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Induction of Interferon in Mice Infected with *Toxoplasma gondii.** (31624)

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Since the observation of Rotem *et al*(1) that interferon can be elicited by non-viral nucleic acids, the recognized number of such non-viral inducers of interferon has steadily increased. About 15 of these inducers have been described. Some of them are macromolecular compounds(2,3), others are microorganisms(4).

Because *Toxoplasma gondii* is an obligate, intracellular parasite, which, *in vivo*, multiplies in cells of the reticuloendothelial system (known to be a rich source of interferon(5)) it was of interest to determine whether *T. gondii* is capable of inducing interferon.

Materials and methods. The parasite. The RH strain(6) of *T. gondii* was maintained in CFW, 3-4-week-old male mice by serial intraperitoneal passage every 4 and 3 days alternately. At the time of each passage the peritoneal exudate was cultured for bacteria in thioglycollate broth, diluted 1:20 with isotonic saline, and inoculated intraperitoneally into healthy mice (0.2 ml per mouse).

Infection of cell cultures. Saline suspensions of peritoneal exudate containing *T. gondii* were centrifuged at 55 *g* for 5 minutes. The supernate was removed, centrifuged at 700 *g* for 10 minutes, and the sediment resuspended in Eagle's minimum essential medium (MEM). The *T. gondii* concentration (as determined by direct counting in a hemocytometer) was 16×10^6 per ml. The suspension was then diluted further 100 × in MEM with 20% heat inactivated calf serum. Clone L-929 (murine fibroblast) cells (ATCC) were grown in 60 mm plastic petri dishes (Falcon) in M199 with 5% fetal calf serum. The cultures were infected with the parasite approximately 1 day prior to reaching a complete monolayer (*i.e.*, 50-100 cells per microscopic field, at 450× magnification).

Infection of mice. Peritoneal exudate from mice infected 3 days previously with toxoplasma, was diluted 1:20 with saline and inoculated intraperitoneally into 3-4-week-old CFW male mice. The exudate was cultured in thioglycollate broth. No bacteria were recovered. A portion of the inoculum was filtered through an 0.45 Millipore filter and applied to murine fibroblast (L-929) monolayers. No cytopathogenic effect was observed. In addition, when cells pretreated in this manner were challenged with vesicular stomatitis virus (VSV) (Indiana strain), there was no inhibition of viral multiplication. These observations weigh against the presence of viral agents capable of multiplication or direct interference in the murine cell system employed. Each mouse received either 0.2 ml of toxoplasma suspension or 0.2 ml of the control solution. At different

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time intervals after the infection, mice were anesthetized with pentobarbital sodium 0.05 mg/g. Using sterile precautions, skin over the thorax was removed, and a cardiac puncture with a #22 needle was done. Blood was aspirated, pooled separately for the infected and the control mice, allowed to clot at room temperature for 30-60 minutes, and centrifuged at 900 *g* for 10 minutes. The sera were separated and stored at 4°C. Sera from infected mice were found not to contain any toxoplasma when applied to the L-929 cells.

Interferon assay. The method of interferon assay has been described in detail previously (3). L-cells were grown in 60 mm petri dishes (Falcon), in M199 with 5% fetal calf serum. When complete cell monolayers were achieved, they were overlaid in duplicate with 2 ml aliquots of serial 2× dilutions of the material to be tested for interferon. Following 6 hours of incubation, dishes were drained, washed with phosphate buffered saline, and challenged with approximately 50 plaque forming units of vesicular stomatitis virus. After a 30-minute adsorption, 5 ml of nutrient agar (1% Ionagar with M199 and 1% fetal calf serum) were added to each dish, and incubation resumed for an additional 48 hours. At that time monolayers were stained with neutral red (0.02%), and the plaques counted. Interferon titer was taken as the highest dilution of the test material which reduced the plaque number to 50% of the control number of plaques.

Results. Attempted production of interferon in L-cell culture. L-929 cell monolayers grown in petri dishes were infected with 8×10^5 toxoplasma in 5 ml of MEM per dish, and control dishes received 5 ml of the media without toxoplasma. Three infected and 2 control cultures were removed at 3, 8, 24, 48, 72 and 96 hours. The supernate from each dish was aspirated, pooled separately for infected and control plates for each time of sampling, filtered through an 0.45 Millipore® filter and stored at 4°C until assayed for interferon. The monolayers were fixed for 1 minute with methanol, stained with Giemsa stain, and then evaluated under oil immersion for the extent of infection.

Kinetics of the *T. gondii* infection are summarized in Table I. Of the cells counted 100% were infected by 72 hours, and no cells remained attached by 96 hours. Prior to filtration, tissue culture fluids at 96 hours contained approximately 5×10^6 toxoplasma per petri dish sampled. When the cell culture fluids were assayed for interferon, no inhibition of the VSV plaque size or number was found even at the lowest (1/4) fluid dilution.

TABLE I. Kinetics of *Toxoplasma gondii* Multiplicaton in Infected L-Cell Monolayers.

Hr after infection*	% of L-cells infected†
3	15
8	10
24	12
48	81
72	100

* Infecting dose was 8×10^5 toxoplasma per cell culture.

† Percent was determined by counting 400 cells at 1000 × magnification.

Production of interferon in mice. In the first experiment 0.2 ml of the saline suspension containing 2×10^6 toxoplasma was inoculated into 8 mice. Four control mice were given 0.2 ml of the saline diluent. At 8, 24, 48 and 72 hours after infection, mice were bled in the described manner. Pooled sera were stored at 4°C and then assayed for interferon.

Sera from the infected mice had significant levels of interferon beginning with the 24-hour sample (Table II). The highest titer (480 units) was found at 24 hours, and thereafter the titer diminished though interferon was detectable for at least 72 hours. Control sera from mice injected with saline showed no inhibition of VSV plaque size or number at the lowest serum dilution tested (1/12).

In the confirmatory experiment, 14 mice

TABLE II. Levels of Serum Interferon in Mice Following Infection with *Toxoplasma gondii.*

	Interferon concentrations*	
Hr after infection	Infected mice†	Control mice‡
8	<24	0
24	480	0
48	384	0
72	346	0

* Units per 2 ml of serum.

† Infected with 2×10^6 toxoplasma intraperitoneally.

‡ Given 0.2 ml saline intraperitoneally.

TABLE III. Physicochemical and Biological Properties of Interferon Induced by *Toxoplasma gondii* Infection in Mice.

Physicochemical properties (treatment of specimens)	Interferon concentrations*
Untreated serum	768
Dialysis against pH 2/24 hr	384
Centrifugation at 105,000 g/2 hr	768
Heating at 56°C/30 min	144
Treatment with trypsin 500 μg/ml at 37°C/60 min	0

Biological properties	Effect of treatment
Destruction of VSV infectivity by incubation with inhibitor at 37°C/60 min	none
Effect in cells of heterologous species	"

* Units per 2 ml of serum.

were each given 1.5×10^6 toxoplasma. Six control mice were inoculated with 0.2 ml of a turbid saline suspension of cells derived from a normal mouse spleen. All mice were bled at 24 hours.

The titer of interferon in the pooled sera from the infected mice was 768 units. Interferon was not demonstrated in control mice.

Characterization of the inhibitor as interferon. In order for a viral inhibitor to be classified as "interferon" it has to possess certain physico-chemical characteristics, and fulfill certain biological criteria. As shown in Table III, the viral inhibitor was not significantly altered by dialysis against a pH 2 buffer for 24 hours, indicating acid stability and nondializability. It was not removed by centrifugation at 105,000 *g* for 2 hours. It was significantly reduced in titer (81%) upon heating at 56°C for 30 minutes. The relative thermolability of mouse interferon, which distinguishes it from those of other species, has been reported previously(4,7). The inhibitor was completely inactivated by trypsin.

The inhibitor was not directly virucidal when incubated with VSV. Finally, the inhibitor did not suppress the formation of VSV plaques even at its lowest dilution (1/12) in cells of human origin (Clone 1-5C-4(8)) thus confirming its species specificity.

Discussion. The data presented here indicate that *T. gondii* is capable of eliciting an interferon in the sera of infected mice. That induction of interferon was due to infection with the toxoplasma, and not to other material in the inoculum is supported by: 1) the absence of demonstrable viable micro-organisms other than toxoplasma in the inoculum; 2) the absence of interferon production in mice injected with normal mouse tissue; and 3) the difference in kinetics of interferon production from that seen with endotoxin—a potential contaminant of animal tissues.

The interferon response following infection with *T. gondii* is considerably slower than that elicited by viral or bacterial agents. In the latter systems interferon serum levels peak at between 2 to 16 hours and are diminished or undetectable by 24 hours(4,9). One explanation for this could be the much slower generation time of toxoplasma in cells (*i.e.*, 5-10 hours and cell burst time of 36-48 hours) when compared to viruses or bacteria.

It is concluded that the parasite, *Toxoplasma gondii,* should be included in the list of non-viral microorganisms, such as *Brucella abortus, Salmonella typhimurium, Serratia marcescens*(4), *Rickettsia tsutsugamushi*(10). and TRIC agents(11), which have been shown to induce interferon. The present studies, and the corroborative report by M. M. Freshman *et al* also appearing in this issue of Proc. Soc. Exp. Biol. and Med.(12), represent the first demonstration of interferon induction by a protozoan.

Summary. Infection of mice with the protozoan *Toxoplasma gondii,* resulted in the appearance in their sera of a viral inhibitor possessing the major physico-chemical and biological properties of interferon. This interferon was undectectable in the sera at 8 hours following infection, reached a peak at 24 hours, and persisted for at least 72 hours. Infection of L-929 (murine) cell cultures with toxoplasma did not elicit the formation of detectable interferon in the culture fluids.

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In vitro and *in vivo* Antiviral Action of an Interferon-Like Substance Induced by *Toxoplasma gondii.** (31625)

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A variety of RNA- and DNA-containing viruses induce interferon production in different animal species. Among these are myxoviruses(1), pox viruses(2), picornaviruses(3), arboviruses(4), papovaviruses(5), herpes viruses(6), and adenoviruses(7). Several nonviral interferon inducers have also been discovered, including bacteria(8), rickettsia(9), Trachoma-inclusion conjunctivitis agents(10), pleuropneumonia-like organisms(11), bacterial endotoxin(12,13), fungal products (Statolon(14) and Helenine(15)), phytohemagglutinin(16), and cyclohexamide(17).

Toxoplasma gondii is an obligate intracellular protozoan parasite which infects most mammali cells except for the non-nucleated red blood cells(18). In many species including man, it can produce either congenital or acquired infection. These studies were undertaken to determine whether this agent is capable of inducing antiviral protection.

Materials and methods. Toxoplasma gondii. Toxoplasma, Sabin strain, (RH) were passaged in 20 to 25 g Swiss-Webster mice by intraperitoneal (IP) injection of 4×10^6 organisms (0.2 ml). At 3 days mice were tapped for peritoneal fluid (PF), and an inoculum of 4×10^6 organisms was injected into a new series of mice (45 g retired breeders) from which the PF for these experiments was obtained. On occasion, the PF was filtered through a Baird-Tatlock filter(19) to eliminate the white blood cells. By 5 days these RH-infected mice were usually dead. The PF was centrifuged at 3500 rpm for 30 minutes, and the supernatant collected for assay of antiviral activity. Serum was collected by bleeding from the axilla. Both PF supernatant and sera were centrifuged at 70,000 $\times$ *g* for one hour before further study to remove toxoplasma.

An avirulent strain of *Toxoplasma gondii* (ME-49) was also used for mouse (20-25 g) inoculation. Some mice chronically infected with this strain have remained alive up to a year. Cysts for use in these experiments were obtained from mouse brain which had been ground and diluted to a concentration of approximately 10 cysts/0.2 ml.

Tissue culture. A Spinner-adapted interferon-sensitive line of cells obtained from Dr. J. Younger of the University of Pittsburgh was grown in Eagle's Minimum Essential Medium (MEM)(20) supplemented with 10% calf serum and penicillin, streptomycin, and mycostatin. A plaque reduction assay (21) was used employing bovine vesicular stomatitis virus (VSV)(22) as challenge virus. Before VSV challenge all monolayers were rinsed with 4 ml of MEM. The titer of antiviral activity was expressed as the reciprocal

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