Prolonged Antiviral Protection by Interferon Inducers (34291)

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(Introduced by T. C. Merigan)
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A variety of interferon inducers including microorganisms as viruses and bacteria, derivatives of living materials, and synthetic anionic polymers were tested for their ability to inhibit vaccinia virus tail lesions in mice. This test system is highly sensitive and reliable for evaluation of antiviral compounds (1, 2), allows quantitation of their protective activity, and could be used for titration of interferon samples (2). The polyanionic interferon inducer, polyacrylic acid, causes a significant reduction of the number of vaccinia virus lesions, when given prior to infection and its antiviral effect persists for a much longer period than interferon can be detected in the blood stream (2, 3). The present study confirmed the prolonged activity of polyacrylic acid, extended it to other interferon inducers and evaluated the role of interferon and other factors in the antiviral protection afforded by these compounds.

Materials. Brucella abortus (strain 19), an attenuated strain used for vaccination of cattle was supplied by Dr. C. Huygelen, R.I.T., Genval, Belgium. *Proteus rettgeri* and *Escherichia coli* were grown in tryptic soy broth (Difco) and washed with saline before use. Newcastle disease virus (Kumarov strain) underwent serial passages in the chorioallantoic cavity of chick embryos. Sindbis virus (strain Egypt AR 339) was propagated in chick embryo fibroblasts. Polyacrylic acid (200,000 mol wt) was prepared and characterized as described previously (4). Pyran copolymer (designated NSC 46015-C; 17,000 mol wt) was supplied by Dr. T. C. Merigan, Stanford University, Stanford, California. Alginic acid was purchased from Light Co., Colnbrook, England. An extract was prepared from *Penicillium chrysogenum* Wisconsin 49408 with the hot (60°) phenol–sodium dodecyl sulfate procedure. Endotoxin was extracted from *P. rettgeri* according to the method of Roberts (5).

Methods. To study the effect of these compounds on the formation of vaccinia tail lesions, young female NMRI mice, weighing 14–16 g, were injected either intraperitoneally or intravenously with 0.5 ml of the tested compound in appropriate medium. One or 8 days later, the mice were inoculated intravenously with 0.1 ml of a dilution of vaccinia virus (commercial calf lymph vaccine received from the Vaccines Office in Brussels, Belgium), giving an average of about 10–20 lesions/tail. Pox lesions were counted 6–8 days after vaccinia challenge. Student's *t* test was used to assess significance of the distribution of the individual results per group.

Results. All substances studied stimulate interferon production in animals either after intravenous or intraperitoneal injection: *B abortus* (6, 7), *E. coli* (7), Sindbis virus (8), Newcastle disease virus (8), polyacrylic acid (3), pyran copolymer (9), alginic acid (3), *P. chrysogenum* extract (10), and *P. rettgeri* endotoxin (11). We found intact *P. rettgeri* bacteria able to induce significant interferon amounts in the mouse. Living and heat-killed *B. abortus* did not differ in interferon-inducing ability. In a representative experiment 1800 units of circulating interferon /ml were obtained in mice 8 hr after intravenous injection of either viable or killed *B. abortus,* as assayed in a plaque inhibition assay on primary mouse embryo fibroblasts against vesicular stomatitis virus.

Table I shows that there was nearly 100% inhibition of vaccinia tail lesion formation by *B. abortus* and Sindbis virus and 70–95%

[1] Aspirant of the Belgian "Nationaal Fonds voor Wetenschappelijk Onderzoek."

TABLE I. Effect of Different Bacteria and Viruses on the Formation of Vaccinia Tail Lesions in Mice.[a]

Expt. no.	Treatment (organism, route, days prior to infection)	No. of lesions: Per individual mouse	No. of lesions: Av	Probability (comparison with control group)
1	*B. abortus*,[b] ip, 8	0, 0, 0, 0, 0, 0, 0, 0, 1, 1	0.18	$p < 0.001$
	ip, 1	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0.00	$p < 0.001$
	iv, 8	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0.00	$p < 0.001$
	iv, 1	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0.00	$p < 0.001$
	P. rettgeri,[c] iv, 8	3, 5, 2, 1, 5, 3, 5, 2, 2, 0, 3, 5	3.00	$p < 0.001$
	iv, 1	0, 0, 0, 0, 1, 1, 2, 2, 2, 1, 0	0.82	$p < 0.001$
	NDV,[d] iv, 8	0, 0, 4, 0, 3, 8, 3, 11, 5, 3, 2, 3	3.50	$p \sim 0.001$
	iv, 1	1, 0, 0, 0, 0, 0, 0, 0, 0, 1, 5	0.58	$p < 0.001$
	Sindbis,[e] iv, 8	0, 1, 0, 2, 0, 1, 0, 0, 0, 0, 1, 0	0.42	$p < 0.001$
	iv, 1	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0.00	$p < 0.001$
	Control	30, 15, 21, 3, 12, 8, 5, 19, 10, 4, 28, 13	14.00	—
2	*E. coli*,[f] iv, 8	1, 0, 2, 6, 1, 6, 1, 1, 2, 3, 13	3.27	$p \sim 0.001$
	iv, 1	0, 1, 0, 0, 0, 0, 2, 3, 10, 12, 3, 11	3.50	$p < 0.01$
	Control	14, 17, 2, 15, 18, 29, 6, 11, 4, 3, 14, 6	11.58	—

[a] Vaccinia virus was injected intravenously; the different interferon-inducing bacteria and viruses were injected intraperitoneally (ip) or intravenously (iv).

[b] *Brucella abortus*: 10^9 living bacteria/mouse.

[c] *Proteus rettgeri*: 10^9 living bacteria/mouse.

[d] Newcastle disease virus: 10^8 EID_{50} (egg infectivity $dose_{50}$)/mouse.

[e] Sindbis virus: 10^8 PFU (plaque-forming units in rat embryo fibroblasts)/mouse.

[f] *Escherichia coli*: 10^8 living bacteria/mouse.

inhibition by *P. rettgeri*, *E. coli*, and Newcastle disease virus. The *B. abortus*, Sindbis virus, and *E. coli* were equally effective when injected 8 days or 1 day before vaccinia virus challenge. The *P. rettgeri* and Newcastle disease virus were somewhat less active

TABLE II. Effect of Synthetic Polycarboxylates on the Formation of Vaccinia Tail Lesions in Mice.[a]

Expt. no.	Treatment (substance, route, days prior to infection)	No. of lesions: Per individual mouse	No. of lesions: Av	Probability (comparison with control group)
1	PAA,[b] ip, 8	1, 1, 5, 0, 1, 0, 2, 2, 2, 4, 0, 3	1.75	$p < 0.001$
	ip, 1	0, 2, 2, 1, 0, 3, 1, 0, 4, 0, 2, 2	1.42	$p < 0.001$
	iv, 8	0, 0, 0, 0, 0, 0, 0, 1, 0, 0, 0, 10	0.92	$p < 0.001$
	iv, 1	0, 0, 0, 0, 1, 1, 0, 0, 3, 0, 0, 7	1.00	$p < 0.001$
	im, 8	2, 4, 0, 1, 2, 0, 1, 4, 3, 0, 0, 15	2.67	$p < 0.005$
	im, 1	0, 2, 4, 1, 1, 3, 2, 1, 0, 1, 1, 2	1.50	$p < 0.001$
	Control	14, 17, 2, 15, 18, 29, 6, 11, 4, 3, 6, 14	11.58	—
2	Pyran,[c] ip, 8	7, 1, 3, 1, 2, 7, 12, 2, 12	5.22	$p < 0.005$
	ip, 1	2, 0, 2, 1, 1, 1, 2, 6, 8, 1	2.40	$p < 0.001$
	Control	6, 16, 27, 15, 27, 6, 18, 12, 19, 26, 6	16.18	—

[a] Vaccinia virus was injected intravenously; the synthetic polycarboxylates were injected intraperitoneally (ip), intravenously (iv) or intramuscularly (im).

[b] Polyacrylic acid: 250 μg/mouse.

[c] Pyran copolymer: 250 μg/mouse.

TABLE III. Effect of Extracts of Living Materials on the Formation of Vaccinia Tail Lesions in Mice.[a]

Expt. no.	Treatment (extract, route, days prior to infection)	No. of lesions: Per individual mouse	No. of lesions: Av	Probability (comparison with control group)
1	Alginic acid,[b] ip, 8	30, 3, 7, 10, 1, 6, 10, 34, 12, 4, 10, 17	12.00	$p < 0.02$
	ip, 1	10, 4, 9, 7, 10, 7, 13, 7, 14, 14, 7, 10	9.33	$p < 0.001$
	Endotoxin,[c] ip, 8	33, 22, 21, 10, 27, 21, 3, 6, 33, 32, 7, 26	20.08	Nonsignificant
	ip, 1	0, 9, 1, 6, 9, 12, 0, 13, 8, 10, 17	7.73	$p < 0.001$
	iv, 8	31, 8, 2, 38, 18, 25, 20, 13, 19, 18	19.20	Nonsignificant
	iv, 1	0, 1, 0, 0, 0, 0, 1, 0, 0	0.22	$p < 0.001$
	Control	18, 11, 15, 30, 26, 31, 20, 22, 15, 23, 24, 19	21.17	—
2	P. Wisc.,[d] ip, 8	1, 3, 0, 1, 26, 15, 25, 1, 1, 0, 0	6.64	$p \sim 0.02$
	ip, 1	0, 0, 0, 0, 0, 3, 7, 0, 0, 3, 1, 2	1.33	$p < 0.001$
	Heat-killed *B. abortus*,[e] ip, 8	16, 23, 13, 13, 14, 13, 21, 18, 15, 11	15.70	Nonsignificant
	Control	1, 14, 13, 15, 33, 13, 16, 14, 20, 24	16.20	—

[a] Vaccinia virus was injected intravenously; the extracts were injected intraperitoneally (ip) or intravenously (iv).

[b] Alginic acid: 5 mg/mouse.

[c] *P. rettgeri* endotoxin: 200 μg/mouse.

[d] *P. chrysogenum* Wisconsin extract: equivalent of 5 mg of crude mycelium/mouse.

[e] *B. abortus* inactivated at 60° for 1 hr: 10^9 killed bacteria/mouse.

when injected 8 days prior to infection. The synthetic polyanion polyacrylic acid (PAA) provided significant protection regardless of the route of injection (intraperitoneally, intravenously, or intramuscularly) (Table II). The related pyran (maleic anhydride–divinyl ether) copolymer was also highly active. The effect of both PAA and pyran persisted for at least 8 days. Alginic acid had a moderate effect on vaccinia virus plaque formation both at 1 and 8 days (Table III). The mold (P. Wisconsin) extract lost a significant part of its activity and *P. rettgeri* endotoxin its whole activity within 1 week. Whereas viable *B. abortus* bacteria exhibited almost complete suppression of tail lesion formation, heat-killed *B. abortus* failed to confer any protection when injected 8 days before infection.

Other polyanionic substances, not listed in Table I, II, and III, as mucopolysaccharides extracted from different rat tissues, carrageenans, copolymers of acrylic acid and 2-ethylhexylacrylate, of methacrylic acid and methylmethacrylate, of methacrylic acid and styrene, and acrylic acid acrylamide copolymers with less than 60% free carboxyl groups (3), neither stimulated interferon production nor resistance to vaccinia tail lesion formation. The polycation diethylaminoethyldextran failed to induce either interferon or resistance to vaccinia virus infection as well. The methyl substituted polyacrylic acid, polymethacrylic acid, reduced the tail lesions by 45% ($p < 0.20$) when injected intraperitoneally 8 days before virus challenge, but increased the number of lesions by 60% ($p < 0.10$) when injected 1 day before infection (2). There is no direct explanation for the latter phenomenon but it might be due to a disaggregating effect of polymethacrylic acid on the virus particles.

Discussion. Several interferon inducing microorganisms and plastic polymers conferred an inhibitory activity on the formation of tail lesions in vaccinia infected mice. The antiviral protection persisted for at least 8 days, and as most compounds did not show any loss of activity within 1 week, their activity might be expected to last for a much longer

period. On the other hand, extracts of biological material as endotoxin, heat-killed *B. abortus* bacteria and a phenol extract from *P. chrysogenum* Wisconsin offered rather transient protection: their antiviral effect disappeared within a few days after injection.

The highly active interferon inducer polyriboinosinic acid/polyribocytidylic acid homopolymer pair (poly rI/rC) (12) has been reported to offer significant protection against a lethal Mengo virus infection in mice but protection diminished quickly within 6–10 days (13); in contrast, pyran copolymer was still effective when given 60 days before Mengo virus (13). The facultative intracellular bacterium *Listeria monocytogenes* and the obligate intracellular protozoa *Toxoplasma gondii* and *Besnoitia jellisoni* also stimulated interferon production during the acute phase of infection (14–17) and conferred prolonged resistance to Mengo virus for 1–3 months (Listeria) or 1 year (Toxoplasma) (17).

It seems as though interferon inducers could be spread over two classes: a first one including living materials as viruses, bacteria, and protozoa, and not readily biodegradable polymers as polyacrylic acid and pyran copolymer, and a second one including extracts or derivatives of living materials and synthetic polyribonucleotides which would be metabolized with greater ease within the animal. The first group should provide prolonged antiviral protection, the second rather transient protection. Continuous presence of the material might be essential for the persistence of protection by microorganisms and plastics. The antiviral effect offered by injection of these substances 1 day prior to infection could readily be explained by interferon circulating in the blood stream. But circulating interferon levels fall soon, (within 5 days (13)), and the interferon produced early can not account for the protection observed against a virus challenge 1 week later (2). Low quantities of circulating interferon, not detectable by presently available methods might be produced at late times after injection of either microorganisms or plastics and confer a protective activity against vaccinia virus. Alternatively, other factors than circulating interferon could explain the beneficial effects of microorganisms and plastics injected 8 days or more before infection. It is possible that the prolonged resistance is cell-bound and results from either a direct or an interferon-mediated interaction of the compound with the virus during critical phases of its multiplication. The microorganisms and plastics compounds tested might exert their prolonged antiviral effect by stimulating the interferon response to the subsequent vaccinia virus challenge (hyperreactivity as observed with Freund's complete adjuvant in animals inoculated with foot and mouth disease virus (18). Another possibility is that certain as yet undetermined humoral factors may be produced and alter the manifestations of the viral disease.

Summary. Several interferon inducers including bacteria, viruses, and synthetic polycarboxylates demonstrated an antiviral effect against vaccinia virus challenge in mice. Various extracts of living materials were equally effective in stimulating interferon production. They provided rather transient protection in contrast with the prolonged protection following injection of either living microorganisms or plastics. The antiviral activity offered by injection of these different interferon inducers 1 day before virus challenge can be explained by interferon production. The protection observed at later times might or might not be mediated by interferon, but seems to depend on the continuous presence of the microorganisms or plastics in the host.

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