lifetime of any single tree (*17*). Although the necessary relations have not yet been established, there is no reason to believe that growth lines on marine invertebrates are less significant.

GEORGE R. CLARK II

Division of Geological Sciences, California Institute of Technology, Pasadena 91109

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Interferon Inducers Protect Mice against Plasmodium berghei Malaria

Abstract. *Injection of mice with two interferon inducers, Newcastle disease virus or statolon, 20 hours after inoculation with* Plasmodium berghei *sporozoites, prevented or delayed the development of detectable malarial parasitemia and death.*

To know whether the antimicrobial action of interferon is limited to viruses or extends to other classes of intracellular parasites is important with respect to the mechanism of action of interferon, to the metabolism and mechanism of reproduction of intracellular parasites, and to the range of potential therapeutic or preventive applications of interferon and of inducers of interferon activity. Interferon inhibits the development of psittacosis and trachoma-inclusion conjunctivitis (TRIC) agents (*1*), a group of intracellular parasites distinct from viruses (*2*). The experiments reported here show that two interferon inducers, statolon (*3*) and Newcastle disease virus (NDV), exert a protective effect in vivo on a protozoal infection, *Plasmodium berghei* mouse malaria.

The cyclical propagation of *P. berghei* in rodents and in *Anopheles stephensi* (*4*) and the method of harvesting sporozoites from the mosquitoes' salivary glands (*5*) have been described. In this system, development of parasites previous to their invasion of erythrocytes takes place in liver cells, chiefly during the first 40 to 46 hours after injection of sporozoites, and detectable parasitemia appears usually from the 3rd to 7th day, depending upon the number of sporozoites injected (*6*). Female CF1 mice (4 weeks old, Carworth Farms) were injected intravenously with 5000 sporozoites in 0.2 ml of a solution of 50 percent saline, and 50 percent human serum. Seventeen to 20 hours later, they were injected with the interferon inducers or with control materials. A 4 percent suspension of statolon (*7*) was prepared and diluted in 1 percent sodium bicarbonate just before use. Control materials included 1 percent bicarbonate, the stabilizer (*7*), and the 4 percent statolon suspension after heating at 95°C for 1 hour, since this procedure has been reported to decrease the interferon-inducing activity of statolon (*3*). Statolon and the respective control materials were injected intraperitoneally in 0.2 ml of 1 percent sodium bicarbonate. Allantoic fluid was harvested from NDV-infected chick embryos and titrated in chick embryo cell cultures by the plaque method (*8*). Dilutions of NDV were prepared in physiological saline. Control materials included physiological saline, normal allantoic fluid from 13-day-old chick embryos, and the supernatant of the infected allantoic fluid after centrifugation at 100,000*g* (most but not all infectious virus was removed by centrifugation). Portions (0.2 ml) of NDV or of the respective control materials were injected intravenously.

Three to six mice were bled for determination of serum interferon 8 hours after the injection of NDV (or of respective control fluids) or 16 hours after the injection of statolon (or of respective control fluids). The pooled serums from NDV-injected mice, and those from the respective control mice, were dialyzed at *p*H 2.0 for 3 to 4 days to destroy residual infectious virus. Interferon activity was determined by the ability of twofold dilutions of the tested pooled serums to inhibit vesicular stomatitis virus (VSV) plaque formation in mouse L cells. The reciprocal of the highest dilution of the tested pooled serum causing a reduction of 50 percent or more in the number of control plaques was considered the interferon titer (*9*). Blood smears obtained from the remaining mice (usually 10 per group) on the 4th, 5th, 6th, 7th, and 8th days after the sporozoite injection and twice a week thereafter were stained with Giemsa. The parasitized erythrocytes per 10,000 erythrocytes were counted, and the following end points were determined: (i) the number of mice that showed detectable parasitemia (patency); (ii) the duration of the mean interval between sporozoite injection and first smear with one or more parasites per 10,000 erythrocytes (prepatent period); and (iii) the mean number of parasitized erythrocytes on a given day. Patent animals were observed daily until they died (up to 35 days) and nonpatent ones for 21 days. Deaths occurred only in patent animals, and all patent animals died.

Allantoic fluid from NDV-injected embryos exerted a protective effect up to dilutions of 10^{-2} to 10^{-3} (Table 1). This effect was not associated with the injection of fluid per se (saline control), nor with the constituents of normal allantoic fluid (allantoic fluid control). The protective effect was decreased by the centrifugation of NDV allantoic fluid. Thus, it was associated either with the infectious virus particle or with a particle present only in infected allantoic fluid and sedimenting at 100,000*g*. The dilution end point of the protective effect of NDV was about the same whether it was measured by the number of surviving animals or whether it was measured by the mean prepatent period. The protective effect of statolon (Table 2) was not due to the diluent or to the stabilizer. It was decreased after heating at 95°C, as was the interferon-stimulating effect of statolon (experiment 4).

From the results shown in Tables 1 and 2, it can be concluded that (i) two interferon inducers of different origin and composition, statolon and NDV, exert a protective effect against sporo-

Table 1. Inhibition of *P. berghei* malaria and induction of serum interferon by NDV in mice. IF, interferon; PFU, plaque-forming units.

Material injected (log dilution)	NDV injected (PFU)	Experiment 1		Experiment 2	
		Serum IF (titer)	Mortality (No. dead per 10)	Serum IF (titer)	Mortality (No. dead per 10)
		NDV-infected allantoic fluid			
0	9.4×10^8	10,240	0	1280	0
−2	9.4×10^6	< 40	2	40	1
−3	9.4×10^5	< 40	7	< 40	0
−4	9.4×10^4	< 40	7	< 40	6
		*Supernatant of NDV-infected allantoic fluid**			
0	2.0×10^7	< 40	5	< 40	3
−1	2.0×10^6	< 40	7	< 40	7
−2	2.0×10^5	< 40	10	< 40	8
−3	2.0×10^4	< 40	8	< 40	9
		Normal allantoic fluid			
0		< 40	8	< 40	8
		Saline			
		< 40	9	< 40	7

* After centrifugation at 100,000*g*.

zoite-induced *P. berghei* malaria and (ii) the protective effect of the interferon inducers is decreased by procedures which decrease their interferon-inducing effect. These results support the hypothesis that the protective effect of statolon and NDV against *P. berghei* mouse malaria was due to the production of interferon or to a closely associated phenomenon, although other mechanisms cannot be entirely excluded. The fact that the interferon inducers were active when administered after the sporozoite challenge does not vitiate this hypothesis, since interferon-mediated protection against certain viruses has been demonstrated when interferon was administered after the onset of viral infection (*10*).

The demonstration that NDV and statolon protect against mouse malaria should stimulate the investigation of other interferon inducers for their effect on malaria. In view of our results, it seems possible that nonspecific inhibitors of experimental malaria such as *Corynebacterium parvum* or Freund's adjuvant (*11*), or even certain antimalarial drugs effective at the preerythrocytic stage, might either possess interferon-inducing activity or, perhaps, potentiate the induction of interferon synthesis by the protozoal agent itself.

The protective effect observed in our experiments could take place during development of *P. berghei* in liver cells, at the time of release of tissue merozoites or during the initial development of the parasite in erythrocytes. If the effect of the interferon inducers takes place within 24 hours of their administration, the affected stage would be the late development phase of preerythrocytic forms. This is a time when considerable increase in the number of nuclei and in the mass of the preerythrocytic forms occurs and when several organelles, including ribosomes, have been observed in their cytoplasm (*12*).

The possibility that interferon may affect the development of protozoa has important implications for students of the mechanism of interferon action. Several variables which cannot be introduced into experiments on virus-infected cells could become amenable to study. For instance, studies of interferon-induced modification of ribosomes (*13*) have been limited, by necessity, to host-cell ribosomes. A protozoal system could allow comparative studies of modifications of the parasite ribosomes and of the host ribosomes.

An effect of interferon on the psittacosis and TRIC agents has been demonstrated (*1*). The possibility that certain protozoa may be added to the list of interferon-susceptible organisms is raised by our study and by the independent demonstration by Remington and Merigan (*14*) that the multiplication of *Toxoplasma gondii* in cell cultures is inhibited by preparations of interferon.

Note added in proof: Since this manuscript was submitted, we have tested a third inducer of interferon activity, the complex of polyriboinosinic acid and polyribocytidylic acid (*15*). We have found that this compound also protects mice against sporozoite-induced *Plasmodium berghei* malaria.

RENÉ I. JAHIEL, JAN VILCEK
RUTH NUSSENZWEIG
JEROME VANDERBERG
Departments of Preventive Medicine and Microbiology, New York University School of Medicine, New York 10016

Table 2. Inhibition of *P. berghei* malaria and induction of serum interferon by statolon in mice. The stabilzer (*7*) was composed of 58 percent glucose and 16 percent $(NH_4)_2CO_3$ in 1 percent $NaHCO_3$; prepatent period (days) mean±S.D.; IF, interferon.

Material injected (mg)	Experiment 3		Experiment 4		
	Mortality (No. dead per 10)	Prepatent period (days)	Mortality (No. dead per 10)	Prepatent period (days)	Serum IF titer
		Statolon			
8	3	7.33 ± 2.52	0		160
0.8	3	6.00 ± 1.00	4	5.75±0.50	
0.08	8	6.25 ± 1.03			
		Heated statolon			
8	5	6.40 ± 0.55	5	6.00±1.00	40
0.8	9	5.56 ± 1.02	5	5.80±0.84	
0.08	9	4.67 ± 0.54			
		Stabilizer (7)			
	9	4.89 0.60			
		1 percent $NaHCO_3$			
	10	4.50 0.57	9	5.00±0.50	< 40

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Interferon: Protection of Cells Infected with an Intracellular Protozoan (Toxoplasma gondii)

Abstract. *Chick and mouse cell monolayers treated with interferon were significantly protected from destruction by toxoplasma. The results suggest that fewer numbers of organisms were released from interferon-treated cells. Interferons were uniformly more active in a viral assay than in a toxoplasma assay, and they had the same properties in both assays. The data suggest that interferon activity may be directed against organisms phylogenetically higher than viruses.*

A significant role for interferon in protection of cells from destruction by obligate intracellular parasites other than viruses is suggested by the observations on its effect in infections with organisms such as psittacosis (*1*) and trachoma-inclusion conjunctivitis (TRIC) agents (*2*, *3*). In that the obligate intracellular protozoan, *Toxoplasma gondii,* induces interferon production (*4*), we set out to determine if treatment of cells in vitro with interferon will alter their resistance to destruction by toxoplasma.

Chick interferon was prepared in 9- to 11-day-old embryonated eggs with the WS strain of influenza (*5*), and mouse interferon was prepared in L cells with Newcastle disease virus (*2*). Antiviral activity of mouse and chick interferon was measured in L cell and chick embryo fibroblast monolayers, respectively, with the use of bovine vesicular stomatitis virus (VSV) (*5*). For evaluation of mouse interferon activity against toxoplasma, 6×10^5 L cells that had been grown in monolayers were suspended in 2.4 ml of various dilutions of interferon in Eagle's minimal essential medium (MEM) supplemented with inactivated fetal calf serum (10 percent), streptomycin (100 μg/ml), and penicillin (50 unit/ml). The fetal calf serum used contained no toxoplasma antibodies, as judged by the Sabin-Feldman dye test. The cell suspensions (0.4 ml) were plated on cover slips (22 mm) attached to supporting glass rings with silicone grease and incubated at 37°C for 16 hours in an atmosphere containing 5 percent of CO_2. After the monolayers were washed twice with Earle's balanced salt solution, 0.5 ml of supplemented MEM containing 5×10^5 toxoplasma was placed on each monolayer (approximately 2.5 organisms per cell). The RH strain of toxoplasma was used in all experiments and was obtained from the peritoneal fluid of mice that had been infected 3 days earlier. The monolayers were then incubated at 37°C for 1 hour, washed with Earle's solution and incubated again in supplemented MEM at 37°C for varying periods of time. Monolayers were fixed in Bouin's solution and were stained with hematoxylin and eosin. Destruction of an interferon-treated monolayer was determined quantitatively by comparison to both uninfected monolayers (value of 0) and untreated infected monolayers (value of 4+). The intermediate degrees of destruction were scored with a value of 1+ for 10 to 30 percent destruction, 2+ for >40 to 70 percent destruction, and 3+ for 75 to 90 percent destruction.

Activity of chick interferon against toxoplasma was tested by a plaque assay in secondary cultures of chick embryo fibroblasts. Cells (5×10^5) were seeded in petri dishes (60 by 15 mm) in a modification (fetal calf serum was used) of the supplemented medium 199 described by Rubin (*6*). When the monolayers were confluent (usually 48 hours), the medium was removed and replaced with 4 ml of the supplemented medium or with dilutions of interferon in this medium. The monolayers were incubated for 8 hours, and washed with Earle's solution, and approximately 300 toxoplasma in 2 ml of suspending medium (*7*) were added to each dish. After incubation for another hour, the medium was removed and replaced with overlay medium (*8*). After 5 days at 37°C in an atmosphere containing 5 percent of CO_2 the cells on the plates were stained with a solution (0.05 percent) of neutral red, and plaques were counted. Microscopic examination revealed the plaque areas to consist of cell debris and free toxoplasma.

Table 1. Assay of interferon activity in L cells with vesicular stomatitis virus and toxoplasma.

Interferon preparations	Activity	
	VSV*	Toxoplasma †
A. Pressure dialyzed crude	1/20,000	1/400
B. Crude (experiment 1)	1/900	1/50
Crude (experiment 2)	1/900	1/50
C. CM-Sephadex purified	1/2200	1/300
D. Lyophilized CM-Sephadex purified ‡	<1/30	<1/10
E. XE-64 purified (with 0.05 percent bovine serum albumin added)	1/560	1/20
F. XE-64 purified	1/275	1/30

* Dilution of interferon preparation which inhibits VSV plaque formation in L cells by 50 percent. † Dilution of interferon preparation which reduces L cell destruction by toxoplasma from 4+ in control-infected monolayer to 2+ destruction. ‡ The original titer of this preparation was 1:100 against VSV and, in contrast to the usually observed stability of such lyophilized preparations, had markedly decreased on storage—this loss in activity was reflected by results obtained in both assays.

Monolayers of L cells treated with mouse interferon before infection were significantly protected from destruction by toxoplasma as compared with untreated infected monolayers (Fig. 1). However, in all interferon-protected monolayers there were intracellular replicative forms of toxoplasma. A preparation of chick interferon which was active in the chick cell plaque-reduction assay with toxoplasma showed no activity in the assay consisting of toxoplasma and L cells. Mouse interferon was not active against toxoplasma infection as judged in a cover slip assay with chick embryo fibroblasts rather than L cells. Samples treated with mouse interferon at various temperatures for 1 hour were stable at 4° and 37°C, partially lost their activity at 56°C, and completely lost activity at 80°C in L cell assay both with virus and with toxoplasma. In both assays lowering of the *p*H to 2 did not destroy interferon activity; the interferon activity did not sediment after cen-