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Radioimmunoassay of Leukocyte (Alpha) Interferon and Its Application to Some Clinical Conditions

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A radioimmunoassay for human leukocyte interferon alpha (alpha IFN) has been developed, using tracer produced by recombinant DNA (IFLrA) and polyclonal rabbit antiserum against partially purified leukocyte IFN (PP alpha IFN). Sensitivity was 4 units/ml using sequential saturation. Only lymphoblastoid IFN showed complete cross reactivity. Serum alpha IFN concentrations were measured in normal subjects and in patients with acute viral infections, bone and joint diseases, and malignancies. Some cases in the first two groups of patients had significantly elevated serum levels compared with controls. The pharmacokinetics of alpha IFN were studied in treated cancerous patients. Radioimmunoassay and biological assay gave similar and closely correlated results. Radioimmunoassay is thus a useful method for the routine assay of alpha IFN, especially in biological fluids containing low concentrations.

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Alpha interferon (alpha IFN) is produced by leukocytes in response to a variety of inducers such as viruses, bacterial products, polynucleotides, tumor cells, and allogenic cells. It has several biological properties: antiviral activity, regulatory actions on the immune system, inhibition of normal and neoplastic cell multiplication, alteration of macromolecular synthesis, and plasma-membrane conformation (*1*,*2*).

For many years, interferons have been measured by biological assays based on antiviral activity in cell culture (*3*). Recently, alpha IFN has been measured by immunological methods: radioimmunometric assay (*4*,*5*), radioimmunoassay (*6*,*7*), and enzymoimmunoassay (*8*). The clinical importance of the biological properties of the interferons has led us to establish a radioimmunoassay for alpha IFN and to apply it to various clinical conditions: viral infections, various neoplasms (some treated with injections of alpha IFN), and immune disorders such as rheumatoid polyarthritis (RA).

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MATERIALS AND METHODS

Antigens. Partially purified leukocyte IFN (PP alpha IFN) kindly provided by Cantell and Hirvonen (*9*) was used to immunize rabbits. A preparation of pure leukocyte IFN (IFLrA), produced by recombinant DNA, was made by Staehelin et al. (*10*) and was used as tracer.

The reference preparation was human leukocyte IFN standard (B 69/19).* Interferon bioactivity was assessed by measuring the dilution that caused a 50% reduction in the virus titer or cell destruction—the original definition of an interferon unit. One unit was equivalent to the biological activity of ±7 pg of pure alpha IFN.

Fibroblastic IFN, immune IFN, and IFN mock (interferon-free preparation containing natural contaminants from cellular production) were obtained from Billiau (*11*).

Lymphoblastoid IFN was kindly provided by Fantes†.

Since IFN has endorphin-like activity (*12*), we tested beta endorphin, beta lipotropin, enkephalin, and ACTH in the IFN radioimmunoassay. Finally, supernatant fluids from lymphocyte cultures containing large quantities of T-cell growth factor (TCGF = interleukin 2, or ITL 2) were obtained from the experimental surgery laboratory.

Antiserum. One rabbit was given an emulsion made up of 0.5 ml complete Freund's adjuvant and 0.5 ml 0.9% NaCl containing 10,000,000 units of PP alpha IFN, administered in ten intradermal injections on either side of the vertebral column using the method of Vaitukaitis et al. (*13*). Two booster injections were also given, with a month between them, using the same amount of IFN emulsified in incomplete Freund's adjuvant. Two weeks after the last booster, the rabbit serum, at a dilution of 10^{-4}, bound 30% of labeled IFN. The affinity constant of this antiserum was 8.2×10^{10} $1/M$, determined by the method of Rodbard and Hutt (*14*).

Labeling. The IFN obtained by the recombinant method, and purified to homogeneity using monoclonal antibodies, was labeled with I-125 using the lactoperoxidase method (*15*).

In order to separate labeled alpha IFN (alpha IFN*) from radioactive iodide, the iodination mixture was filtered on Sephadex G25 (0.9 by 30 cm) in 0.05 *M* Sorensen phosphate buffer, pH 7.5, containing 0.05 g % NaN_3 and 0.5% bovine serum albumin (BSA). The alpha IFN was eluted in the first peak. Additional purification was obtained by filtration on a column of ACA 54 (0.9 by 65 cm) in the same buffer as used for Sephadex G25 filtration. Two peaks of radioactivity were eluted: a small peak corresponding to aggregated material, followed by a large second peak of radioactivity in the elution zone corresponding to molecules with a molecular weight of approximately 20,000 daltons, i.e., alpha IFN. Only this second peak was used as tracer in the IFN radioimmunoassay.

The mean specific activity, calculated by the method of Berson and Yalow (*16*), was 130 ± 35 $\mu Ci/\mu g$, equivalent to approximately 1 atom of I-125 per molecule of alpha IFN.

Assay method. *Competitive binding in one step.* The incubation medium consisted of 0.05 *M* phosphate buffer, pH 7.5, containing 0.5% BSA and normal rabbit serum at a dilution of 2.5×10^{-3}. To 0.4 ml of this buffer were added 20,000 cpm of alpha IFN, antiserum to a final dilution of 2.5×10^{-5}, and either increasing amounts (5, 10, 20, 37, 75, 150, 310, 620, and 1250 units) of the unlabeled standard alpha IFN (B69/19), or 50 to 100 μl of the medium to be assayed. Incubation was for 24 hr at 18°C, since it had been shown that maximum tracer binding to antibody was reached at that time. At 4°C the time required for similar binding was 48 hr.

The free alpha IFN was separated from that bound to antibody by double precipitation. To the incubation medium was added 1 ml of 0.05 *M* phosphate buffer, pH 7.5, containing 5% polyethyleneglycol (PEG) 6,000, 0.02% (W/V) microcrystalline cellulose, 0.5% Tween 20, and sheep antirabbit globulin antiserum diluted 1:150 (*17*). After a 20-min incubation and 15 min of centrifugation, the supernatant was discarded and the precipitate counted in a gamma counter. In the absence of antiserum, less than 2% radioactivity was found in the precipitate.

Sequential saturation. The first step consisted of mixing 0.2 ml of incubation buffer containing unlabeled alpha IFN (either standard or sample) and 0.1 ml antiserum diluted 10^{-4}, then incubating at 18°C for 24 hr: The tracer (20,000 cpm) was added to the medium for a further 24-hr incubation, and separation was as described above.

Bioassay. Patients' sera were assayed for interferon activity by a cytopathogenic inhibition test using the method of Havell and Vilcek (*17*). The test used Wish cells with vesicular stomatitis virus (VSV) as challenge. Fifteen thousand cells per well were seeded in 96 well microplates in 100 μl of Eagle's minimal essential medium supplemented with nonessential aminoacids and 10% fetal calf serum. After 24 hr, the medium was replaced by 100 μl of the test-serum dilution. Twenty-four hours later, plates were drained and 100 plaque-forming units (PFU) of VSV were added and 100 μl medium containing 5% fetal calf serum. Titers were expressed in international units as the reciprocal of the dilution giving a 50% cytopathogenic effect. Under these circumstances, one international unit of alpha IFN was equivalent to one laboratory unit.

Clinical investigations. *Normal subjects.* Alpha IFN levels were measured in the sera of 365 clinically normal blood donors. There were 183 men and 182 women, between the ages of 10 and 70 yr.

Cancer patients treated with alpha IFN and beta IFN. Seven patients with metastatic malignancies (three breast cancers, three malignant melanomas, one hypernephroma), had been given intramuscular injections of alpha IFN 2, produced by recombinant DNA technology[‡].

Increasing doses of alpha IFN were given at 3-day intervals, 30 million and 100 million units, respectively. Blood samples were taken 1, 2, 4, 6, 12, 24, 36, and 48 hr after injection. In addition, a patient with progressive breast cancer was treated with subcutaneous injections of beta IFN, 6,000,000 U. Blood samples were taken in the same manner.

Patients with viral infections. Serum was obtained from 23 patients during febrile viral illnesses and 10 to 40 days after the symptoms had resolved. Serology readout on the second sample showed that the subjects had been infected either by influenza B virus (13 patients) or adenovirus (three patients), or by herpes vi-

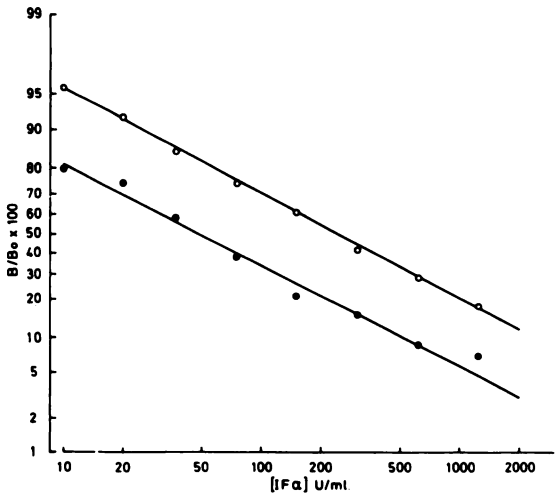

FIG. 1. Standard curves of IFN alpha assay obtained by both equilibrium (O) and sequential saturation (●) methods. Logit-log data. Reference preparation is standard 69/19 M.R.C.

ruses such as cytomegalovirus (*3*), varicella-zoster (*2*) and herpes simplex (*2*).

Patients with malignancy. IFN was also measured in a group of patients with solid tumors with or without clinically detectable metastases: 14 adenocarcinomas of the breast (11 with metastases, M+), seven ovarian cancers (7 M+), seven gastrointestinal cancers (6 M+), four epidermoid carcinomas of the larynx (four without metastases, Mo), three melanomas (3 M+), two prostatic adenocarcinomas (2 M+), one cancer of the cervix (M+) and one of endometrium (Mo), one sarcoma (M+), one hypernephroma (M+), and two epidermoid carcinomas of the bronchus (2 M+). All patients were being treated by radiotherapy or chemotherapy.

We also studied a group of 32 subjects who had been treated for solid malignant tumors but who had no current evidence of disease: 22 carcinomas of the breast, six gastrointestinal carcinomas, three endometrial cancers, one prostatic adenocarcinoma, one epidermoid carcinoma of the larynx, and three Hodgkin's lymphomas. Sera were collected after completion of chemo- and/or radiotherapy during the first 24 mo of follow-up.

Patients with osteoarticular disorders. Alpha IFN was measured in the sera of 24 patients. Eight had osteoarthrosis and 16 had inflammatory arthropathies: eight rheumatoid polyarthritis, two juvenile polyarthritis, one psoriatic arthritis, two polymyalgia rheumatica, one polychondritis, one scleroderma and, one ankylosing spondylitis. Blood sedimentation rate and fibrinogen levels were measured in the same sample of blood that was used to measure alpha IFN.

Statistical analysis. Differences were assessed using the paired t-test and analysis of variance (*19*).

RESULTS

Validation of the RIA. Figure 1 shows the standard curves obtained by both the equilibrium and the sequential saturation methods. The latter curve showed displacement with smaller amounts of unlabeled alpha IFN; when subjected to logit-log transformation, the two standard curves were completely parallel. Parallel inhibition curves of alpha-IFN binding to antibody were found with PP alpha IFN, IFLrA and the standard

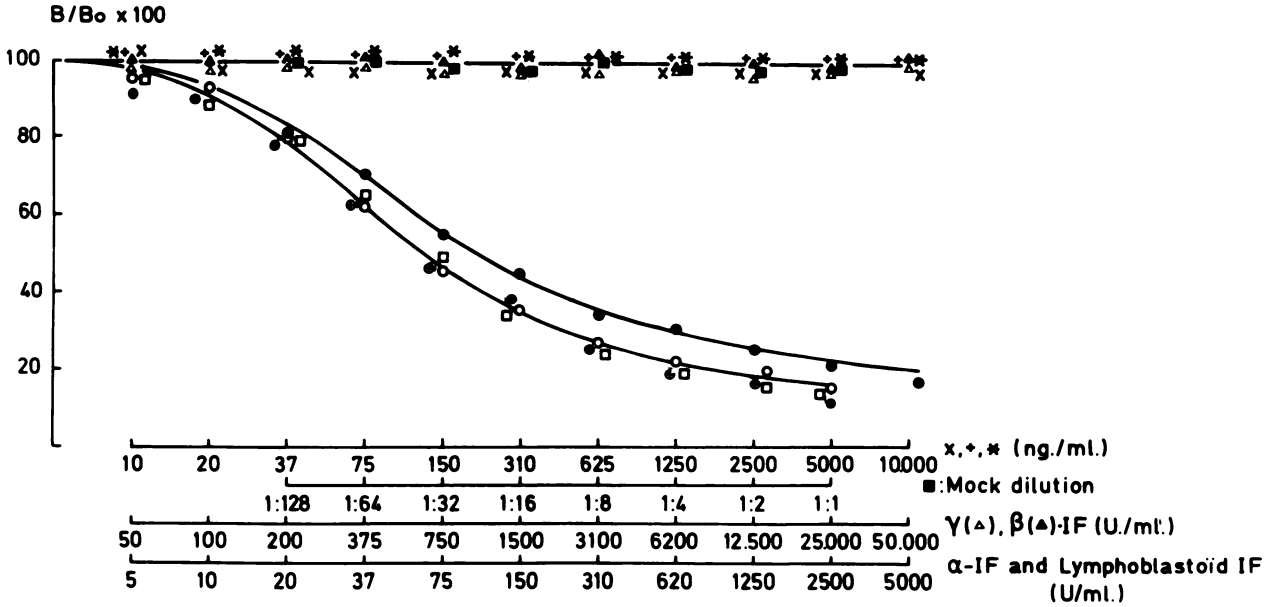

FIG. 2. Assay specificity using sequential saturation. Superimposition of inhibition curves is observed with partially purified leukocyte interferon (PP IFN alpha □), recombinant DNA-produced interferon (IFLrA ⊕) and standard 69/19 MRC°. Lymphoblastoid type ⊕ completely cross-reacts. There is no cross-reaction with A.C.T.H. *, beta endorphin +, beta lipotrophin ×, leu and met enkephalins O, gamma △, beta ▲, and mock ■ interferons.

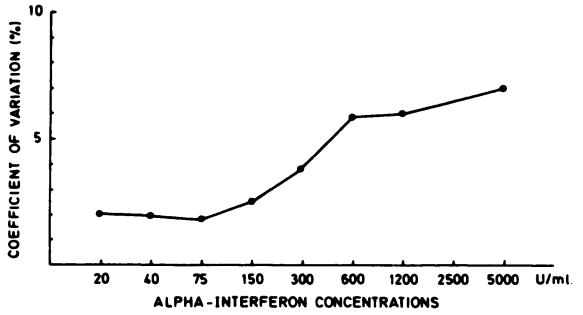

FIG. 3. Profile of precision obtained for IFN alpha assay using sequential saturation method. CV based on ten assays estimation.

(69/19 MRC) (Fig. 2). In contrast, no displacement was seen with IFN beta and gamma in doses of 50,000 units u, interferon Mock in undiluted solution, beta endorphin, beta lipotrophin, the enkephalins, and ACTH in doses up to 10 μg. There was no cross-reactivity in culture media rich in TCGF (ITL 2). Lymphoblastoid IFN showed complete cross-reactivity (Fig. 2).

Assay sensitivity (defined as the smallest amount of unlabeled antigen capable of significantly reducing the binding of alpha IFN to antibody in the absence of unlabeled alpha IFN) was 2.2 units/tube, i.e., 22 units/ml using the competitive binding method in one step, and 0.4 unit/tube, i.e., 4 units/ml using the sequential saturation method.

Precision, expressed as within-assay coefficient of variation, increased with the amount of unlabeled IFN added, but was less than 6% between 0 and 1,200 units. The 0.04 Gaddum index (*20*) was highly satisfactory. Between-assay cv was less than 12% (Fig. 3).

Accuracy was measured as the recovery of known amounts of added IFN to a human serum that had been shown to be IFN-free. Regression analysis of the relationship between amount added and amount measured gave r = 0.99, slope = 0.97, and an intercept below assay sensitivity (2.4 units/ml compared with 4 units/ml) (Fig. 4).

Levels of alpha IFN in normal and pathological sera. *Normal subjects.* Mean alpha IFN concentration was 6.6 U/ml (Fig. 5). Nonparametric calculation of the 95th percentile of the observed frequency distribution determined that 10 units/ml was the endpoint of the normal range.

Patients treated with IFN. The kinetics of alpha IFN injected into patients with breast cancer depended on the amount given. Peak concentrations were found earlier as the amount injected was increased: it came between 6 and 12 hr following 30 million units, and between 4 and 6 hr after 100 million units (Fig. 6). The mean maximum serum concentration obtained was proportional to the amount injected: 338 ± 62 (mean ± s.e.m.) units/ml following 30 million units, and 1248 ± 265 following 100 million units. Higher doses of IFN had cumulative effects, since activity was still detected 48 hr after the intramuscular injection of 100 million units, whereas levels after 30 million units were normal at that time. The calculated half-times for exogenous alpha IFN were 6½ hr for 30,000,000 and 10 hr for 100,000,000 units of exogenous alpha IFN were injected.

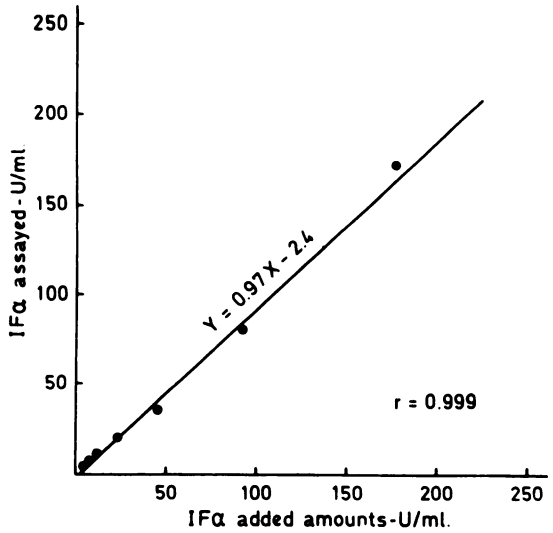

FIG. 4. Relationship between amount added and amount measured in IFN alpha-free serum.

There was a close correlation between the log values of measured IFN by either biological or radioimmunologic assay: log Y = 0.98 log X −0.035; r = 0.95; p <0.001 (Fig. 7).

Patients with viral infections. During the acute phase of viral infection, IFN levels were greater than 10 units/ml in 13 of 23 patients. The mean value for this group was significantly greater than that in control normal subjects. Alpha IFN levels fell significantly during the period of convalescence at the time when proof of viral infection was indicated by the sero-conversion test (Table 1). Mean levels then were not significantly different from normal.

Patients with tumors not treated with alpha IFN. None of 43 patients with various malignancies had IFN levels greater that 10 units/ml. Only one patient in the group of subjects with malignancy who had been treated

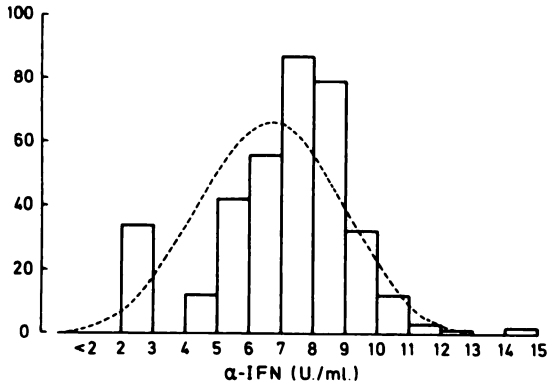

FIG. 5. Normal distribution of IFN alpha for 365 normal subjects.

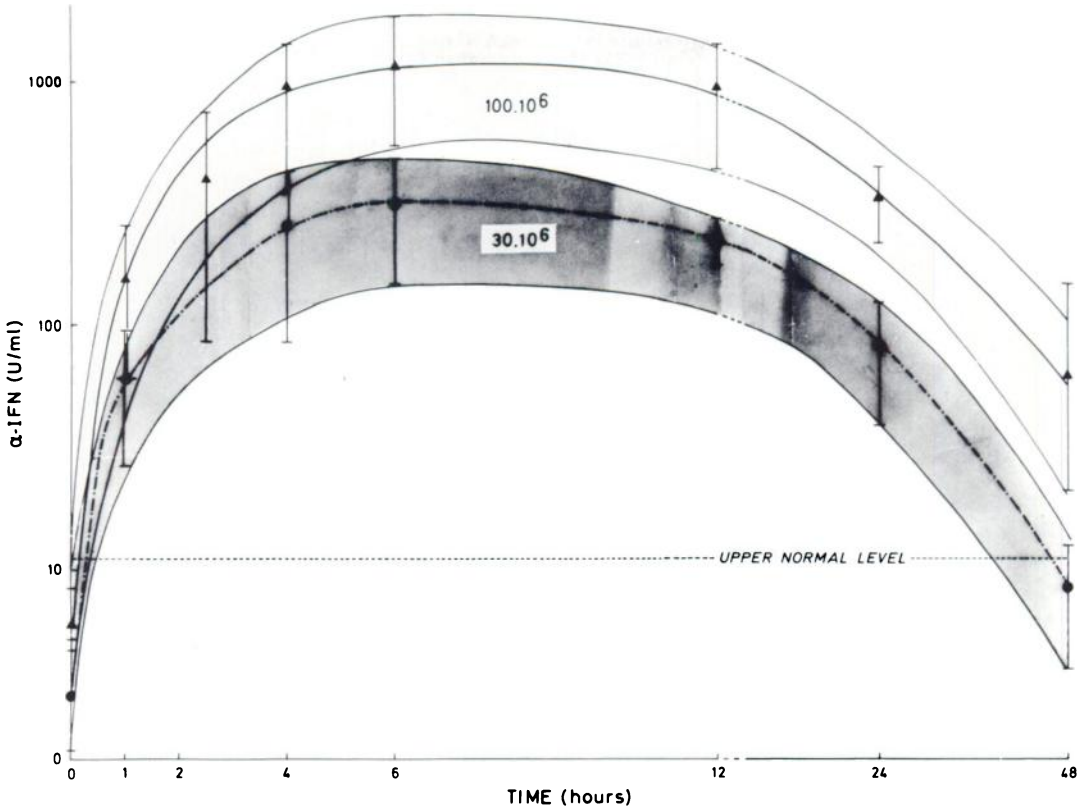

FIG. 6. Mean IFN alpha levels ±1 SEM when 30.10^6U (●) and 100.10^6 (▲) of IFN alpha are injected intramuscularly in 7 cancerous patients at 3-day intervals.

and cured had an increased IFN level (13 units/ml). The mean levels of serum leucocyte IFN (evolving solid-tumor group: 5.6 units/ml; cured solid-tumor group: 6.2 units/ml) and their variances (respectively 6.1 and 4.5) did not differ from normal.

Patients with autoimmune disorders. Patients with arthrosis had normal alpha IFN levels, and their mean did not differ from normal (7.8 ± 3.2 units/ml). In contrast, increased levels were found in seven of 16 patients with inflammatory arthropathies, and the mean

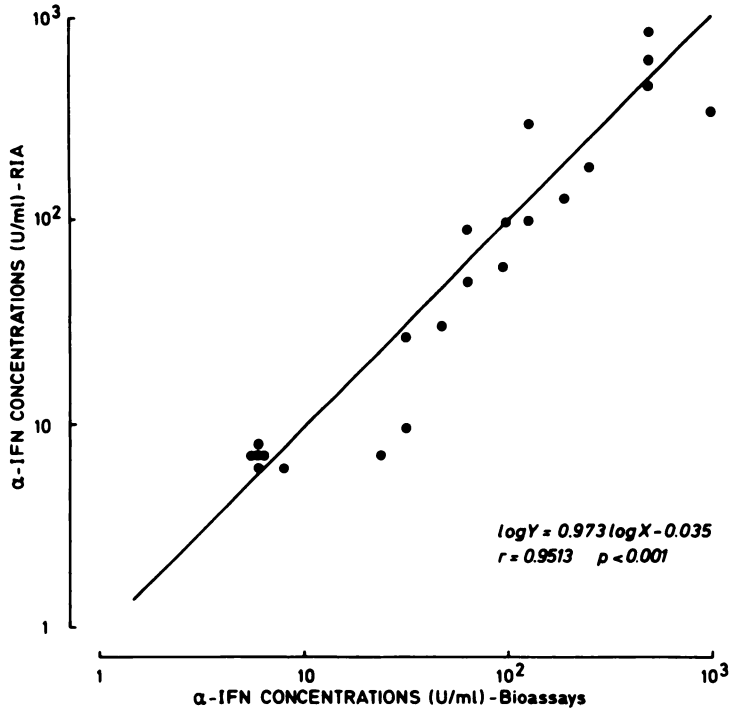

FIG. 7. Correlation between log values of measured IFN alpha by biological or radioimmunological assay for cancerous patients treated with intramuscular injections of interferon.

TABLE 1. ALPHA INTERFERON CONCENTRATIONS DURING AND AFTER VIRAL INFECTIONS

Nature of the infection	Acute viral phase (units/ml)	10–40 days after resolution of symptoms (units/ml)
Adenovirus	11	9
Adenovirus	5	6
Adenovirus	6	7
Cytomegalovirus	12	4
Cytomegalovirus	9	6
Cytomegalovirus	14	11
Herpes	7	6
Herpes	12	11
Varicella	12	10
Varicella	13	9
Influenza B	6	2
Influenza B	44	10
Influenza B	5	3
Influenza B	34	5
Influenaz B	30	6
Influenza B	9	5
Influenza B	8	4
Influenza B	9	4
Influenza B	18	9
Influenza B	12	3
Influenza B	11	6
Influenza B	21	5
Influenza B	7	7
	Mean: 17.7* s.d. ± 9.9	Mean: 6.4† s.d. ± 2.7

* Significantly different from IFN level in normal population ($p < 0.0001$; unpaired t-test).

† Significantly different values seen during febrile phase ($p < 0.001$; paired t-test).

(9.6 ± 3.8) was significantly different from that of normals ($p < 0.0001$) and patients with arthrosis ($p < 0.05$). There was no correlation between alpha IFN levels and sedimentation rate or fibrinogen concentration (Table 2).

DISCUSSION

The radioimmunoassay for alpha IFN demands well-defined experimental conditions in order to obtain the sensitivity, precision, and accuracy that will allow the measurement of the small amounts present in serum. In order to attain these, we labeled the pure antigen with lactoperoxidase and purified it with successive gel filtrations on Sephadex G25 and Aca 54. Tracer corresponding to nonpolymerized alpha IFN was present in the second peak following Aca 54 filtration. The sequential saturation technique gave excellent sensitivity: 0.4 units/tube (±2.8 pg), i.e., 4 units/ml (±28 pg/ml). Precision and accuracy were highly satisfactory, as indicated by the precision profile (Fig. 3) and the recovery of known amounts of alpha IFN added to an interferon-free serum (Fig. 4).

Specificity was established by the lack of cross-reaction between alpha IFN and beta, gamma, and mock interferons, TCGF, beta endorphin, beta lipotrophin, the enkephalins, and ACTH.

In contrast, complete cross-reactivity was seen between alpha IFN and lymphoblastoid IFN, as would be expected from their 85% identity in amino acid sequence (*21*). Such complete cross-reactivity was also noted by Secher (*4*) and Yonehara (*22*), who showed that alpha IFN cross-reacted in the RIA for lymphoblastoid IFN.

The use of a polyclonal anti-alpha IFN antibody, as in our system and that of Daubas and Mogensen (*6*), may have a number of advantages over the use of monoclonal antibodies—as used in the radioimmunometric assays of Secher (*4*) and of Walker (*5*), in the RIA of Meurs et al. (*7*), and in the enzymoimmunoassay of Gallati (*8*). On the one hand, polyclonal antibodies have a high affinity constant (8.2×10^{-10} l/*M*), and on the other hand they react identically with different preparations of alpha IFN (IFLrA and PP alpha IFN), in contrast to certain monoclonal antibodies that give nonparallel curves with different types of alpha IFN (*8*).

The results found by RIA and by biological assay in the sera of patients treated with alpha IFN were similar and closely correlated. There is thus no dissociation between immunological and biological activity in our experience. The RIA, nevertheless, has a number of advantages over bioassay; on the one hand, RIA is specific for alpha IFN, whereas the bioassay cannot distinguish between alpha and beta IFN. Furthermore, the bioassay is subject to many other substances that influence viral growth—in particular, components other than IFN present in the assay sample. Further, the bioassay has inadequate sensitivity and precision to allow measurement of the small amounts of IFN that circulate in normal subjects (*7*). Finally, the bioassay method is long, laborious, and poorly reproducible.

Interferon levels in the sera of normal subjects were of the same order of magnitude as those described by other investigators (*4–6*). Treatment with alpha IFN leads to a considerable increase in its concentration in the blood of cancer patients. The injection of 100 million units causes increased levels for more than 48 hr. Further, the maximal concentration found and the latent period between injection and maximum concentration, together with the persistence of increased interferonemia beyond 24 hr, varied with the amount of alpha IFN injected. Our results confirmed those previously obtained using bioassay and enzymoimmunoassay (*23–25*) and

provide kinetic data useful in designing IFN treatment in various disorders such as cancer. It remains unclear why the half-time for exogenous alpha IFN was shorter when larger doses were injected intramuscularly. Various possibilities, including the degree of local resorption in muscles and the effectiveness of catabolic mechanisms, may affect the uptake and elimination pattern, as is suggested by marked variations in individual values seen in the pharmacokinetic curves in the same subject.

Viral infections are able to cause the release of IFN (*26*), particularly the alpha type (*27*). Using our RIA, we have shown values above normal in the sera of some patients during the acute phase of well-documented viral infection. This interferonemia is found before the development of specific antibody or cell-mediated immune responses, and has been demonstrated by others (*28*,*29*). In contrast, during the early or later convalescent phases, alpha IFN levels have returned to normal. As was found in the study of Green et al. (*27*), there was no correlation between the amount or persistence of alpha IFN and either the degree of clinical illness or the rate of recovery. In cases where alpha IFN levels were normal during the acute phase, it is quite possible that the increase might have occurred during the febrile period preceding blood sampling. Another explanation would be the lack of systemic release of alpha IFN during these viral infections. As suggested by Stiehm et al. (*29*), the local production of IFN within the affected target organ may be more important than circulating IFN. This "in situ" production could be sufficient to limit virus yield and its spread from its site of origin. Finally, we cannot exclude the possibility that some viral infections cause IFN release while others do not. Like Wagner (*30*) and Stiehm et al. (*29*), we believe that the increase of IFN levels during the acute phase is one of the responses of the organism to limit the multiplication and expression of the viral infection.

The range of basal endogenous IFN levels in the sera of patients with cancer has not yet been well documented. The fact that there was no significant increase in IFN levels in patients with evolving solid tumors, and in those cured of cancer without evidence of persistent disease, would be consistent with data reported by other authors (*31*,*32*), who showed that patients with advanced lymphoid malignancies could have deficiencies in alpha IFN or gamma IFN production in vitro. In contrast, patients with certain hematopoietic malignancies such as chronic myelocytic leukemia may produce increased amounts of alpha IFN (*29*). Moreover, T-cell chronic lymphocytic leukemia is associated with spontaneous gamma IFN production by lymphocytes.

In the inflammatory arthropathies, IFN levels were increased in some patients, and the mean value for the group was greater than that of normal patients and those with osteoarthrosis. There was no correlation between the severity of the inflammation, as indicated by sedimentation rate and fibrinogen levels, and the presence of normal or increased alpha IFN levels. This observation should be compared with the data of Hooks et al. (*33*) who noted increased IFN levels in six of 11 patients with rheumatoid arthritis, and six of ten with scleroderma. The biological assay methods used by these authors, however, do not allow the identification of whether the IFN was of the alpha and/or gamma type. The significance of this observation remains unclear. In inflammatory arthropathies—such as rheumatoid polyarthritis and juvenile and ankylosing spondylitis—there are immunological abnormalities consisting of B-lymphocyte hyperactivity and depressed T-lymphocyte function. The hyperactivity of the B cells is shown by increased gamma globulin levels and the presence of autoantibodies and of immune complexes, including rheumatoid factor. Moutsopoulos and Hooks (*2*) showed that mitogens and viruses stimulate IFN production in vitro by mononuclear cells from RA patients. Furthermore, the increase in IFN levels could result from a normal and appropriate reaction during the acute phase of these inflammatory arthropathies, from polyclonal activation of B cells, and/or the persistence of a viral infection that may or may not be associated with the causes of these inflammatory disorders.

From another point of view, the possibility cannot be excluded that the increased IFN production might play an etiopathological role in the production of the immunological abnormalities of these autoimmune disorders (*34*). Studies of a much larger number of patients will be required to determine increases in alpha IFN levels as a function of the clinical state, treatment, and immunological tests, and to try to understand their significance.

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FOOTNOTES

* From the Medical Research Council, National Institutes of Biological Standards and Controls, London.

† Wellcome Research Laboratories, England.

‡ Schering-Plough.

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