasitemia in thin blood smear. The larger sizes of both organs in 0 Gy-injected mouse is due to the increasing number of leucocytes leading to splenomegaly during malaria infection and increasing the activity of erythropoiesis in spleen from abnormal immune response to repeated attacks of malaria [13].

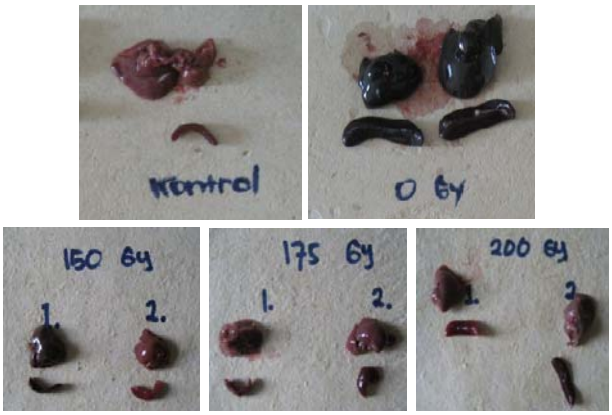

Fig. 2. Macroscopic observations of liver and spleen of mouse injected with 0, 150, 175 and 200 Gy irradiated parasites on day 28 post injection directly (0 h) after irradiation. Control is non infected mouse.

In the study on the time of injection after irradiation, there was no difference found in the infectivity of irradiated parasite injected 1 and 2 h post 150 Gy irradiation in terms of parasitemia except for day 8 to 12, but it became more infectious to the host at 16 and 18 days post injection. It is known that the injection of irradiated parasites is best conducted within 1 h, preferably within 0-30 minutes, after irradiation, as seen in Fig. 3. However, no significant difference was found between times of injection.

The percentages of parasitemia in the blood of mice injected with 175 Gy irradiated parasites whenever injected were lower than 12% in all cases and all mice survived during the experiment (Fig. 4). Again, this result indicates that irradiation of 175 Gy was more effective in attenuating parasites compared to 150 Gy. However, injection at 4 h post irradiation resulted in no parasitemia in any

mice, which was most probably due to the mortality of parasites before injection. For injections with 200 Gy-irradiated blood which were injected 2 h post irradiation, there was no parasitemia found in the blood of animals which died at times varying from 1 to 4 weeks (there was no data available for the extension times of 3 and 4 h). This implies that higher dose of irradiation and longer extension time before injection would result in the early death of parasites.

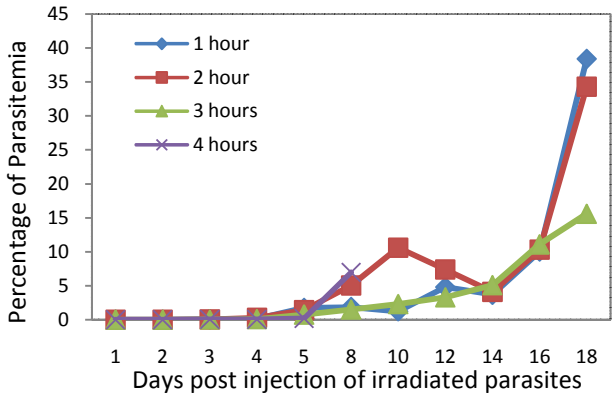

Fig. 3. Percentage of parasitemia in mouse blood injected with 150 Gy irradiated parasites at various times post gamma irradiation.

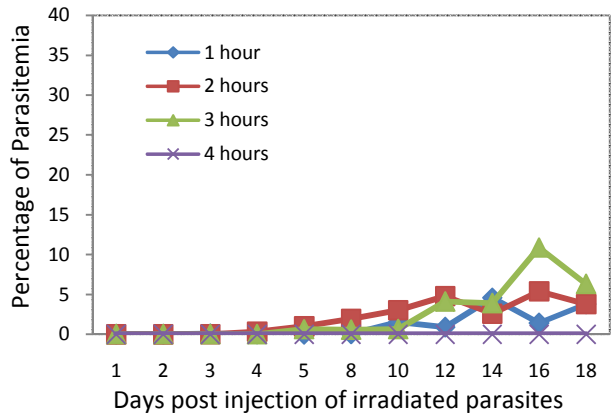

Fig. 4. Percentage of parasitemia in mouse blood injected with 175 Gy irradiated parasites at various times post gamma irradiation.

We are developing live-attenuated vaccines containing a version of the living microbe which has been weakened by ionizing radiation in the lab so it cannot cause disease. Because a live-attenuated vaccine injection is the most similar event to a natural infection, these vaccines are a good learning model for the immune system. They elicit strong cellular and antibody responses and often confer lifelong immunity. Despite the advantages of live-attenuated vaccines, there are also some disadvantages [14]. As found in research activities for doses more than 200 Gy, it is the nature of living things to change, or mutate, and the organisms used in live-attenuated vaccines are no different. The remote possibility exists that an attenuated microorganism in the vaccine could revert to a virulent form and cause disease. Also, not everyone can safely receive live-attenuated vaccines. For their own protection, people who have damaged or weakened immune systems cannot be given live vaccines. Another limitation is that live-attenuated vaccines usually needs to be refrigerated to stay potent. If the vaccine needs to be shipped overseas and stored by health care workers in developing countries that lack widespread refrigeration, a live vaccine may not be the best choice. Live-attenuated vaccines are relatively easy to create for certain microorganisms. As they evolve to adapt to the new environment, they become weaker with respect to their natural host, namely human beings [15].

Gamma radiation is widely used by many researchers to inactivate parasite for the preparation of vaccines, instead of traditional heat or chemical methods of inactivation. Gamma irradiation appears to create a vaccine which is more effective than so-called "killed" vaccines against disease, and has the added advantage of a longer storage life than "live" vaccines. Irradiation is a technically simple process which retains the structural features of the microbial pathogen without destroying the natural antigens or the intrinsic adjuvants. Therefore, a strong immune response is induced in the vaccinated host. Irradiation destroys the DNA, making the microorganism unable to replicate so it cannot establish an infection, but some residual metabolic activity may survive, so the irradiated microorgan-ism can still find its natural target in the host [16].

The dose of radiation with gamma rays used to attenuate plasmodium is the most crucial factor. Therefore, determination of the dose of gamma-irradiation which will produce attenuated parasites without affecting the pre-erythrocytic and erythro-cyte stabilities is the ultimate goal of many studies on irradiation vaccine. It is thought that the amount of radiation required to render the parasites non-viable is about 150 Gy for an inoculum of certain number of parasites [17,18]. It means that a most important step is to determine minimum dose of irradiation required to adequately attenuate each sporozoite.

A critical focus of vaccine development process has been to determine the minimum dose of radiation that adequately attenuates all sporozoites, and thus ensures that the vaccine will not cause malaria. Researchers are also interested at determining the highest dose of radiation that leaves the parasites able to elicit optimal protective

immunity. For this, Chattopadhyay R. *et al.* (2008) have studied in the *Plasmodium yoelii* rodent model system [19]. Exposure to 100 Gy completely attenuated *P. yoelii* sporozoites after direct injection of irradiated parasites. They also demonstrated that intravenous immunization of mice with *P. yoelii* sporozoite which had received 200 Gy, double the radiation dose required for attenuation, resulted in 100% protection. These results support the contention that a radiation-attenuated sporozoite vaccine for malaria will be safe and effective at a range of radiation doses and time of injection.

CONCLUSION

Irradiation with 150 and 175 Gy at dose rate of 380.0 Gy/h was effectively attenuate parasites and resulted in longer pre-patent period and the survival of mouse. This is supported by macroscopic observations. No difference occured in the infectiveness of irradiated parasite injected 1 and 2 h post irradiation of both doses. It is known that the injection of irradiated parasites into mouse should be conducted within 1 h or less after irradiation. Higher dose of irradiation and longer extension time before injection would result in the early death of parasites.

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