Infective *Toxoplasma gondii* Trophozoites Attenuated by Ultraviolet Irradiation

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Both the virulence and infectivity of *Toxoplasma gondii* trophozoites are affected by increasing fluences of ultraviolet irradiation. All mice receiving unirradiated virulent trophozoites intraperitoneally died in an average of 5 days. All mice inoculated with trophozoites irradiated with 20 J m^{-2} died in an average of 8 days. Fluences of 35, 55, and 70 J m^{-2} resulted in 90, 20, and 0% deaths, respectively. Fluences up to 720 J m^{-2} did not increase the number of nonviable trophozoites. The minimum fluence which prevented mouse deaths (70 J m^{-2}) also prevented trophozoite proliferation in baby hamster kidney (BHK-21) cell cultures. Minimum fluence-irradiated trophozoites attached to or entered BHK-21 cells or both at a rate similar to that for controls. BHK-21 cells fixed and stained 18 h after inoculation with unirradiated trophozoites contained both single and multiple trophozoites. In contrast, cell cultures inoculated with minimum fluence-irradiated trophozoites contained only single, nonreplicated trophozoites. No proliferation of trophozoites in these cultures was found as long as observed, 14 days after inoculation. Fluences of ultraviolet irradiation can therefore be determined which allow infection of cells but prevent trophozoite proliferation.

Blocking of cell division by irradiation (8) has proven useful for the development of vaccines against various pathogens, including obligate intracellular protozoan parasites (17). *Toxoplasma gondii* trophozoites exposed to ionizing radiation have been used as experimental vaccines against acute primary infections in mice (1, 2, 15). The protection reported (2, 15), although of short duration, was more complete against virulent challenge than has been obtained from killed trophozoite vaccines or by immunization by passive transfer (13). Seah and Hucal (15) and Chhabra et al. (2) did not determine whether their vaccine trophozoites were still capable of infecting nonprofessionally phagocytic cell lines. However, Lund et al. (9) earlier reported that trophozoites exposed to as high as 30 krads were still capable of entering HeLa cells without proliferating.

The ability to produce attenuated (unable to proliferate) infective (nonphagocytic) cells from a virulent strain of *T. gondii* is investigated in the present work. Nonionizing ultraviolet (UV) light instead of the higher energy ionizing sources is used to irradiate trophozoites obtained from cell culture rather than from the mouse peritoneal cavity. The recent development of a simple procedure (4) to efficaciously separate cell culture-propagated trophozoites from host cell debris has enabled us to produce adequate numbers of organisms which may prove qualitatively superior to animal propagation for vaccine development studies.

MATERIALS AND METHODS

Mice. The Nya:NYLAR strain of mice was used for routine intraperitoneal propagation of parasites and for studies on the effects of UV irradiation upon viability and virulence.

Cell cultures. Baby hamster kidney cells (BHK-21) cultured as monolayers in plastic culture flasks were also used for propagation of trophozoites and for experimentation. The culture medium was modified Eagle medium (MEM), with Earle basic salt solution (GIBCO Laboratories) plus NaHCO_3 (0.023 M, final concentration), penicillin (10,000 U/ml), streptomycin (100 $\mu\text{g/ml}$), inactivated fetal bovine serum (15% GIBCO), and tryptose phosphate broth (10%, Difco Laboratories).

Propagation of trophozoites was initiated by inoculating BHK-21 cultures with peritoneal exudate from mice infected 3 to 4 days earlier with *T. gondii* (RH). The inoculum, in a final volume of 0.2 ml, was adjusted with sterile phosphate-buffered saline (PBS, pH 7.2) to deliver 1 trophozoite for every 10 BHK-21 cells. The culture flasks were then incubated at 37°C until the host cell monolayer had become completely detached (4 or 5 days).

Parasite isolation. Cell culture-propagated trophozoites were separated from host cell components by glass fiber filtration and further purified by differential centrifugation, as described previously (4), except that the glass fiber filters were formed in 500- cm^3 plastic separatory funnels (Kartel) and filtrate centrifugation was reduced to $650 \times g$. A hemacytometer was used to count trophozoites.

UV irradiation. The purified trophozoites in the final pellets were suspended in PBS to contain 10^7 organisms per ml. Each suspension was placed in a glass petri dish or tray to a depth of $\leq 1 \text{ mm}$ and was

stirred continuously by rotation at 120 rpm during UV exposure at room temperature. The organisms received a fluence (measured with a Blak-Ray UV meter calibrated to measure 254 nm) of ca. $2.4 \text{ J m}^{-2} \text{ s}^{-1}$ from an unfiltered germicidal lamp (General Electric Co.) emitting primarily 254-nm light. Total fluences from 0 to $4,320 \text{ J m}^{-2}$ were used. The irradiated trophozoites were used for inoculation or study within 1 h after exposure.

Effect of various fluences upon the ability to proliferate. To determine the effect of various fluences upon trophozoite proliferation, we injected 0.5-ml suspensions containing 10^5 irradiated parasites intraperitoneally into mice. The mortality rate was used as an indicator of the effectiveness of various fluences in preventing proliferation leading to acute toxoplasmosis.

Once the minimum fluence (MF) necessary to reduce mortality to zero was established, the loss of proliferation of MF trophozoites was examined in cell cultures. Trophozoites irradiated at MF were inoculated into plastic culture flasks (25 cm^2) containing 3- or 4-day-old BHK-21 cultures in 40 ml of MEM. The cultures were incubated at 37°C for 18 h, at which time the medium (containing the extracellular trophozoites) was removed. Each flask was rinsed four times with 10 ml of fresh MEM. The cultures were then examined daily for 7 days with an inverted-stage microscope for the presence of intracellular parasites and plaque formation. Unirradiated trophozoites, virulent and heat killed (56°C , 1 h), were processed identically as controls.

Two weeks after the cell cultures had been inoculated, 0.5-ml suspensions of BHK-21 cells scraped from the monolayers of the MF-irradiated cultures and MEM from the same cultures were inoculated intraperitoneally into five mice. Five days later the peritoneal cavities were washed with 1 ml of PBS and examined directly with a microscope for the presence of trophozoites, and then subinoculated intraperitoneally into five more mice, each receiving 0.2 ml. There was no evidence of acute toxoplasmosis during the 28 days of observation.

Physical stability and disappearance from inoculum suspension. Irradiated and unirradiated trophozoite suspensions (each 1×10^6 in 0.1 ml of PBS) were inoculated into duplicate Lab-Tek tissue culture slide chambers containing either 0.2 ml of MEM alone or a 2-day-old culture of BHK-21 cells in MEM. At the end of each incubation time (0, 15, 30, 45, and 90 min; 37°C), the slides were rotated at 90 rpm for 2 min to resuspend any sedimented, unattached trophozoites, sample portions were taken, and the counts from the duplicate cultures were averaged.

Trypan blue exclusion. The trypan blue exclusion test for mammalian cell viability was used as a means of assessing cell damage due to irradiation. The irradiated organisms were tested for the ability to exclude trypan blue immediately after exposure and 1 h later at room temperature.

Oxygen consumption. Once the MF was established, the rate of oxygen consumption by trophozoite suspensions after MF irradiation was measured with a Clark-type oxygen electrode attached to an oxygen monitor and linear recorder. Samples of MF-irradiated

and unirradiated trophozoites were concentrated by centrifugation at $650 \times g$ for 5 min at 4°C immediately after irradiation. The trophozoite pellets were resuspended in Hanks balanced salts solution and combined to give 3×10^8 trophozoites per ml. Oxygen consumption at 37°C was measured immediately and after 1 h at 37°C .

Presence of irradiated trophozoites in BHK-21 cells. Culture slides (eight chambers per slide) were prepared with 4.5×10^4 BHK-21 cells in 0.2 ml of MEM per chamber. After 2 days of incubation these microcultures were each inoculated with 10^6 trophozoites in 0.1 ml of PBS. Each duplicate chamber received MF or $4,320 \text{ J m}^{-2}$ irradiated trophozoites or PBS only.

After incubation for 18 h at 35°C in an atmosphere of 5% CO_2 , the cultures were rinsed with Dulbecco PBS (GIBCO) and fixed with 5% glutaraldehyde (Tousimis) in Dulbecco PBS for 45 min at 4°C . The plastic chambers were removed, and the cells were stained with toluidine blue O (10). The gasket on each slide was removed with a razor blade, and the cells were mounted with Permount (Fisher) and a cover slip. Visualization of trophozoites surrounded by a vacuole (5) demonstrated the presence of parasites within host cells.

RESULTS

Proliferation in mice; MF. Mice receiving virulent unirradiated trophozoites intraperitoneally all died, as did those receiving trophozoites irradiated with a total fluence of ca. 20 J m^{-2} (Table 1). Fluences of 35, 55, and 70 J m^{-2} produced 90, 20, and 0% deaths, respectively. For this reason the MF was established as 70 J m^{-2} . Two mice from the group injected with MF-irradiated trophozoites were killed 4 weeks after inoculation and examined microscopically. No trophozoites were found in washings of the peritoneal cavities, and no cyst-like structures were found in brain tissue smears.

TABLE 1. Effect of ultraviolet irradiation on *T. gondii* trophozoite (RH) virulence in mice and BHK-21 cell cultures

Fluence (J m^{-2})	No. of mice dead ^a	Mean survival (days) ^a	Days to destruction ^b (mean)
0	20	5	5
20	20	8	5
35	18	11	7
55	4	13	8
70	0	— ^c	— ^d

^a 10^5 trophozoites per mouse were given intraperitoneally; there were 20 mice per group.

^b 10^5 trophozoites per BHK-21 culture; there were four cultures per group.

^c —, All were living after 180 days of observation.

^d —, No evidence of monolayer destruction, due to trophozoite proliferation within 14 days.

None of the mice receiving heat-treated trophozoites died.

Proliferation in cell culture. The attenuation of virulence by UV irradiation, as demonstrated in mice, was correlated with plaque formation and monolayer destruction in BHK-21 cell cultures (Table 1). Monolayer cultures inoculated with trophozoites either unirradiated or irradiated at 20 J m^{-2} were completely destroyed in 5 days. Trophozoites receiving intermediate fluences (35 and 55 J m^{-2}) also proliferated in culture, and complete monolayer destruction occurred after 7 and 8 days, respectively. MF-irradiated trophozoites did not produce plaques up to 14 days after inoculation and did not produce lethal infection in mice.

Dye exclusion test. All heat-treated trophozoites were unable to exclude dye; by the same test only about 1% of the purified unirradiated trophozoites accumulated dye. Trophozoites attenuated by the MF were still able to exclude dye (Fig. 1). Only at the highest fluence ($4,320 \text{ J m}^{-2}$) was 100% of the organisms stained when examined immediately after exposure.

Rate of attachment and/or entry into host cells. MF irradiation had no effect upon the attachment or entry of trophozoites into BHK-21 cells or both. In MEM only, the number of irradiated or unirradiated trophozoites remained almost constant for 90 min (Fig. 2). In the presence of cells, both irradiated and unirradiated trophozoites were removed from the medium at essentially the same rate.

MF-irradiated trophozoites were intracellular but not proliferating. Light microscopy revealed unirradiated and MF-irradiated trophozoites within the BHK-21 cells. The unirradiated trophozoites had reproduced, but multiple trophozoites contained within single vacuoles were not observed in cell cultures inoculated with MF-irradiated parasites. Trophozoites

FIG. 1. Effect of UV irradiation upon the ability of *T. gondii* trophozoites to exclude trypan blue. Each data point represents the mean of four counts per duplicate experiment ($n = 8$).

FIG. 2. Effect of MF (70 J m^{-2}) upon the rate of attachment or entry of *T. gondii* trophozoites into BHK-21 cells at 37°C or both. Each data point represents the mean of two counts per duplicate culture from two experiments ($n = 8$).

killed by $4,320 \text{ J m}^{-2}$ irradiation were not found in the cells.

Oxygen consumption. MF irradiation had no effect upon oxygen consumption, measured immediately after exposure and centrifugation, as compared to unirradiated, centrifuged controls. Both irradiated and unirradiated trophozoites experienced a 50% decline of oxygen consumption after a 1-h incubation in MEM at 37°C .

DISCUSSION

An effect of UV irradiation upon *T. gondii* trophozoites has been shown in only a few earlier reports (3, 14). Further investigations are facilitated by our purified virulent *T. gondii* trophozoites, which can be suspended at a standardized density in a simple salts medium. Since the trophozoites are unicellular and relatively small ($2\text{-}\mu\text{m}$ diameter) (5), the parasite chromosomes are likely to be critical target structures for photobiological damage (6).

T. gondii trophozoites attenuated by ionizing irradiation (9)—but not heat-killed or glutaraldehyde-fixed trophozoites (7)—can enter non-phagocytic cell lines. *T. gondii* trophozoites divide only within the host cells (5). Extracellularly they have a limited capacity for in vitro nucleic acid synthesis (11, 12). When photodamage is sufficient to block normal deoxyribonucleic acid replication, one might expect the damage to be expressed when cell division is attempted rather than by interfering events leading to host cell entry.

We have established an MF of UV irradiation which renders the trophozoites attenuated (non-

proliferative) but still infective, as revealed by their presence in BHK-21 cells. Heat-killed trophozoites were not found in BHK-21 cells, confirming the earlier report by Jones et al. (7). In contrast, the entry of virulent trophozoites was reported (16) to begin within minutes after inoculation of the cell cultures. We also found a rapid decrease in the number of virulent and attenuated trophozoites remaining extracellular or unattached to host cells. Further evidence that the MF-irradiated trophozoites are attenuated but infective, rather than killed, was provided by the results of the trypan blue exclusion experiments. Protection against virulent, lethal murine toxoplasmosis afforded by vaccination with the attenuated trophozoites is now being studied.

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