4. Stinebring, W. R., Youngner, J. S., Nature, 1964, v204, 712.
5. Kono, Y., Ho, M., Virology, 1965, v25, 162.
6. Sabin, A. B., J.A.M.A., 1941, v116, 801.
7. Merigan, T. C., Science, 1964, v145, 811.
8. Sugiura, A., Kilbourne. E. D. Virology, 1965, v26, 478.
9. Rytel, M. W., Kilbourne, E. D., J. Exp. Med., v123, 767.
10. Hopps, H. E., Kohno, S., Kohno, M., Smadel, J. E., Bact. Proc., 1964, p115.
11. Merigan, T. C., ibid., 1966, p119.
12. Freshman, M. M., Merigan, T. C., Remington, J. S., Brownlee, I. E., Proc. Soc. Exp. Biol. and Med., 1966, v123,

Received June 14, 1966. P.S.E.B.M., 1966, v123.

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

MONIQUE M. FRESHMAN, THOMAS C. MERIGAN, JACK S. REMINGTON, AND INEZ E. BROWNLEE

Division of Infectious Diseases, Department of Medicine, Stanford University School of Medicine, and the Division of Allergy, Immunology, and Infectious Disease, Palo Alto Medical Research Foundation, Palo Alto, Calif.

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

* This research was supported by grants AI 05629 and AI 04717 from Nat. Inst. Health.

TABLE I. Antiviral Activity Observed in Peritoneal Fluid and Serum After Intraperitoneal Injection of *Toxoplasma gondii* (RH).

Hr after injection	Units of antiviral material: Peritoneal fluid	Serum
24	N-S*	10
28	29	24
48	77	48
72	59	50
96	50	77
120	34	N-S
Uninjected controls	<3	<3

* Not studied.

of the highest dilution of the material which reduced by 50% the number of virus plaques which developed as compared to control plates.

After overnight contact of the PF with the cell monolayer, the formation of a fibrin clot over the monolayer was occasionally observed. Prior treatment of the PF with heparin in a concentration of 0.5 unit/ml prevented this and did not alter the antiviral properties of the PF.

Mengo virus. Dr. H. Levy of the National Institutes of Health provided this agent which was passed in our laboratory in L cell monolayers, and stored at −70°C. In all experiments the dosage inoculated was approximately 2×10^8 pfu, as measured on L cell monolayers. This was found to be between one and two logs higher than the LD_{50} of this strain of Mengo virus in our mice.

To study the protective effect of avirulent toxoplasma against a pathogenic virus, mice were injected IP with 0.2 ml of a suspension containing toxoplasma brain cysts (strain ME-49), and challenged 48 hours later by IP injection of Mengo virus. The time to death was determined and compared to that of control animals injected IP with Mengo virus alone. In a later experiment, a group of mice was inoculated subcutaneously (SC) with 0.2 ml of ME-49 cysts. Control mice received normal mouse brain by the same route. Seven days later both experimental and control mice were challenged IP with 2×10^8 pfu of Mengo virus.

Results. Demonstration and characterization of an antiviral substance in tissue culture. Table I presents the time of appearance of antiviral activity elicited by IP injection of RH toxoplasma. The antiviral activity of the PF at 2 days after infection in several experiments varied between approximately 70 and 300 units.

Table II outlines the characteristics of the antiviral substance induced by IP injection of mice with *T. gondii* (RH). Two day PF was used for this study with an activity of 180 units. If the PF was allowed to remain in contact with L cell monolayers for only one hour instead of the usual 12 hours, no antiviral activity could be demonstrated. In addition, no diminution in plaque-forming ability of VSV was observed when the antiviral material was incubated directly with VSV for one hour. The PF had the same amount of antiviral activity in a plaque-assay on L-cells performed with Mengo virus as the challenge. Finally, when the PF was assayed against VSV in both chick embryo fibroblasts and L cells, the titer of antiviral activity was 300 units in L cells as compared to less than 10 units in the heterologous chick cells. The level of the serum antiviral activity was somewhat lower and will be characterized in subsequent experiments.

After demonstration of the production of an antiviral material induced by *T. gondii in vivo,* it was important to ascertain whether this was induced in response to the infecting parasites or to a contaminating virus in the preparations. Mice were inoculated with approximately 4×10^6 RH organisms (3 day PF). After 3 days the PF was collected and

TABLE II. Characterization of *Toxoplasma gondii*-Induced Antiviral Material.*

Procedure	Loss in activity
Trypsin	90%
Deoxyribonuclease	0
Ribonuclease	0
Dialysis	32%
Ultracentrifugation	0
38°C	0
56°C	73%
70°C	90%
Short contact with cells	86%
Acid treatment	Marked below pH 4.0

* PF was treated with enzymes at a concentration of 0.5 mg/ml for one hour at 37°C. It was dialyzed for 20 hr at 4°C. It was ultracentrifuged at 100,000 × *g* for 2 hr. Heat or acid treatment was carried out for one hr.

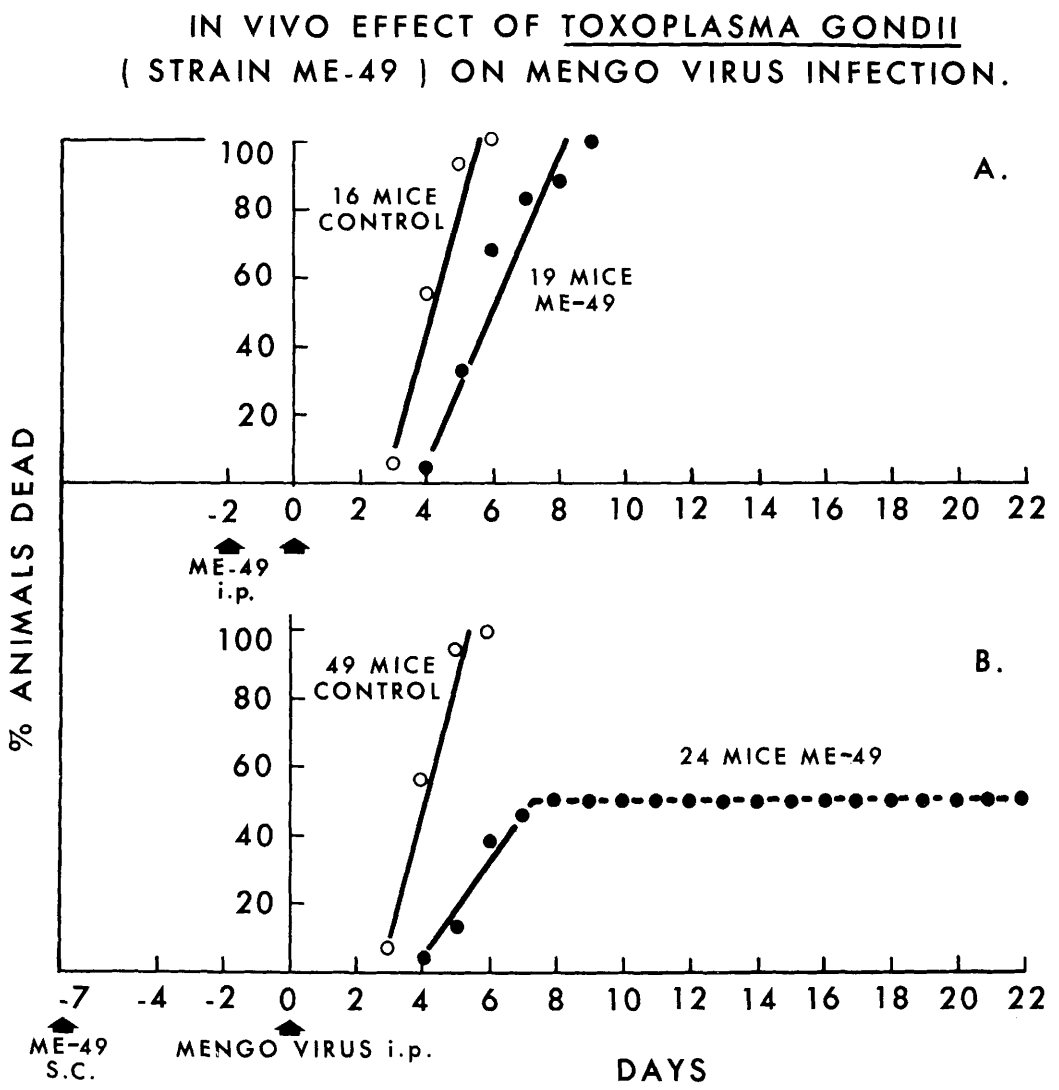

FIG. 1-A SW mice were pre-injected IP with 0.2 ml *Toxoplasma gondii* (strain ME-49). Two days later the mice received an IP challenge injection of Mengo virus (2×10^8 pfu). Controls received only Mengo virus.

FIG. 1-B SW mice pre-injected SC with 0.2 ml of *T. gondii* (ME-49) or normal mouse brain. Seven days later they received an IP challenge injection of Mengo virus (2×10^8 pfu).

centrifuged at 4000 rpm for 1 hour. Mice were then injected IP with either precipitate containing parasites or supernatant fluid. After 72 hours the animals were bled, the PF collected and both sera and PF were assayed in tissue culture. The antiviral inducing activity was found in the infranatant; none was found in the supernatant.

In vivo *protection studies.* A virulent strain ME-49 was used for mouse inoculation in experiments designed to demonstrate protection afforded mice against challenge with virulent Mengo virus. Fig. 1 illustrates the degree of protection attained by prior inoculation of avirulent *T. gondii* with either of two routes of inoculation. The control slopes in both Parts A and B of the Figure are similar. In the IP injection experiments (Fig. 1-A), the curve of the ME-49 injected group is displaced to the right by about 2 days, *i.e.,* the mice are protected to some extent by the IP M-49 injection.

A more dramatic effect is evident in the SC injected group (Fig. 1-B). Only 50% of the experimental group died and, usually, deaths occurred later than in the control group. The survivors were alive 4 weeks later.

Discussion. These studies indicate that *Toxoplasma gondii* can induce an antiviral substance *in vivo* that is active both in tissue culture against VSV and Mengo virus challenge and *in vivo* against Mengo virus. A peak in antiviral activity of the PF was seen at 2 days, whereas the activity in serum appears to peak somewhat later (4 days). It would seem that some portion of the early serum antiviral activity represents material which has diffused into the circulation from the peritoneal cavity, as described by Gresser *et al*(23), in experiments with IP injected concentrated interferon. However, the antiviral activity in the serum at 3 and 4 days after infection may come from extraperitoneal sites, as we have noted progressively increasing parasitemia and generalized infection prior to death of these mice. Subsequent to these investigations, it came to our attention that others(24) had independently observed similar antiviral activity induced by *T. gondii* in mouse serum.

Characterization of the antiviral material in the PF has shown that it fulfills many of the criteria used to define interferon. It appears to be a protein as its biologic activity is destroyed by treatment with trypsin. Neither treatment with DNAse or RNAse affected its antiviral activity, nor did ultracentrifugation at 100,000 $\times$ *g* for 2 hours. However, the activity was markedly reduced by heating above 56°C. The antivirla material inhibited both VSV and Mengo virus replication *in vitro* to the same extent. Furthermore, no loss in virus titer was observed when virus was incubated directly with the PF-containing antiviral material. This confirmed the lack of direct action of the material against virus, and clearly distinguished it from antibody. The antiviral action was species specific in tissue culture. One hour's incubation of the antiviral substance in contact with the monolayer did not provide sufficient time to protect the cells from subsequent virus challenge. Thus the protection resulting from the toxoplasma-induced antiviral material is mediated through prolonged contact with the cells, and is similar to virus induced interferon in this respect.

Less pH stability of this antiviral material is noted in comparison to viral induced mouse interferon, which is stable to pH 2. This may be due to co-precipitation of interferon with other acid precipitable materials in the PF. Experiments are in progress to characterize the antiviral material found in both the serum and peritoneal fluid by other methods including G-100 Sephadex gel filtration to measure molecular weight(25).

Various investigators have shown that avirulent or inactivated viruses are better interferon inducers than live viruses(26,27). An avirulent or chronic strain of *T. gondii* was used for *in vivo* stimulation of antiviral activity. Subsequently, mice were challenged with a neurotropic virus, Mengo virus. The results shown in Fig. 1 indicate definite *in vivo* antiviral protection. Subcutaneous inoculation of ME-49 7 days prior to virus inoculation resulted in better protection than did the intraperitoneal inoculation 2 days prior to virus challenge. It appears that the slow, sustained release of ME-49 (and/or the induced antiviral substance) over 7 days resulted in more solid protection.

In recent unpublished studies in this laboratory, antiviral activity was observed in the serum of mice at 7 days, but not at 2 days after IP inoculation of ME-49. Also, *in vivo,* good protection was demonstrated against Mengo virus in ME-49 IP injected mice challenged after 7 days, which was identical to that observed in animals in which the ME-49 was SC injected. From these data it appears that the degree of *in vivo* antiviral protection correlates with serum antiviral activity and is determined by the length of time elapsed following toxoplasma inoculation, rather than the route of injection.

It would be of interest to determine the interferon sensitivity of toxoplasma as many agents which stimulate interferon production are also inhibited by interferon. Present knowledge of the mechanism of action of interferon allows us to speculate on this question. Interferon appears to act on the ribo-

somal level of protein synthesis by preventing the formation of polysomes from host ribosomes and viral messenger RNA(28,29). TRIC agents appear to be sensitive to the action of viral induced interferon(30) and it is not yet clear whether they, like viruses, employ the host cell's or their own ribosomes for synthesis of their proteins. However, as bacteria and protozoa possess their own ribosomes(31,32), we would predict that those protozoa and bacteria which grow intracellularly are not sensitive to interferon action. Bacteria and protozoa, moreover, both have been reported to support virus replication (33,34). Therefore their biosynthetic pathways might be expected to resemble those of the host cell in its apparent lack of change in the presence of interferon(35,36).

Summary. *Toxoplasma gondii* has been shown to induce *in vivo* an antiviral substance whose action is demonstrable both in tissue culture and *in vivo*. Characterization of this material has shown it to fulfill many of the criteria used to define interferon. Inoculation of an avirulent strain of *T. gondii* resulted in protection against later challenge by a virulent neurotropic virus.

We wish to give special thanks to Mrs. Jean McIntosh and Miss Beverly Koops for excellent technical assistance.

1. Isaacs, A., Lindenmann, J., Proc. Royal Soc. London, 1957, v147, 258.
2. Nagano, Y., Kojima, Y., Compt. Renod. Soc. de Biol., 1958, v152, 1627.
3. Ho, M., Enders, J. F., Proc. Nat. Acad. Sci., 1959, v45, 385.
4. Vilcek, J., Nature, 1960, v187, 73.
5. Allison, A. C., Virology, 1961, v15, 47.
6. Fruitstone, M. J., Waddell, G. H., Sigel, M. M., Proc. Soc. Exp. Biol. and Med., 1964, v117, 804.
7. Béladi, I., Pusztai, R., personal communication, 1966.
8. Youngner, J. S., Stinebring, W. R., Science, 1964, v144, 1022.
9. Hopps, H. E., Kohno, S., Kohno, N., Smadel, J. E., Bact. Proc., 1964, 115 (Abstract).
10. Merigan, T. C., Hanna, L., Proc. Exp. Biol. and Med., 1966, v122, 421.
11. Stinebring, W. R., Youngner, J. S., personal communication, 1966.
12. ———, Nature, 1964, v204, 712.
13. Ho, M., Science, 1964, v146, 1472.
14. Kleinschmidt, W. J., Cline, J. C., Murphy, E. B., Proc. Nat. Acad. Sci., 1964, v52, 731.
15. Rytel, M., Shope, R. E., Kilbourne, E. D., J. Exp. Med., 1966, v123, 577.
16. Wheelock, E. F., Science, 1965, v149, 310.
17. Youngner, J. S., Stinebring, W. R., Taube, S. E., Virology, 1965, v27, 541.
18. Jacobs, L., Ann. Rev. Microbiol., 1963, v17, 429.
19. Fulton, J. D., Spooner, D. F., Trans. Royal Soc. Trop. Med., and Hyg., 1957, v51, 123.
20. Eagle, H., Science, 1959, v130, 432.
21. Merigan, T. C., ibid., 1964, v145, 811.
22. Wagner, R., Virology, 1961, v13, 323.
23. Gresser, I., Fontaine, D., Coppey, J., Falcoff, R., Falcoff, E., personal communication, 1966.
24. Rytel, M., Jones, T., personal communication, 1966.
25. Merigan, T. C., Winget, C. A., Dixon, C. B., J. Mol. Biol., 1965, v13, 679.
26. Isaacs, A., Adv. Vir. Res., 1963, v10, 1.
27. Burke, D. C., Isaacs, A., Brit. J. Exp. Path., 1958, v39, 452.
28. Joklik, W., Merigan, T. C., Proc. Nat. Acad. Sci., 1966, in press.
29. Levy, H., Carter, W., personal communication, 1966.
30. Hanna, L., Merigan, T. C., Jawetz, E., Proc. Soc. Exp. Biol. and Med., 1966, v122, 417.
31. Microsomal Particles and Protein Synthesis. Proc. of 1st Symposium of Biophysical Society, R. B. Roberts, Ed., Pergamon Press, 1958.
32. Goodwin, T. W., Lindberg, O., Eds. Biological Structure and Function, II, 1961, Prescott, D. M., p527.
33. Abel, P., Trautner, T. A., Z. Vererbungsl., 1964, v95, 57.
34. Zech, L., IX Intern. Congress Microbiol., 1966, 492 (Abst).
35. Merigan, T. C., Baron, S., McKerlie, M. L., Proc. Soc. Exp. Biol. and Med., 1966, v121, 50.
36. Merigan, T. C., Levy, H. B., ibid., 1966, v121, 53.

Received September 9, 1966. P.S.E.B.M., 1966, v123.