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Human Interferon-Gamma Increases Resistance of Tobacco Hornworm (*Manduca sexta*) Larvae to *Galleria mellonella* Nuclear Polyhedrosis Virus

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Abstract: In the present study we wanted to investigate if human interferon-gamma (hIFN-γ) is functional in insect cells and performed experiments to test the possibility of interferon antiviral activity. Interferon-like molecules have not yet been identified in insects. We investigated if molecule(s) with functional and structural similarities to hIFN-γ could be involved in antiviral defense of *Manduca sexta* larvae in protection studies using hIFN-γ. Fifth-instar larvae of tobacco hornworm were inoculated with *Galleria mellonella* Nuclear Polyhedrosis Virus (NPV) that was lethal to 88% of larvae. Inoculation of larvae with recombinant hIFN-γ prior to infection with NPV induced an increased resistance to NPV infection when compared with larvae which received hIFN-γ following NPV infection and to controls which received only NPV. The most significant survival rate (67%) was observed in larvae which received hIFN-γ 6 h before NPV infection. Data obtained by different salt fractionations of hemolymph using Cibacron Blue agarose chromatography indicated possible isolation of an interferon-like insect molecule. This indicated that cytokine molecule(s) with activity similar to that of hIFN-γ could be important in antiviral defense by this insect.

Key words: *Manduca sexta,* Interferon-gamma, polyhedrosis virus, protection

INTRODUCTION

Interferons are cytokines that help cells resist viral infections. Mammalian interferon-gamma (IFN-γ) is a homodimer lymphokine molecule with a molecular weight of 17-19 kDa, consisting of 156-167 amino acids depending on the species[1,2]. It is synthesized by T-lymphocytes and Natural-killer (NK) cells and has a variety of antiproliferative and antiviral properties in its role as an immunomodulator in the mammalian adaptive immune response. It is a powerful activator of various cells involved in the vertebrate immune response, including macrophages, neutrophils and NK cells. It also facilitates the differentiation of T- and B- lymphocytes of vertebrates.

Galleria mellonella Nuclear Polyhedrosis Virus (NPV) was previously found to be lethal to *Manduca sexta* larvae[3]. No interferons have yet been identified in insects[4]. However, evidence for the existence of invertebrate IFN-γ-like molecules has been presented[4]. Homologues of the interferon consensus response elements are known to enhance the activity of insect antibacterial-peptide gene promoters[5]. Molecules possessing structural similarities to IFN-γ have been found in various invertebrates, including a nematode *Caenorhabditis*[6] and an insect *Drosophila*[7]. Also, mammalian IFN-γ has been shown to act as a growth factor in the development of some invertebrates[8]. Previous study has demonstrated the existence of high-affinity binding sites for hIFN-γ in the hemolymph of *M. sexta* larvae[9,10].

The present study was conducted to investigate if recombinant human interferon-gamma (hIFN-γ) is functional in insect cells and performed experiments to test the possibility of interferon antiviral activity. We present evidence here for a protective activity of hIFN-γ in larvae of the tobacco hornworm (*M. sexta*) infected with *Galleria mellonella* NPV. We also investigated the possibility of identifying an interferon-like molecule in this insect larva.

MATERIALS AND METHODS

Protection of larvae from nuclear polyhedrosis virus by human interferon-gamma: *Manduca sexta* eggs and growth medium were obtained from Carolina Biological Supply Co. (Burlington, NC). Immediately upon hatching, the larvae were transferred to the *M. sexta* growth medium. Upon reaching the fifth instar, 84 larvae were divided into sets of 6 larvae (sets A-G; Table 1) which received different schedules of treatments with hIFN-γ (Gibco, Grand Island, NY) and NPV (Carolina Biological Supply Co.). For each set treated with hIFN-γ and NPV, a corresponding control set of 6 larvae received only NPV.

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Table 1: Survival of *Manduca sexta* larvae treated with hIFN-γ and NPV

Time (h)	% A1/	% B	% C	% D	% E	% F	% G	% Ctrl
36	100**	83.3	100.0**	66.7*	66.7	66.7	83.3	76.2
48	100*	66.7*	66.7*	50.0	16.7	33.3	16.7	31.0
60	66.7*	50.0	66.7*	16.7	0.0	0.0	0.0	23.8
72	33.3	33.3	66.7*	0.0	0.0	0.0	0.0	14.3
84	16.7	0.0	66.7	0.0	0.0	0.0	0.0	11.9
96	0.0	0.0	66.7	0.0	0.0	0.0	0.0	11.9
108	0.0	0.0	66.7	0.0	0.0	0.0	0.0	11.9
120	0.0	0.0	66.7	0.0	0.0	0.0	0.0	11.9

1/ The percentage columns (A-G) show the % of larvae surviving NPV infection in each set of 6 larvae. The set labels (A-G and Ctrl.) and the schedule of hIFN-γ and NPV treatments were as follows: A: hIFN-γ given 24 h prior to NPV; B: hIFN-γ given 12 h prior to NPV; C: hIFN-γ given 6 h prior to NPV; D: hIFN-γ and NPV given simultaneously; E: hIFN-γ given 6 h after NPV; F: hIFN-γ given 12 h after NPV; G: hIFN-γ given 24 h after NPV; Control (initially, 42 larvae): only NPV given; no hIFN-γ given. Asterisks indicate significance in the Bonferroni t test vs. the corresponding control point at the level of 99% confidence (**) or 95% confidence (*), respectively.

One-half or 42 of the larvae were used as controls. All treatments were in 0.14 M NaCl-0.01 M sodium phosphate buffer, pH 7.4. Prior to each treatment, the larvae were anesthetized by cooling at -5°C for 5 min. The NPV treatment consisted of ventrally microinjecting each larva between the prolegs using a microinjector apparatus with approximately 10^6 polyhedra-containing viruses in a volume of 10 μL. The occlusion bodies were suspended in carbonate-chloride solution, pH 10.4 for 45 min at room temp to release virions prior to injection[11]. The hIFN-γ treatment consisted of microinjecting each larva with 20 ng of hIFN-γ in a volume of 10 μL. The treatment schedules and results are shown in Table 1.

Fractionation of hemolymph and added ^{125}I-hIFN-γ by Cibacron Blue Agarose: Following removal of cells by centrifugation, supernatants of hemolymph were applied to a preparative (4 mL) column of HiTrap Cibacron Blue agarose (Pharmacia, Uppsala, Sweden) for fractionation of hemolymph. ^{125}I-hIFN-γ was also applied to the column. The column was then eluted with 1.5 M NaCl+50% ethylene glycol, 0.1 M NaCl+50% ethylene glycol, 2.0 M NaCl, 1.5 M NaCl, 1.0 M NaCl, 0.75 M NaCl, 0.5 M NaCl and 0.3 M NaCl (all in 0.02 M sodium phosphate buffer, pH 7.4). Twenty mililiter of each eluent were passed through the column in fractions of 4 mL. After protein quantitation[12], the pooled eluates for each of the concentrations used were dialyzed against 0.07 M NaCl-0.01 M phosphate buffer, pH 7.4. The dialyzed pools were aliquoted into 1.5 mL fractions and then evaporated by vacuum centrifugation at a trap temp of -80°C. The dryed residues were then stored at -80°C until assayed for ^{125}I-hIFN-γ activity. Human IFN-γ (Gibco, Grand Island, NY) was iodinated with ^{125}I (Du Pont/New England Nuclear, Cambridge, MA) as previously described[9]. The 1.5 M NaCl+50% ethylene glycol eluate fraction was applied to a Sephacryl S-300 column in 0.1 M NaCl-0.1% SDS-1 mM EDTA buffer, pH 7.4 for further analysis.

RESULTS

Survival of the larvae was monitored at 12 h intervals over a period of 120 h following the treatments (Table 1 and Fig. 1). There was no mortality in any group at 12 and 24 h, indicating that the treatment procedure itself (cold anesthesia followed by microinjection) did not contribute to larval mortality. Therefore, all deaths can be attributed to infection by NPV. All treated larvae (including the controls) exhibited the characteristic signs of NPV infection, including cessation of feeding activity, torpor and dark spots over the larval body. Polyhedra were seen by microscopic examination of dead larvae.

All sets of six larvae receiving hIFN-γ before infection with NPV (sets A, B and C were more resistant to infection by NPV when compared with larvae treated with hIFN-γ simultaneously with (set D), or after (sets E, F and G) NPV infection (Table 1 and Fig. 1). Sets A-C were also more resistant to NPV infection than the control larvae (also infected with NPV, but not treated with hIFN-γ). This was particularly true of set C (hIFN-γ given 6 h before NPV), where 4 of the 6 larvae (66.7%) survived and recovered from the NPV infection (Table 1 and Fig. 1). Upon sacrificing, the hemolymph of larvae in set C did not have the cloudy appearance characteristic of larvae infected with NPV, indicating that the virus may have been eliminated from these larvae. The larvae in set C also displayed signs of NPV infection later than did larvae in any other set. Treatments D-G, where hIFN-γ was given at the same time as NPV, or following infection with NPV, showed essentially no protection by hIFN-γ at 48 h or more after completion of the treatment (Table 1 and Fig. 1). As for the 42 control larvae used, only 12% (5 out of 42) survived. Beginning at 84 h, the number of surviving control larvae remained constant (Table 1 and Fig. 1).

Cibacron Blue agarose chromatography resulted in adsorption of a significant fraction of protein presumed to contain most of the basic cytokine-like molecules present in *M. sexta* hemolymph, including IFN-γ-like molecules (Fig. 2). This conclusion was supported by our finding that over 80% of ^{125}I-hIFN-γ strongly adsorbed to another Cibacron Blue agarose column used to evaluate the efficiency of this adsorption. A desorption of ^{125}I-hIFN-γ was best accomplished by elution with 1.5 M NaCl+50% ethylene glycol (Fig. 2). This procedure resulted in recovery of about 50% of ^{125}I-hIFN-γ applied to the Cibacron Blue agarose column, most of which was eluted then with 1.5 M NaCl+50% ethylene glycol (Fig. 2).

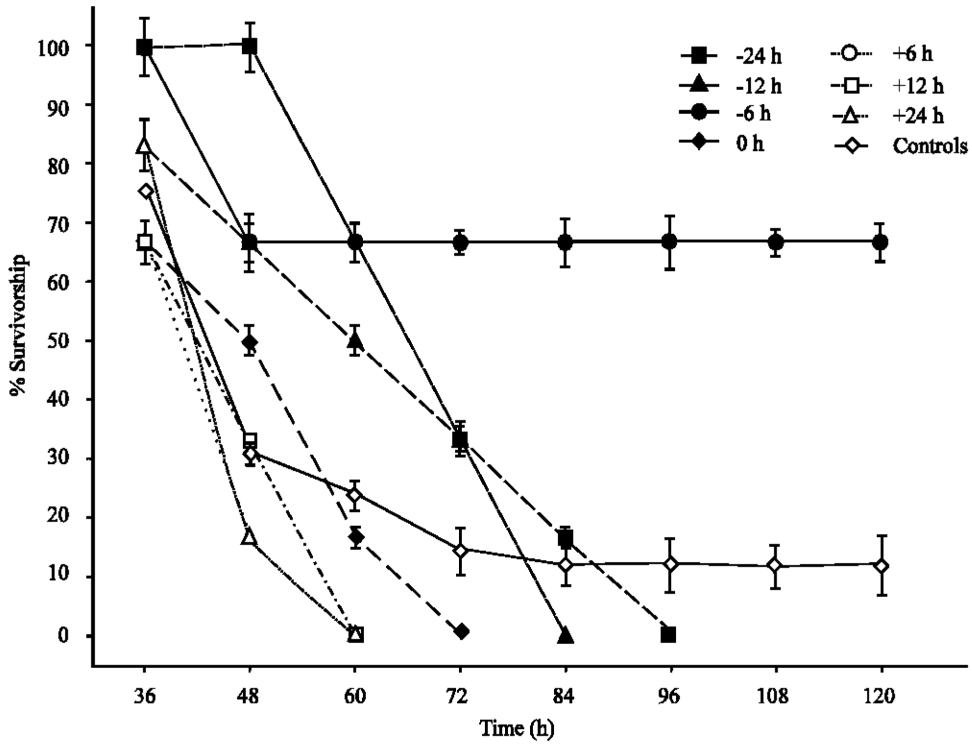

Fig. 1: Protection of *Manduca sexta* larvae from NPV by hIFN-γ

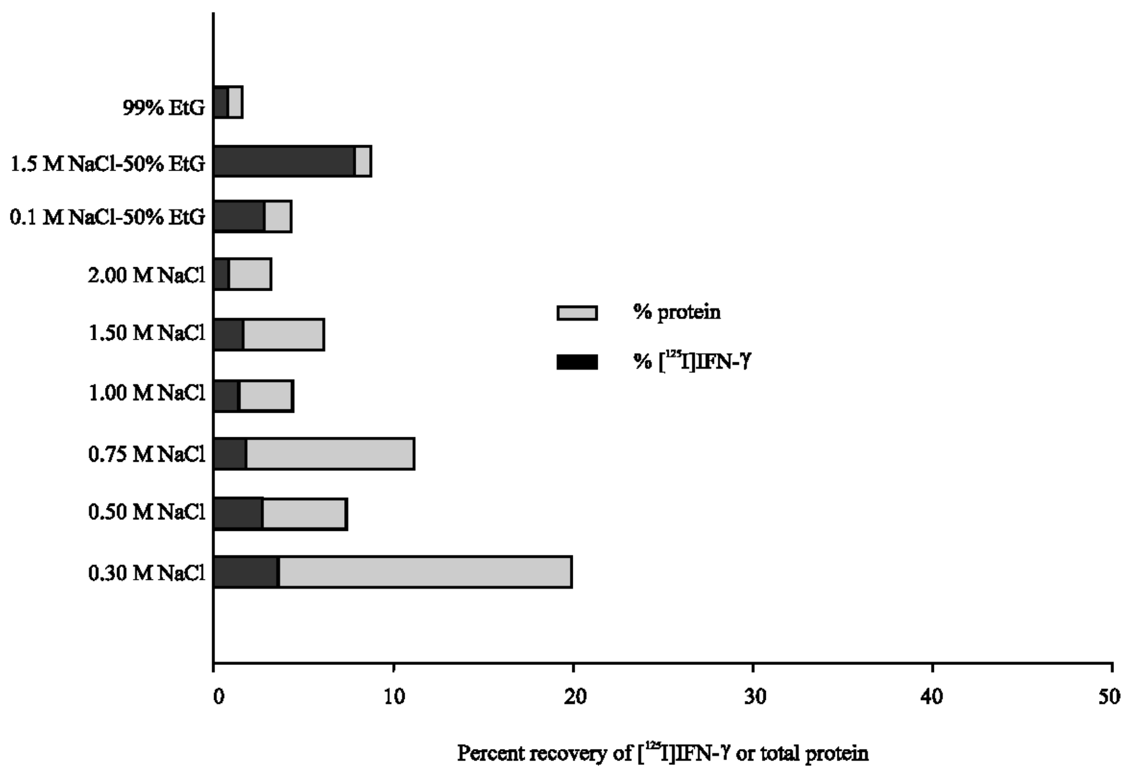

Fig. 2: Fractionation of hemolymph extract and added [^{125}I]hIFN-γ by Cibacron Blue agarose chromatography; EtG = Ethylene glycol

The ^{125}I-hIFN-γ and hemolymph protein recovered by 1.5 M NaCl+50% ethylene glycol eluted with an Rf value of 0.7 from Sephacryl S-300 in 0.1 M NaCl-0.1% SDS-1 mM EDTA buffer, pH 7.4. This elution position is identical with that of the peak of unfractionated radioactive cytokine and so the recovered IFN-γ should be considered as native protein.

DISCUSSION

In view of our discovery of high-affinity binding sites for hIFN-γ in the hemolymph of *M. sexta* larvae[9,10], we were interested in determining the possibility of protective activity by hIFN-γ in *M. sexta* larvae infected with NPV. Nuclear polyhedrosis viruses can cause high mortality in insect larvae[13]. We were also interested in possibly identifying an IFN-γ-like molecule in *M. sexta* larvae.

Molecules similar to mammalian interleukins and fibroblast growth factors have been identified in insect tissues[14,15]. Since some of these cytokines possess significant sequence identities with IFN-γ, cross-reactivity of IFN-γ binding at the respective binding sites may occur. These receptor sites may be more pleiotropic than usually thought, based on our evidence regarding the heterogeneity in molecular size of the IFN-γ receptors in *M. sexta* larvae[9] .

The results indicate that hIFN-γ has a statistically significant protective effect against infection by NPV in *M. sexta* larvae (Table 1). The protection of *M. sexta* larvae by hIFN-γ against NPV infection indicated a specific biological activity by this cytokine. The mechanism of this protective effect could be connected to induction, activation or mobilization of cells (possibly plasmatocytes, which are invertebrate hemolymph cells equivalent to vertebrate macrophages) and/or cytokines in the insect by hIFN-γ. This further implies that molecule(s) with functional and structural similarities to IFN-γ could be involved in the antiviral defense of *M. sexta* larvae. An action of IFN-γ through induction of heterologous cytokine receptors has been documented[16]. An additional reason may be a short-term or transient response to IFN-γ produced in synergy with other cytokines, possibly similar to the synergy that is frequently found between IFN-γ and tumor necrosis factor-alpha (TNF-α)[17]. Molecules similar to TNF-α are found in invertebrates[18]. The synergy of IFN-γ and TNF-α in mammalian systems, which involves the sharing of receptors, transducers and transporters, could well be found here in insect systems, which possess structural analogues of both classes of cytokines[19]. The data imply that an *M. sexta* receptor for hIFN-γ, which we have previously described in hemolymph[9,10], could recognize an insect molecule similar to hIFN-γ (Table 1 and Fig. 1). This could result in the *in vivo* inhibition of NPV infection of *M. sexta* larvae and also suggests the presence of an interferon-like molecule which was also investigated here.

Fractionation of hemolymph employing Cibacron Blue agarose was done to isolate possible *M. sexta* proteins similar to IFN-γ (Fig. 2). Cibacron Blue agarose and heparin-agarose are matrices that have been previously used by other researchers[20,21] for fractionation of basic growth factors including IFN-γ. The greatest ^{125}I-hIFN-γ activity was seen after elution with 1.5 M NaCl+50% ethylene glycol (Fig. 2). This eluate fraction (eluted then with 50% ethylene glycol in 1.5 M NaCl) would be expected to contain the most basic proteins like hIFN-γ and also insect IFN-γ. This elution fraction was also applied to a Sephacryl S-300 column. Recovery of hIFN-γ and basic hemolymph protein from Cibacron Blue agarose and by Sephacryl S-300 chromatography in the form corresponding to the input cytokine indicates that IFN-γ-like insect proteins could be recovered here in native form. The data likely indicate then the initial isolation of an interferon-like molecule from *M. sexta* insect larvae. Interferon-like molecules have not before been identified in insects. Elucidation of the immune mechanisms of insects could prove important in possibly controlling insect populations by regulation of their cytokine expression.

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