Re-examination of resistance of *Toxoplasma gondii* tachyzoites and bradyzoites to pepsin and trypsin digestion

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SUMMARY

The effect of digestion in trypsin and acid pepsin on *Toxoplasma gondii* tachyzoites and bradyzoites was re-evaluated because of recent use of this method to distinguish tachyzoites from bradyzoites. *Toxoplasma gondii* tachyzoites survived better in 0·5% trypsin solution for 1 h than in 1·0% solution, and occasionally survived for 2 h in acid pepsin solution. Extracellular tachyzoites (⩾ 1000) were also infectious orally to mice and cats. Bradyzoites survived equally in trypsin and acid pepsin solutions but the digestion of brain tissue in 0·5% trypsin solution was better than in acid pepsin solution. The resistance to digestion in acid pepsin solution is not a reliable method to distinguish tachyzoites from bradyzoites.

Key words: *Toxoplasma gondii*, tachyzoites, bradyzoites, trypsin, pepsin, resistance.

INTRODUCTION

Tachyzoites and bradyzoites are structurally and biologically different stages of *T. gondii*. Post-natally, humans or animals become infected with *T. gondii* by ingesting food or water contaminated with oocysts from infected cat faeces or by the ingestion of tissue cysts from uncooked or undercooked infected meat. After ingestion, sporozoites or bradyzoites convert to tachyzoites within 18 h inside the host tissues (Dubey, 1997; Dubey *et al*. 1997). Tissue cysts begin to form the first week post-infection (p.i.) and are thought to persist for life (Dubey & Frenkel, 1976). Reactivation of latent infection, for example, in patients with acquired immunodeficiency syndrome (AIDS), can lead to fatal toxoplasmosis. The reactivation is thought to be due to rupture of tissue cysts, and formation of new tachyzoites and bradyzoites. It is not known whether bradyzoites from older tissue cysts can directly give rise to new tissue cysts or have to go through the tachyzoite stage first. Bradyzoites are less susceptible to chemotherapy that is effective against tachyzoites. Therefore, the fate of bradyzoites in host tissues is of clinical significance.

Recently, there has been great interest in studying conditions needed for stage conversion of *T. gondii* (for review see Frenkel, 1996; Gross *et al*. 1996). Several criteria are used to distinguish tachyzoites from bradyzoites. Structurally, tachyzoites have a centrally located nucleus, few or no periodic acid–Schiff (PAS)-positive granules, and are found during acute infection whereas bradyzoites have a terminally located nucleus, many PAS-positive granules, are enclosed in a resistant cyst wall, and are more prevalent during the chronic stage (Dubey & Frenkel, 1976). Recently, several tachyzoite and bradyzoite-specific monoclonal antibodies have become available (Weiss *et al*. 1995; Lane, Dubremetz & Smith, 1996). However, the transition stages between tachyzoites and bradyzoites and *vice versa* are not well defined structurally or antigenically (Frenkel, 1996).

Bradyzoites are resistant to gastric digestion and thus are infectious orally whereas tachyzoites are destroyed by gastric juice (Jacobs, Remington & Melton, 1960). The resistance of tachyzoites and bradyzoites to digestive enzymes is used as a criterion to differentiate between these stages (Frenkel, 1996; Gross *et al*. 1996). However, there are conflicting reports with respect to the susceptibility of tachyzoites to acid pepsin digestion (Dubey & Frenkel, 1976; Hoff *et al*. 1977; Pettersen, 1979, 1984; Sharma & Dubey, 1981; Lindsay, Toivio-Kinnucan & Blagburn, 1993; Popiel, Gold & Booth, 1996). The objective of the present investigation was to re-examine the effect of trypsin and acid pepsin on tachyzoites and bradyzoites of *T. gondii*.

MATERIALS AND METHODS

Toxoplasma gondii *strains*

Four strains of *T. gondii* (P89, RH, VEG, GT-1) were used. The RH strain, originally isolated by Sabin (1941), and used by researchers world-wide, belongs to genetic type I (Howe & Sibley, 1995) and is highly pathogenic for mice. The VEG strain

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isolated in the laboratory of Dr J. S. Remington from the blood of an AIDS patient (Parmley *et al.* 1994; Dubey *et al.* 1996), belongs to type III in the classification of Howe & Sibley (1995) and is mildly pathogenic to mice based on tachyzoite or bradyzoite-induced infections. The P89 strain, isolated from the heart of a pig, is mildly pathogenic to mice (Dubey, Thulliez & Powell, 1995) and is a genetic recombinant of type I and III organisms. The GT-1 strain, isolated from muscles of a goat, is highly pathogenic to mice (Dubey, 1980) and belongs to genetic type 1.

To obtain tachyzoites of P89 and VEG strains, tissue cysts from donor mouse brains were inoculated i.p. into immunosuppressed (2·5 mg of methylprednisolone acetate given once or twice i.m.) mice or gamma-interferon knockout (KO) mice. This was done because tissue cysts of these strains do not produce many tachyzoites in peritoneal fluid of outbred albino mice. The RH strain was maintained by i.p. inoculation into mice. On days 3–15 p.i., 2–5 ml of saline (0·85% NaCl solution) or phosphate-buffered saline (PBS, pH 7·2) were inoculated into the peritoneal cavity and the peritoneal exudate (pex) was aspirated. After microscopical examination, pex was used for various experiments with or without passage through a 27-gauge needle, unless stated otherwise. To obtain extracellular tachyzoites, pex was mixed with saline or PBS to a volume of 10–20 ml, and filtered through a 3 μm membrane filter (Nucleopore, CA, USA). The filtrate contained approximately 10^6 tachyzoites/ml and no host cells. There were at least 10^5 tachyzoites present in pex in each experiment.

Digestion in pepsin or trypsin solutions

Acid pepsin solution (pepsin 2·6 g, NaCl 5·0 g, HCl 7·0 ml and water to 500 ml, pH ~ 1·0) was made 1 h before use. The pepsin was from porcine stomach (1:10000 biological activity, Sigma Chemical Co., St Louis, MO 63178, USA). An equal volume of this acid pepsin solution, pre-warmed to 37 °C, was mixed with an equal volume of the test material pre-warmed to 37 °C; the pH of the mixture of pex and acid pepsin was between 1·4 and 1·6. The final concentration of the acid and pepsin in the mixture was the same as that used by Jacobs *et al.* (1960). In the latter part of our investigation, the acid pepsin digest was neutralized with 1·2% sodium bicarbonate (pH ~ 8·3); this neutralization did not affect *T. gondii* infectivity. The same batch of pepsin was used for all experiments.

The trypsin solution (Grand Island Biological Co., Grand Island, NY 14072, USA, 1:300 biological activity) solution was prepared in saline or PBS, pre-warmed to 37 °C, and then mixed with an equal volume of test material. The same batch of trypsin was used for all experiments.

In 1 experiment, 0·5% solution of pancreatic enzyme from porcine pancreas (Lot 1440877, Sigma Chemical Co., St Louis, MO, USA) was used. Incubations in trypsin, pepsin, and pancreatic enzymes were performed at 37 °C.

Bioassay of material for T. gondii

Trypsin, pepsin, or pancreatic enzyme digests in various experiments were centrifuged at 1200 ***g*** for 10 min in 50 ml tubes. The supernatant was carefully decanted and the sediment was washed again in saline or PBS or was bioassayed directly in mice after suspension in 1000 units of penicillin and 100 μg of streptomycin/ml. For bioassay, the inoculum, either undiluted, or diluted 10-fold was inoculated s.c. or i.p. into 2–5 female 20–25 g Swiss-Webster mice (Taconic Farms, Germantown, NY, USA).

In certain experiments, undigested pex was bioassayed in mice and cats by the oral route. For this, mice were fed pex using a 4 cm long, 22-gauge feeding needle with a bulbous end (Biomedical Needles, Popper and Sons, Inc., NY, USA). For feeding weaned cats, pex was poured in the mouth by holding of the cat head in an upward position. Newborn kittens were fed by a stomach tube. Faeces of cats were examined daily for *T. gondii* oocysts both microscopically and by bioassay in mice as described (Dubey, 1995).

All mice used for bioassay were examined microscopically for *T. gondii* infection. Impression smears of pex, lungs or brains of mice that died were examined for *T. gondii* stages. Survivors were bled 2 months later and a 1:50 dilution of serum from each mouse was tested for antibodies to *T. gondii* using the modified agglutination test (MAT) as reported previously (Dubey & Desmonts, 1987). Surviving mice were killed 2 months p.i. and a 3 mm^2 piece of cerebrum was placed between a glass slide and cover-slip and examined microscopically for tissue cysts (Dubey & Beattie, 1988). Mice were considered infected when *T. gondii* organisms were found in their tissues.

Experimental design

Experiment 1. The purpose of this experiment was to compare the effect of trypsin and acid pepsin digestion on *T. gondii* stages in pex, lungs, and brains of mice in relation to duration of *T. gondii* infection. For this, 9 mice were inoculated i.p. with tissue cysts of the P89 strain from murine brains. On days 7, 10, and 15 p.i., pex, lungs and brains were pooled separately. Lungs and brains were homogenized separately in saline. On days 7 and 10 p.i. approximately 10^6 tachyzoites were present in pex whereas on day 15 p.i. only a few tachyzoites were seen. Whole organ homogenates of lungs, brains, and an aliquot of pex were incubated in pepsin for 1 h. In

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addition, an aliquot of pex was also incubated in 1% trypsin for 1 h. Digested tissues were diluted with saline, centrifuged, and bioassayed into 2–4 mice.

Experiment 2. This experiment was performed to study the effect of 3 concentrations of trypsin (0·25, 0·5, 1·0%) on *T. gondii* in pex. Two trials were performed. In the first trial, 3 mice were inoculated i.p. with bradyzoites of the P89 strain. All 3 mice were killed on day 7 p.i., and their pex harvested in saline, pooled, and divided equally into 4 aliquots. All aliquots were centrifuged, the supernatant discarded and the sediment suspended in 0·25, 0·50 or 1·0% trypsin in PBS, or in PBS alone. After 60 min incubation, parasite suspensions were diluted 10-fold with PBS to minimize the action of trypsin, centrifuged at 1200 ***g***, the sediment suspended in PBS, and 10-fold dilutions were bioassayed in mice.

In the second trial, the effect of the 3 concentrations of trypsin (1, 0·5, 0·25%) was examined using extracellular RH strain tachyzoites. For this, pex obtained on day 5 p.i. was suspended in PBS, passed first through a 27-gauge needle, then through a 3 μm filter. The number of tachyzoites was counted and adjusted 900000/ml in PBS, and divided into 4 aliquots (groups A–D) of 5 ml each. To groups A–D, 5 ml of PBS or 2, 1 and 0·5% trypsin in PBS were added. After 60 min incubation (without shaking) at 37 °C, 0·5 ml of the material from each group was mixed with 4·5 ml of RPMI tissue culture medium containing 10% horse serum (RPMIS); this dilution was considered 10^{-1}. Six further serial 10-fold dilutions (10^{-2}–10^{-7}) were made in RPMIS and an aliquot (0·5 ml) was inoculated i.p. into 5 mice for each dilution for each group.

Experiment 3. The purpose of this experiment was to study the effect of acid pepsin on the viability of RH strain tachyzoites. The pex was obtained 3, 4, 8, 9 or 10 days after tachyzoite inoculation and the same procedures were followed as in Exp. 1.

Experiment 4. The purpose of this experiment was to study the effect of acid pepsin digestion on VEG strain tachyzoites and to test oral infectivity of tachyzoites to cats and mice. Four trials were performed. In trials 1 and 2, pex obtained on days 4 and 7 p.i., was incubated in acid pepsin solution. In trial 3, pex obtained on day 6 p.i. was incubated in acid pepsin solution and also bioassayed in cats. In trial 3, four mice treated with cortisone were inoculated i.p. with mouse brain containing tissue cysts of the VEG strain of *T. gondii* obtained from mice inoculated 9 months earlier. The mice became ill 5 days p.i. and 1 died day 6 p.i. The pex was divided into 6 equal aliquots of 3 ml each. Material from 1 aliquot was inoculated orally by a stomach tube into an 8-day-old kitten and an aliquot was fed to the mother (queen) of the kitten by pouring 3 ml of the material into the mouth of the queen held in an upward position. The kitten was born in our facility and the history and management of our cats has been described previously (Dubey, 1995). The faeces of the queen were collected daily from the litter pan, floated in sugar solution, examined microscopically, and bioassayed in mice as described (Dubey, 1995). The remaining aliquots of pex were digested in acid pepsin solution for 30, 40 or 60 min.

In trial 4, nine outbred cortisone-treated mice and 1 KO mouse were inoculated i.p. with tissue cysts of the VEG strain. On the 5th day p.i. mice were killed, and the pex aspirated. Out of a total volume of 12 ml pex, 1 ml was fed to 2 *T. gondii*-free weaned cats (0·5 ml for each cat) and 5 ml was fed to 10 mice (0·5 ml for each mouse). To the remaining 6 ml of the pex, 6 ml of acid pepsin solution was added, incubated for 30 and 60 min at 37 °C, then neutralized with $NaHCO_3$, centrifuged, and inoculated s.c. into 8 mice.

Experiment 5. The purpose of this experiment was to compare the effect of trypsin, pepsin and pancreatin on digestibility of brain tissue and of bradyzoites. Two trials were performed. In trial 1, 75 g brain tissue from 2 pigs that were fed approximately 200 oocysts of the VEG strain of *T. gondii* 2 months earlier and of 2 rats infected with GT-1 strain of *T. gondii* 12 months earlier were homogenized in a blender in 5 vols of saline and filtered through gauze to remove large particles. Then 150 ml of brain homogenate (containing 11·5 g of brain tissue) were divided equally into 3 aliquots (A–C) of 50 ml each; to aliquot A 50 ml of 0·5% trypsin in PBS were added, to aliquot B 50 ml of acid pepsin solution were added, and to aliquot C 0·5% pancreatic enzyme in PBS was added. The homogenates were incubated at 37 °C for 1 h and then centrifuged, the sediment diluted with PBS, centrifuged again, and the supernatant discarded. The volume of sedimented pellet in each digest was estimated. The sediment from each tube was suspended in PBS to make a total of 15 ml. From each homogenate 0·5 ml of the suspension was mixed with 4·5 ml of PBS (considered 10^{-1} dilution) and 5 further serial 10-fold dilutions were made. Aliquots (0·5 ml) from each homogenate were inoculated s.c. into 4 mice each.

In the second trial, the brain of a rat inoculated 443 days earlier with GT-1 strain was homogenized in saline and divided into 3 equal aliquots. One aliquot was mixed with an equal volume of 0·5% trypsin, the second aliquot was mixed with acid pepsin solution and the third aliquot was mixed with an equal volume of saline. After incubation for 30 min at 37 °C, all aliquots were serially diluted 10-fold and aliquots of each dilution were inoculated s.c. into 2 mice.

Table 1. Effect of acid pepsin and trypsin digestion on *Toxoplasma gondii* stages in tissues of mice in relation to duration of infection with P89 strain

Day of infection in donor mice	Acid pepsin			Trypsin
	Pex	Lungs	Brain	Pex
7	0/2*	2/4	0/2	2/2
10	0/2	0/2	2/2	2/2
15	0/2	2/2	2/2	2/2

* No. of mice infected with *T. gondii*/No. of mice injected.

Table 2. Effect of incubation of extracellular *Toxoplasma gondii* RH strain tachyzoites in different concentrations of trypsin and PBS

Dilution of pex	Trypsin			PBS
	1 %	0·5 %	0·25 %	
10^{-3}	5/5*	5/5	5/5	5/5
10^{-4}	4/5	5/5	4/5	5/5
10^{-5}	0/5	2/5	1/5	5/5
10^{-6}	0/5	0/5	0/5	4/5
10^{-7}	0/5	0/5	0/5	0/5

* No. of *T. gondii*-positive mice/No. of mice injected.

Experiment 6. The purpose of this experiment was to compare the infectivity of RH strain tachyzoites to mice by oral and parenteral routes and to study the quantitative survival of tachyzoites after acid pepsin digestion *in vitro*. Two trials were performed.

In trial 1, pex was obtained from mice incubated i.p. 7 days earlier with the RH strain tachyzoites. An aliquot of pex was passed through a 27-gauge needle and a 3 μm filter to free all tachyzoites from host cells; the remaining pex was not filtered. After counting the tachyzoites in a haemacytometer, the number of extracellular tachyzoites was adjusted to 200000/ml (considered 10-^{1} dilution). Six serial 10-fold dilutions were made in RPMI tissue culture medium with 10 % foetal calf serum. From each dilution 10 mice were inoculated (0·5 ml/mouse), 5 mice orally and 5 mice s.c.

The major portion of pex containing approximately $2{\cdot}2 \times 10^7$ tachyzoites was incubated in acid pepsin solution at 37 °C. After 1 h, the pex digest was neutralized with $NaHCO_3$, diluted 10-fold in RPMI tissue culture medium, and inoculated i.p. into 4 mice.

RESULTS

Experiment 1

Toxoplasma gondii in pex did not survive digestion in pepsin solution, irrespective of the duration of infection, whereas organisms from lungs and brain did survive in pepsin (Table 1). Organisms in pex survived in trypsin digestion for 1 h irrespective of the duration of infection.

Experiment 2

In trial 1, there was a 100-fold reduction in infectivity of tachyzoites digested in 1 % trypsin compared with digestion in 0·25 % trypsin for 60 min. The dilution of tachyzoites that was infective to mice was 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} after incubation in PBS, 0·25 % trypsin, 0·5 % trypsin and 1 % trypsin, respectively.

In trial 2, the infectivity of trypsin incubated tachyzoites was $\geqslant$ 10-fold lower than that incubated in PBS (Table 2).

Experiment 3

The RH strain tachyzoites survived pepsin digestion in 6 of 9 preparations up to 120 min but the results were not consistent (Table 2). The survival was not related to the number of tachyzoites in the inoculum and protection within cells because extracellular organisms also survived in acid pepsin solution (Table 2). Mice infected with the RH strain died within 13 days after i.p. inoculation. Antibodies to *T. gondii* were not found in mice that survived for 4 weeks p.i.

Experiment 4

In trials 1 and 2, tachyzoites of the VEG strain were killed by incubation in acid pepsin solution for 15, 30 or 60 min. In trial 3, tachyzoites survived incubation for 40 and 60 min in acid pepsin solution (Table 3) and both cats fed pex of the VEG strain became infected with *T. gondii*. Both the queen and her kitten developed a MAT antibody titre of 1:400. *Toxoplasma gondii* lesions were seen in the brain of the kitten euthanized on day 21 p.i. (data not shown). The queen shed *T. gondii* oocysts starting at day 17 after feeding tachyzoites and oocysts were shed for 7 days.

In trial 4, tachyzoites were infective orally to cats and mice but did not survive *in vitro* incubation in acid pepsin solution (Table 4). Both cats shed *T. gondii* oocysts 15 or 16 days after ingesting tachyzoites. All 10 mice fed tachyzoites became infected with *T. gondii*.

Experiment 5

In the first trial, the volume of pellet from digests in trypsin, pepsin, and pancreatin solutions was estimated to be 5·0, 10·5, and 5·0 ml, respectively. The pellets in trypsin and pancreatin digests were solid

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Table 3. Effect of incubation in acid pepsin mixture (37 °C) on *Toxoplasma gondii* RH strain tachyzoites obtained from peritoneal exudates of mice

Incubation time (min)	Day pex obtained and whether pex was filtered (F) or not filtered (NF)									Total
	3, NF	3, NF	3, F	4, NF	8, NF	8, NF	9, F	9, NF	10, NF	
10	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	4*	N.D.	N.D.	4/4†
15	N.D.	N.D.	N.D.	N.D.	N.D.	4	N.D.	N.D.	N.D.	4/4
20	N.D.	N.D.	2	N.D.	N.D.	N.D.	4	N.D.	N.D.	6/8
30	4	4	0	0	0	4	0	0	N.D.	12/32
40	0	N.D.	N.D.	2	N.D.	N.D.	N.D.	0	N.D.	2/12
50	0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0/4
60	0	1	1	0	0	0	2	0	0	4/36
90	N.D.	N.D.	N.D.	N.D.	0	N.D.	N.D.	N.D.	N.D.	0/4
120	N.D.	4	N.D.	N.D.	0	N.D.	N.D.	N.D.	0	4/12
180	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0	0/4

* No. of mice positive for *T. gondii* of 4 mice inoculated.
† No. of mice positive for *T. gondii*/No. of mice inoculated.
N.D., Not done.

Table 4. Effect of incubation in acid pepsin on *Toxoplasma gondii* VEG strain tachyzoites from peritoneal exudate of mice

Incubation time (min)	Pex day 4 (Trial 1)	Pex day 7 (Trial 2)	Pex day 6* (Trial 3)	Pex day 5* (Trial 4)
15	0†	0	N.D.	N.D.
20	N.D.	N.D.	0	N.D.
30	0	0	0	0/4
40	N.D.	N.D.	4	N.D.
60	0	0	4	0/4

* Pex was infective orally to 2 cats.
† No. of mice positive for *T. gondii* of 4 mice inoculated.
N.D., Not done.

whereas that in pepsin was not solid. The *T. gondii* infectivity of bradyzoites in 3 digests was identical. The 10^{-6} dilution of brain from each digest was not infective to mice and 10^{-4} dilution was infective to all 4 mice inoculated with each digest. The 10^{-5} dilution was infective to 1 of 4 mice inoculated from each digest.

In the second trial, the 10^{-4} dilution of brain from all 3 aliquots was infective to 2 of 2 mice. The 10^{-5} dilutions of trypsin and pepsin-digested brains were infective to 2 of 2 mice whereas the undigested brain was infective to 1 of 2 mice. The 10^{-6} dilutions of trypsin and pepsin-digested brains were infective to 1 of 2 mice, whereas undigested brain was not infective to 2 mice.

Experiment 6

In trial 1, 12 of 15 mice inoculated orally with 10^3–10^5 tachyzoites became infected with *T. gondii* (Table 5). The estimated count of tachyzoites paralleled infectivity (Table 5) by the s.c. route indicating that virtually all tachyzoites injected into mice were viable. All $2{\cdot}2 \times 10^7$ tachyzoites were killed by incubation in acid pepsin solution for 1 h.

In trial 2, results were similar to those in trial 1 (Table 5).

DISCUSSION

The resistance of bradyzoites to digestion by gastric juice has been known for more than 36 years. Jacobs *et al*. (1960) found that bradyzoites can survive in acid pepsin solution for 2 h or more whereas tachyzoites can not. Jacobs *et al*. (1960) digested homogenates of liver, spleen, and lungs of mice inoculated i.p. with RH strain in acid pepsin solution for 60 min and found that tachyzoites were rendered non-infective for mice. Direct microscopical examination of tachyzoites in acid pepsin solution revealed that tachyzoites were damaged immediately; they were more granular, less refractile, and became ghost-like within 15–30 min (Jacobs *et al*. 1960). Unlike acid pepsin solution, tachyzoites survived in 1% trypsin for 3 h (Jacobs *et al*. 1960).

Sharma & Dubey (1981) quantitatively studied survival of bradyzoites in pepsin and trypsin solutions. They found that bradyzoites survived in acid pepsin solution for 2 h but not for 3 h, and although they survived in 1% trypsin for 3 h there was a > 10-fold reduction in infectivity between 1 and 3 h.

The susceptibility of tachyzoites and bradyzoites to trypsin and pepsin has been used as a bioassay to detect bradyzoites in tissues with different conclusions or interpretations. Dubey & Frenkel (1976) compared biological criteria with histology to detect early tissue cysts in mice inoculated parenterally with tachyzoites. They found that cats fed mouse tissues containing tachyzoites either shed no oocysts or did so after a long (> 14 days) pre-patent period whereas tissues containing bradyzoites were highly

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Table 5. Comparison of infectivity of extracellular *Toxoplasma gondii* RH strain tachyzoites to mice by i.p., s.c., and oral routes

	Trial 1		Trial 2		
Dilution	s.c.	oral	i.p.	s.c.	oral
10^{-1}	5* (7–9)†	4 (9–10)	5 (7)	5 (7–9)	5 (9–11)
10^{-2}	5 (8–9)	4 (8–11)	5 (7–8)	5 (8–9)	4 (10–11)
10^{-3}	5 (9–10)	4 (9–10)	5 (7–9)	5 (9–11)	3 (11–14)
10^{-4}	5–(10–11)	0	5 (8–9)	5 (7–11)	0
10^{-5}	5 (10–11)	0	5 (8–10)	5 (10–12)	0
10^{-6}	3 (11–12)	0	3 (9–10)	4 (11–14)	0
10^{-7}	4 (12–16)	0	1 (10)	0	0
10^{-8}	N.D.	N.D.	0	0	0

* No. of mice infected with *T. gondii* of 5 mice inoculated. All *T. gondii*-infected mice died between days 7 and 16 p.i.
† Day of death (range).
N.D., Note done.

infective to cats, and cats shed oocysts with a short pre-patent period ($\leqslant$ 10 days). Using this criterion, they established that tachyzoites can convert to bradyzoites between 2 and 3 days in tissues of mice inoculated with tachyzoites. They then compared infectivity for cats and mice of brain tissues of mice infected 7, 10, or 30 days earlier with tachyzoites. Brains of mice infected for 7 and 10 days were more infective to cats, and cats shed oocysts with a short pre-patent period whereas the same inocula were rarely infective to mice by the oral route, indicating that either few bradyzoites were present at 5 and 7 days p.i. or early tissue cysts were less infective for mice than for cats. The infectivity of brains of mice inoculated 30 days earlier was comparable in mice and cats.

Results of this investigation indicate that incubation for 1 h in both trypsin and pepsin has little deleterious effect on bradyzoites from tissue cysts from chronically infected mice. However, bradyzoites/tissue cysts produced during acute infection were more susceptible to digestion in pepsin (Dubey, 1997; Dubey *et al*. 1997).

Tachyzoites of *T. gondii* are fragile and highly susceptible to salt concentration. For example, tachyzoites survive better in PBS than in saline and survival is even better in tissue culture medium with serum. The differences in the results of the 2 trials in Exp. 2 may be related to the method of bioassay. In trial 1, dilutions were made in PBS, whereas in trial 2, dilutions were made in tissue culture medium with 10% serum. The deleterious effect of 1% trypsin on *T. gondii* tachyzoites reported by Sharma & Dubey (1981) may be because tachyzoites were incubated in saline instead of PBS. The results of trial 2 in Exp. 2, leave no doubt that even extracellular tachyzoites survive in 0·5% trypsin for 1 h.

Derouin & Garin (1991) followed the course of *T. gondii* infections in mice inoculated with tachyzoites or tissue cysts. They digested tissues in 0·25% trypsin for 120 min and reported what appeared to be trypsin-resistant and trypsin-sensitive organisms during the course of infection. They reported that trypsin-resistant parasites increased with duration of infection and interpreted these findings as meaning that an increase in infectivity was due to the development of bradyzoites. This interpretation can not be correct because there are no trypsin-sensitive or trypsin-resistant tachyzoites. In the present study, tachyzoites from pex survived in trypsin for at least 1 h where results of pepsin digestion were variable, irrespective of the duration of infection.

Pettersen (1979) made 2 important observations with respect to trypsin and pepsin digestion. Firstly, the destruction of tachyzoites in acid pepsin solution was due to acid not pepsin because no differences were found in survival when tachyzoites were incubated in acid pepsin solution at room temperature compared with 37 °C. Secondly, tachyzoites of 2 virulent strains (RH and strain 119) survived in acid pepsin solution for 20 min but not 25 min. Pettersen (1979) thought that bradyzoites were present in the peritoneal exudate (pex) of mice inoculated with bradyzoites of 2 avirulent strains of *T. gondii*. The pex was obtained 4 or 6 days after i.p. inoculation with bradyzoites of strain DUE; the organisms in pex survived 90 min in acid pepsin solution at 37 °C. However, Dubey & Frenkel (1976) did not find bradyzoites in pex of mice inoculated with tachyzoites of the M-7741 strain. They inoculated pex into the jejunal lumen of cats (to avoid digestion in the stomach); the cats became infected but did not shed oocysts until $\geqslant$ day 21 p.i. indicating that only tachyzoites were present in the inoculum (Dubey & Frenkel, 1976). Moreover, mice inoculated i.p. with bradyzoites of the M-7741 strain, 2 to 6 days earlier were not infective to cats orally indicating that bradyzoites had converted to tachyzoites within 2 days of i.p. inoculation. Whether

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bradyzoites are ever present in pex is of significance because pex is used as a source of tachyzoites for various experiments. The results of the present study suggest that bradyzoites are not produced in the pex. The results obtained by Pettersen (1979) are probably related to unexplained factors because somewhat similar results were obtained in the present study with the RH strain which does not form bradyzoites in mouse pex.

Pettersen (1984) also proposed that bradyzoites can be excreted in milk of mice 5 days after i.p. inoculation of lactating mice with bradyzoites. He based this conclusion on the observation that milk treated with HCl for 60 min at room temperature produced *T. gondii* infection in mice. The results of this experiment are contrary to the conclusion reached by Dubey & Frenkel (1976) who found that bradyzoites were not formed until day 7 p.i. in any tissue of mice after bradyzoite inoculation, let alone being excreted in milk. Again, results obtained in the present study do not support Pettersen's conclusion.

Popiel *et al*. (1996) recently used a pepsin digestion procedure to quantitate the development of bradyzoites *in vitro* using cell culture as the bioassay. Tachyzoites of the T-263 strain, obtained after growth for 2 days in cell culture were killed after a 10 min digestion in pepsin. 'Bradyzoites' produced in cell culture survived pepsin digestion for 30 to 60 min. They concluded that organisms that resisted 30 min pepsin digestion were bradyzoites and not tachyzoites.

Lindsay *et al*. (1993) compared the appearance of pepsin-resistant organisms with the development of tissue cysts by transmission electron microscopy (TEM) in cell cultures inoculated with 21 isolates of *T. gondii*. All but 2 isolates of *T. gondii* produced tissue cysts as confirmed by TEM. The 2 exceptions were the RH strain and a temperature sensitive (tS-4) mutant derived from it; tissue cysts were not seen in cell cultures inoculated by these 2 strains but organisms were pepsin resistant (30 min). These results appeared paradoxical at that time because the tS-4 strain does not form tissue cysts and does not persist in tissues of mice, hamsters, pigs, monkeys, cotton rats (reviewed by Lindsay *et al*. 1993). The line of the RH strain from which the mutant tS-4 was derived does not produce tissue cysts (Waldeland, Pfefferkorn & Frenkel, 1983). Their findings can now be interpreted differently in the light of the present study.

Of all the methods currently available to distinguish tachyzoites from bradyzoites, the most reliable method is by bioassay in cats. Cats fed tachyzoites may become infected and may shed oocysts with a long pre-patent period ($\geqslant$ 15 days), whereas cats fed bradyzoites shed oocysts with a short ($\leqslant$ 10 days) pre-patent period. Oral infectivity by itself is not a reliable method to distinguish tachyzoites from bradyzoites. In the present study, all 4 cats fed tachyzoites became infected. The method of feeding may or may not have a bearing on results. The kitten was fed tachyzoites by a stomach tube, whereas for the queen and weaned cats, tachyzoites were poured into the mouth. In the present study whether cats or mice became infected by tachyzoites that entered through pharyngeal mucosa or from those that survived digestion in gut was not determined but it is academic with respect to natural infections.

In the present study, higher doses ($\geqslant$ 1000) of extracellular tachyzoites of *T. gondii* were infectious to mice and cats by the oral route. Therefore, infectivity of *T. gondii* by the oral route should not be used as a criterion to distinguish tachyzoites from bradyzoites. The oral infectivity of tachyzoites observed in the present study might explain the recent case of toxoplasmosis in a breast milk-fed child whose mother had recently acquired toxoplasmosis (Bonametti *et al*. 1997). Riemann *et al*. (1975) had reported toxoplasmosis in a child fed unpasteurized goat milk.

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