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Role of Interferon in the Antiviral State Elicited by Selected Interferon Inducers (40737)

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In a previous study we demonstrated that mice could be protected against viral infection by a second injection of endotoxin administered during the period of interferon "tolerance" (1). The tolerance period is that interval following interferon production during which restimulation results in little or no interferon synthesis (2, 3). Those earlier studies suggested, but did not conclusively show, that protection against viral infection by endotoxin was not dependent on interferon induction. In the present study, splenectomized mice and anti-interferon gamma globulin were used to investigate further the relationship between the circulating interferon produced in response to interferon inducers and the antiviral state. The results indicate that protection by endotoxin is not mediated by interferon while that of poly(I:C) and pyran can at least be partially attributed to interferon induction.

Materials and methods. Animals. Young adult female Swiss Webster mice, weighing 18–20 g were purchased from commercial sources and housed in groups of 10. Splenectomies were completed 4 days prior to the injection of endotoxin (4). The animals were maintained in accordance with the "Guide for Laboratory Animal Facilities and Care" as published by the National Academy of Sciences National Research Council.

Viruses. Stock suspensions of Encephalomyocarditis virus (strain MM) and Mayaro viruses were prepared and titrated by the methods previously described (5). The mean lethal dose (LD_{50}) of each virus preparation was computed by the method of Reed and Muench (6) by using the intraperitoneal (ip) route of inoculation. In experiments, each virus was injected ip into mice at the dose indicated and the number of deaths recorded daily for a period of 14 days.

Statistical analysis. Significance of the difference was determined by χ^2. Differences were considered significant if $P < 0.05$.

Interferon inducers. Endotoxin (*Escherichia coli* 0:128:B12 lipopolysaccharide B) was purchased from Difco Laboratories Inc., Polyinosinic polycytidylic acid (poly(I:C)) was obtained from Sigma Chemical Company and Maleic acid-divinyl ether copolymer (Pyran) was generously supplied by Hercules Inc. Each inducer was administered intraperitoneally in 0.2-ml volumes which contained 100 μg of endotoxin, 100 μg poly(I:C), or 200 μg of pyran respectively.

Interferon. Production, purification, and concentration of L-cell interferon was accomplished by the method described by Knight (7). Final preparations of the purified interferon were assayed for 50% plaque reduction (PR_{50}) units. Tenfold serial dilutions of the preparations were made in Eagle's minimal essential medium. Each dilution was added to confluent L-cell monolayer cultures in duplicate. After 6 hr of incubation at 37°C, the cell cultures were washed and each culture was challenged with approximately 100 plaque-forming units (PFU) of MM virus. The PR_{50} titer of the preparation was the reciprocal of the highest dilution which inhibited the number of plaque-forming units by 50%. It was determined that the interferon preparations contained approximately 200,000 PR_{50} units/mg protein.

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TABLE I. PROTECTION OF MICE BY ENDOTOXIN AGAINST MAYARO OR MM VIRUS INFECTION

Treatment Hour:			
0	24	48[a]	Dead/total
HBS	HBS	Mayaro virus	15/39
HBS	HBS	MM virus	25/40
HBS	Endo	Mayaro virus	6/40
HBS	Endo	MM virus	4/40
Endo[b]	HBS	Mayaro virus	18/40
Endo	HBS	MM virus	5/40

[a] Mice were challenged intraperitoneally with one LD_{50} of either MM or Mayaro virus.
[b] Endotoxin (Endo) given intraperitoneally at a dose of 100 μg per mouse.

Anti-interferon gamma globulin. New Zealand white rabbits weighing approximately 2.5 kg were given injections of the concentrated interferon preparation at 2-week intervals for a period of 16 weeks. The first injection was given intramuscularly (im) and contained approximately 100,000 PR_{50} units of interferon in Freunds complete adjuvant. At each 2-week interval, the animals were given 50,000–150,000 PR_{50} units of interferon in Hanks' balanced salt solution by both the im and intravenous routes of inoculation. The rabbits were bled by heart puncture 7 days after the final injection, the serum collected, and cell specific antibody removed by adsorption with L cells. The gamma globulin was extracted by three precipitations with ammonium sulfate at a final concentration of one-third saturation (15). The precipitate was dissolved in borate-buffered saline. After removing the salts by dialysis, the gamma globulin preparation was passed through a 0.45 μm Millipore filter. Tenfold serial dilutions of this antibody preparation were made using Hanks' balanced salt solution. Each dilution was incubated at 37°C for 30 min with an equal volume of L-cell interferon containing 400 PR_{50} units. It was determined that a 1:10 dilution of the antibody preparation completely neutralized the plaque reducing ability of the interferon.

Results. Protection by endotoxin. When endotoxin was administered 24 hr before virus challenge (Table I), the mice were protected against lethal infection by either Mayaro ($P < 0.05$) or MM virus ($P < 0.001$). When the inducer was given 48 hr prior to the virus, however, the mice were no longer protected against Mayaro virus while protection against MM virus remained unchanged. These data demonstrate that endotoxin induces a relatively short protection period against Mayaro virus infection.

The results in Table II again show that endotoxin protected the mice against Mayaro virus infection only when the inducer was given 24 hr prior to virus challenge (group 8 compared to groups 1–4; P

TABLE II. EFFECT OF SINGLE AND MULTIPLE INJECTIONS OF ENDOTOXIN ON INTERFERON INDUCTION AND PROTECTION OF MICE AGAINST MAYARO VIRUS INFECTION

	Treatment at Day[a]				Interferon (PR_{50} Units) at Day[b]				
Group	0	1	2	3	0	1	2	3	Dead[c]/total
1	HBS	HBS	HBS	HBS	—	—	—	—	35/40
2	Endo	HBS	HBS	HBS	80	—	—	—	37/40
3	Endo	Endo	HBS	HBS	—	<1	—	—	36/40
4	Endo	Endo	Endo	HBS	—	—	<1	—	39/40
5	Endo	Endo	Endo	Endo	—	—	—	<1	34/40
6	Endo	HBS	HBS	Endo	—	—	—	<1	19/40
7	Endo	Endo	HBS	Endo	—	—	—	<1	32/40
8	HBS	HBS	HBS	Endo	—	—	—	166	10/40
9	HBS	HBS	Endo	Endo	—	—	—	<1	38/39
10	HBS	Endo	Endo	Endo	—	—	—	<1	38/39

[a] Groups of mice were treated with either endotoxin (Endo; 100 μg/mouse) or HBS as indicated.
[b] Two hours after endotoxin inoculation, 10 mice from appropriate groups were bled and the pooled plasma assayed for interferon content.
[c] All of the mice were challenged with mayaro virus (2 LD_{50}) on Day 4.

< 0.05 in each instance). This protection was nullified by prior exposure of the animals to the same inducer (groups 5, 7, 9, and 10). However, when the first exposure to endotoxin occurred three days prior to the second administration of endotoxin (group 6), protection against lethal infection by the virus was evident although circulating interferon could not be detected.

Table III contains results obtained when splenectomized mice were used. These data indicate that normal and splenectomized mice were protected when the endotoxin was given by either the intraperitoneal or intravenous routes of inoculation. In normal mice, measurable amounts of circulating interferon were elicited by both routes of inoculation. In splenectomized mice, however, interferon was detected only when the inducing endotoxin was given intraperitoneally. The data in Tables II and III are consistent with the hypothesis that protection by endotoxin against viral infection can be independent of demonstrable interferon production.

Effect of anti-interferon gamma globulin on protection. Anti-interferon gamma globulin was used to explore the relationship between the protective and interferon-inducing properties of endotoxin, poly(I:C), and pyran. The data in Table IV show that when anti-interferon gamma globulin was given shortly after the administration of the interferon inducers, the protective effects of poly(I:C) and pyran against MM virus infection were inhibited ($P < 0.05$) while that of endotoxin was not affected. These data indicate that at least some of the protection by poly(I:C) and pyran can be attributed to interferon. Protection by endotoxin, however, does not appear to be mediated by interferon.

TABLE III. EFFECT OF SPLENECTOMY ON INTERFERON INDUCTION AND PROTECTION BY ENDOTOXIN AGAINST MAYARO VIRUS INFECTION

Group	Treatment[a]	Interferon[b]	Dead[c]/total
1	HBS (ip)	<1	34/40
2	Endo (ip)	120	8/40
3	Endo (iv)	50	10/40
4[d]	HBS (ip)	<1	24/30
5[d]	Endo (ip)	240	5/30
6[d]	HBS (iv)	<1	26/30
7[d]	Endo (iv)	<1	9/30

[a] Groups of mice were treated with either endotoxin (Endo; 100 μg/mouse) or HBS by either the intraperitoneal (ip) or intravenous (iv) route as indicated.

[b] Two hours after endotoxin inoculation, five mice from each group were bled and the pooled plasma assayed for PR_{50} units of interferon.

[c] All of the mice were challenged ip with mayaro virus (2 LD_{50}) 24 hr after treatment with either endo or HBS.

[d] Mice were splenectomized 4 days before the start of the experiment.

TABLE IV. EFFECT OF ANTI-INTERFERON GAMMA GLOBULIN ON PROTECTION BY INTERFERON INDUCERS AGAINST MM VIRUS INFECTION

Treatment at:		
0 hr[a]	+15 min[b]	Dead/total[c]
HBS	HBS	17/30
Poly(I:C)	HBS	1/30
Poly(I:C)	Anti-IF	8/30
Pyran	HBS	1/30
Pyran	Anti-IF	8/30
Endo	HBS	8/30
Endo	Anti-IF	9/30

[a] Inducer or HBS (0.2 ml) injected intraperitoneally; poly(I:C), 200 μg; pyran, 400 μg; endotoxin (Endo), 100 μg.

[b] Fifteen minutes after inducer, HBS or anti-interferon gamma globulin (Anti-IF) diluted 1:10 given intraperitoneally (0.2 ml).

[c] Mice were challenged with MM virus (about 1 LD_{50}) 24 hr after inducer treatment and deaths recorded for 14 days.

Protection by circulating interferon. An experiment was done to investigate more directly the role of circulating interferon in protection against an intraperitoneal injection of virus. Mice were given a protective dose of interferon by either the intraperitoneal or intravenous route of inoculation. Both groups of animals were then challenged intraperitoneally with MM virus. The data in Table V show that there was no protection when the interferon was given intravenously. These results are consistent with the hypothesis that levels of circulating interferon do not reflect the degree of antiviral protection.

Discussion. The data in this study indicate that protection by endotoxin was not mediated by interferon. This conclusion was indirectly demonstrated by the protection of mice against Mayaro virus infection

TABLE V. INABILITY OF CIRCULATING INTERFERON TO PROTECT MICE AGAINST MM VIRUS INJECTED BY THE INTRAPERITONEAL ROUTE

Treatment[a]	Route of injection	Dead[b] total
HBS	ip	10/30
IF	ip	2/30
IF	iv	10/30

[a] HBS or 3500 PR_{50} units of interferon (IF) given either intraperitoneally (ip) or intravenously (iv).

[b] Mice were challenged with an LD_{50} of MM virus given ip 24 hr after initial treatment. Deaths recorded for a period of 14 days.

in the absence of demonstrable levels of circulating interferon (Tables II and III), and directly by the absence of an inhibitory effect by anti-interferon gamma globulin on the antiviral state resulting from the endotoxin inducer (Table IV). In contrast, protection following the administration of either poly(I:C) or pyran was significantly reduced by treatment with anti-interferon gamma globulin. These data suggest that at least a portion of the protective state elicited by poly(I:C) or Pyran can be attributed to interferon and are in agreement with those reported by Gresser *et al.* (8). It is probable, however, that the circulating interferon induced by poly(I:C) or pyran is not involved in establishing the antiviral state. This conclusion is supported by the data in Table V which show that interferon injected directly into the blood stream (circulating interferon) did not protect the animals against MM virus injected intraperitoneally. In similar studies Gresser *et al.* (9, 10) reached the same conclusion. The site of interferon production in response to poly(I:C) or pyran stimulation has not been determined, and as suggested by Sharpe *et al.* (11), circulating interferon might be an "overspill" and not involved in protection. This would explain the lack of correlation between detectable levels of circulating interferon and the degree of protection against viral infections reported previously (12). Thus, the level of circulating interferon appears to be a poor indicator of the potential of a substance as an antiviral agent.

A possible mechanism of action of endotoxin, as well as other interferon inducers, in establishing the antiviral state is the stimulation of macrophage activity. Rabinovitch *et al.* (13) reported increased stimulation of mouse peritoneal macrophage activity following treatment with various inducers. Endotoxin caused the most activation followed by pyran, with poly(I:C) exhibiting the least amount of stimulation. Intraperitoneal injection of interferon itself has been reported to stimulate the phagocytic activity of peritoneal macrophages (14).

These studies support our previous conclusion that the antiviral protection which results in response to interferon inducers is probably due to several mechanisms of action and not to the interferon system alone (1).

Summary. Data are presented which show that protection of mice by endotoxin against viral infection is not mediated by interferon while that of poly(I:C) and pyran can be at least partially attributed to interferon induction. The results also suggest that circulating interferon does not protect against a virus administered intraperitoneally.

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