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Susceptibility of Artificially Released Zoitcs of *Toxoplasma gondii*, to Gamma Irradiation*

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Abstract: Bioassay in mice revealed, that tachyzoites, bradyzoites and sporozoites that had not been manipulated or artificially separated from their natural environment, were capable of resisting a dose of 250 Gy, whereas tachyzoites of a strain of *Toxoplasma* of low pathogenicity for mice (ME- 49), obtained by culture in the peritoneal cavity of immunodepressed mice, sporozoites artificially released from oocysts and bradyzoites artificially released from brain tissue cysts, resisted doses of only 150 and 100 Gy, respectively. These irradiated zoites were able to protect mice against infection and brain cyst formation. It is important to ascertain the sublethal dose of γ irradiation of zoites of *Toxoplasma gondii* for experimental immunization, so that zoites are capable of penetrating host cells without further multiplication, to elicit immunity without persisting in the host tissues.

Key words: Susceptibility, *Toxoplasma gondii*, artificially released zoites, gamma irradiation

Introduction

Toxoplasma gondii is a cosmopolitan protozoan parasite. It can cause fetal damage in humans and abortion in species such as sheep, goats, pigs and rabbits if first contracted during pregnancy (Dubey and Beattie, 1988). The incidence of human congenital toxoplasmosis has been shown to be 1 to 6 per 1,000 births. Although most infected newborns are asymptomatic at birth, adverse sequelae often develop later in life in a significant proportion of these congenitally infected children (Remington and Desmonts, 1990). In addition, reactivation of latent *T. gondii* infection often is fatal in patients who are immunosuppressed due to acquired immunodeficiency syndrome, cancer therapy or organ transplantation (Luft and Hafner, 1990).

Gamma irradiation of zoites of *Toxoplasma gondii* might be used for (a) decontamination of meat or vegetables (Dubey *et al.*, 1986; 1994, 1998 a) or for the development of (b) vaccines (Chhabra *et al.*, 1979; Dubey *et al.*, 1996, 1998b; Mas Bakal and In't Veld, 1979; Mitsuyoshi *et al.*, 2002; Omata *et al.*, 1996; Seah and Hucal, 1975). In the latter situation, it is important to ascertain the minimum dose of irradiation, so that zoites are capable of penetrating host cells, without further multiplication as demonstrated by light microscopy in tissue culture (Lund *et al.*, 1961) and by electron microscopy and bioassay (Mitsuyoshi *et al.*, 2002), to ensure that the host will not have a latent *Toxoplasma* infection.

There are three infectious stages of *T. gondii*: Tachyzoites, bradyzoites (in tissue cysts) and sporozoites (in oocysts) (Dubey and Beattie, 1988). Several investigations have been carried out to

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determine this critical dose of γ rays with tachyzoites of the RH strain of *Toxoplasma* (Dubey *et al.*, 1994, 1996, 1998a; Mitsuyoshi *et al.*, 2002). To compare the immunogenicity of RH tachyzoites, tachyzoites from tissue cyst-producing strains of *Toxoplasma*, bradyzoites and sporozoites of *Toxoplasma*, the zoites must be artificially released from macrophages, tissue cysts and oocysts, respectively. Since these procedures might not be completely harmless for the zoites, it was the aim of the present work to compare the sensitivity to γ rays, of zoites such as they are in nature and after the liberation, as free individuals in suspensions, so that they can be used in prototype vaccines.

The lethal dose of γ irradiation has been found for RH tachyzoites (Chhabra *et al.*, 1979; Kobayashi and Jacobs, 1963; Lund *et al.*, 1961; Mas Bakal and In 't Veld, 1979; Mitsuyoshi *et al.*, 2002). However, in the present study, tachyzoites of the complete (cyst and oocysts producing) strain ME-49 of *Toxoplasma* will be irradiated. To our knowledge, this has not been done before and to achieve it, mice must be artificially immunodepressed and tachyzoites liberated from macrophages from peritoneal exudates and irradiated. The lethal dose of γ irradiation for tissue cysts has been determined by Dubey *et al.* (1986), Dubey and Thayer (1994) and Song *et al.* (1993), but in the present study the lethal dose of bradyzoites of *Toxoplasma gondii* released from tissue cysts will be determined, something not performed before. For this, cysts will be separated from brain tissue of mice and the cysts wall will be digested with the technique developed by one of the authors (Freyre, 1995). The lethal dose of γ irradiation for sporulated oocysts has been estimated by Dubey *et al.* (1996, 1998a, b). In the present study, however, free sporozoites will be irradiated after liberation from oocysts, according to the method of Freyre and Falcón (2004). The lethal dose of γ irradiation for free sporozoites of *Toxoplasma gondii* has not been determined before, to our knowledge.

Materials and Methods

Animals

Twenty grams CF-1 mice were used to cultivate *Toxoplasma* in the peritoneal cavity, as donors of tissue cysts of *Toxoplasma* and for bioassays. A weaned cat of the European breed was used to obtain *Toxoplasma* oocysts. It was obtained from the breeding colony of the Laboratory for Toxoplasmosis, College for Veterinary Sciences, Montevideo. Both mice and the cat were free of *Toxoplasma* infection, as ascertained with the Direct Agglutination (DA) assay of Desmonts and Remington (1980), using 1:64 as the threshold titer indicative of *Toxoplasma* infection. The experiments performed comply with current laws of Uruguay.

Toxoplasma

Strains RH and ME49 of *Toxoplasma* were used. To obtain RH tachyzoites, the parasite was passed i.p. in mice every 3 days. To obtain tissue cysts of strain ME49, mice were inoculated s.c. with 10^3 tachyzoites. Mice were used as a source of tissue cysts 30-60 days after inoculation. Brains were removed and homogenized in 0.9% NaCl by passing them 12 times with a syringe through a 19 gauge needle. Cysts were counted under the microscope in $4 \times 25 \mu\text{L}$ aliquots of brain homogenate in 0.9% NaCl.

ME49 oocysts were obtained by feeding the brain and the carcass of a mouse with a persistent *Toxoplasma* infection to a weaned cat. Fecal specimens were collected 4-7 days after inoculation and oocysts were separated by sugar flotation (Frenkel, 1977). Oocysts were incubated in 2% sulfuric acid at room temperature with agitation for 4 to 7 days.

Separation of Toxoplasma Tissue Cysts from Brain Tissue and to Liberate Viable Bradyzoites

For this purpose, the method of Freyre (1995) was used. Brains were blended in a Waring blender with 20% dextran solution and the homogenate was centrifuged at 4,000 for 10 min. Cysts present in the sediment were digested by adding an equal amount of a solution containing 1 g NaCl, 1.4 mL HCl and 1 mg pepsin (1:60,000 assay activity) per 100 mL water and incubated at 37°C for 1 min.

Excystation of Viable Sporozoites

For this purpose, the method of Freyre and Falcón (2004) was used. Shortly: sporulated oocysts were incubated at 40°C for 2 h in an ultrasonic bath in a 1.4 % Na₂CO₃ solution in distilled water with 1% phenol red, 1,000 IU mL⁻¹ penicillin, 0.1 mg mL⁻¹ streptomycin and 10 µg mL⁻¹ amphotericin B that had been gassed with CO₂, to free sporocysts. Sporocysts were then resuspended in PBS pH 11 with 600 mg% bovine albumin, 10% bovine bile, 1,000 IU mL⁻¹ penicillin, 0.1 mg mL⁻¹ streptomycin and 10 µg mL⁻¹ amphotericin B and 1% phenol red. CO₂ was gassed into the tube until the solution turned yellow and the mixture was incubated at 40-42°C for 1 h. Sporozoites were then resuspended in PBS pH 7.2 with 600 mg% bovine albumin.

Method to Obtain ME-49 Tachyzoites

To obtain ME-49 tachyzoites, ME-49 cysts were i.p. inoculated in mice injected with 4 mg cortisone acetate (Depomedrol, Upjohn). Four to six days later, tachyzoites were recovered by washing the peritoneal cavity with PBS pH 7.2.

Irradiation of Toxoplasma

Tachyzoites, bradyzoites and sporozoites were suspended in PBS pH 7.2 with 600 mg% bovine albumin at a concentration of 5×10⁴ zoites mL⁻¹. Tissue cysts were suspended in the same fluid at a concentration of 10² mL⁻¹. Oocysts were suspended in distilled water at a concentration of 5×10³ oocysts mL⁻¹. These samples were irradiated in a Co₆₀ Gamma Chamber 4000 A (Isotope group, Bhabha Atomic Research Centre, Trombay, Bombay, India), to receive 100-300 Gy (Table 1).

Bioassays

Immediately after irradiation, each of 5 mice per irradiation dose, was inoculated i.p. with approximately 10⁴ tachyzoites, bradyzoites or sporozoites, or 10³ oocysts or 20 tissue cysts of *Toxoplasma* in 0.2 mL fluid. Thirty days after inoculation the mice were killed by cervical dislocation and their brains removed. Squash preparations were made with one of the hemispheres of the brain in 5×5 cm glass slides, made upon request and screened for *Toxoplasma* cysts. When cysts were not seen by microscopy, the other hemisphere was homogenized by passing brain tissue 12 times in a 3 mL hypodermic syringe with a 19 gauge needle, in 1 mL NaCl 0.9%. The emulsion was i.p. inoculated in 2 mice. Thirty days later, the mice were bled and their sera screened for *Toxoplasma* specific antibodies with the DA test.

Immunization of Mice With Irradiated Zoites

To test if irradiated zoites were immunogenic, 41 mice were intraperitoneally (i.p.) inoculated with 10⁶ tachyzoites of strain ME-49 of *Toxoplasma* irradiated with 250 Gy and 38 mice were i.p. inoculated with 10⁶ bradyzoites of the same strain, irradiated with 150 Gy. Three immunizations were performed with each group, at 15 days intervals. Fifteen days after the last immunization, the mice in both groups and those in a control group of 10 mice, were challenged with 20 sporulated oocysts of strain ME-49 of *Toxoplasma*. Mice were killed 30 days later and toxoplasma brain cysts were counted at 200 x by squashing each hemisphere between two 5×5 glass slides.

Results

Results obtained are from Table 1 and 2 shows that all the zoites survived after receiving 150 to 250 Gy. Three hundred Gy were lethal for tachyzoites, bradyzoites and sporozoites. The zoites that had not been the subject of manipulations (RH tachyzoites, bradyzoites in intact cysts and sporozoites contained in sporulated oocysts), survived after receiving 250 Gy (with the exception

Table 1: Inactivation of tachyzoites, bradyzoites and sporozoites of *Toxoplasma gondii* by gamma irradiation

Stage	Dose of gamma rays (Gy)				
	100	150	200	250	300
RH tachyzoites	+	+	+	+	-
ME49 tachyzoites ⁽¹⁾	+	+	+	-	-
	+	+	-	-	-
ME49 bradyzoites ⁽¹⁾	+	-	-	-	-
	+	-	-	-	-
ME49 cysts in brain ⁽¹⁾	+	+	+	+	-
tissue, emulsified	+	+	+	+	-
ME49 Sporozoites	+	+	-	-	-
ME49 sporulated oocysts ⁽¹⁾	+	+	+	-	-
	+	+	+	+	-

+ = positive bioassay; - = negative bioassay. ⁽¹⁾ each line a different trial

Table 2: Protection against brain cyst formation in mice immunized with tachyzoites and bradyzoites of strain ME-49 of *Toxoplasma gamma* irradiated and later challenged with 20 oocysts of the same strain

	Experimental groups		
	Immunized with tachyzoites	Immunized with bradyzoites	Non immunized controls
Nr mice	41	38	10.000
% mice with brain cysts	12	24	50.000
Average Nr of brain cysts among all the mice	3	2	1.290
Average Nr of brain cysts among mice with brain cysts	23	7	2.584

of one of the trials with sporulated oocysts). Conversely, the zoites that were obtained after artificial rupture of macrophages (ME-49 tachyzoites), after artificial liberation from oocysts, or after artificial digestion of tissue cysts, were inactivated after receiving doses of 250, 200 and 150 Gy, respectively.

The number of brain cysts formed in mice immunized with irradiated tachyzoites or bradyzoites was reduced as compared to a control group of non immunized mice that had been similarly challenged with toxoplasma oocysts (Table 2).

Discussion

The objectives of the present study have been accomplished, as it was determined that when tachyzoites of a strain of *Toxoplasma* of low pathogenicity for mice (ME-49), obtained by culture of 4 to 6 days duration in the peritoneal cavity of immunosuppressed mice, sporozoites artificially released from oocysts and bradyzoites artificially released from tissue cysts, resisted doses of only 150-200, 150 and 100 Gy, respectively. On the other hand, it was also found in the present work that 300 Gy is lethal for RH tachyzoites, ME-49 cysts in brain emulsion and ME-49 sporulated oocysts. It is likely that the differences observed between naive zoites and zoites artificially obtained or separated, with regards to their susceptibility to γ rays, are due precisely to the processes they have been subjected to.

Other authors have found that the lethal dose of γ irradiation is between 150 and 300 Gy for RH tachyzoites (Chhabra *et al.*, 1979; Kobayashi and Jacobs, 1963; Lund *et al.*, 1961; Mas Bakal and In 't Veld, 1979; Mitsuyoshi *et al.*, 2002); from 400 to 600 Gy for tissue cysts (Dubey *et al.*, 1986; Dubey and Thayer, 1994; Song *et al.*, 1993) and 200-400 Gy for sporulated oocysts (Dubey *et al.*, 1996, 1998 a, b). The reason for the differences observed is difficult to ascertain, since inactivation of zoites by γ rays is independent of the strain of *Toxoplasma* (Dubey and Thayer, 1994; Song *et al.*, 1993) and of temperature, in the range of -4 to 16°C (Dubey and Thayer, 1994). According to Dubey and Thayer (1994), minor differences between irradiation studies are probably related to the technique of measuring the irradiation dose, the thickness of the samples irradiated and the presence or absence of air in the irradiated samples.

Aside from showing the reduction in viability of zoites of *Toxoplasma gondii* separated from their natural environment, the immunogenicity of irradiated zoites was demonstrated in the present work. The strong reduction in the number of toxoplasma brain cysts formed in mice immunized with zoites artificially released and then irradiated, as compared to a control group of non immunized mice (Table 2), is an indication of the immunogenicity of the zoites. It is also an indication that zoites were alive, since killed *toxoplasma* zoites are not immunogenic (Elsaid *et al.*, 1999; Waldeland and Frenkel, 1983). Zoites were not recovered by mouse inoculation, however, indicating that their reproduction was abrogated.

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