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**ABSTRACT**

Interferon is a protein which is classically designated as inducing an antiviral state in cells. In a variety of systems, other cytokines have occasionally been observed to exhibit apparent antiviral activity. We have examined 25 cytokines for protection of A549 human lung carcinoma cells from the cytopathic effect (CPE) of encephalomyocarditis virus. Four cytokines, IL-1 $\beta$ , TNF- $\alpha$ , IL-4 and IL-13 appear to induce an antiviral state in these cells in the 100 to 800 pg/ml range independent of exogenous IFNs. None of the other cytokines enhance CPE alone or enhance or inhibit the CPE activities of IFN- $\alpha$ , - $\beta$  or - $\gamma$ . Antibodies specific for the 4 cytokines neutralize the effect as does an interferon receptor chain 2 specific neutralizing antibody, suggesting that type I IFN plays a role in the effect. A sheep polyclonal antibody which neutralizes all the human alpha subtypes has no effect on the CPE activity of these 4 cytokines, while a sheep polyclonal which neutralizes human IFN- $\beta$  effectively inhibits the antiviral activity. Incubation of the cells with the cytokines alone leads to no measurable production of IFN, while addition of virus to the cells after cytokine incubation leads to significant IFN- $\beta$  production, but little or no IFN- $\alpha$  production when measured by ELISA. Cytokines which exhibited no apparent CPE did not induce interferon beta either before or after viral challenge in these assays. This suggests that the 4 cytokines may be priming the cells to produce IFN when pattern recognition receptors are stimulated by the virus. The finding that IL-4 and IL-13 appear to prime IFN production is surprising since these cytokines often act antagonistically with IFN. Additional cell types were examined and endothelial cells were found to upregulate ISGs in response to TNF- $\alpha$  and IL-1 $\beta$  while a proprietary mesothelioma cell line was found to be relatively insensitive to addition of any of the 25 cytokines. These results suggest caution is necessary when interpreting cell-based assay results in situations where other cytokines may be present.

**INTRODUCTION**  
All classes of Interferon, Type I, II and Type III, induce an antiviral state in at least a subset of cell types. This activity is due to the expression of a number of proteins which interfere with one or more stages of the viral life cycle. Assays for interferon generally rely on the ability of IFN to specifically protect cells from viruses or are based on the upregulation of mRNA from one of the genes induced by IFN treatment. Over the years other cytokines have been shown to exhibit apparent antiviral activity, primarily IL-1, TNF- $\alpha$  and IL-6. Some studies have demonstrated that IL-1 and TNF- $\alpha$  can work by upregulating IFN in the cells.

In this study we extend these results by demonstrating that cytokines which lead to protection from viral challenge in A549 cells work primarily through priming the cells to produce interferon-beta upon viral challenge. Little or no interferon-beta is produced prior to viral challenge and cytokines which do not protect induce no interferon. Additionally we demonstrate that IL-4 and IL-13 which are often thought of as inhibitors of IFN action also prime A549 cells to produce IFN on viral challenge. This may be due to the role IL-4 and IL-13 play in the lung where they seem to have both pro- and anti-inflammatory functions.

**METHODS**  
**Cell lines and viruses.**  
Human epithelial A549 ATCC (Manassas, Va.), DMEM with 10% FBS (Invitrogen) was used to maintain A549 cells.  
Encephalomyocarditis virus (VR-123B) and vesicular stomatitis virus (VR-123B) were also from ATCC and were grown in Vero (CCL-81).  
**Reagents.**  
Human IFN- $\alpha$ 2a was obtained from PBL inventory. Other cytokines were from RnD Systems. Sheep anti-human interferon- $\alpha$ , anti-human interferon- $\beta$  and the IFNAR2 monoclonal antibody were from PBL inventory. Monoclonal antibodies to TNF- $\alpha$ , IL-1 $\beta$ , IL-4 and IL-13 were from RnD systems.

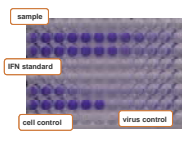
**Cytopathic Effect Assay (CPE).**  
All assays were established by plating cells in microtiter plates, allowing them to adhere for 2-4 hours followed by addition of test IFN. After an overnight incubation dilutions of EMCV was added. After 40-48 hours cytopathic effect in wells containing no IFN was determined. The dilution of the viral stock to be used in the assay was empirically determined as 2-4 fold more concentrated than the dilution which killed 90 to 100% of the cells in the time frame. Viable cells were stained with crystal violet and excess dye was washed off with tap water. After drying the plates for at least 2 hr, the dye was solubilized with 70% methanol and absorbance was determined at 562 nm in a Molecular Devices UVXmax plate reader. Inhibition CPE was calculated by setting 100% inhibition as cells incubate with virus but no IFN (virus control) and 0% inhibition as cells incubate without virus (cell control). Six wells were used for both controls. Generally the cell control was 6-10 times the value of the virus control. All samples data points were run in duplicate wells in at least two independent assays.

For experiments looking at the effects of various cytokines on IFN activity, the cytokines were held at suboptimal concentrations and the IFN was titrated 2-fold across the plate. Data was analyzed as for the A549 CPE assay.

**Neutralization Experiments.**  
A set concentration of cytokine or IFN was incubated for 1 hour with serial dilutions of polyclonal or monoclonal antibodies. This mixture was then added to 0.1 ml of A549 cells in a 96 well microplate. After overnight incubation EMCV was added and CPE allowed to develop for 40-48 hours. Plates were stained and analyzed as for the AV assay.

**ELISA Analysis.**  
ELISA for the detection of Human Interferons were from PBL inventory. The products # 41105, 41410 and 41415 were used as recommended.

**Figure 1. Cytopathic Effect Assay.**



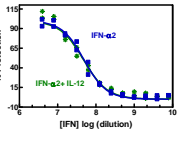
IFN cytopathic effect assay  
Human IFN usually examined on A549 or WISH cells  
IFN protects cells from the cytopathic effect of viruses such as encephalomyocarditis (EMCV) and vesicular stomatitis (VSV)

**Table 1. Cytokines Examined**

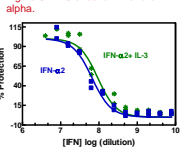
IFN- $\alpha$	IL-2	IL-18
IFN- $\beta$	IL-3	IP-10
IFN- $\gamma$	IL-4	ISG-15
IFN- $\lambda$	IL-6	MIG
IFN- $\lambda$	IL-8	MIP-1 $\alpha$
HGH	IL-10	MIP-1 $\beta$
IL-1 $\beta$	IL-12	RANTES
	IL-15	TNF- $\alpha$
	VIP	

Some, in red, are known to have apparent antiviral activity

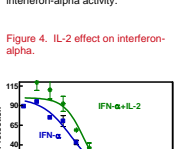
**Figure 2. Antiviral effect of Interferon.**



**Figure 3. IL-3 effect on interferon- $\alpha$ 2.**

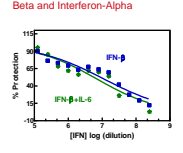


**Figure 4. IL-2 effect on interferon- $\alpha$ 2.**

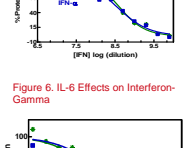


Again there may be a small effect.

**Figure 5. IL-6 Effects on Interferon-Beta and Interferon-Alpha**



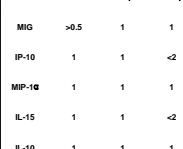
**Figure 6. IL-6 Effects on Interferon-Gamma**



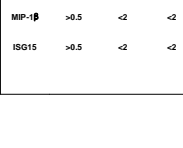
**Table 2. Effects of Cytokines on Interferon Activities**

	IFN- $\alpha$	IFN- $\beta$	IFN- $\gamma$
IL-2	<2	1	1
IL-3	1	<2	1
IL-6	1	1	>0.5
IL-12	1	1	1
IL-18	>0.5	1	1
HGH	1	1	1
RANTES	<2	1	1
VIP	1	1	1
IL-8	>0.5	>0.5	<2

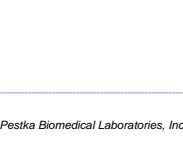
**Figure 7. Effect of TNF- $\alpha$ , IL-1 $\beta$  and IL-4 in the AV assay.**



**Figure 8. Suboptimal IL-4 and IL-13 effects on IFN- $\alpha$ 2 protection**

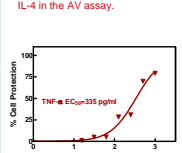


**Figure 9. Neutralization of TNF- $\alpha$  AV Activity**

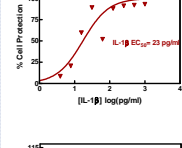


Overall, these effects are relatively small.

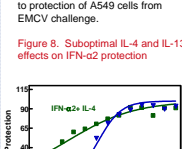
**Figure 10. IL-1 $\beta$  AV Activity is neutralized by Sheep anti-Human IFN- $\beta$  but not by anti-Human IFN- $\alpha$ .**



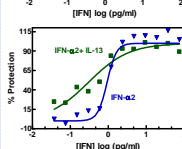
**Figure 11. ELISA Characterization of A549 supernatants treated with cytokines.**



**Figure 12. ISG15 mRNA Expression in response to TNF- $\alpha$ .**



**Figure 13. IFN Sensitivity of A549 and a Mesothelioma Cell Line**



IL-4, IL-13, TNF- $\alpha$ , and IL-1 $\beta$  give rise to protection of A549 cells from EMCV challenge.

IL-4 and IL-13 enhance IFN- $\alpha$ 2 activity

TNF- $\alpha$  antiviral activity is neutralized by TNF- $\alpha$  specific monoclonal antibody but not IL-1 $\beta$  monoclonal antibody.

Little or no IFN is produced prior to viral addition.

IFN- $\alpha$ 2 and IL-4

IL-4 and IL-13 enhance IFN- $\alpha$ 2 activity

IFN- $\alpha$ 2

IFN- $\alpha$ 2+IL-13

IFN- $\alpha$ 2+IL-4

IFN- $\alpha$ 2

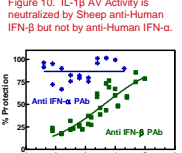
IFN- $\alpha$ 2+IL-13

IFN- $\alpha$ 2+IL-4

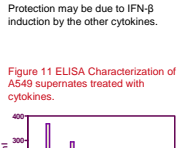
IFN- $\alpha$ 2

IFN- $\alpha$ 2+IL-13

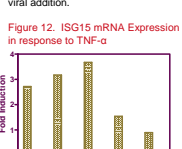
**Figure 14. IL-1 $\beta$  AV Activity is neutralized by Sheep anti-Human IFN- $\beta$  but not by anti-Human IFN- $\alpha$ .**



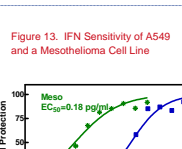
**Figure 15. ELISA Characterization of A549 supernatants treated with cytokines.**



**Figure 16. ISG15 mRNA Expression in response to TNF- $\alpha$ .**



**Figure 17. IFN Sensitivity of A549 and a Mesothelioma Cell Line**



IL-4, IL-13, TNF- $\alpha$ , and IL-1 $\beta$  give rise to protection of A549 cells from EMCV challenge.

IL-4 and IL-13 enhance IFN- $\alpha$ 2 activity

TNF- $\alpha$  antiviral activity is neutralized by TNF- $\alpha$  specific monoclonal antibody but not IL-1 $\beta$  monoclonal antibody.

Little or no IFN is produced prior to viral addition.

IFN- $\alpha$ 2 and IL-4

IL-4 and IL-13 enhance IFN- $\alpha$ 2 activity

IFN- $\alpha$ 2

IFN- $\alpha$ 2+IL-13

IFN- $\alpha$ 2+IL-4

IFN- $\alpha$ 2

IFN- $\alpha$ 2+IL-13

IFN- $\alpha$ 2+IL-4

IFN- $\alpha$ 2

IFN- $\alpha$ 2+IL-13