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Contents

Purification and Characterization of Interferons

Ernest Knight, Jr

I Introduction	1
II Purification of Interferons	2
III Characterization of Pure Interferons	4
A Chemical and Physical Properties	5
B Biologic Properties	9
IV Conclusions	10
V References	11

Effects of Interferons on Cell Growth and Function

Joyce Taylor-Papadimitriou

I Background and Introduction	13
II Interferon as a Hormone	16
III Interactions of Interferons with the Cell Membrane	17
A Evidence for Interaction with Membrane receptors	17
B Changes in Membranes of Interferon-treated Cells	19
C Potential Interest of Interferon-Membrane Interactions	20
IV Inhibition of Cell Proliferation	21
A General Features of Inhibition of Cell Growth <i>In Vitro</i>	21
B Role of Inhibition of Cell Growth by Interferon in Host Defence Mechanisms	22
C Role of Growth Inhibition in Antitumour Effect of Exogenously Administered Interferons	23
D Inhibition of Normal Cell Growth <i>In Vivo</i>	24

E Mechanisms Involved in Interferon-induced Inhibition of Cell Proliferation	26
V Effects of Interferons on Other Cell Functions	36
A Inhibition of Cell Functions	37
B Enhancement of Cell Functions	39
C Relevance to <i>In Vivo</i> Effects	39
VI How Many Interferons?	40
VII Prospects and Predictions	41
VIII What Sort of Hormone?	42
IX References	42

The Type I Human Interferon Gene System; Chromosomal Location and Control of Gene Expression

Derek C. Burke

I Introduction	47
II Localization of the Interferon Gene(s)	48
A The Interferon Gene is a Nuclear Gene	48
B Chromosomal Localization of the Human Interferon Gene(s)	49
III Control of Interferon Production in Somatic Cell Hybrids	55
A Mouse-Human Hybrids Produce Human Fibroblast Interferon	55
B Repressed Interferon Genes can be Switched On in Hybrids	56
C Priming and Superinduction in Cell Hybrids	57
D Glycosylation of Interferon in Cell Hybrids	58
IV Control of Transcription of the Human Interferon Gene(s)	58
A Control of Transcription in Superinduction	58
B Increased Rate of Transcription after Treatment with 5-Bromodeoxyuridine	60
C Increased Rate of Transcription after treatment with Sodium Butyrate	61
V Translation of Human Interferon mRNA	62
A In Cell-free Systems	62
B In <i>Xenopus</i> Oocytes	62
VI Prospects	63
VII Acknowledgements	63
VIII References	63

The Purity and Safety of Interferons Prepared for Clinical Use: The Case for Lymphoblastoid Interferon

N. B. Finter and K. H. Fantes

I Introduction	65
II Sources of Interferon for Clinical Use	68
A Human Leukocyte Interferon	68
B Human Fibroblast Interferon	68
C Human Lymphoblastoid Interferon	68
III Problems with Namalwa Cell Interferon	70
A Viruses	72
B Nuclei Acids	72
C Scrapie Agent	72
D Endotoxin	72
IV Impurities of Preparations Intended for Clinical Use	73
A Contaminants Derived from the Cells	73
B Contaminants Derived from the Medium	76
C The Inducer and Contaminants from the Inducer Preparation	77
D Chemicals Used to Increase Interferon yields	77
E Chemicals Used in the Purification	78
V Concluding Remarks	78
VI References	79

Lymphokines, Cytokines and Interferon(s)

Stanley Cohen and Pierluigi E. Bigazzi

I Introduction	81
II Nonantiviral Effects of Interferon	83
A Effects on the Immune System	83
B Nonimmune Effects	84
C General Considerations	84
III The Cytokines: Lymphokine Activities Produced by Nonlymphoid Cells	85
IV Relation between Cytokines and Lymphokines	89
V Relation between Cytokines and Antiviral Interferons	89
VI Possible Relationships among Cytokines, Lymphokines and Interferons	91
VII References	93

Interferon Nomenclature: Report from the Committee on Interferon Nomenclature

Committee

I Introduction	1
II Sources of Interferon for Clinical Use	2
A Human Leukocyte Interferon	2
B Human Fibroblast Interferon	3
C Human Lymphoblastoid Interferon	3
III Problems with Nomenclature of Interferon	4
A Virus	4
B Genetic Origin	4
C Source of Cells	4
D Induction	4
IV Induction of Interferon for Clinical Use	5
A Components Derived from the Cells	5
B Components Derived from the Medium	5
C The Inducer and Components from the Inducer	5
D Components Used to Induce Interferon	5
E Chemicals Used in the Induction	5
F Concluding Remarks	5
V References	6
VI Glossary	7
VII Appendix I: Problems and Interferon(s) and Interferon(s)	8
A	8
B	8
C	8
D	8
E	8
F	8
G	8
H	8
I	8
J	8
K	8
L	8
M	8
N	8
O	8
P	8
Q	8
R	8
S	8
T	8
U	8
V	8
W	8
X	8
Y	8
Z	8
VIII Appendix II: Problems and Interferon(s) and Interferon(s)	9
A	9
B	9
C	9
D	9
E	9
F	9
G	9
H	9
I	9
J	9
K	9
L	9
M	9
N	9
O	9
P	9
Q	9
R	9
S	9
T	9
U	9
V	9
W	9
X	9
Y	9
Z	9
IX Appendix III: Problems and Interferon(s) and Interferon(s)	10
A	10
B	10
C	10
D	10
E	10
F	10
G	10
H	10
I	10
J	10
K	10
L	10
M	10
N	10
O	10
P	10
Q	10
R	10
S	10
T	10
U	10
V	10
W	10
X	10
Y	10
Z	10

Purification and Characterization of Interferons

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I Introduction	1
II Purification of Interferons	2
III Characterization of Pure Interferons	4
A Chemical and Physical Properties	5
B Biologic Properties	9
IV Conclusions	10
V References	11

I INTRODUCTION

The best way to describe the progress in purification and characterization of the interferon proteins is that it has just begun. As in the study of other biological molecules and in the pursuance of religion, purity must precede understanding. Moreover, characterizing an interferon before it is pure is like characterizing a child before it is born, some things may change. The purification to homogeneity and characterization, beyond generalities, of the interferons has been a long and tortuous journey. However, considerable progress has been made in the last few years and it should accelerate in the future.

The purpose of this article is two-fold: (1) the presentation of some facts, opinions (biased), and speculations on the purification and characterization of those interferons that have been obtained pure; (2) the description of some experiences and interactions with other scientists in the pursuit of pure interferon during the past decade. In some cases where personal comments are used, names will be omitted to protect the innocent (and my personal safety). This article is not intended to be a thorough review of the literature on this subject. Reviews are available and the reader is recommended to them for a more comprehensive survey (Stewart, 1979a). My intent is to review only

those interferons that have been obtained pure and to compare and evaluate their chemical and biological properties. The purification procedures for obtaining pure interferons are varied and ingeneous and the reader is referred to the original publications for details.

II PURIFICATION OF INTERFERONS

Pure is: (1) having a homogeneous or uniform composition, not mixed: (2) free from adulterants or impurities; full-strength: (3) free from dirt, defilement, or pollution; clean: (4) free from foreign elements: (5) containing nothing inappropriate or extraneous.

The American Heritage Dictionary of the English Language

For our knowledge is imperfect and our prophecy is imperfect; but when the perfect comes, the imperfect will pass away.

St Paul, First Corinthians 14:9, 10

The discoverers and early workers with interferon realized the necessity to obtain pure interferon. Little did they know of the long and arduous task ahead to obtain the minute quantities of the pure interferons that we have today. Why has it been difficult to obtain pure interferons? First, the biological activity of an interferon is extremely potent (effective at 10^{-12} – 10^{-13} M on cells in culture). A cell does not make very much, thus there is very little starting material. Second, it is an inducible system and not a constitutive one; therefore it has to be obtained from animals or cells growing in culture since the usual sources of polypeptide hormones, animal organs, contain no interferon. Clearly there is no chance of obtaining sufficient quantities of a pure interferon if there is insufficient starting material. Third, there is the assay; it is slow and imprecise. Very few scientists will undertake the purification of a protein whose assay requires 2–3 days. For example, a purification step may be performed and if the interferon is unstable, the activity may disappear by the time the results of the assay are obtained. Thus it is possible not to get beyond the first step in a purification since most of the interferon may be lost by the time its activity is determined.

Although the word interferon is often used in the singular (*Time Magazine*, 1980) there is more than one protein with interferon activity. The early research on interferon indicated some species specificity, suggesting that all interferons are not the same protein. The first attempts to purify an interferon were with chick interferon, the system where interferon was discovered. In 1967, Karl Fantes and I. G. S. Furminger (Fantes and Furminger, 1967) reported results which were to foretell the future of interferon purifications for the next 10 years. After extensive purification of chick interferon they discovered that on electrophoretic analysis of the proteins and interferon activity, the activity did not reside in any detected protein! All persons who

have attempted to purify an interferon have experienced the same result, activity but no identifiable protein. Such results have sent the experimenters into the depths of depression and have caused them to consider other lines of work. After I had had a similar experience someone said to me, "Well, maybe we will never characterize or understand an interferon". Other indignities have been suffered. Comments such as "I believe you have extensively purified the interferon but that band on the gel isn't really interferon", were received with sullen resignation. However, in spite of painfully slow progress, and pessimistic predictions from their friends, those that purify interferons (masochists that they are) have persisted and prevailed.

Probably no one who has been involved in the purification of an interferon believed initially that it would be as difficult as it has been. Little did I know of the problems ahead when, in 1973, Ed Havell and Jan Vilček gave me some human fibroblast cells for the production of interferon. As I departed their laboratory with the cells they shook my hand and wished me luck in the purification. I did not realize at that time that the cells in the two small flasks that they gave me would later produce so many cells and so little interferon.

A further complicating factor in the purification of the interferons has been the discoveries that more than one interferon can be produced by a cell or by different cells from the same animal. There appear to be at least three mouse interferons and four human interferons (for a review of these interferons see Stewart, 1979a). Those interferons that have been obtained pure are shown in Table I. The methods of their purification have been

TABLE I Pure Human and Mouse Interferons

Type of interferon	Molecular weight	Reference
Human fibroblast	20 000	Knight (1976a, b)
Human fibroblastoid	19 000	Berthold <i>et al.</i> (1978)
Human leukocyte	18 000	Rubinstein <i>et al.</i> (1979)
Human leukocyte	18 400–22 100 (5 proteins)	Berg and Heron (1980a)
Human lympho- blastoid	18 500	Zoon <i>et al.</i> (1979)
Mouse L cell	22 000–38 000 (9–10 proteins)	Knight (1975)
Mouse C-243 cells	22 000, 35 000 (2 proteins)	De Maeyer-Guignard <i>et al.</i> (1978)
Mouse L cells	24 000, 40 000 (2 proteins)	Iwakura <i>et al.</i> (1978)
Mouse Ehrlich ascites tumour cells	35 000, 26 000, 20 000 (3 proteins— A, B, C)	Kawakita <i>et al.</i> (1978)

omitted but the details are available in the references listed. All the recent purifications have focused on the human and mouse interferons. These interferons have held the most scientific interest and cell culture methods for their production have been developed. The reader should be aware that those pure interferons listed have been obtained only in microgram quantities.

However, the old question returns. How do you know it is pure? How can you be sure that the protein you are analysing is *really* interferon? These are tiresome but appropriate questions. First, those interferons listed in Table I migrate as one protein band coincident with the biological activity when subjected to electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate. This is a widely accepted method for determining the purity of a protein. Second, all have been purified to similar final specific activities. In my opinion, arguments about specific activities and absolute purity are futile, given the unavoidable imprecision and inaccuracy in making the measurements for specific-activity calculations. (Once at a scientific meeting a friend said that he believed that the specific activity of pure interferon was about 10^{11} units/mg. I am eternally thankful that he was not right or we would now have nanograms of pure interferons instead of micrograms). Third, *N*-terminal amino acid sequences of some human and mouse interferons have been obtained. The data show that there is, in each case, only one protein in the preparation (i.e. only one sequence of amino acids). Thus, I believe it has been demonstrated beyond doubt that both human and mouse interferons have been obtained pure, albeit in very small amounts. On the other hand, a die-hard sceptic might say that an inert protein has been isolated with physical properties very similar to the interferon and that coincidentally the interferon activity purifies with it (in other words, the ultimate specific activity really is 10^{11} units/mg).

III CHARACTERIZATION OF INTERFERONS

Since some interferons can now be obtained pure (Table I) it is essential that chemical and physical properties be characterized. Furthermore, since the discovery of interferon, numerous nonantiviral effects have been attributed to the interferons albeit mostly with interferons in impure preparations (Stewart, 1979b). As with the chemical and physical properties it is also essential to determine which biologic effects attributed to interferons in impure preparations can be demonstrated with pure interferons. I will briefly review and comment first on the chemical and physical properties of the pure interferons and second on the biologic effects caused by the pure interferons.

A Chemical and Physical Properties of Pure Interferons

The only physical property of the pure interferons that has been determined is their molecular weights (shown in Table I). They have been calculated from mobilities observed after electrophoresis in polyacrylamide gels containing sodium dodecylsulphate. These molecular weights are probably not an accurate reflection of amino acid composition since interferons are glycoproteins containing an unknown amount of carbohydrate. The molecular weight of one human leukocyte interferon has been calculated from the amino acid composition (Rubinstein *et al.*, 1979) and that of human lymphoblastoid interferon has been obtained (by electrophoresis) after extensive deglycosylation of the interferon (Zoon *et al.*, 1979). The molecular weights of the human interferons cluster in the range 15 000–25 000. The pure mouse interferons range from 22 000 to 38 000 (Table I). As mentioned earlier, a complication in the purification of some of the interferons has been their size heterogeneity. For instance one group (Rubinstein *et al.*, 1979) purified one leukocyte interferon to homogeneity, but their purification procedure (high performance liquid chromatography) revealed the existence of other leukocyte interferons. Another group purified five leukocyte interferons to homogeneity (Berg and Heron, 1980a). The relationships among these leukocyte interferons that have been purified by different procedures is not clear and further work must be done to clarify them. Human fibroblast and human fibroblastoid interferons have been obtained pure (Table I) from diploid fibroblasts and transformed fibroblasts respectively and recent experiments (Okamura *et al.*, 1980) indicate that they may be identical proteins.

The pure mouse interferons show considerable size heterogeneity. One report (Knight, 1975) stated that 9–10 interferons could be obtained pure. These interferons were obtained from mouse cells induced by MM virus. Other pure mouse interferons have been obtained from mouse C-243 cells (De Maeyer-Guignard *et al.*, 1978), mouse L cells (Iwakura *et al.*, 1978) and mouse Ehrlich ascites tumour cells (Kawakita *et al.* 1978); the inducer in each case was Newcastle disease virus (NDV). All three cell lines produced two or three interferons when purified to homogeneity (Table I). Why mouse L cells induced with MM virus should produce more interferons than other mouse cells induced by NDV is not clear at present. We will have to wait on the answer to this question as no one, to my knowledge, is using MM virus as an inducer for the large scale preparation of interferon (Knight became interested in human interferon). All characterization of mouse interferons is being done with interferons produced by NDV-induced cells.

The identity (or lack of identity) of human fibroblast, fibroblastoid, leukocyte and lymphoblastoid interferons in amino acid sequence has been the subject of considerable speculation. It has been suggested that some of

the differences in physical (molecular weights) and chemical (antibody cross reactivity) properties could be the result solely of differences in carbohydrate content rather than differences in amino acid sequence. The amino acid composition has been determined for one human leukocyte interferon (Rubinstein *et al.*, 1979), human lymphoblastoid interferon (Zoon *et al.*, 1979), human fibroblastoid interferon (Tan *et al.*, 1979) and human fibroblast interferon (Knight *et al.*, 1980). There are significant differences in the amino acid compositions indicating different amino acid sequences. Furthermore, amino acid sequences at the *N*-terminal of human fibroblast (Knight *et al.*, 1980) and human lymphoblastoid (Zoon *et al.*, 1980) interferon have been determined (Table II). The *N*-terminal sequences of the two interferons are different, therefore they are different proteins.

First, the *N*-terminal amino acid sequencing and how it was performed. When scientists that sequence proteins by standard methods are asked how much protein they need to begin their experiments, they respond by saying 1 mg, minimum. This is a very modest request to them but 1 mg of pure interferon still boggles the minds of those that purify interferons. In 1978, however, the development at the California Institute of Technology of methods for obtaining substantial amino acid sequence on sub-nanomole quantities of a protein was reported (Hunkapiller and Hood, 1978; 1980). This technology seemed ideally suited for the sequencing of interferons and collaborations were established between the California Institute of Technology and groups that had small amounts of pure interferons. These collaborations led to publication in February, 1980 of the *N*-terminal sequences of human fibroblast and human lymphoblastoid interferons and two mouse interferons. Table II shows a comparison of *N*-terminal sequences of these interferons. It is clear that human fibroblast and lymphoblastoid interferons are not identical proteins since there is only one homologous amino acid (leucine at position 9) in the first 13 residues. Recent experiments show that the first 13 amino acids at the *N*-terminal of human fibroblastoid interferon (Okamura *et al.*, 1980) have the same sequence as those at the *N*-terminal of human fibroblast interferon (Table II). This *N*-terminal homology suggests that they are the same proteins, but further sequence must be obtained from both to prove it. In the first 20 *N*-terminal residues of the mouse interferons there is homology at only two positions (glutamine at 5 and lysine at 16) thus proving that they are different proteins. This conclusion had been suggested earlier by tryptic mapping of the two mouse interferons (Iwakura *et al.*, 1978; Cabrer *et al.*, 1979). The homology that was the least expected was that between the human and mouse interferons (Table II). Thirteen of the 20 amino acids at the *N*-terminal of human lymphoblastoid and mouse C interferons are identical while three of 13 of human fibroblast and mouse A interferons are identical. What these homologies mean for biological activity or for evolution we cannot even speculate but what is

TABLE II *N*-Terminal Amino Acid Sequences of Human and Mouse Interferons

Interferon	<i>n</i> -Terminal sequence	References
Human fibroblast	H-Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu ¹⁰ -Gln-Arg-Ser-Ser	Knight <i>et al.</i> (1980)
Human lymphoblastoid	H-Ser-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu ¹⁵ -Gly-Asn-Arg-Arg-Ala-Leu-Ile-Leu-Leu-Ala-Gln ²⁰	Zoon <i>et al.</i> (1980)
Mouse A and B	H-Ile-Asn-Tyr-Lys-Gln-Leu-Gln-Leu-Gln-Glu-Arg-Thr-Asn-Ile-Arg-Lys-? -Gln-Glu-Leu-Leu-Gln-Leu	Taira <i>et al.</i> (1980)
Mouse C	H-Ala-Asp-Leu-Pro-Gln-Thr-Tyr-Asn-Leu-Gly-Asn-Lys-Gly-Ala-Leu-Lys-Val-Leu-Ala-Gln	Taira <i>et al.</i> (1980)
Human fibroblast	H-Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Gln-Arg-Ser-Ser	
Mouse A and B	H-Ile-Asn-Tyr-Lys-Gln-Leu-Gln-Leu-Gln-Glu-Arg-Thr-Asn	
Human lymphoblastoid	H-Ser-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Gly-Asn-Arg-Arg-Ala-Leu-Ile-Leu-Leu-Ala-Gln	
Mouse C	H-Ala-Asp-Leu-Pro-Gln-Thr-Tyr-Asn-Leu-Gly-Asn-Lys-Gly-Ala-Leu-Lys-Val-Leu-Ala-Gln	

strongly suggested is that we are investigating a family of proteins. Furthermore, this demonstrates to the next to last sceptic that we really have purified and sequenced interferons. The last sceptic will not be convinced yet, but read on, there is more to come.

The use of recombinant DNA technology for the cloning of eukaryotic genes into bacteria has developed rapidly and is being widely used. Considerable publicity has been devoted in the past year to the cloning of an interferon gene. Recently, the cloning of human fibroblast (Taniguchi *et al.*, 1980a) and human leukocyte (Nagata *et al.*, 1980) interferons has been reported. The fibroblast interferon cDNA synthesized from interferon messenger RNA, was cloned into a bacterial plasmid. The DNA sequence for the fibroblast interferon in the bacterial plasmid was identified by sequencing a plasmid DNA (identified as containing a probable fibroblast gene by biological method.) and locating a DNA sequence that could be translated into the 13 amino acid sequence which had been obtained from the fibroblast protein (Table II). The gene for human leukocyte interferon was cloned in a similar manner and was identified by isolating a bacterium that produced leukocyte-like interferon activity (antiviral). The plasmid DNA of the leukocyte gene has also been sequenced (Mantei *et al.*, 1980). Its sequence when converted into amino acid sequence is homologous in 15 of 20 amino acids to the *N*-terminal sequence obtained from the lymphoblastoid interferon protein. These data show that the leukocyte and lymphoblastoid interferons are very similar at their *N*-terminals. In both fibroblast (Taniguchi *et al.*, 1980a) and leukocyte (Mantei *et al.*, 1980) genes the DNA sequence predicts interferon polypeptides of 166 amino acids. There are no data yet available on the amino acid sequence obtained from the leukocyte protein nor are sequence data available from the DNA of a cloned lymphoblastoid interferon. Whether the complete amino acid sequences obtained from the interferon proteins will be identical to that derived from the cloned DNAs remains to be determined.

Human fibroblast interferon DNA and human leukocyte interferon DNA have been compared (Taniguchi *et al.*, 1980b) and the authors point out that there is 45% homology at the nucleotide and 29% homology at the amino acid level. Moreover, this is a clear indication to them that these two interferons are from the same family of proteins. The identical homology between the fibroblast interferon *N*-terminal sequence obtained from the protein and that derived from the fibroblast DNA should silence the last critic, but I doubt it. At a future meeting someone may ask, "Well, do you really believe that that protein is interferon?"

Is the carbohydrate moiety of those interferons that have been obtained pure necessary for activity *in vitro*? Probably not, but a definite answer must await further experimentation. Human lymphoblastoid interferon has been extensively deglycosylated and it retains activity (Zoon *et al.*, 1979). Several

interferons that have been obtained pure, human leukocyte (Rubinstein *et al.*, 1979), mouse interferon (Knight, 1975; De Maeyer-Guignard *et al.*, 1978), contain very little or no carbohydrate but yet are active *in vitro*. The results should be taken only as indications and not proof of active interferons without carbohydrate. The carbohydrate composition of an interferon may vary from preparation to preparation since a cell controls the polymerization of carbohydrates less rigidly than the polymerization of amino acids.

B Biologic Properties of Pure Interferons

The varied biologic effects of the interferons have been recently reviewed (Stewart, 1979a) and it is my intention only to point out those effects which have been shown to be caused by a pure interferon. All the pure interferons shown in Table I were purified as antiviral activities, thus it is important to ask if the pure interferons will (or will not) cause some or all of the biologic effects that have been attributed to interferon preparations. In 1976 it was reported that homogeneous human fibroblast interferon would inhibit the growth of cells in culture (Table III). It has taken 14–17 years to prove

TABLE III Inhibition of the Growth of Cells in Culture by Pure Interferons

Interferon	Cells inhibited	Reference
Human fibroblast	Human fibroblast	Knight (1976a, b)
Human leukocyte	Human lymphoblastoid	Evinger <i>et al.</i> , (1980)
Human leukocyte	Human lymphoblastoid	Berg and Heron (1980b)
Mouse	Murine leukaemia L1210	De Meyer-Guignard <i>et al.</i> (1978)
Mouse	Mouse L cells	Iwakura <i>et al.</i> (1978)

that the original observation (Paucker *et al.*, 1962) was in fact caused by interferon. There have been no exceptions, all the interferons that have been obtained pure, and have been tested, inhibit the growth of cells in culture (Table III). For those who have been associated with this area of interferon research this is a satisfying answer and conclusion to a major question.

There are other nonantiviral effects caused by interferons. Those non-antiviral effects, other than the inhibition of cell growth, that have been caused by pure interferons are shown in Table IV. These effects had been observed earlier with impure preparations of interferons and these experiments and observations have been reviewed (Stewart, 1979b). The most extensive study of non-antiviral activities has been made with pure mouse interferon (Gresser *et al.*, 1979; Table IV). Similar observations have been made with pure human leukocyte interferon (Berg and Heron, 1980c; Table

TABLE IV Nonantiviral^a Activities/of Pure Interferons

Interferon	Activity	Reference
Mouse	Inhibition of growth of a transplantable tumour in mice	Gresser <i>et al.</i> (1979)
Mouse	Enhancement of the expression of histocompatibility antigens	
Mouse	Inhibition of antibody formation <i>in vitro</i>	
Mouse	Inhibition of sensitization to sheep erythrocytes and the expression of delayed type hypersensitivity in mice	
Mouse	Enhancement of natural killer cell activity <i>in vivo</i> and <i>in vitro</i>	
Mouse	Enhancement of cell sensitivity to the toxicity of polyI: polyC	Einhorn (1980)
Human fibroblast	Enhancement of spontaneous cytotoxicity of lymphocytes	
Human leukocyte	Enhancement of natural "killer" lymphocytes	Berg and Heron (1980c)
Human leukocyte	Enhancement of mixed lymphocyte reaction	
Human leukocyte	Enhancement of HLA expression on lymphocytes	

^a Nonantiviral activities other than inhibition of cell growth which is shown in Table III.

IV). A most important finding in the contribution by Gresser *et al.* (1979) is that pure mouse interferon inhibits the growth of a transplantable tumour in mice. It seems quite clear (finally) that the same protein that induces the antiviral activity causes a variety of biologic effects. The molecular mechanisms by which the interferons cause the various nonantiviral effects are unknown. The investigation of these mechanisms will undoubtedly be an area of very active research in the future as more pure interferons become available.

IV CONCLUSION

My main interest during my participation in interferon research has been purification and characterization which explains this somewhat prejudiced account. Currently, there is excellent research ongoing in these areas in a number of laboratories and the acquisition of knowledge should accelerate. There are now two conclusions about the interferons with which there can be no argument: (1) interferons can be obtained pure and the protein studied;