

Interferon and Its Inducers—A Never-Ending Story: “Old” and “New” Data in a New Perspective

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The discovery of interferon (IFN), as an antiviral substance, by Isaacs and Lindenmann in 1957 [1] attracted the attention of a number of investigators, including Pieter De Somer at the Rega Institute in Leuven (Belgium) and Tom Merigan at Stanford University (California). De Somer's original attempts were aimed at determining the mode of action of IFN [2], whereas Merigan's work focused on trying to understand the role of IFN in human viral diseases [3], by use of the mouse as an animal model.

Of pioneering importance was the demonstration by Merigan [4] in 1967 that IFN could be induced in mice by a synthetic polyanion called maleic divinyl ether copolymer (or pyran copolymer). In further experiments, pyran copolymer was also shown to induce IFN in humans [5], although the serum IFN titers obtained in humans were considerably lower than those in mice and, moreover, were accompanied by a considerable increase in body temperature.

Meanwhile, in De Somer's laboratory, I had identified 2 other polyanions, polyacrylic acid (PAA) and polymethacrylic acid, as antiviral agents [6, 7] and attributed their antiviral action to either the induction of IFN or a direct interference with virus adsorption to the cells. Overall, the structures of PAA and polymethacrylic acid are quite similar to that of pyran copolymer (figure 1), the prominent features being the -C-C-C-C-C-C- or -C-C-C-O-C-C- backbone and the negatively charged carboxylic acids mounted on this backbone.

In writing about the potential role of IFN in clinical medicine in 1967, Merigan [8] had added an addendum: “recently, a significant report appeared describing the induction of IFN and antiviral effects in animals with double-stranded synthetic ribonucleotide homopolymers.” This report [9], by Maurice Hilleman's group at Merck, was the first of the induction of IFN by what later could be hailed as the most potent inducer of IFN- β ever described, poly(I)·poly(C).

DOUBLE-STRANDED RNA INDUCERS OF IFN

Guided by the discovery of poly(I)·poly(C) (figure 2) as a potent inducer of IFN [9], we further examined the structural requirements to which polyribonucleotides should adhere to induce IFN production and resistance to viral infection. Our initial studies indicated that a stable secondary, preferably multistranded, structure was required for antiviral activity [10]. This antiviral activity of these polyribonucleotides could be dramatically enhanced by thermal activation [11, 12], a remarkable phenomenon that has remained unexplored after all these years.

Starting from the alternating double-stranded RNA poly(A-U), we were able to significantly increase the IFN-inducing ability through substitution of the thiophosphate for the phosphate moieties [13]. The resulting poly(sA-sU) (figure 3) also gained increased resistance against degradation by nucleases, compared with that of its parent compound, poly(A-U), but the initial expectation that this modified RNA would ever aid in the fight against viral diseases [14] was eventually not fulfilled.

With poly(I)·poly(C) as the double-stranded RNA inducer of IFN, we [15] demonstrated that, in an animal model of virus-induced encephalitis (i.e., on intranasal challenge of mice with vesicular stomatitis virus

Potential conflicts of interest: none reported.

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The Journal of Infectious Diseases 2006;194:S19–26

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0022-1899/2006/19406S1-0004\$15.00

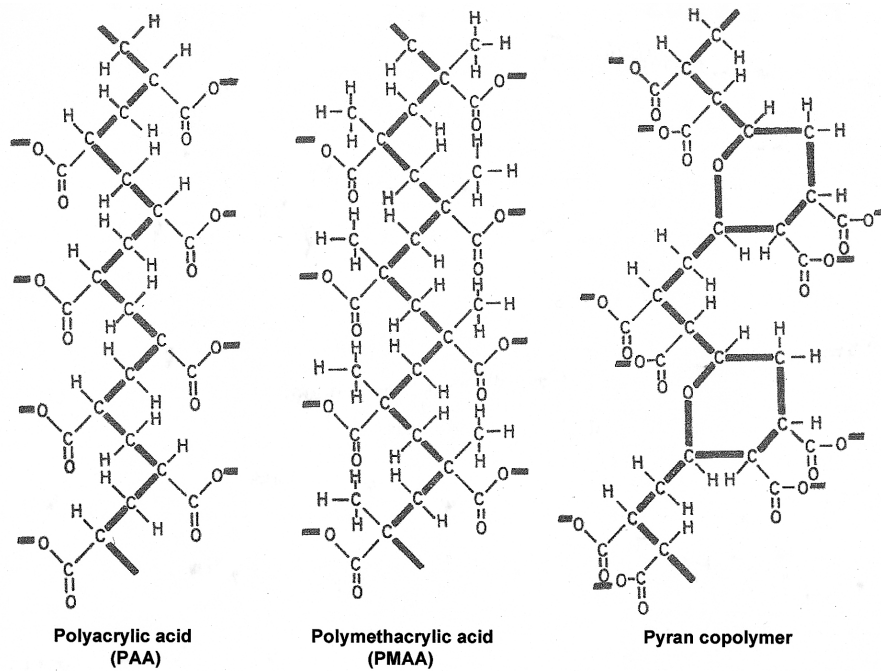


Figure 1. Structures of polyacrylic acid, polymethacrylic acid, and pyran copolymer

[VSV], a rhabdovirus), the whole protective effect conferred by the double-stranded RNA could be accounted for by IFN production. In those early days of IFN (and IFN inducer) research, we also developed an animal (mouse) model for induction of tumors by Moloney murine sarcoma virus, which is still used today to study the *in vivo* antiretroviral activity and which allowed us to demonstrate the inhibitory effect of

poly(I)·poly(C) on Moloney murine sarcoma virus-induced tumor formation [16].

Later on, it was shown that, of the 2 strands of poly(I)·poly(C), the poly(I) strand plays the predominant role and that, for the induction of IFN, poly(I) and poly(C) may be added in sequential order—that is, poly(I) followed by poly(C) [17]—assuming that, under these conditions, the double-stranded

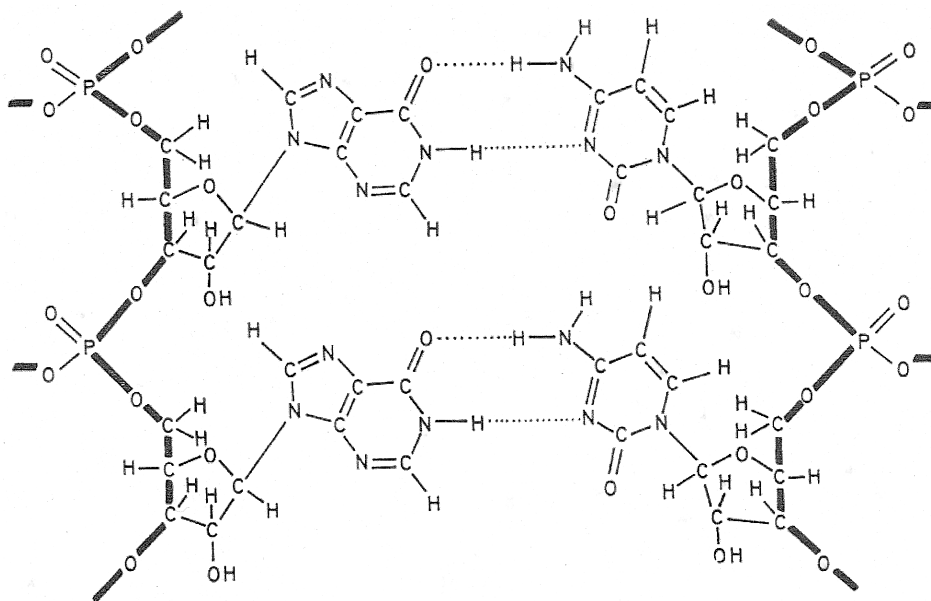


Figure 2. Structure of poly(I)·poly(C)

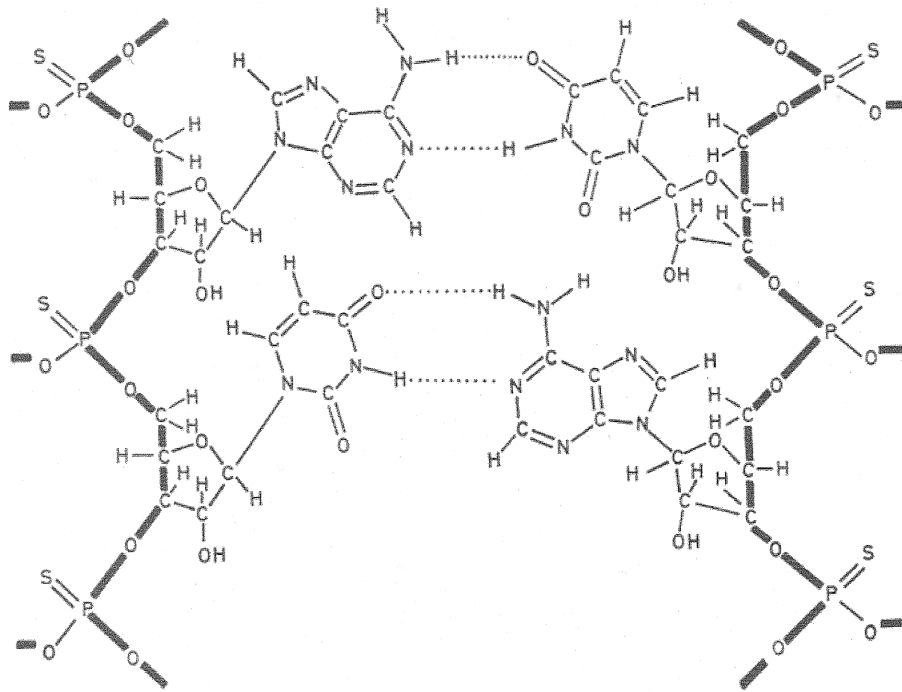


Figure 3. Structure of the thiophosphate analogue of poly r(A-U)

poly(I)·poly(C) complex would be assembled at the cell surface. Also, modifications in the poly(I) strand (i.e., 7-N substitution by a CH group) could be introduced without detrimental effects on the IFN-inducing ability of the resulting double-stranded RNA complex [18]. Moving in the other di-

rection, by introducing modifications in the poly(C) strand (i.e., interruption of this strand every twelfth or thirteenth cytidine residue by uridine), poly(I)·poly(C) analogues such as poly(I)·poly(C₁₂U) were constructed that still induced IFN while being subject to faster degradation by nucleases [19]. This

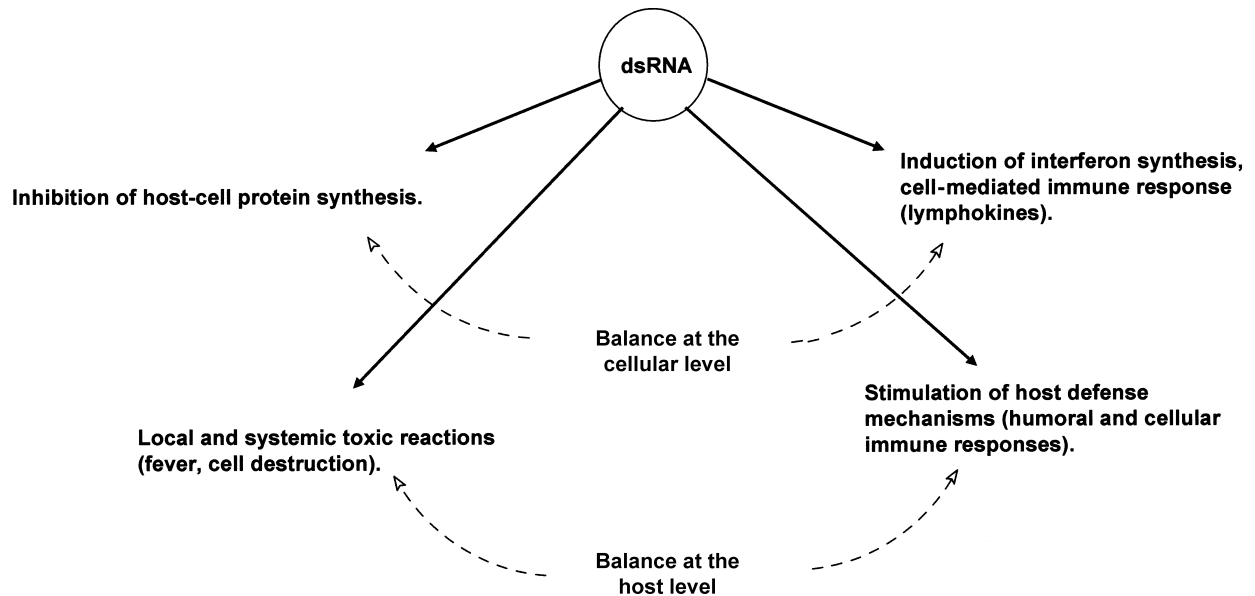
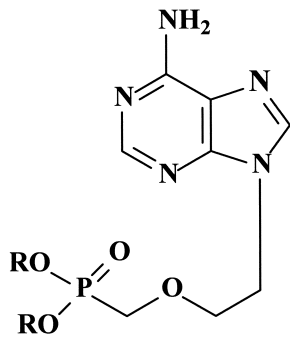


Figure 4. Modulatory role of double-stranded (ds) RNA (modified from [20])



R = H: Adefovir (PMEA)
R = (CH₃)₃C-CO-O-CH₂-: Adefovir dipivoxil [Bis(POM)-PMEA]

Figure 5. Structure of adefovir and adefovir dipivoxil. PMEAs, 9-(2-phosphonomethoxyethyl)adenine.

type of “mismatched” double-stranded RNA still survives today under the name Ampligen.

Double-stranded RNA has a double-modulatory role (figure 4), as originally postulated by Carter and De Clercq [20]. At the cellular level, it induces IFN synthesis but inhibits host cell protein synthesis, and, at the host level, it stimulates host defense mechanisms but induces both local and systemic toxic side effects [20]. Recently, double-stranded as well as single-stranded RNAs have been postulated to interact with Toll-like receptors 3 and 7 (see, e.g., [21]), but the authenticity of these interactions remains to be further established.

IFN- α IN THE TREATMENT OF HEPATITIS B AND C

A landmark observation, which laid the basis for the later use of IFN- α in the treatment of hepatitis B, was made by Merigan’s

group in 1976 [22], when they showed that parenteral (human leukocyte) IFN administration at a dosage between 6.0×10^3 and 17×10^4 U/kg/day was associated with a rapid and reproducible decrease in all Dane particle markers in 3 patients with chronic active hepatitis B. Long-term IFN therapy was associated with a marked decrease in hepatitis B surface antigen levels in 2 of 3 patients and a disappearance of e antigen in 2 of 2 patients. It was concluded that IFN may be useful in limiting carrier infectivity or eradicating chronic hepatitis B virus (HBV) infection.

Four drugs have been formally approved for the treatment of chronic HBV infections: pegylated IFN- α , lamivudine, adefovir dipivoxil [23], and entecavir. Whether combinations of these drugs provide incremental benefit in the treatment of hepatitis B has not been established, although it deserves further exploration. Adefovir dipivoxil corresponds to the bis(pivaloyloxymethyl)ester of 9-(2-phosphonomethoxyethyl)adenine (PMEA; figure 5), a compound that was first mentioned for its antiviral properties in 1986 [24]. Adefovir dipivoxil has proved to be efficacious in the treatment of both e antigen-negative and e antigen-positive chronic hepatitis B [25, 26]. It has been firmly established that adefovir acts as a chain terminator in the reverse-transcriptase (RNA-dependent DNA polymerase) reaction [27], and this by itself could explain the reductions in HBV DNA titers achieved in vivo by the therapeutic doses used for adefovir dipivoxil (10 mg/day orally).

It should be mentioned in this context that adefovir (PMEA) has also been shown to enhance NK cell activity and IFN production, at least in mice [28, 29], and more-recent studies with N⁶-substituted PMEAs derivatives [30] have indicated that this type of compound can also stimulate the secretion of cytokines and chemokines. Whether such potential side effects could con-

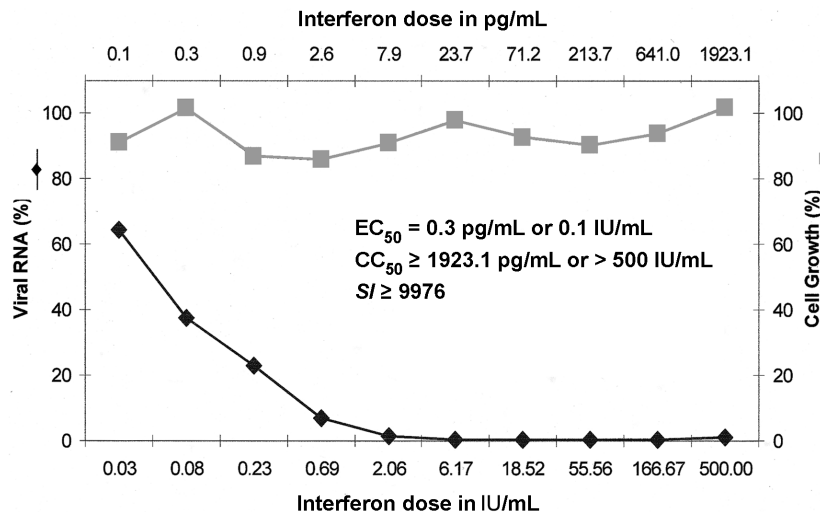


Figure 6. Effect of intron A on the replication of hepatitis C virus (genotype 1b) replicon in Huh-7 cells. Unpublished data (average values for 2 experiments) are from J. Paeshuyse, E. De Clercq, and J. Neyts (2004). SI, selectivity index.

Table 1. Effect of time of a single polyacrylic acid (PAA) injection on the formation of vaccinia virus tail lesions.

Time of PAA injection ^a before virus infection	Lesions, no.		P (vs. controls)
	Per individual mouse	Mean	
Control	9, 3, 8, 1, 6, 2, 5, 11, 4, 0, 3, 2, 0, 11, 15, 10, 30, 4	6.89	
4 weeks	0, 1, 0, 5, 1, 0, 2, 0, 3, 1, 0, 2, 1, 3, 0, 11, 4, 3, 7, 1, 0	2.14	.005 < P < .01
3 weeks	0, 0, 1, 3, 5, 3, 0, 0, 0, 9, 3, 1, 0, 0, 1	1.73	.01 < P < .02
2 weeks	3, 1, 2, 1, 2, 1, 6, 1, 0, 0, 0, 1, 2, 1	1.50	P ≈ .01
1 week	0, 1, 1, 0, 0, 3, 2, 1, 1, 0, 2, 2, 1, 0, 1	1.00	.001 < P < .002

NOTE. Vaccinia virus was injected intravenously. Data are from [39].

^a Intraperitoneally, 0.25 mg.

tribute to the efficacy of adefovir dipivoxil in the treatment of chronic hepatitis B has not been demonstrated, however.

IFN- α (in its pegylated form), in combination with ribavirin, has become the standard treatment for chronic hepatitis C virus (HCV) infections. In a recent study, Hadziyannis et al. [31] demonstrated that patients infected with HCV genotype 1 required treatment with pegylated IFN- α 2a (180 μ g/week) plus ribavirin (1000 or 1200 mg/day) for 48 weeks, whereas patients infected with HCV genotypes 2 or 3 seemed to be adequately treated for only 24 weeks with the same dose of pegylated IFN and a lower dose (800 mg/day) of ribavirin. Therefore, at least in the long term, hepatitis C may be better managed by IFN and ribavirin when caused by HCV genotypes 2 or 3 rather than by genotype 1. The mechanistic basis for this differential behavior remains to be unraveled.

Of note, IFN has a strong antiviral effect on HCV, as we have demonstrated in the HCV replicon system in Huh-7 cells, in which IFN- α (intron A) was found to inhibit the replication of HCV (genotype 1b) at an EC₅₀ of 0.3 pg/mL, whereas no cytotoxicity for the host cells was noted at a 10,000-fold higher concentration (figure 6). Ribavirin had relatively weak antiviral activity in the HCV (genotype 1b) replicon system (we have not yet examined the effects of IFN and ribavirin in HCV replicon systems with other genotypes). Thus, I surmise that IFN- α , which mainly acts as an immunosuppressant in the treatment of chronic hepatitis B, primarily acts as an antiviral in the treatment of hepatitis C, whereas ribavirin, which is best known for its antiviral properties, may be assumed to primarily act as an immunosuppressant in the case of hepatitis C.

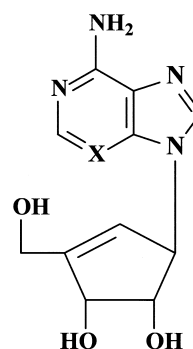
IFN- β IN THE TREATMENT OF MULTIPLE SCLEROSIS

In 1980, we succeeded in cloning human IFN- β and bringing it to expression through DNA recombination technology [32, 33]. Now, 20 years later, IFN- β has become the standard treatment for multiple sclerosis. It has been shown to be an effective

treatment, in a dose-related manner, for relapsing-remitting multiple sclerosis in terms of relapse rate, defined disability, and all magnetic resonance imaging outcome measures [34].

In a recent overview, Revel [35] noted that the role of IFN- β in the treatment of relapsing-remitting multiple sclerosis is now well established, and its efficacy has been demonstrated unequivocally in large-scale clinical trials. Recent trials underline the importance of both dose and dosing frequency and indicate that, for improved efficacy in relapsing-remitting multiple sclerosis, IFN- β therapy should be administered frequently at the highest tolerable and, thus, most effective dose.

The mechanism of action of human IFN- β in the treatment of multiple sclerosis has not been firmly established but may more likely be mediated by an immunosuppressant rather than antiviral effect. Human IFN- β has traditionally been used in the treatment of multiple sclerosis, and human IFN- α has been used in the treatment of chronic hepatitis B and C. There are no scientific reasons to believe that it will not work the other way around, but direct comparative studies of IFN- α versus IFN- β in the treatment of either hepatitis B or C or multi-



Neplanocin A (X = N)
3-Deazaneplanocin A (X = CH)

Figure 7. Structure of neplanocin A and 3-deazaneplanocin A

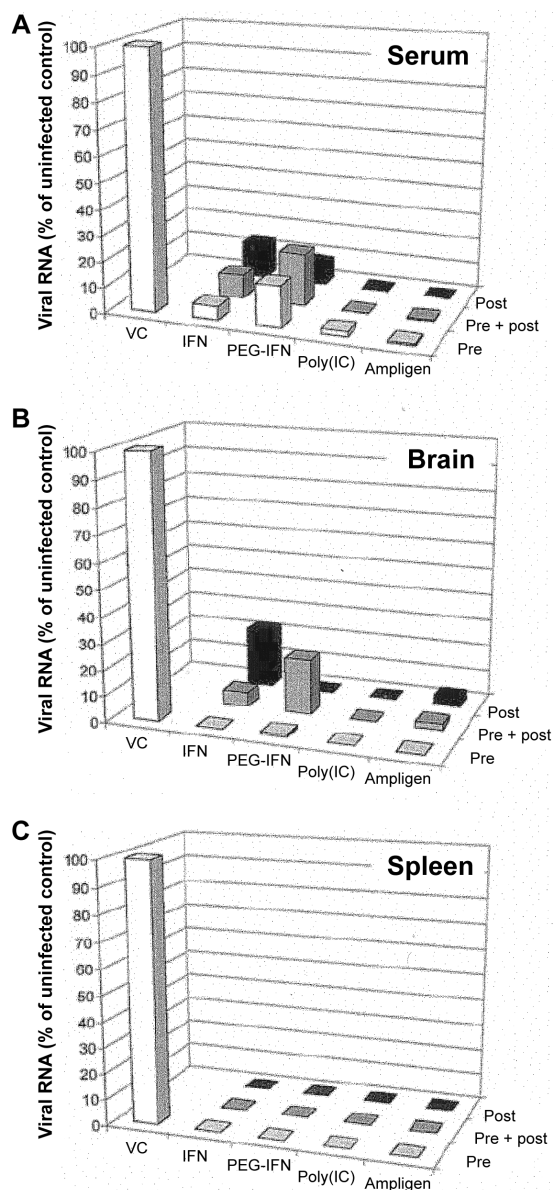


Figure 8. Effect of treatment with interferon (IFN)- α 2b, pegylated IFN- α 2b (PEG-IFN), poly(I)·poly(C) (poly(IC)), and Ampligen on virus titers (determined by quantitative reverse-transcription polymerase chain reaction) in serum, brain, and spleen at 7 days after infection of SCID mice with the flavivirus Modoc. Post, after infection; Pre, before infection; VC, virus control (data are from [47]).

ple sclerosis have, to the best of my knowledge, not been performed.

IFN IN THE POTENTIAL TREATMENT OF SEVERE ACUTE RESPIRATORY SYNDROME (SARS)

IFN- α , - β , and - γ have been found, *in vitro*, to be effective in inhibiting the replication of the SARS coronavirus (SCV) [36]. IFN- α effectively inhibited SCV replication, but with a selec-

tivity index 50–90 times lower than that of IFN- β . IFN- γ was slightly better than IFN- α in Vero cell cultures but was completely ineffective in Caco2 cell cultures.

In vivo, prophylactic treatment of SCV-infected macaques with pegylated IFN- α was found to significantly reduce viral replication and excretion, viral antigen expression in type 1 pneumocytes, and pulmonary damage [37]. Pegylated IFN- α may, therefore, be considered a candidate drug for the prophylaxis and therapy of SARS.

IFN IN THE POTENTIAL TREATMENT OF POXVIRUS INFECTIONS

Should smallpox ever pose a threat following a bioterrorist attack with variola virus [38], IFN and its inducers, among many other compounds (such as cidofovir [23]), may be considered as a possible means to counteract such an attack. In this perspective, we had already shown in 1968 [39] that IFN and its inducers are able to strongly act prophylactically against poxvirus infections. In the vaccinia virus tail lesion model in mice, IFN and PAA were able to markedly suppress poxvirus-induced lesions, the most remarkable finding being that a single injection of PAA, 4 weeks before challenge with virus, was able to significantly reduce the number of vaccinia virus-induced tail lesions (table 1) [39].

IFN IN THE POTENTIAL TREATMENT OF FILOVIRUS INFECTIONS

The filoviruses, Marburg and Ebola, are classified as category A biowarfare agents by the US Centers for Disease Control and Prevention. Most known human infections with these viruses have been fatal, and no vaccines or effective therapies are currently available [40]. The filovirus disease syndrome resembles that caused by other hemorrhagic fever viruses, necessitating studies in a biocontainment laboratory to confirm the diagnosis. Some progress has been made in developing vaccines and

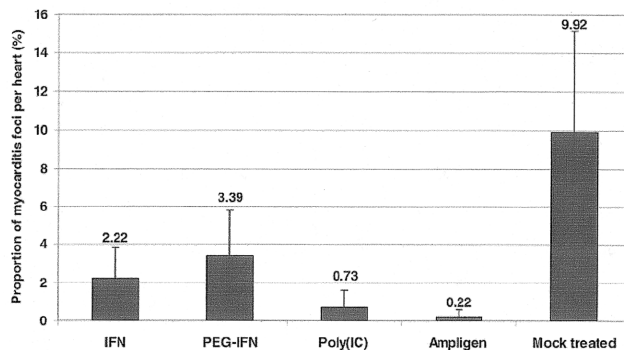


Figure 9. Effect of treatment with interferon (IFN)- α 2b, pegylated IFN- α 2b (PEG-IFN), poly(I)·poly(C) (poly(IC)), and Ampligen on coxsackie B3 virus-induced myocarditis in C3H/HeNHsd mice (data are from [48]).

antiviral drugs, but efforts are hindered by the limited number of maximum-containment laboratories.

As mentioned by Bray et al. [41], the recombinant B/D chimeric form of human IFN- α has proven to be highly protective against Ebola virus in mice: a single dose given on the day of challenge only delayed death, but a 5- to 7-day course of the same dosage, begun on day 0, 1, or 2 after infection, was highly protective. However, a recent trial of IFN- α 2b therapy in Ebola virus-infected cynomolgus monkeys resulted only in a delay in the onset of viremia, fever, and illness; all animals finally died of the infection [42].

That IFN may be effective in the treatment of filovirus (i.e., Ebola virus) infections could be anticipated from our initial results [43] on the protective effects obtained with IFN and IFN inducers (such as PAA) in newborn mice infected with VSV, a rhabdovirus related to the filoviruses. In fact, VSV could be considered a surrogate virus of the filoviruses, in that anti-VSV activity may be predictive of activity against filoviruses. This premise has been borne out with *S*-adenosylhomocysteine hydrolase inhibitors such as 3-deazaneplanocin A (figure 7); on the basis of its activity against VSV [44], it was tested in treating Ebola virus infections in mice, and a single dose protected the mice against a lethal challenge with Ebola virus [41].

How might 3-deazaneplanocin A exert its antiviral action against Ebola virus *in vivo*? On one hand, 3-deazaneplanocin is a potent inhibitor of *S*-adenosylhomocysteine hydrolase [45]; on the other hand, 3-deazaneplanocin has been shown to massively stimulate the production of IFN- α in Ebola virus-infected mice [46]. Considering the unequalled potency of double-stranded RNAs in inducing IFN, I hypothesize that, as an *S*-adenosylhomocysteine hydrolase inhibitor, 3-deazaneplanocin leads to the accumulation of *S*-adenosylhomocysteine, which, being a product and inhibitor of methyltransferase reactions using *S*-adenosylmethionine as methyl donor, will inhibit these methylation reactions, including those that are required for the 5'-capping of the viral mRNA [45]. In the case of negative-stranded RNA viruses (such as Ebola), this means that the positive-stranded RNA (transcribed from the minus strand by the RNA replicase) is not processed and remains attached to the negative-stranded RNA, thus resulting in increased double-stranded RNA formation and, consequently, IFN induction. Further experiments should be undertaken to validate this hypothesis.

IFN AND ITS INDUCERS IN THE POTENTIAL TREATMENT OF FLAVIVIRUS-INDUCED ENCEPHALITIS AND COXSACKIE B3 VIRUS-INDUCED MYOCARDITIS

IFN- α 2b, pegylated IFN- α 2b, poly(I)·poly(C), and poly(I)·poly(C)₁₂U (Ampligen) have been evaluated against Modoc virus encephalitis in a mouse model of flavivirus infections. All

compounds significantly delayed virus-induced morbidity (paralysis) and mortality (due to progressive encephalitis). Virus load was significantly reduced, by 80%–100%, in serum, brain, and spleen of mice that had been treated with IFN- α 2b, pegylated IFN- α 2b, poly(I)·poly(C), or Ampligen (figure 8) [47]. IFN and double-stranded RNA inducers of IFN may, therefore, be considered for further studies of their potential in therapy for or prophylaxis against flavivirus infections in humans.

Similarly, IFN and the double-stranded RNA inducers of IFN may seem promising candidate drugs for therapy for or prophylaxis against coxsackie B virus-induced myocarditis (figure 9) [48]. Ampligen, at 20 mg/kg of body weight/day, was found to reduce by 98% the severity of coxsackie B3 virus-induced myocarditis in mice, as assessed by morphometric analysis. When poly(I)·poly(C) was administered at 15 mg/kg/day, it reduced the severity of virus-induced myocarditis by 93%. IFN- α 2b and pegylated IFN- α 2b were less effective and reduced the severity of virus-induced myocarditis by 78% and 66%, respectively.

SUMMARY

IFN- α (whether pegylated or not) has acquired a definitive place in the treatment of chronic hepatitis B and C, as has IFN- β in the treatment of multiple sclerosis. Further potential indications for pegylated IFN- α and - β include SARS, and those for pegylated IFN- α and IFN inducers, such as poly(I)·poly(C) and poly(I)·poly(C)₁₂U (Ampligen), include filovirus, poxvirus, flavivirus, and coxsackie B virus infections.

Acknowledgments

I thank my “old time” tutors and advisers (Pieter De Somer and Thomas C. Merigan) and colleagues (Walter Fiers, Jean Content, and Rik Derynck) and “new time” collaborators (Johan Neyts, Pieter Leyssen, Nathalie Charlier, Elizaveta Padalko, Jan Paeshuyse, and Armando De Palma) for the invaluable role they have played (or are still playing) in the unfolding story of interferon and its inducers. Special thanks are also due to Christiane Callebaut for her dedicated editorial assistance.

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