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Standardization of interferons

Many papers appearing in *Antiviral Research* are on interferon or interferon inducers. In such publications interferons have until recently, always been quantified in biological units. One biological unit of interferon is usually defined as the amount which, when dissolved in 1 ml of cell culture supernatant, partially inhibits virus replication in this cell culture. In this definition there are so many biological variables that it cannot, by itself, provide a sufficient basis for comparability of results between laboratories.

In the last three years assay procedures have been developed which do not rely on biological activity but rather on antigenic mass, e.g., radioimmunometric assays. The widespread use of these assays will bring some relief to the confusion, but will also create new problems in comparing results from different laboratories. Besides, whatever assay procedure might be developed to measure 'weight' of interferon in preparations, investigators will never be exempt from also quantifying in a standardized fashion the biological activity of a given weight of interferon (i.e., specific biological activity). Thus, there will remain a need for internationally accepted reference preparations with a certified biological activity, as well as for a certain discipline in using these preparations.

In 1969 the World Health Organization assumed the responsibility to coordinate the efforts done by various national agencies and of individual research groups, to bring uniformity in assaying biological activities of interferons and in reporting results obtained by such assays. A number of reports of expert committees were published, 'working reference preparations' were established, and 'international reference preparations' were adopted.

The latest WHO report is entitled 'Standardization of Interferons'. It contains a comprehensive body of information on the characterization of the available reference preparations as well as unambiguous instructions as to their use. Most importantly, it also contains a section on 'Reporting of Results in Scientific Publications', which is highly recommended reading for authors submitting papers to *Antiviral Research*.

As a service to our readers we have obtained permission of the World Health Organization to reprint this report, originally published as Annex 1 to the 33rd Report of the WHO Expert Committee on Biological Standardization (WHO Technical Report Series, Nr. 687, 1983).

A. BILLIAU
Editor

STANDARDIZATION OF INTERFERONS

Report of a WHO Informal Consultation[1]

WHO EXPERT COMMITTEE
ON BIOLOGICAL STANDARDIZATION

A WHO informal consultation on the standardization of interferons took place in Geneva from 14 to 16 June 1982. The meeting was opened on behalf of the Director-General by Dr F.T. Perkins, Chief, Biologicals, who noted that great progress has been made in the production and testing of interferons since the international reference preparations were established in 1978. He noted also that interferons now had many more applications, and that it was essential to review the preparations and the techniques used, in order that the results from different countries could be compared.

1. INTRODUCTION

Since their discovery in 1957, interferons (IFN) have been studied as antiviral agents. More recently, with the demonstration of their possible anti-cancer activity and effects on the immune system, interest in interferons has multiplied (*1*). This heightened interest, together with the greater availability of the material and the recognition of different types of human interferon (Table 1), have made its necessary to compare in different laboratories the materials tested and the potencies of the preparations intended for clinical use. To this end, the international reference preparations of human leukocyte, human fibroblast, rabbit, mouse, and chicken interferons (*2*, *3*) (see Table 2) have been established by the World Health Organization; the responsibility for the distribution of these preparations rests with the National Institute for Biological Standards and Control (NIBSC), London, England, and the Research Resources Branch,

[1] Held in Geneva from 14 to 16 June 1982.

Table 1. Interferons produced in human cells

Interferon preparation	Common production system	Major constituents[a]	Old nomenclature
leukocyte interferon	buffy coat leukocytes and virus[b]	HuIFN-α different subtypes	Le(leukocyte) type 1, pH 2, stable, foreign-cell induced
lymphoblastoid interferon (Namalwa)	B lymphoblastoid cells and virus[b]	HuIFN-α different subtypes[c]	Ly(lymphoblastoid) type 1, pH 2, stable, foreign-cell induced
fibroblast interferon	fibroblasts and double-stranded RNA	HuIFN-β	F (fibroblast), Fi, type 1, pH 2, stable
"immune" interferon	buffy coat leukocytes, or T lymphoblastoid cells } and mitogen	HuIFN-γ	HF (immune), type II, T, pH 2, labile, antigen-induced, mitogen-induced

[a] The designations IFN-α, IFN-β, and IFN-γ are used to classify the interferons according to their structure. In contrast, the commonly used terms leukocyte interferon, lymphoblastoid interferon, and fibroblast interferon refer to preparations made from the cells concerned. The term "immune" or 'type II" interferon was formerly used to denote crude preparations of IFN-γ, which probably also contained other lymphokines. Both leukocyte and lymphoblastoid interferons contain several alpha-type human interferons. Preparations of leukocyte interferon made and purified in different laboratories may differ in the number or proportion of alpha-type interferons present. Similarly, lymphoblastoid interferon preparations may differ according to the cells and purification process used.

[b] Sendai virus or Newcastle disease virus.

[c] Crude preparations also contain HuIFN-β.

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Interferon preparations are usually complex mixtures of proteins, and even those intended for clinical use may contain contaminating proteins as well as some interferon molecules inactivated during processing, purification, and storage.

Since, until recently, the only means of quantifying interferon activity was by measuring its biological effects, and since the various assays available for doing so were developed soon after its discovery, it became apparent that some mechanism for the comparison of results from different laboratories was necessary. The need for research on reference preparations was clear. The first meeting on the subject, at which such preparations were proposed, was held in London in 1969 (*3*); the working reference materials were also established at that meeting. In 1978, the World Health Organization, having recognized their general acceptance, need, and use, adopted these preparations and a human fibroblast interferon reagent as international reference preparations (*4*).

The importance of these materials is shown by the demand for them (Table 3), the absence of controversy regarding their unitage, and the reporting of results in the literature in terms of international units (IU). The purpose of these reference materials is to calibrate interferon assays. To do this, a laboratory reference preparation should be compared directly with the appropriate international reference preparation so that its activity can be expressed in international units.

Table 2. Characteristics of freeze-dried interferon reference material

Interferon preparations	Catalogue number	Producer cells	Predominant interferon type	Titre assigned as units/ ampoule (or Log_{10})	Predicted stability (years to lose 1000 units) at:		Inducer	Inactivation or removal of inducer
					20 °C	4 °C		
WHO international reference preparations	69/19 (MRC RES STD B) (NIBSC)[a]	Human leukocytes	α	5 000 (3.7)	>1.5	>29	Sendai virus	pH 2
	G023-902-527 (NIH)[b]	Human fibroblasts	β	10 000 (4.0)	>1.5	>29	Poly I - poly C	washing
	G019-902-528 (NIH)	Rabbit kidney	Unknown	10 000 (4.0)	1.0	6.3	Bluetongue virus	pH 3.5
	G002-904-511 (NIH)	Mouse L-929	α + β	12 000 (4.1)	5.5	110	Newcastle disease virus	$HClO_4$
	67/18 (NIBSC)	Chicken embryo fibroblasts	Unknown	80 (1.9)	[c]		Influenza B virus	pH 2
WHO international working reference preparations	G023-901-527 (NIH)	Human leukocytes	α	20 000 (4.3)	1.4	28.8	Sendai virus	pH 2
New reference research preparations in progress	Gg23-901-530 (NIH)	Human lymphocytes	γ	Unknown	Unknown		Staphylococcal enterotoxin A	porous glass chromatography
	Gg02-901-533 (NIH)	Mouse spleen	γ	Unknown	Unknown		*Lens culinaris*	–
	Ga23-901-532 (NIH)	Human lymphoblastoid	α	Unknown	Unknown		Sendai virus	pH 2
	82/A (NIBSC)	*E. coli* (with human gene)	α_2 (αA)	Unknown	Unknown		None	–
	Gxa01-901-535 (NIH)	*E. coli* (with human gene)	α_2 (αA)	Unknown	Unknown		None	–
	82/B (NIBSC)	Human lymphocytes	γ	Unknown	Unknown		Phytohaemagglutinin (*Phaseolus vulgaris*)	porous glass chromatography

[a] NIBSC – National Institute for Biological Standards and Control, London, England.
[b] NIH – National Institutes of Health, Bethesda, MD, USA.
[c] 67/18 held for three months at 37 °C was found to have retained potency when compared with the same preparation held at −20 °C for the same period.

Table 3. Number of ampoules of human and mouse international reference preparations of interferon distributed by the National Institute for Biological Standards and Control, London, England, and the NIH (National Institute of Allergy and Infectious Diseases), Bethesda, MD, USA

Period	Human interferon		Mouse interferon
	Leukocyte	Fibroblast	
Prior to 1979	191[a]	61[b]	518[c]
In 1979	258	104	140
In 1980	241	107	80
In 1981	274	145	115
In first 6 months of 1982	150	54	40

[a] 1973–1978 (inclusive).
[b] 1976–1978 (inclusive).
[c] 1972–1978 (inclusive).

The more recent surge of interest in "immune" or gamma-interferon has again indicated a need for new reference preparations. The NIH are currently supporting the development of appropriate reagents for both human and mouse gamma-interferons. Hopefully, these should be available shortly.

Other reagents, distributed by the NIH, which have proved to be of immense value are antisera to mouse and human leukocyte and human fibroblast interferons (Table 4). These materials were prepared by repeatedly injecting interferons into sheep or bovines until significant interferon neutralizing activity could be measured in their sera.

Recombinant DNA technology has yielded major discoveries in the structure of interferon at an unprecedented rate. This has led to the realization that there may be as many as 14, or even more, different alpha-interferon subtypes. It is possible also that there are subtypes of beta-interferon. It may be possible to develop monoclonal antibodies to each of these interferons, and the question may be asked whether it will eventually be useful to make such reagents available.

There is considerable interest in the anti-tumour effects of interferon and in its immunomodulatory effects, such as increases in T-cell cytotoxicity, natural killer-cell activity, antibody-dependent-cell cytotoxicity, and macrophage function. Therefore, the question should be considered whether it would be helpful to develop new assay systems to quantify these biological activities and to define the reference preparations in such terms, as well as by antiviral activity.

2. STANDARDIZATION OF ASSAYS

There are many different kinds of biological assay for the meas-

Table 4. Antisera to interferon available from the NIH (National Institute of Allergy and Infectious Diseases), Bethesda, MD, USA

NIH antiserum or control antiserum	Antigen preparation	Catalogue No.	Neutralizing titre[a]
Sheep anti-human fibroblast interferon	Diploid cells: poly I:C – purified to specific activity of 1×10^6 U/mg protein	G028-501-568	12 000 against 8–10 U IFN
Control sheep anti-human fibroblast interferon	Void material from above glass-filter column	G029-501-568	< 50 against 8–10 U IFN[1]
Sheep anti-human leukocyte interferon	Buffy coat: Sendai virus – purified to specific activity of 1×10^4 U/mg protein	G026-502-568	750 000 against 8–10 U IFN[2]
Control sheep anti-human leukocyte interferon	Void material from above Sepharose 4B column	G027-501-568	< 50 against 8–10 U human IFN
Bovine anti-human lymphoblastoid (Namalwa) interferon	Namalwa: Sendai virus[b]	G030-501-553	40 000 against Namalwa[3]
Control bovine anti-human lymphoblastoid (Namalwa) interferon	Normal calf serum[b]	G031-501-553	< 25 against Namalwa IFN[4]
Sheep anti-mouse L-cell interferon	L_{929}-NDV – purified to specific activity of 1×10^6 U/mg protein	G024-501-568	300 000 against 8-10 U IFN[5]
Control sheep anti-mouse L-cell interferon	Void material from above Sepharose 4B column	G025-501-568	< 50 against 8-10 U mouse IFN

Heterologous antibody activity:

[1] May be toxic at ≤ 1:100 dilutions
[2] 2000 titre against 8–10 U human fibroblast IFN
[3] 40 000 titre against primary leukocyte IFN
10 000 titre against fibroblast IFN
[4] < 25 titre against leukocyte IFN
< 25 titre against fibroblast IFN
[5] Low levels of antibody to human leukocyte IFN

[a] These titres are those stated in the NIH Reference Reagent notes describing each preparation.
[b] Both sera were passed through a "scrubber" column consisting of Sepharose with Namalwa cell protein and Sendai virus attached.

urement of interferon activity, and such assays utilize a variety of cell cultures and viruses. For any assay chosen by a particular laboratory, every effort must be made to standardize the conditions and materials that relate to the use of viruses and cell cultures. Several important practical considerations in the performance of an assay have been detailed, including: (*a*) familiarity with the basic cell culture methods, sterile handling techniques, and the characteristics of the virus being used; (*b*) techniques for making serial dilutions of interferon samples (such as twofold or threefold steps); (*c*) approaches to obtaining optimum precision, such as by increasing the number of titrations on different occasions; (*d*) determination of dose–response curves and their parallelism and reproducibility when the potency of two or more preparations is to be compared; and (*e*) statistical analysis of quantitative data. Careful attention should be paid to the control of quality of the virus batches, the animal sera used in cultures, and the water used in the medium. Particular attention should also be paid to ensuring that these are free from mycoplasma, fungi, and bacteria or their products; also, the cells used should be free from latent viruses. Suitable methods for verifying sterility are described in the Requirements for the Sterility of Biological Substances (*5*).

2.1 Interferon unitage

The biological activity of interferon preparations is expressed in units. The unitages used, in order of preference, are: (*a*) an international unit, as defined by an appropriate international reference preparation, if one exists; (*b*) a research unit as defined by a research standard preparation, if available from a national agency; or (*c*) if no research preparation exists, a laboratory unit such as the minimum amount of interferon producing an arbitrarily defined degree of activity in a given test system.

2.2 Calibration of assays and standards

The purpose of interferon assays is to obtain and express results in a unit of activity that is constant within any particular laboratory and to relate such activity to that of the appropriate international reference preparation, if available (*6*). Not only should assay conditions be standardized as far as practicable, as stated above, but the variations between each set of assays and the sensitivity of those assays should be monitored by the inclusion of an appropriate laboratory reference preparation. Each of the international reference preparations mentioned in the preceding section has been defined as containing a certain amount of interferon per container; the amount was decided upon on the basis of repetitive testing in different laboratories by a number of workers expert in the use of the appro-

priate techniques. Since the slopes of dose–response curves of human alpha, beta, and gamma interferons[1] differ, the reference preparation of one cannot be substituted for that of another. The use of reference preparations has been complicated by the recognition of interferon subtypes; for example, HuIFN-α preparations of different origin contain a number of interferon proteins in different proportions. These different interferons may have different biological properties, since data obtained on purified molecular subtypes of HuIFN-α show that they may differ in their relative antiviral activities in different cells, depending on the particular cell and, possibly, also on the virus used in an assay system.

Because of the great care taken in their preparation and calibration, international reference preparations are very costly materials and therefore should be used only for the calibration of national or laboratory standards in international units and not as general laboratory reagents.

When received, an international reference preparation should be handled according to the instructions on the data sheet sent with each shipment. After use, any excess material can be stored at 4 °C for a few days, provided it is sterile; small, accurately measured aliquots of, for example, 0.1 ml, should be stored at −70 °C or colder. Usually these can be thawed later and used to calibrate or recalibrate a laboratory reference preparation; however, it should be noted that repeated freezing and thawing can inactivate the interferon.

Each laboratory should prepare its own laboratory reference preparation, which should be homologous with the corresponding International Reference Preparation; the laboratory reference preparation should be shown by statistical analysis to give a parallel dose–response curve. For example, a preparation of HuIFN-α can be made from human buffy-coat cells or from cells of the Namalwa human lymphoblastoid line; the latter preparation, however, should be purified sufficiently to eliminate the HuIFN-β present in the crude harvest.

The laboratory reference preparation should be clarified by centrifugation, stabilized (if necessary) by adding, for example, human or bovine serum albumin to a final dilution of 4 mg/ml, distributed in a large number of containers, and stored frozen, preferably at −70 °C. A sufficient number of containers should be stored to provide one for each assay envisaged for a period of 2–3 years. The activity of the laboratory reference preparation must then be calibrated with great care in terms of the corresponding international

[1] Hereafter the human alpha, gamma, and beta interferons are referred to as HuIFN-α, HuIFN-β, and HuIFN-γ, respectively.

reference preparation in parallel assays on at least five occasions, and preferably on as many as 20 occasions. In each assay, a value of the activity of the International Reference Preparation is obtained, and the ratio between this and its defined potency expressed in international units gives a measure of the sensitivity of that particular assay. By applying the same correction factor to the laboratory reference preparation, its potency may be expressed in international units.

As an example, suppose that the International Reference Preparation of Interferon, Human Leukocyte, (preparation 69/19, with a defined potency of 5000 IU per ampoule) has, because of the sensitivity of the assay on that occasion, an end-point titre of $-3.8 \log_{10}$ (or 1:6300), and that in the same assay, the laboratory reference preparation of leukocyte interferon has an end-point titre of $-4.1 \log_{10}$ (or 1:12600). The potency of this preparation in IU is then calculated as $(12600/6300) \times 5000 = 10\,000$ IU. The geometric mean of the values, thus expressed in IU, for the potency of the laboratory reference preparation obtained on 5–20 occasions is computed, and the preparation with its potency thus calibrated can now replace the international reference preparation in subsequent titrations. A similar calculation is made when measuring test samples in relation to the laboratory standard in order to express those results in IU.

Unless accelerated degradation tests provide evidence that the laboratory reference preparation is very stable, the possibility that it may deteriorate during storage should not be overlooked. Its potency should therefore be rechecked from time to time against a sample of the International Reference Preparation that has been stored frozen.

The potency of a preparation is usually expressed as units per ml, or, if possible, as described above, in IU per ml. The term "specific activity" relates the potency to the total amount of protein present (before the addition of a stabilizing protein, if any) and thereby indicates the relative purity of the preparation.

2.3 Reference bioassay

There are many interferon assays that measure the degree of resistance of cells to viruses. Diverse attributes are used to measure viral activity (infectivity, haemagglutinin, enzymes, effect on cell cultures, etc.). Also, many different viruses and cells are used and these can differ widely in their sensitivity to a single type of interferon as well as to different types of interferon. Although the somewhat chaotic situation resulting from this has been relieved by the introduction of international reference preparations of interferons, the way of defining the values of potency to such reference materials

has never been clarified. The current practice of WHO for the establishment of the unitage of a national or international reference material is to request a number of experts to determine the activity of the material by any assay method that is in current use in their laboratory. The results obtained by the pooling of such data for interferons have often given arithmetic or geometric means with very large standard deviations, sometimes with a range of values of as much as ± 10-fold, reflecting the different sensitivities of the various assay methods. Nevertheless, these means have been used to define a value in international units for the potency of a given reference preparation (*2*). However, in the case of two early reference preparations—those of chicken and rabbit interferons—the potencies were defined on the basis of a single method of assay with a particular virus and cell.

Although the definition of the potency of a first standard can be arbitrary, its unitage is chosen to provide a generally acceptable value. When replacement standards of the same biological substance are needed, their unitage must be assigned as accurately as possible by comparison with the appropriate first standard. Such comparisons may sometimes present difficulties. For example, the first International Reference Preparation of Interferon, Human Leukocyte, contains a mixture of different HuIFN-α subtypes, and these vary in their relative activity on different cells–for example, different results are obtained on human cells compared with bovine cells. Therefore, the results of potency comparisons between these standard preparations and a candidate replacement standard may be influenced both by the assay system used and the number and proportions of the various subtypes present in the two preparations. Although it is desirable that such differences be identified by testing the preparations in a variety of different assays, it is suggested that a single assay with stipulated components should also be used by all the participating laboratories.

A reference bioassay suitable for this purpose has been proposed (*2*). It is important that this bioassay fulfils the following criteria:

(*a*) It should be relatively simple and easy to carry out. The reagents should be available to any laboratory involved in modern virus research or clinical studies in virology, and the challenge virus must be usable in all countries (e.g., vesicular stomatitis virus cannot be used in some countries). The cell system and viruses to be used should be easily transportable from one laboratory to another, and the propagation and storage of these materials should not present any special major problem.

(*b*) Within the limits of the biological tests, the assay should be relatively rapid.

(*c*) The assay must be reproducible, and the reading of end-points objective and unequivocal. The assay end-point should be measured

directly by a significant reduction in the yield of virus—for instance, 0.5 $\log_{10}$ in infectivity—when compared with the yield of virus in control cultures not treated with interferon.

(*d*) The measurement and yield of a one-step growth cycle should be employed to test the antiviral activity of interferon preparations. The possible effects of priming and of endogenous interferon production in multiple-step growth cycles should be avoided by using multiplicities of virus challenge consistent with obtaining infection of the entire cell population. However, very high virus multiplicities should be avoided; they could increase the possibility of residual unadsorbed virus that might preclude accurate estimation of the final yield and also possibly decrease the apparent effectiveness of interferon treatment.

Concordant with these criteria, a reference bioassay for interferon is proposed for collaborative studies on standards. Encephalomyocarditis (EMC) virus is recommended as the challenge virus because of the ease with which EMC virus can be grown and assayed. Furthermore, EMC virus is stable and has low pathogenicity for man. It is acceptable for use worldwide, it is sensitive to interferon, and has a very wide host range. The virus strain recommended is that currently in use at the Medical College of Wisconsin, USA. The cells recommended are L_{929} cells (MCW line) for mouse interferon and the RK-13 cell line for rabbit interferon. Although the diploid fibroblast, BUD-8 strain, was previously recommended for human interferon, in view of the relatively slow growth of diploid human fibroblasts and their limited longevity, a continuous line of human cells should be used—for example, the NIH A549 line of lung carcinoma cells, which are sensitive to all three types of human interferon. Technical details of the assay are given in Appendix 1.

3. REPORTING OF RESULTS

3.1 Scientific publications

In scientific publications, there should be a brief description of the bioassay. The important technical elements of the bioassay (i.e., cells, virus, and end-point) should be described. The names and assigned unitage of the international reference preparation (IRP), together with the catalogue number, should be stated along with the logarithm of the observed geometric mean titre, its standard deviation, and the number of titrations done to obtain this result, thereby indicating the sensitivity of the assay.

Example description: HuIFN-β preparations were assayed in a dye-uptake assay with WISH cells using EMC as the challenge virus. The IRP of human fibroblast interferon (GO23-902-527 with a

defined potency of 4.0 $\log_{10}$ IU per vial) when reconstituted in 1.0 ml, had a geometric mean titre of 3.7 $\log_{10}$ IU/ml (SD = 0.25; n = 5). In each assay an internal laboratory standard was included which had been calibrated against the IRP in 20 titrations yielding a potency of 4.3 $\log_{10}$ IU/ml (SD 0.16). All titres are reported in IU/ml and are means of three or more titration results.

3.2 Protocols submitted to regulatory agencies

The essential information to be given to a national health authority or to any other regulatory agency is the same as that required for a scientific publication. However, in order to enable the agency to evaluate the antiviral potency of the individual interferon preparation the bioassay method should be described in greater detail.

If an international reference preparation exists, the following information should be reported:

(*a*) a brief description of the bioassay and the calculation of interferon activity in international units;

(*b*) a description of the materials used in the assay (i.e., cells and virus, including their sources, as well as international and laboratory reference preparations);

(*c*) data showing that in the assays the dose–response curves of the IRP, the laboratory reference preparation used, and the interferon sample assayed are parallel, and

(*d*) the number of titrations carried out of the individual interferon preparation, and the logarithms of the geometric mean of the titres and the standard deviation.

If a laboratory reference preparation has been used, the above details should be reported for the laboratory reference preparation. In addition, data should be given which show that the laboratory reference preparation is stable under the conditions of storage in the laboratory and that the material has remained stable during the period between its preparation and use.

If no appropriate international reference preparation exists or if in the assay system used the dose–response curves of the international reference preparation and the interferon preparation to be tested are not parallel, the antiviral activity can be expressed only as an arbitrary laboratory unit. In this case the agency should be supplied with the internal reference preparation of the producer, and the information given should enable the agency to repeat precisely the assay used. This information should include:

(*a*) a *detailed* description of the assay, the calibration of the assay with an internal interferon reference preparation, and the calculation of the antiviral potency in laboratory units;

(*b*) a description of the materials used (cells, source of cells, virus and source of virus), a description of the preparation of the internal interferon standard, and data showing that the internal standard is

stable under the conditions of storage in the laboratory and that the material has remained stable during the period between its preparation and use; and

(*c*) the number of titrations carried out of the individual interferon preparation, the geometric mean of the titres and the standard deviation, expressed as the logarithm of the antiviral activity.

4. CHARACTERIZATION OF STANDARDS

Interferon reference preparations should be demonstrated to have the properties generally ascribed to interferons. These include: (*a*) solubility, i.e., the preparation should be shown to be non-sedimentable at 100 000 g; (*b*) the protein nature, as demonstrated by the loss of activity after incubation with proteolytic enzymes such as trypsin; (*c*) species specificity of action characteristic for the given type of interferon. In addition, physicochemical characteristics of the active component (such as molecular weight and isoelectric point) should be included. Whenever possible, a complete physicochemical and antigenic characterization of any subtypes that might be present should be made. The antigenic nature of the interferon type should be determined using the available reference preparations of antisera. The degree of stability at pH 2 over a period of 24 hours should be reported. Relative heat stability, as demonstrated by partial resistance to inactivation after incubation for one hour at 56 °C, is an unreliable characteristic and is influenced greatly by the protein content of the preparation.

Descriptions of the preparation and processing of the standard material should include (*a*) nature of producing cell and of the inducer; (*b*) methods of removal or of inactivation of the inducer; (*c*) steps used in purification and concentration; (*d*) specific activity expressed as units of biological activity per milligram of protein; (*e*) additives and solutions utilized; (*f*) methods of freeze-drying; and (*g*) nature of the gaseous atmosphere in the glass container, which, for WHO purposes, must be sealed by fusion of the glass, with demonstration of the adequacy of the seal (*8*). The final product should be demonstrated to be free from bacteria, fungi, mycoplasma, and viruses by appropriate culture methods (*5*). The final analysis of the potency of the product should be done on cells of homologous as well as heterologous species. Information concerning the predicted stability of the standard should be provided; such prediction can be derived from the data obtained in accelerated stability tests, such as the multiple isothermal stability test (*7*) (see Appendix 2).

5. REFERENCE ANTISERA

Antibodies to interferons are useful for many purposes including: (*a*) characterization of interferon molecules, in particular distinguishing types and subtypes; (*b*) purification of interferon from other proteins; (*c*) isolation of individual molecular variants of interferons from mixtures of different interferons; (*d*) performing quantitative interferon assays using immunoprecipitation or immunosorbent assay systems (RIA, ELISA); (*e*) characterization of the antigenic structure of interferon, including the description and localization of the principal antigenic domains; (*f*) performing certain steps in molecular cloning of interferon (e.g., isolation of ribosome-bound mRNA) by immuno-adsorbent techniques, (*g*) localizing interferon molecules in cells by immunofluorescence or immuno-electron-microscopy; and (*h*) serving as reference preparations for the calibration of IFN antibodies prepared in various laboratories.

5.1 Polyclonal antibodies

Several antisera to interferons are available from the NIH, Bethesda, USA (see Table 4). One important characteristic of these antisera is their specificity for particular types of interferon. The antiserum to human lymphoblastoid (Namalwa) IFN (GO30-501-553) is known to react with HuIFN-α and, to a lesser extent, with HuIFN-β. Similarly, antiserum to human leukocyte interferon (GO26-502-568) also reacts with human HuIFN-β. The probable reason for this lack of specificity is the presence of small amounts of HuIFN-β in the preparations of either Namalwa or leukocyte interferon used for the immunization of animals. In contrast, antiserum to human fibroblast HuIFN-β (GO28-501-568) is specific for HuIFN-β and does not cross-react with HuIFN-α. Antiserum to mouse L cell interferon (MuIFN) (GO24-501-568) reacts with both MuIFN-α and MuIFN-β. It is known that most mouse interferon preparations contain mixtures of MuIFN-α and MuIFN-β in various proportions. The relative potency of this serum was found to be somewhat higher against isolated MuIFN-α than against MuIFN-β (*9*).

Additional antisera and control globulins have been prepared, and are now being tested by various laboratories. These are a rabbit anti-HuIFN-γ and its corresponding control globulin, and rabbit anti-MuIFN-γ and its corresponding control globulin. Preliminary indications are that these anti-IFN-γ sera are less potent than the reference antisera prepared with other interferons. However, since there is an acute need for antisera to HuIFN-γ, even these relatively low-titre preparations should be of great value. Since sera specific for HuIFN-α, lacking neutralizing activity against HuIFN-β, can now be produced, future efforts should be undertaken to prepare

such monospecific anti-HuIFN-α sera as well as antisera specific for MuIFN-α and MuIFN-β.

5.2 Monoclonal antibodies

Monoclonal antibodies against HuIFN-α are now becoming available from several private sources. Monoclonal antibodies against other interferons are also being produced in various laboratories.

One clear advantage of monoclonal antibodies compared with polyclonal antisera is their greater specificity and uniformity. For example, it is possible to use monoclonal antibodies for characterizing interferon subtypes (such as various subtypes of alpha interferon). Since monoclonal antibodies recognize only one epitope they may be more useful for the purification of mixtures of interferons with a common antigenic determinant. On the other hand, monoclonal antibodies often lack any neutralizing capacity, making the demonstration of their reactions more complicated (a binding assay, or RIA, or enzyme immunoassay would then be required). Therefore, monoclonal antibodies might not be as useful as polyclonal antibodies produced against pure interferons in as much as polyclonal antibodies recognize *various* antigenic determinants of proteins belonging to the same family. In addition, the fact that the specificity of monoclonal antibodies may be limited to some subtypes is a disadvantage in certain situations. Clearly, both polyclonal and monoclonal antibodies to interferons will be required.

6. NEUTRALIZATION ASSAYS

The potency of antisera to interferon has mostly been determined by performing neutralization assays. The term "neutralization" implies that the antibody to interferon abolishes the biological activity of a given interferon preparation. The term "binding" implies that the antibody combines with the interferon molecule with or without abolishing biological activity. Thus a negative neutralization assay does not rule out that binding has occurred. These considerations should be taken into account in deciding whether antisera contain antibodies to interferon.

Neutralization assays can be carried out in various ways. The definitions of the neutralization titre adopted by different investigators are not uniform, and in general it is difficult to correlate the results obtained in different systems. It is, therefore, proposed that the neutralization titre of an antiserum be defined as the reciprocal of the antiserum dilution that reduces the potency from 10 interferon units/ml to 1 interferon unit/ml. The assays should be carried out using the same bioassay system as used for interferon titration,

but without antiserum. The interferon unitage in this context is the laboratory unit (LU), and not the international unit (IU), i.e., 10 actual antiviral units should be used in the test. Although it might be desirable to define the neutralization titre in terms of IU, it is proposed that the LU be used as the basis of neutralization assays, because the definition in terms of IU has attendant theoretical difficulties.

For the determination of the neutralization titre, a series of interferon–antiserum mixtures are prepared containing a constant concentration of interferon and varying dilutions of antiserum (e.g., with two-fold dilution steps). The mixtures are incubated for one hour at 37 °C, and put on the assay cells for observation of the antiviral effect; interferon titration without antiserum should be done in parallel. In view of the variations in the interferon sensitivity between assays, it is advisable to use at least two or three interferon concentrations in each neutralization assay, and the concentration of interferon used for the determination of the antiserum titre should approach as closely as possible 10 LU/ml. The neutralization titre is then expressed as the reciprocal of the antiserum dilution, which reduces 10 LU of interferon to 1 LU/ml. It must be noted that the concentrations of interferon and of antiserum are those in the final mixture in the culture fluid of assay cells.

Neutralization assays may also be done using a constant antiserum concentration and varying concentrations of interferon. However, if the purpose is to neutralize a certain quantity of interferon, the antiserum concentration needed must be empirically determined using the constant interferon method. On the other hand, the constant antibody method can be a sensitive test to detect neutralization, if the precision of the interferon assay is high. The latter is the method of choice for analysing interferon preparations that contain non-neutralizable components, e.g., a crude sample containing both alpha and beta interferons.

Neutralization assays may also be carried out by mixing an appropriately diluted antiserum with an excess of interferon, and then titrating the residual uncombined interferon by making a dilution series of the mixture. The assays described above that do *not* require the dilution of the interferon–antiserum mixtures are recommended since dilution will cause some dissociation of the interferon–antibody complex once formed, unless the antibody affinity is extremely high. Therefore, the apparent interferon titre obtained does not necessarily represent the concentration of residual interferon in the original mixture.

7. IMMUNOASSAYS

Unlike the interferon biological assays described above, the measurement of interferon antigen in a mixture of proteins and other antigens does not involve cell culture methods. Immunoassays generally require either pure interferon or antibody to pure interferon without contaminating antibodies to other antigens. Either the interferon or the specific interferon antibody must be labelled in some appropriate way. Calibration of the antigen content in relation to units of biological activity remains a major problem to be resolved. Indeed, it is possible on theoretical grounds that interferon molecules could still be antigenic and compete in an RIA or ELISA and at the same time be biologically inactive; therefore, it is important to establish that there is a correlation between biological activity and the results obtained by the immunoassay.

Four immunoassay methods have recently been described. An immunoradiometric assay for HuIFN-α utilizes ^{125}I monoclonal NK2 antibody to HuIFN-α (*9*). The sensitivity of this assay is such that it can measure levels of interferon of 50 units/ml or more. Linearity is obtained with low but not with high concentrations of interferon. This assay can detect only the species of HuIFN-α that have the NK2 antigenic site; it is now known that at least 6 natural subtypes of HuIFN-α have the NK2 antigenic site, and that at least 2 do not. In addition to this assay a sandwich-type assay using a polyclonal anti-HuIFN-β (*11*, *12*) and an interferon displacement assay using polyclonal anti-HuIFN-α antibodies (*13*) have also been described. Another sandwich-type assay using two different monoclonal antibodies LI-1 and LI-9 has been described, and it detects certain HuIFN-α subtypes (*14*); this assay can detect concentrations of HuIFN-α (αA) of at least 5 units/ml.

Immunoassays may be very useful in detecting a specific interferon in patients injected with an HuIFN-α subtype. However, caution should be exercised in the use of an immunoassay for the detection of endogenous HuIFN-α in biological fluids, since only certain subtypes may be detected by these assays. More experience must be gained with such potentially useful assays.

8. NEW STANDARDS

The existing interferon standards have been prepared from low-purity interferon preparations, but have nevertheless proved to be very useful for the calibration of bioassays. The existing standards should be retained as long as the supply lasts; however, when new standards are prepared, they should contain interferons that have been purified.

8.1 HuIFN-α

There are at present sufficient stocks of the 69/19 international reference preparation (1300 vials on 31 December 1981) for at least another 5 years. This preparation was derived from Sendai-virus-induced human buffy coat cells, and is a mixture of alpha-interferons; in addition, there may be a small amount (less than 1%) of beta-interferon. Since 69/19 and the international working reference preparation GO23-901-527 are a mixture of HuIFN-α subtypes in undetermined proportions, it is not clear whether calibrations using 69/19 are valid when applied to an individual alpha subtype, or to another undefined mixture of alpha-interferons; it is assumed that the relative potencies obtained in an antiviral assay will be determined in particular by the alpha subtype that is most potent in that cell-virus system, even though this may be, in terms of weight relative to the other subtypes, a minor component in the reference preparation. To overcome in part this attendant theoretical problem in the use of these standards, it has been recommended that a reference bioassay be used in the development of replacement or new HuIFN-α standards.

Since 69/19 may not be an appropriate standard for individual subtypes, it is possible that the calibration of a particular subtype of HuIFN-α may require a standard containing only that particular subtype. Thus, a separate reference preparation may eventually be needed for each of the many (14 or more) individual HuIFN-α subtypes. At the present time, it is not clear whether all of these can be made available, or indeed whether they will actually be needed. However, interim reference preparations of two subtypes, namely HuIFN-α_1(D) and IFN-α_2(A), are being prepared, and these will be developed and evaluated by the National Institute of Biological Standards and Control, London, and the National Institutes of Health (NIH), Bethesda, USA. Their potencies will be determined in reference units relative to the international reference preparation. These standards can then be used to calibrate other preparations of the corresponding single subtype in reference units.

Until other HuIFN-α subtype reference preparations are available, it is suggested that individual manufacturers and research groups should prepare an internal laboratory reference preparation of the subtype on which they are working: this should meet all the criteria set out in this document for a reference preparation. This laboratory reference preparation should be assigned a potency (the basis for which must be fully documented) based on the potency of 69/19 in the particular assay system(s) used.

All new alpha-interferon subtype reference preparations should be purified to contain 90–95% interferon protein, diluted approximately 4.0 $\log_{10}$ units/ml, and stabilized by the addition of a suitable protein before it is distributed into ampoules and freeze dried.

Since the same considerations may apply to preparations of mixed alpha-interferons derived from human lymphoblastoid cells (e.g., Namalwa cells), a reference preparation of Namalwa interferon is being prepared by the NIH.

Clinicians should be prepared for the possibility that when reference units are established for different human alpha-interferons the same nominal doses in reference units of, for example, IFN-α_1(D), IFN-α_2(A), and Namalwa interferon may be found to produce different responses in patients.

8.2 HuIFN-β

The current International Reference Preparation for HuIFN-β appears to be appropriate. However, the existing reference preparation is of low purity and should eventually be replaced by one of high purity.

8.3 HuIFN-γ

Since there are so many investigators involved in active research with interferon and its possible application to cancer treatment, there is some urgency to establish a reference preparation. A reference research preparation of HuIFN-γ is to be made available shortly.

8.4 Other mammalian interferon standards

There is a current recognized need for research on individual murine-alpha, beta, and gamma interferons. Currently a reference preparation containing a mixture of murine alpha and beta interferons is available, and a murine gamma interferon reference preparation will be made available in the near future.

9. CALIBRATION OF INTERFERON STANDARDS IN ACTIVITIES OTHER THAN ANTIVIRAL ACTIVITY

Even though interferons have various biological effects, there is at present no consensus on the need for, or feasibility of, standardizing tests for the non-antiviral effects, and the international reference preparations have not been calibrated for this purpose. Furthermore, the international reference preparations of interferon have not been analysed in terms of their antigenic content or of the proportions of the subtypes present.

Research in the development and quantification of new and appropriate techniques for testing non-antiviral effects should be encouraged.

10. SUMMARY AND CONCLUSIONS

The great degree of current scientific and clinical interest in interferons emphasizes the importance of reliable measurements of the activity of these substances in generally accepted units. These measurements are best achieved by antiviral bioassays. For this purpose reference materials for potency have already been developed for certain human and animal interferons, and some of these have been established by WHO as international reference preparations. Such preparations are now extensively used in the calibration of bioassays. However, with the realization that there are three main types of human interferon (alpha, gamma, and beta), as well as several subtypes, the question has been posed whether new or additional interferon standards are needed.

The primary purpose of an international reference preparation is to calibrate the assay used in each laboratory. The first step is the calibration of an appropriate laboratory standard preparation in terms of the international reference preparation: in this step and in the subsequent use of the laboratory standards in the assay of test samples, the various dose–response curves must be strictly parallel for valid results to be obtained and for the reporting of titres in international units. According to WHO procedure, the unitage for international reference preparations has been assigned from the analysis of the results obtained in a collaborative study involving several workers, each using an individual bioassay method. Usually a mean from these data has been taken to define the unitage, but a wide range of values is often obtained in such a study. It is therefore suggested that in future collaborative studies a single reference bioassay method should be used by all the participants in addition to their own method. Such an assay was proposed for future evaluations.

In reporting data on assays for publication, scientists should give a certain minimum amount of technical and statistical data pertinent to their assay results, including an outline of the bioassay method, details relating to the international reference preparation used to calibrate the assay, standard deviation, and the number of determinations used in the calibration of the laboratory standard preparation. When data are to be submitted to regulatory agencies, more details should be provided, including data on parallelism of dose–response curves of the sample and the appropriate standard preparations.

Stocks of the first Reference Preparation of Interferon, Leukocyte should last for about 5 more years. When a new reference preparation of human leukocyte interferon is needed eventually to replace the existing one (69/19), some difficulties may arise: the latter contains a mixture of HuIFN-α subtypes in unknown proportions, and

it may not be possible to produce an exactly matching candidate new reference preparation. Since 69/19 contains a mixture of subtypes it may not be appropriate for the standardization of any individual subtype. It is not at present clear whether a reference preparation for each of the 14 or so individual HuIFN-α subtypes will be needed. However, it is recommended that those producing a particular HuIFN-α subtype for clinical use should prepare in sufficient quantity a laboratory standard: this should fulfil the criteria mentioned in the document for a reference preparation and be highly purified, i.e., 90–95% pure. It seems likely that at some later date, such a preparation may be officially adopted as a reference preparation by a national agency or by WHO. There is an urgent need for a standard of HuIFN-γ and a research reference preparation should be available fairly soon. Research standards for certain animal interferons, especially individual MuIFN-α, MuIFN-β, and MuIFN-γ will be useful.

The existing international reference preparations have been evaluated only in antiviral tests. There is no consensus on the need for, or feasibility of, developing standard tests for other biological activities.

Polyclonal antibodies to a number of interferons are currently available as research reagents. Among other uses, these can be employed for the characterization of interferon antigenic types and for the calibration of laboratory antisera. It is emphasized that immunoassays and other non-biological methods for the assay of interferons may also recognize inactive or inactivated interferon molecules, and they are thus not a complete substitute for bioassays. Many of the existing polyclonal antibodies are not specific for a single interferon type. Some monoclonal antisera to interferons have now been developed which can be used, for example, for characterizing individual HuIFN-α subtypes. These monoclonal antibodies, however, may lack neutralizing capacity. Both monoclonal and specific polyclonal sera will probably be needed in the future. Some procedures for carrying out neutralization assays with interferon and ways of calculating the titre have also been outlined in this report.

It is concluded that although significant advances have been made in the standardization of interferons by virtue of the availability of the international reference preparations, many problems regarding standardization of materials and methods remain to be solved by future research.

Appendix 1

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Appendix 2

REFERENCE BIOASSAY FOR CALIBRATING INTERFERON STANDARD PREPARATIONS

The assay can be done in test-tubes (using three tubes per interferon dilution) (*2*) or in microtitre plates. After incubating the cells overnight with the interferon to be tested, the medium containing the interferon is removed and the cells washed twice before being infected (one hour adsorption) with a virus-to-cell multiplicity of 5–10 infectious units per cell. The cells are then washed twice more and incubated to permit a single-cycle of viral growth (7–8 hours or more). The cell cultures are then frozen and thawed, serial 10-fold dilutions of the resulting suspension are each titrated in six wells in a microtitre plate containing L cells, and the end-point of infectivity is determined by the cytopathic effect. The titres of virus yield from each of the tubes are then determined by a statistical method (either Reed-Muench or Spearman-Kärber). The interferon titre is taken as the reciprocal of the dilution as determined from dose-response

curves to produce a 0.5 $\log_{10}$ (70%) reduction in virus yield. The assay should be repeated on at least 4 occasions and tested in parallel with the method of titration of interferon activity usually employed in the laboratory. The results obtained for the reference materials by all the methods should be reported to the agency responsible for the assignment of unitage of the standard.

Appendix 3

ACCELERATED DEGRADATION TESTS

Two types of heating test, designated as multiple isothermal stability (MIS) methods, are recommended for determining the stability of freeze-dried interferon preparations. These are the predictive MIS test, and the confirmatory MIS test (*7*). The predictive MIS test, used primarily to predict the residual activity of a given interferon lot at any given temperature of storage, consists in placing about 10 ampoules at each of 3 temperatures (usually 52 °C, 60 °C, and 68 °C, but depending upon the anticipated stability of material from preliminary tests the range can be extended up to 76 °C or down to 44 °C). Duplicate samples are taken over a period of 10 days to 12 months, and thereafter held at −70 °C together with control samples, also kept continuously at −70 °C. The samples are then rehydrated, titrated for activity simultaneously, and the results plotted (log interferon titre against time in days). Arrhenius plots are then derived and a curve is constructed that shows future possible activity loss at any temperature. The confirmatory MIS test consists in determining over a period of months to years whether losses occur in samples kept at 56 °C, 37 °C, 22 °C, 4 °C, −20 °C, and −70 °C (as control). Results of tests on the NIH standard preparations stored during a period of several years indicate that the Arrhenius plots provide a conservative prediction of the long-term stability when samples of the preparations are stored at lower temperatures (*7*).

A third heating test, known as the linear mono-isothermal stability (LNS) test, is useful for the rapid evaluation of the alteration in the composition of the product, such as stabilizing additives, or in the conditions of the freeze-drying process (*7*). In the LNS test, temperatures are raised continuously at a rate of 1.5 °C/hour over the range of 50–90 °C, and samples are taken periodically for the assay of residual activity (*7*). Stable freeze-dried preparations usually maintain their activity up to 85–90 °C, whereas labile preparations may begin to lose activity between 50 and 70 °C.

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