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INFORMAL CONSULTATION ON THE STANDARDIZATION OF INTERFERONS

Geneva, 7-9 November 1983

CONTENTS

INTRODUCTION

A WHO Informal Consultation[1] on the Standardization of Interferons took place in Geneva from 7 to 9 November 1983. The meeting was opened by Dr F. T. Perkins, Chief, Biologicals, who noted the further progress that had been made particularly in the testing of the new interferons. He wished to take this opportunity to thank all the scientists who have given so much of their time working towards the establishment of appropriate international standards by the measurement of activity of these preparations.

I. RESULTS OF THE INTERNATIONAL COLLABORATIVE ASSAY STUDIES - 1983

Eight investigators in five countries measured the activity of one human interferon gamma (HuIFN-γ) and also compared the activity of three sorts of human interferon alpha (HuIFN-α) preparations with the International Reference Preparation for HuIFN-α leucocyte. It was noted that this study represented the first international collaborative effort to measure interferon standards utilizing a single bioassay with cells and virus obtained from a single source.

All four of the preparations had been shown to be suitable as international reference materials and a recommendation was made that three of them be established as international standards. It was recommended that the fourth, a preparation of human leucocyte HuIFN-α should be recognized as an international working reference preparation. Since the three proposed international standards have been hitherto unavailable it was important that they be established without delay in order that laboratories throughout the world could compare their results. The report of the findings of the participants is attached (Annex II). It was anticipated that more studies investigating additional proposed standards would be carried out in the future.

II. REFERENCE BIOASSAY

The Group agreed that a reference bioassay suitable for the three types of human interferon was desirable for two main reasons. First, such an assay would be most useful for the standardization of interferon preparations containing mixtures of various subtypes, i.e. mainly for preparations derived from fresh white blood cells or lymphoblastoid cells. Second, the availability of such an assay that is reliable, accurate and relatively easy to perform will be of value especially for laboratories that do not specialize in interferon research. However, it is important to realize that no single bioassay may be suitable for all kinds of interferon studies. Furthermore, not all problems inherent in the evaluation of multicomponent interferon preparations would be solved by the use of a standard assay procedure.

Attempts to design a reference bioassay applicable for all situations have already been made in the past. A single cycle encephalomyocarditis (EMC) virus yield reduction assay in A549 cells was recently used for the calibration of several human interferon reference preparations (see Annex II Table 5, "Results of the international collaborative assay studies"). Although satisfactory results were obtained, most investigators who participated in the international collaborative study felt that this assay was too cumbersome to serve as a permanent reference assay.

The Group felt that another reference bioassay should be selected and suggested that the US National Institutes of Health consider convening a meeting of experts devoted to a more thorough analysis of this subject.

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[1] A list of participants is shown in Annex I.

III. IMMUNOASSAYS

Interferons can be quantified by immunoassay in a variety of ways as outlined in the previous report. These assays require either pure interferon, highly specific polyclonal antibody or monoclonal antibody to interferon. One of these reagents is usually labelled, either with a radioisotope, such as ^{125}I, or conjugated to an enzyme, such as horseradish peroxidase.

Although calibration of antigen content in relation to units of biological activity remains a problem,[1] available evidence indicates that a high degree of correlation can be achieved. Thus, two of the available immunoassays have proven very useful in quantifying interferon in serum of patients treated with HuIFN-α_2 and indications are that these assays may replace bioassays for this particular application.[2] It is important to note that these two immunoassays do not detect all HuIFN-α subtypes and are therefore not suitable for quantifying multicomponent HuIFN-α preparations.

In addition to these current methods, new immunoassays designed to detect HuIFN-γ are being developed. In a sandwich-type assay using two different monoclonal antibodies to HuIFN-γ, as little as 0.3 international units/ml can be detected.

Recent development of immunoassays should allow standardization by weight of pure interferons (e.g. individual HuIFN-α subtypes). This will require the availability of International Standards (ISs) with interferon contents defined by weight; these ISs could serve to calibrate other immunoassays. However, an assay for antiviral activity will still be required for preparations considered for clinical use.

IV. MEASUREMENT OF ANTIBODIES TO INTERFERON IN SERA OF PATIENTS

When assaying for the presence of antibodies to interferon in serum samples from patients, it is desirable to use the most sensitive procedure feasible. One of the procedures described in "Standardization of Interferons",[2] for neutralization assays is the use of constant interferon and varying dilutions of serum should be followed.

In order to allow for the inter-laboratory comparisons of assay sensitivities and antibody content of serum specimens, the following parameters of the procedure used should be reported: final concentration of interferon and serum in the reaction mixture, final volume of reaction mixture, lowest final dilution of serum tested. It should be noted that when a mixture of interferon subtypes is used in a neutralization assay, complete neutralization will only occur if the patient serum contains neutralizing antibodies against all subtypes present.

When highly purified radiolabelled interferon is available, an additional procedure that detects non-neutralizing as well as neutralizing antibodies may be used. Aliquots of serum are incubated with a defined quantity of labelled interferon, and putative interferon-antibody complexes are precipitated by the use of a second antibody (e.g. goat anti-human IgG). The second antibody may be immobilized on an inert substance, such as agarose, if appropriate. If alternative tests are used such as an immunoradiometric assay of interferon not neutralized by serum, they must be properly validated.

V. ASSAYS OF ANTIPROLIFERATIVE AND OTHER CELLULAR EFFECTS OF INTERFERONS

In addition to their antiviral effect, interferons have various other biological actions. Each of these actions can be assayed by one or more procedures. Comparisons of experimental results between laboratories are often difficult because different methods are used and some form of standardization would seem advisable.

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[1] WHO Technical Report Series No. 687, 1983, p. 52.

[2] WHO Technical Report Series No. 687, 1983.

1. The cell growth inhibitory effect of interferons

Interferons can inhibit the proliferation of cells. Whilst it is assumed that this effect plays some role in the anticancer potential of interferons, its importance relative to other biological actions is not certain. Therefore, assays of the antiproliferative effect of interferon preparations cannot at present be said to have predictive value for their clinical antitumour potential. With this reservation, it would be desirable that laboratories include a reference antiproliferative bioassay in their panel of assays. Therefore studies to develop an antiproliferative assay applicable to all available types and subtypes of human interferon are encouraged. Currently, the most widely used assay utilizes the lymphoblastoid cell line, Daudi, which is among the most sensitive to antiproliferative effects of HuIFN- α and β . However this cell line is poorly sensitive to the antiproliferative effect of HuIFN- γ .

2. Activation of mononuclear cells

Interferons activate the cytocidal effect of lymphocytes in various assay systems (natural killer activity; antibody-dependent-cellular cytotoxicity; cytotoxicity of macrophages or adherent mononuclear cells). The predictive value of these assays for the clinical antitumour potential of a given interferon preparation is uncertain. Since results obtained in these assays are highly variable depending on the origin and physiological state of the mononuclear cells as well as the target cells it is not possible to recommend a reference bioassay at present.

3. Other assay systems

In the future, assays relying on various other effects of interferons may become of increasing interest to laboratory workers or clinicians, e.g. induction of cellular enzymes (2', 5'-oligoadenylate synthetase, protein kinase), enhanced expression of Fc receptors, and of various cell surface antigens, or modulation of cellular differentiation. Some form of standardization of assays for these effects may be useful.

VI. NEW INTERFERON STANDARDS

1. Human standards

In addition to the three HuIFN- α and one HuIFN- γ proposed standards, proposed standards for HuIFN- α_1 and HuIFN- β , have been developed and await evaluation.

Further research standards for a number of human interferons are likely to be required. Certain points should be noted. For example, there are the following preparations of HuIFN- β : glycosylated molecules derived from human cells; non-glycosylated molecules derived from E. coli; and modified molecules with serine instead of cysteine in position 17, also derived from E. coli. There are preliminary indications that different relative potencies may be obtained for an E. coli-derived HuIFN-β and the International Reference Preparation of HuIFN-β depending on the assay conditions. It may be necessary therefore to have homologous standards for individual recombinant interferons. In the meantime units can be assigned to preparations of such interferons by comparison with the IRPs. However, such an assignment of units must not be taken to imply biological equivalence in all systems.

2. Animal standards

Since the 1960s, chicken, hamster, mouse, rabbit and rat interferons have been used in antiviral or antitumour studies in the homologous animal host. It is recognized that further animal studies are required to show how best to use interferons clinically. Appropriate standards will enable data from different laboratories to be compared in a meaningful way. International standards for some animal interferons are already available, and for others, purified mouse α , β , and γ interferons are in the course of preparation. Standards for other animal interferons are likely to be needed but the impetus for their provision must come from

the workers directly involved. The group suggested that those interested in a particular standard material should contact the Antiviral Substances Programme, National Institute of Allergy and Infectious Diseases[1] (NIAID), or the Division of Viral Products, National Institute for Biological Standards and Control[2] (NIBSC). If enough workers express an interest in, for example, a rat interferon standard, and if the scientists can make material available, then it may prove useful as a working standard. It will be necessary, however, for the donating laboratory to describe the method of assay and to provide data confirming the stability of the material. If the preparation when freeze-dried is shown to be suitable, it will then be provided on request as a working standard, by the above-mentioned institutions.

VII. SUMMARY AND CONCLUSIONS

With the continuing increase in clinical and experimental studies on interferons, greater emphasis must be placed on the precise measurements of the activity of these substances, especially in terms of appropriate international standards. To this end, additional proposed standard preparations have been prepared, characterized, and tested: three purified HuIFN-α preparations and one HuIFN-γ preparation were titrated for potency and were compared with the HuIFN-α International Reference Preparation of Interferon, Human Leucocyte 69/19 by eight laboratories in five countries. A reference bioassay as well as the routine assays used in the individual laboratory concerned were included. Since the data summarized herein established their suitability, it is recommended that the following be accepted as WHO international standards for interferons.

(1) Preparation Ga23-901-532[3] as the international standard for interferon, human, lymphoblastoid (Namalwa) (HuIFN-α(Ly)) having an activity of 25 000 (4.40 $\log_{10}$) IU per ampoule;

(2) Preparation Gxa01-901-535[3] as the international standard for interferon, human, recombinant HuIFN α_2 (αA) having an activity of 9000 (3.95 $\log_{10}$) IU per ampoule; and

(3) Preparation Gg23-901-530[3] as the international standard for interferon, human, HuIFN-γ, having an activity of 4000 (3.6 $\log_{10}$) IU per ampoule.

In view of the urgent need of these standards, the Group requested the WHO Secretariat to take the necessary steps to establish these reference materials as soon as possible.

It is further recommended that the WHO adopt as an international working standard the preparation Ga23-902-530[3] for interferon, human, leucocyte (HuIFN-α(Le)) having an activity of 12 000 (4.08 $\log_{10}$) IU per ampoule.

Other purified interferon preparations suitable for possible future use as international standards should be available soon for international collaborative testing. These include HuIFN-α_1, a replacement HuIFN-β and mouse MuIFN-α, β and γ preparations.

Additional standard preparations for both human and animal interferons are likely to be needed.

The recommendations of the previous report[4] emphasizing the need for details of the assay methods and results either for publication or for submission to regulatory agencies are strongly reaffirmed.

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[1] National Institutes of Health, Bethesda, USA

[2] Hampstead, London, England.

[3] The description of the preparation and the results of testing these materials are attached as Annex II, Part 2.

[4] WHO Technical Report Series No. 687, 1983, Annex 1, pp. 35-60.

It was agreed that a reference antiviral bioassay suitable for measuring all three types of human interferon is desirable. A virus infectivity yield-reduction method, the previously proposed reference bioassay employed in the recent international collaborative assay, gave satisfactory results but was found to be cumbersome and uneconomical. Another reference bioassay should be selected as soon as a meeting of experts can be arranged to analyse suitable alternative methods.

Progress continues to be made in the development of radiometric and enzyme-linked interferon immunoassays, as well as of monoclonal antibodies and other suitable reagents. Further work needs to be done on these rapid and reproducible assays, particularly to establish whether a suitable correlation exists between biological potency and the specific antigenic content of interferon preparations and of clinical samples.

The necessity of utilizing the most sensitive procedures for the detection of antibodies to interferon in the sera of patients was stressed. Although interferons are known to inhibit cell proliferation, to induce certain cellular enzymes, to alter expression of cellular surface antigens, and to activate mononuclear cells, the correlation of such effects with the course of a disease remains to be established. There is a need to develop tests to measure the nonantiviral, biological activities of interferons that can have predictive or clinical value.

ANNEX I

LIST OF PARTICIPANTS

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ANNEX II

PART 1. RESULTS OF THE INTERNATIONAL COLLABORATIVE ASSAY STUDIES - 1983

A. Human interferon reference preparations

An international collaborative study to test the potency of one proposed standard for HuIFN-γ and three proposed standards for HuIFN-α was completed in 1983. Each participating laboratory was asked to utilize its routine bioassay as well as a proposed reference bioassay to titrate these preparations and the IRP of human leucocyte interferon (69/19), on five different occasions. The reference bioassay was an infectivity yield-reduction method employing encephalomyocarditis virus (EMCV) in the human lung carcinoma cell line A549; infectivity yields were measured in mouse L cells by a cytopathic effect endpoint. The virus and cell lines were sent by Sidney Grossberg to each participating laboratory along with details of the appropriate procedures. The raw data obtained in each laboratory were analysed by Pat Jameson and John Kalbfleisch (the biostatistician involved in all previous collaborative testing of freeze-dried interferon standards prepared at the Medical College of Wisconsin for the National Institutes of Health). This study represents the first international collaborative effort to measure interferon standards utilizing a single bioassay with cells and virus obtained from a single source; the great efforts of all the laboratories involved is recognized as a service to the entire interferon research community.

The following four human samples containing one or more alpha interferon (HuIFN-α) were supplied and distributed by the NIH:

Ga23-902-530, a preparation produced in leucocytes and partially purified by Kari Cantell, Helsinki, and subsequently diluted, freeze-dried and characterized at the Medical College of Wisconsin;

Ga23-901-532, a preparation produced in Namalwa lymphoblastoid cells, highly purified and characterized by Norman Finter, Wellcome Laboratories, Beckenham and freeze-dried at the Medical College of Wisconsin;

The International Reference Preparation of Interferon, Human, Leucocyte 69/19 (IRP 69/19);

GxaO1-901-535 HuIFN-α2 (αA) produced in E. coli by a recombinant DNA technique and highly purified and freeze-dried at Hoffmann-La Roche, Nutley; and

The HuIFN-γ preparation, Gg23-901-530, was produced, in peripheral blood lymphocytes partially purified, freeze-dried, and characterized at the Medical College of Wisconsin.

More detailed information on each of the four proposed standards is given in the Reference Reagent Notes attached to this report.

HuIFN-α standards. Data were analysed from those eight laboratories in five countries that submitted results of at least two titrations performed with the reference bioassay method. Dose-response curves were constructed by linear regression analysis of the data from each reference bioassay titration performed by each laboratory. The endpoint was taken to be the 0.5 $\log_{10}$ reduction in virus yield. The results are presented in the table included with each reference reagent note. The slopes of the response curves for a given sample were much less variable within a laboratory than among laboratories, and no statistically significant differences in slopes were obtained among the different HuIFN-α preparations.

Tables 1, 2 and 3 summarize the observed, uncorrected titres obtained by the reference bioassay. From these, a biological activity expressed in International Units per ampoule was assigned to the three NIH reference preparations by comparison with the IRP 619 (Table 5). It is recognized that this first assignment of units to the lymphoblastoid and α_2 recombinant

interferon preparations is arbitrary, since they are not homologous with the IRP 69/19. However, the assigned values are likely to be in the range obtained by most workers in their bioassays.

HuIFN- γ preparation. Table 4 summarizes the test results obtained with the HuIFN- γ proposed standard Gg23-901-530. In this first assignment of units to a HuIFN- γ proposed standard, the potency of 4000 (3.6 $\log_{10}$) Reference Units per ampoule has been chosen on the basis of the results obtained with the reference bioassay and it is proposed that this unitage be adopted as the potency in international units.

B. Reference bioassay

The results obtained on the human interferon reference preparations with the previously proposed reference bioassay and with the assays routinely used in each of the participating laboratories are presented in the tables included in each Reference Reagent Note.

The major reasons for selecting the EMC virus yield-reduction assay derived largely from the recommendations of the Woodstock Conference on Standards[1] which were endorsed with minor amendments at the WHO meeting in Geneva in June 1982.[2] EMC virus was selected because it is readily propagated, it is stable, it has a wide host range, it is acceptable for use worldwide, and it is relatively non-pathogenic for humans. The method, reduction in yield of infectious virus, was selected because it gives unequivocal endpoints (as do other yield-reduction assays), was considered to be relatively sensitive, and would satisfy the definition that interferon action should be manifest as a reduction in virus infectivity. The A549 cell line was selected in preference to other cell lines or strains because it is sensitive to α, β, and γ interferons, it has not changed in its sensitivity to interferons during a large number of passages over a period of several years, and it is easier to handle and grows faster than diploid cell strains. Although originally used with mouse interferon, the proposed reference bioassay for human interferons using an EMC/A549 yield-reduction method was demonstrated by collaborative testing to be feasible and to have relatively high sensitivity in that it measured a greater number of units than that assigned to the IRP 69/19. However, this infectivity yield-reduction assay was shown to be relatively slow and uneconomical.

Simultaneously with the reference assay the participating laboratories also employed their routine assays testing the interferons. Their data suggest some candidate assays of sufficient sensitivity and reproducibility for future collaborative testing of standards. Such assays include the dye-uptake method using FL cells, Sindbis virus with an objectively determined endpoint (Lab No. 6 method), and the haemagglutinin yield-reduction assay (Lab No. 5 method) using A549 cells and EMC virus. In the selection of components for an assay, consideration should be given to the fact that different sublines of continuous cell lines can vary considerably in their sensitivity to interferon. The results from laboratories 1 and 6 obtained by the same routine assay method illustrate this point.

C. Establishment of the HuIFN- α and γ preparations as International Interferon Standards. The group agreed that it was urgent for the Secretariat of WHO to take the necessary steps to obtain the approval for the establishment of the following proposed standards as WHO international standards for interferons:

(1) Preparation Ga23-901-532 as the international standard for interferon, human, lymphoblastoid (Namalwa) (HuIFN- α having an activity of 25 000 (4.40 $\log_{10}$) IU per ampoule;

(2) Preparation Gxa01-901-535 as the international standard for interferon, human, recombinant HuIFN- α_2 (α A) having an activity of 9000 (3.95 $\log_{10}$) IU per ampoule;

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[1] J. Biol. Stand. 7, 383, 1979.

[2] WHO Technical Report Series, No. 687, 1983.

Annex II

(3) Preparation Gg23-901-530 as the international standard for interferon, human, HuIFN-γ, having an activity of 4000 (3.6 $\log_{10}$) IU per ampoule.

The Group further requested that WHO adopt the preparation Ga23-902-530 as an international working reference preparation for Interferon, Human, Leucocyte, (HuIFN-α) having an activity of 12 000 (4.8 $\log_{10}$) IU per ampoule.

TABLE 1. SUMMARY OF RESULTS OF THE INTERNATIONAL COLLABORATIVE STUDY OF THE HUMAN LYMPHOBLASTOID (NAMALWA) INTERFERON REFERENCE PREPARATION NIH CATALOGUE NUMBER Ga23-901-532

Assay method	Results obtained per laboratory								Summary per total	
	1	2	3	4	5	6	7	8	labs[b]	tests[c]
MCV yield-reduction[a]										
Number of titres	10	5	2	6	8	5	5	3	8	44
GMT (log)	5.16	5.08	4.49	4.73	4.27	4.23	4.16	4.60	4.59[d]	4.64[d]
SD (log)	0.44	0.15	0.33	0.44	0.16	0.44	0.31	0.40	0.38	0.51
Other assay methods										
Number of titrations	24	5	NT	NT	7	6	5	5	6	
GMT (log)	3.69	4.70	-	-	4.57	4.95	3.80	3.99	4.28	
SD (log)	0.12	0.12	-	-	0.33	0.16	NA	0.11	0.52	

[a] The reference bioassy method measured the reduction in yield of encephalomyocarditis virus (EMVC) in the human A549 cell line; yield of infectious EMCV was measured by titrations in L cells using a cytopathic-effect endpoint. A standard protocol modified from that originally recommended (4,9) detailing the steps in the microtitre method was provided all participants. EMCV and both cell lines were also provided by Dr Sidney Grossberg's laboratory at the Medical College of Wisconsin.

[b] In this column the geometric mean titre (GMT) and standard deviation (S.D.) are based on the GMT values obtained from titres calculated from the raw data provided by each laboratory. An S.D. value calculated so as to provide a combined estimate of random variation between titrations within the individual laboratory is 0.38 log, corresponding to about 2.4-fold variation.

[c] In this column the GMT and S.D. are based on the total number of titres obtained without regard to laboratory.

[d] The assigned potency of Ga23-901-532, in relation to the International Reference Preparation of Human Leucocyte Interferon 69/19, is 25 000 or 4.4 $\log_{10}$ International Units/ampoule (see Table 5).

TABLE 2. SUMMARY OF RESULTS OF THE INTERNATIONAL COLLABORATIVE STUDY OF THE HUMAN RECOMBINANT INTERFERON ALPHA$_2$ (ALPHA A) REFERENCE PREPARATION NIH CATALOGUE NUMBER Gxa01-901-535

Assay method	Results obtained per laboratory								Summary per total	
	1	2	3	4	5	6	7	8	labs[b]	tests[c]
EMCV yield-reduction[a]										
Number of titres	10	5	2	5	8	5	5	3	8	43
GMT (log)	4.60	4.44	4.22	4.13	4.00	3.90	3.59	4.19	4.13[d]	4.17[d]
S.D. (log)	0.44	0.21	0.48	0.31	0.49	0.97	0.34	0.53	0.31	0.57
Other assay methods										
Number of titrations	24	5	NT	NT	7	6	5	7	6	
GMT (log)	2.92	4.11			4.04	4.46	3.23	3.52	3.71	
S.D. (log)	0.18	0.11			0.30	0.25	NA	0.19	0.59	

[a] The reference bioassay method measured the reduction in yield of encephalomyocarditis virus (EMCV) in the human A549 cell line; yield of infectious EMCV was measured by titrations in L cells using a cytopathic-effect endpoint. A standard protocol modified from that originally recommended(6,7) detailing the steps in the microtitre method was provided all participants. EMCV and both cell lines were also provided by Dr Grossberg's laboratory at the Medical College of Wisconsin.

[b] In this column the geometric mean titre (GMT) and standard deviation (S.D.) are based on the GMT values obtained from titres calculated from the raw data provided by each laboratory. An S.D. value calculated so as to provide a combined estimate of random variation between titrations within the individual laboratory is 0.31 log corresponding to about 2-fold variation.

[c] In this column the GMT and S.D. are based on the total number of titres obtained without regard to laboratory.

[d] The assigned potency of Gxa01-901-535, in relation to the International Reference Preparation of Human Leucocyte Interferon 69/19, is 9000 or 3.95 $\log_{10}$ International Units/ampoule (see text).

TABLE 3. SUMMARY OF RESULTS OF THE INTERNATIONAL COLLABORATIVE STUDY OF THE HUMAN LEUCOCYTE INTERFERON REFERENCE PREPARATION NIH CATALOGUE NUMBER Ga23-902-530

Assay method	Results obtained per laboratory								Summary per total	
	1	2	3	4	5	6	7	8	labs[b]	tests[c]
EMCV yield-reduction[a]										
Number of titres	10	5	2	6	8	5	5	3	8	44
GMT (log)	5.11	4.52	4.54	4.35	4.02	4.27	3.75	3.68	4.28[d]	4.37[d]
S.D. (log)	0.36	0.15	0.18	0.15	0.13	0.75	0.46	0.82	0.47	0.61
Other assay methods										
Number of titrations	24	5	NT	NT	7	6	5	7	6	
GMT (log)	3.27	4.26	-	-	4.22	4.71	3.26	3.84	3.93	
S.D. (log)	0.13	0.09	-	-	0.29	0.18	NA	0.12	0.58	

[a] The reference bioassay method measured the reduction in yield of encephalomyocarditis virus (EMCV) in the human A549 cell line; yield of infectious EMCV was measured by titrations in L cells using a cytopathic-effect endpoint. A standard protocol modified from that originally recommended (4,9) detailing the steps in the microtitre method was provided all participants. EMCV and both cell lines were also provided by Dr Grossberg's laboratory at the Medical College of Wisconsin.

[b] In this column the geometric mean titre (GMT) and standard deviation (S.D.) are based on the GMT values obtained from titres calculated from the raw data provided by each laboratory. An S.D. value calculated so as to provide a combined estimate of random variation betweeen titrations within the individual laboratory is 0.47 log corresponding to about 3-fold variation.

[c] In this column the GMT and S.D. are based on the total number of titres obtained without regard to laboratory.

[d] The assigned potency of Ga23-902-530, in relation to the International Reference Preparation of Human Leucocyte Interferon 69/19, is 12 000 or 4.08 $\log_{10}$ International Units/ampoule (see text).

TABLE 4. SUMMARY OF RESULTS OF THE INTERNATIONAL COLLABORATIVE STUDY TO CALIBRATE THE HuIFN-γ REFERENCE PREPARATION NIH CATALOGUE NUMBER Gg23-901-530

Assay method	Results obtained per laboratory								Summary per total	
	1	2	3	4	5	6	7	8	labs[b]	tests[c]
EMCV yield-reduction[a]										
Number of titres	10	2	7	6	6	5	5	5	8	46
GMT (log)	3.91	3.90	3.67	3.63	3.59	3.43	3.37	3.36	3.61[d]	3.62[d]
S.D. (log)	0.31	0.21	0.11	0.37	0.16	0.16	0.15	0.32	0.22	0.31
Other assay methods										
Number of titres	12	-	7	-	6	5	-	6	5	30
GMT (log)	2.60	-	3.79	-	4.20	3.54	-	3.20	3.47	3.33
S.D. (log)	0.15	-	0.33	-	0.13	0.19	-	0.45	0.61	0.65

[a] The reference bioassay method measured the reduction in yield of encephalomyocarditis virus (EMCV) in the human A549 cell line; yield of infectious EMCV was measured by titrations in L cells using a cytopathic-effect endpoint. A standard protocol modified from that originally recommended (3,7) detailing the steps in the microtitre method was provided all participants. EMCV and both cell lines were also provided by Dr Grossberg's laboratory at the Medical College of Wisconsin.

[b] In this column the geometric mean titre (GMT) and standard deviation (S.D.) are based on the GMT values obtained from titres provided by each laboratory. An S.D. value calculated so as to provide a combined estimate of random variation between titrations within the individual laboratory is 0.22 log, corresponding to about 1.7-fold variation.

[c] In this column the GMT and S.D. are based on the total number of titres obtained without regard to laboratory.

[d] The assigned potency of Gg23-901-530 is 4000 or 3.6 $\log_{10}$ Reference Units (RU)/ampoule.

Annex II

TABLE 5. SUMMARY OF RESULTS OF 1983 INTERNATIONAL COLLABORATIVE STUDY OF HUMAN α AND γ INTERFERON STANDARD PREPARATIONS AND ASSIGNMENT OF POTENCY TO EACH (n=8 LABORATORIES)

HuIFN sample	IFN type	Interferon Geometric Mean Titre			
		Observed (LU[a]/ml/ampoule)		Corrected[b] or assigned (IU[a]/ml/ampoule)	
		Log_{10} ± S.D.	Antilog	Log_{10}	Antilog
MRC 69/19 (Le)	α	3.886 ± 0.288	7 691	3.70	5 000
Ga23-902-530 (Le)	α	4.279 ± 0.468	19 011	4.08[b]	12 000[b]
Ga23-901-532 (Ly)	α	4.590 ± 0.381	38 905	4.40[b]	25 000[b]
Gxa01-901-535 (α2 (αA))	α	4.134 ± 0.313	13 614	3.95[b]	9 000[b]
Gg23-901-530	γ	3.61 ± 0.25	4 044	3.60	4 000

[a] IU = International Units; LU = Laboratory Units as determined by the proposed reference bioassay (EMCV infectivity yield-reduction in human A549 cells).

[b] Corrected against the assigned titre (3.70 log_{10} or 5000 International Units) of the WHO International Reference Human Leucocyte Interferon Preparation MRC 69/19.

Annex II

PART 2. DESCRIPTION OF PREPARATION OF PROPOSED INTERNATIONAL STANDARDS FOR INTERFERONS

1. Freeze-dried Human Lymphoblastoid (Namalwa) Interferon Alpha Reference (Ga23-901-532)

Preparation: Human lymphoblastoid interferon alpha [HuIFN-α(Ly)] was prepared by The Wellcome Research Laboratories, The Wellcome Foundation Ltd., Beckenham, England. Cultures of the human lymphoblastoid Namalwa cell line were infected with Sendai virus (1). After one day of incubation, the supernatant fluids were collected following centrifugation, and the IFN was purified by a series of differential precipitation (2) and chromatographic steps to obtain a purity of 87.4%, with a specific activity of 10^8 IU/mg, in a preparation composed of at least eight different alpha interferons as distinguished by physico-chemical characterization (1). After shipment to the Medical College of Wisconsin, the material was stored at -70°C. Subsequently, the sterile IFN preparation, containing 44.56 µg of HuIFN-α(Ly), was aseptically diluted into ice-cold, sterile buffer solution composed of 0.1 M sodium phosphate buffer, pH 7, supplemented with 5 mg/ml human serum albumin (Travenol "Buminate"). The vessel was packed in wet ice to keep the solution chilled during the process of filling the ampoules; 1.00 ml portions, containing 13.5 ng of HuIFN-α(Ly), were dispensed into borosilicate glass ampoules using a high-precision Hamilton dispenser. The reproducibility of the fill, as measured by the weight of liquid dispensed into 25 preweighed vials (distributed throughout the fill), was 0.61 (coefficient of variation). Ampoules were filled in groups of 19, and held on ice until five groups were filled and were then placed in the refrigerated chamber of the freeze-dryer. After all ampoules were filled they were frozen at -30°C, and the material was dried to a residual moisture of about 3%. The ampoules were then backfilled with argon and heat-sealed at atmospheric pressure. The last ampoule filled in each group of 19 was marked for testing of sterility and antiviral activity after freeze-drying. Ampoules are stored at -70°C but can be shipped at ambient temperatures.

Recommendations for reconstitution: 1.0 ml of sterile distilled water should be added to the lyophilized powder, with care being taken to avoid loss of any material in the neck or stem of the ampoules. Small portions of the reconstituted IFN may be stored at -70°C for dilution at another time. However, a suitable amount of an appropriate dilution, based on the known sensitivity of the assay being used, should be made in the freeze-drying buffer (see above) supplemented with HSA, 5 mg/ml, or in serum-containing culture medium used in the biological assay. Aliquots of the diluted IFN should preferably be stored at -70°C in volumes each sufficient for a single titration. It may be possible to store enough material in a single container at -70°C for use in as many as 3 titrations: more extensive repeated thawing and freezing can result in loss of activity. All liquid samples should be stored at -70°C or lower.

Stability: The freeze-dried reference preparation was tested twice by the linear non-isothermal accelerated degradation test (3) in which material is progressively heated from 50°C to 90°C over a 28-hour period. In replicate titrations of these two tests there was a range of inactivation from 0 to 90% between 50 and 80°C; but the observation that some of the degradation curves showed no loss of activity gave evidence that in spite of variability of results, the IFN was sufficiently stable. From the results of the predictive multiple isothermal accelerated degradation test (3), involving storage of ampoules at 52°, 60°, 68°, and 76°C, with samples being removed at appropriate intervals over the course of 11 months, the product is estimated to be stable at -70°C for several decades. The length of time required to lose 1 log of activity at higher temperatures was estimated from these data to be 0.34 years at 56°C, 1.58 years at 37°C, 6.79 years at 20°C, 11.16 years at 4°C, and 24.42 years at -20°C, and 233 years at -70°C.

Test results: No mycoplasma, bacteria or fungi were detected in 43 samples tested from the 162 different groups of ampoules composing the reference lot. The IFN used for freeze-drying was diluted to contain 1 mg HSAml and characterized as follows: it was non-sedimentable at 100 000 x g, more than 99% inactivated by trypsin in 1 hr, inactivated about 50% during heating at 56°C for up to 3 hr, and not inactivated during 48 hr of pH 2 dialysis at 4°C.

The product was not neutralized by antisera to HuIFN-γ (either provided by Irwin Braude, Meloy Labs, Springfield, VA, or prepared at MCW against purified HuIFN-γ), or by anti-HuIFN-β serum (NIH G028-501-568); but it was neutralized completely by anti-HuIFN-α serum (NIH G026-502-568). The IFN was composed of one molecular size of 15 500 daltons as estimated by sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis in phosphate buffer by the method of Weber and Osborne. Analysis of HuIFN-α by isoelectric focusing revealed one major peak of activity at pH 5.8, with a shoulder at pH 6.2.

Potency was determined from the data contributed by eight international laboratories which had performed two or more titrations of the preparation using a microtitre modification of the proposed reference bioassay technique (Table 1) (4,5). The reference bioassay involves the reduction in yield of infectious EMCV in the A549 line of human lung carcinoma cells; EMCV yields were measured in L cells. The geometric mean titre (GMT) calculated as the mean of the GMT values reported from each laboratory (total number of titrations = 44) was 4.59 log units/ml/ampoule (with a standard deviation, S.D., 0.38 log corresponding to about 2.4-fold variation). Titration of the HuIFN-α(Ly) by routinely used bioassays of different types with various cell-virus combinations, mostly dye-uptake measurements of cytolysis, gave GMT values ranging from 3.69 to 4.95 log units/ml, with mean of 4.20 log units/ml (S.D. 0.53). Additional information is provided in Table 1. There was considerable activity on cells of heterologous species, characteristic of this type of IFN, with the following observed unadjusted titres obtained by the EMCV haemagglutination yield-reduction method (6): 1.2×10^5 Laboratory Units (LU)/ml in human A549 cells, 1.9×10^5 LU/ml in bovine EBTr cells, 7×10^4 LU/ml in feline FEA cells, and 320 LU/ml in murine L cells.

Titre assignment: The assigned titre of the HuIFN-α(Ly) (Namalwa) NIH Reference Reagent Ga23-901-530 is derived from the test results of an international collaborative study using the reference bioassay by proportional relationship to the International Reference Preparation, Human Leucocyte Interferon, British MRC 69/19 having an assigned potency of 5000 IU/ml (see Table 1). As a basis for expressing unitage of HuIFN-α(Ly) in terms of IU established for MRC 69/19 HuIFN-α(Le), slopes of dose-response curves for HuIFN-α(Ly) and MRC 69/19 were analysed; there was no significant difference in slopes for the two IFNs whether calculated from the 44 individual titrations (1.165 [S.D. 0.18] and 1.137 [S.D. 0.18], respectively) or from the average slopes observed in each of the eight laboratories (1.142 [S.D. 0.24] and 1.074 [S.D. 0.26], respectively). Therefore, the assigned potency of Ga23-901-532 is 25 000 International Units or 4.40 log IU/ampoule (see Table 5).

Use of Reference Interferon: The purpose of the HuIFN-α(Ly) (Namalwa) Reference Interferon Reagent is to provide a comparison of the sensitivities of bioassays that measure the antiviral activity of HuIFN-α(Ly) in different laboratories. This preparation should be used only for the calibration of laboratory preparations of HuIFN-α(Ly) which have dose response curves parallel to that of the Reference Reagent (4.5,7-9). It should be noted that if the number or proportion of different IFN-α subtypes in a given lymphoblastoid IFN preparation under test are known to differ significantly from that in this reference preparation, then the use of this reference preparation may not be appropriate. Each laboratory should measure the HuIFN-α(Ly) Reference Reagent simultaneously with an internal laboratory standard in five or more titrations done on separate occasions, and should report the observed logarithm of the geometric mean titre (GMT) or Laboratory Unit (LU) and its standard deviation along with the assigned titre (as the logarithm) of the Reference Reagent Interferon according to recommendations by the World Health Organization (4,5,7-9). The number of International Units (IU)/ml in the laboratory standard (lab std.) should be calculated by proportional relationship to the Reference Reagent (Ref. IFN) as follows:

$$(1)\quad \frac{\text{NIH Ref. IFN assigned IU}}{\text{GMT of NIH Ref. IFN observed LU}} \times \text{GMT lab std. observed LU} = \text{lab std. IU}$$

Similarly, the laboratory standard may be used to determine the titre of test samples in IU.

$$(2)\quad \frac{\text{lab std. IU [from (1)]}}{\text{GMT of lab std. observed LU}} \times \text{GMT test sample observed LU} = \text{test sample IU}$$

Annex II

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2. Freeze-dried Human Recombinant Interferon α 2 (α A) Gxa01-901-535

Preparation: Recombinant Human Alpha α 2 (Alpha α A) Interferon (HuIFN- α 2 (α A) was prepared at the Roche Research Center in Nutley, New Jersey. E. coli cells transformed with a plasmid derived from pLeIFA25 (1) were grown in nutrient medium under conditions that permitted the organism to produce HuIFN- α 2 (α A). The cells containing HuIFN- α 2 (α A) were killed by treatment at low pH (ca. 1.8), harvested, passed through a mechanical grinder and suspended in extraction buffer. The resultant cell debris and nucleic acids were flocculated and removed by centrifugation. The HuIFN- α 2 (α A) in the supernatant fluid was purified by a series of procedures involving affinity chromatography on immobilized anti-interferon monoclonal antibodies, cation exchange chromatography (2) and molecular exclusion chromatography.

The highly purified HuIFN- α 2 (α A) was diluted to the required concentration with a solution containing sodium chloride (9 mg/ml) and human serum albumin (5 mg/ml). The pH was adjusted to 6.9 with sodium hydroxide and the solution was filter sterilized. One ml aliquots were aseptically dispensed into sterile ampoules. The contents of the ampoules were lyophilized and the ampoules sealed under nitrogen. The reproducibility of the fill, as measured by determination of the protein content of 20 ampoules, was $\pm$ 8.8% (coefficient of variation).

Recommendations for reconstitution: 1.0 ml of sterile distilled water should be added to the lyophilized powder, care being taken to avoid loss of any material in the neck or stem of the ampoule. Portions of this reconstituted material can be stored at -70°C, or an appropriate (e.g., 1:10) dilution can be made, preferably in 0.1 M sodium phosphate buffer pH 7 containing

5 mg/ml human serum albumin (HSA); Hanks' salt solution with 5 mg/ml HSA or serum-containing culture medium may be substituted. For optimum, long-term preservation of stability, storage of samples of the liquid material should be at -70°C.

Stability: The freeze-dried reference preparation did not lose any activity in the linear non-isothermal accelerated degradation test (2) in which material is progressively heated from 50°C to 90°C over a 28-hour period. From the results of the predictive multiple isothermal accelerated degradation test (3), involving storage at 52°C, 60°C, 68°C, and 76°C for periods up to 1 year, the product is estimated to have unlimited stability at -20°C, and -70°C. The time predicted to lose 1 log of activity at temperatures above freezing was estimated from these data to be 2.46 years at 56°C, 16 years at 37°C, 94.5 years 20°C, and 679 years at 4°C.

Test results: Forty containers were tested for sterility according to the USP Direct Plant Method. The vials were reconstituted with sterile water and 20 vials were tested in fluid thioglycollate medium and 20 in trypticase soy broth. No evidence of microbial growth was observed during the incubation period.

The amino acid sequence of the HuIFN- α 2 (α A) used to prepare this standard differs from that predicted from the DNA sequence reported by Streuli et al., (4) in that the amino acid at position 23 is lysine instead of arginine. The purity was 99% as determined by photometric scanning of gels following non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular weight of the HuIFN- α 2 (α A) was estimated to be 18 500. It was non-sedimentable at 100 000 xg for 90 minutes, stable at pH 2, and inactivated by trypsin. The specific activity of the HuIFN- α 2 (α A) used was 2×10^8 International Units/mg protein as determined in a cytopathic effect-reduction assay (5) with WISH cells and vesicular stomatitis virus (VSV) as challenge.

Potency was determined from data contributed by eight international laboratories which had performed two or more titrations of the preparation using a microtitre modification of the proposed reference bioassay technique (Table 2) (6,7). The reference bioassay involves the reduction in yield of infectious EMCV in the A549 line of human lung carcinoma cells; EMCV yields were measured in L cells. The geometric mean titre (GMT) calculated as the mean of the GMT values reported from each laboratory (total number of titrations = 43) was 4.13 log units/ml/ampoule (with a standard deviation (S.D.) of 0.31 log corresponding to about 2.0-fold variation). Titration of the HuIFN- α 2 (α A) by routinely used bioassays of different types with various virus-cell combinations, mostly dye-uptake measurements of cytolysis, gave GMT values ranging from 2.92 to 4.46 log units/ml, with a mean of 3.71 log units/ml and S.D. of 0.59. Greater detail is provided in Table 2 and its footnotes. Antiviral activity expressed as a % of that observed in human WISH ammion cells was observed in following cell lines: monkey, Vero 9%; bovine, MDBK 150%; guinea-pig transformed (30%); and feline Felung 130%. Negligible activity (< 1% of that in human WISH cells) was observed on cell lines of the following species: mouse, rat, rabbit, hamster, and horse.

Titre assignment: From the test results of an international collaborative study using the reference bioassay, the assigned titre of the HuIFN- α 2 (α A) Reference Preparation Gxa01-901-535 is 9000 International Units/ampoule or 3.95 $\log_{10}$ International Units/ampoule (see Table 5).

Use of reference interferon: The purpose of the HuIFN- α 2 (α A) Reference Preparation is to provide a comparison of the sensitivities of bioassays that measure the antiviral activity of HuIFN- α 2 (α A) in different laboratories. Each laboratory should measure the HuIFN- α 2 (α A) Reference Preparation in comparison with its own HuIFN- α 2 (α A) internal laboratory standard preparation. WHO recommends that five or more titrations of the reference and the laboratory standard preparations should be done, and that the observed logarithm of the geometric mean titre and its standard deviation be reported in each publication along with the assigned titre (as the logarithm) of the Reference Interferon Standard (6-10). A large number of aliquots of the calibrated laboratory standard material should be kept frozen at -70°C and titrated every time an assay of HuIFN- α 2 (α A) sample