TREATMENT OF HAIRY CELL LEUKEMIA WITH RECOMBINANT ALPHA INTERFERON: II. IN VIVO DOWN-REGULATION OF ALPHA INTERFERON RECEPTORS ON TUMOR CELLS

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Interferons (IFNs) initiate their effects by interacting with specific high-affinity cell surface receptors, but little is known about the physiology of IFN receptor interaction in vivo. Treatment of patients suffering from hairy cell leukemia (HCL) with human recombinant alpha IFN results in significant tumor regression, with clinical improvement in a high percentage of cases. To investigate a possible relevance of binding parameters as response markers, IFN receptor interaction on tumor cells responsive to IFN in vivo was studied. Binding of human alpha 2 IFN to circulating hairy cells was analyzed before and during IFN therapy in ten patients selected on the basis of high numbers of peripheral hairy cells. Binding experiments were carried out on Ficoll-Paque fractionated peripheral cell samples containing a majority of hairy cells. All patients reacted to recombinant alpha IFN treatment with a striking decrease in binding capacity within 12 hours after the first injection. As demonstrated by using a monoclonal antibody able to recognize alpha 2 IFN bound to its receptor, this decreased binding capacity was not due to blocking by circulating IFN but rather to a decrease in receptor number. This receptor "down-regulation" was partially reversible after the first IFN injection. However, upon prolonged IFN therapy, all patients displayed a stable state of decreased receptor expression. Down-regulation of IFN receptors can be regarded as a response marker to IFN treatment. This response marker, however, was not correlated with the clinical response within the first months of IFN therapy.

INTERFERONS (IFNs) are proteins with the common property of protecting cells from viral infection. In addition they exert a variety of pleiotropic effects, such as modulation of various immune functions and inhibition of cell growth. Like polypeptide hormones or growth factors, IFN action is initiated by binding to specific high-affinity cell surface receptors. IFNs comprise three types (alpha, beta, and gamma), and the alpha type is further subdivided into at least 12 subtypes. While pathways of gamma IFN action seem independent from those of alpha and beta IFN, the latter initiate their effects by interaction with presumed common receptors. Despite a considerable amount of data on the initial receptor interaction of IFNs and the subsequent steps, including internalization and degradation, major questions on the signal initiated by receptor binding are unresolved. Likewise, the necessity of ligand and/or receptor internalization for interferon action has not been demonstrated unequivocally, and the role of decrease of receptor number induced by IFN binding (called receptor "down-regulation") remains unclear. The antitumor potential of IFNs can be mediated by a direct cytostatic action on tumor cells as well as indirectly through stimulation of immune response; in both cases, the mechanisms involved are elucidated only in part. Beyond comparative binding data on various lymphoid cells, little is known as yet on the initial steps of IFN action in vivo. Recently, alpha IFN was reported to induce remission in hairy cell leukemia (HCL). To investigate the role of cell surface receptors as markers for responsiveness of HCL to IFN therapy, IFN binding was studied on tumor cells from HCL patients treated with recombinant alpha IFN. Five of the 17 patients studied in part I of this report and five additional patients who entered the same clinical trial were selected on the basis of high numbers of circulating hairy cells before onset of IFN therapy. Number of receptor sites for alpha IFN and the affinity constant were determined before and during IFN therapy by studying the binding of $^{125}I$-labeled human alpha 2 IFN. In addition, the degree of receptor occupation under IFN treatment was studied with a monoclonal antibody that recognizes alpha 2 IFN bound to its receptor.

MATERIALS AND METHODS

Patients and treatment protocol. Experiments were carried out with ten patients with hairy cell leukemia who entered the trial described in part I of this report. The patients were selected on the basis of high number of peripheral hairy cells before onset of therapy ($>10 \times 10^8$ leukocytes/L). Based on peripheral blood and bone marrow data, clinical progress of patients 2, 4, 8, 9, and 21, for whom treatment has exceeded 4 months, is described in part I of this study. All patients were advised of procedures and attendant risks in accordance with institutional guidelines and gave informed consent. Patients 2, 4, 25, and 29 were treated with daily subcutaneous injections of $3 \times 10^6$ units of recombinant human alpha 2 IFN (Hoffman-La Roche, Basel, Switzerland). Patients 4, 8, 21, 27, 36, and 38 were treated with $2 \times 10^6$ U/m$^2$ of recombinant human alpha 2 IFN (Schering Corp., Kenilworth, N.J.) in three weekly subcutaneous injections. These two IFNs differ in only one amino acid and are referred to as recombinant alpha IFN in the following text.

Binding assay. Peripheral blood cells, including a majority of hairy cells, were enriched by fractionation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) according to the manufacturer's protocol. The percentages of hairy cells obtained under these conditions for each binding experiment are shown in Table 1. Subsequently, cells were washed three times at 4°C with Hank's balanced salt solution (HBSS) containing 10 mmol/L HEPES with 10% fetal calf serum (KC Biological, Lenexa, Kan.) and resuspended in this buffer at a density of $2 \times 10^7$ cells/mL. To determine saturation curves, binding experiments were carried out in U-bottomed microtiter plates at a cell density of $10^7$ cells/mL (200 μL/well) as described elsewhere. All incubations with $^{125}I$-labeled human alpha 2 IFN were for 90

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and at cells kept at 0°C to 4°C in balanced salt solution, receptor expression and binding affinity remained virtually unchanged for periods up to 48 hours. Also, cell fractionation on Ficoll-Paque did not modify the binding data (data not shown).

**Mouse monoclonal antibody against human recombinant alpha 2 IFN.** Mouse monoclonal antibodies against human recombinant DNA alpha 2 IFN were raised as described elsewhere.19 These antibodies were characterized with regard to IFN subtype specificity, neutralization of antiviral activity, and the capacity to recognize human alpha 2 IFN specifically bound to its cell surface receptors.19 For the indirect binding assay described below, an antibody (1/I1) specific for alpha 2 IFN in a solid-phase radioimmunoassay was selected.19 Although this antibody did not neutralize the biological activity of alpha 2 IFN, it was able to recognize alpha 2 IFN specifically bound to its cell surface receptors on human lymphoid Daudi cells pretreated with alpha 2 IFN. Nonspecific binding was determined by simultaneous addition of excess human beta IFN, which binds to the same receptors as alpha IFN but is not recognized by the monoclonal antibody 1/I1. Binding of monoclonal antibody 1/I1 was revealed by addition of an 125I-labeled rabbit antimaxine IgG antibody as described below.

**Indirect binding assay.** Peripheral blood cells were prepared as described above and adjusted to 2 x 10^6 cells/mL in the same buffer. To detect receptor-bound alpha 2 IFN, the cells were incubated for 90 minutes at 4°C with the mouse monoclonal antibody 1/I1 capable of recognizing alpha 2 IFN specifically bound to its cell surface receptors, which has been described above and elsewhere.19 Control experiments were carried out with a nonspecific mouse monoclonal antibody (1/O). Cells were then washed twice, and bound mouse antibody was revealed by incubation for 90 minutes at 4°C with an 125I-labeled rabbit antiserum IgG antibody in the presence of 1% normal rabbit IgG to block Fc receptors. This rabbit antiserum IgG antibody was purified by affinity chromatography on mouse IgG coupled to Affigel-10 (BioRad, Richmond, Calif) and labeled with 125I sodium iodide (CEA, Gif sur Yvette, France) to a specific radioactivity of 15 to 30 μCi/μg protein. Briefly, 1 mCi was added to 25 μg IgG in 100 μL phosphate-buffered saline (PBS) and the iodination initiated by addition of 10 μL of chloramine T adjusted to 500 μg/mL in PBS. After 45 seconds the reaction was stopped by addition of 10 μL of sodium metabisulfite at 500 μg/mL in PBS. Unreacted iodine was eliminated by chromatography of the reaction mixture on a prepacked Sephadex G-25 PD-10 column (Pharmacia). To control the capacity of antibody 1/I1 to recognize receptor-bound alpha 2 IFN, cells were preincubated for 90 minutes at 4°C with unlabeled alpha 2 IFN at a saturating concentration of 10,000 U/mL prior to incubation with mouse monoclonal antibodies 1/I1 and 1/O, respectively. All incubations were carried out as for the direct binding assay in U-bottomed microtiter plates at cell density of 10^6 cells/mL in a volume of 200 μL/well. The labeled rabbit antibody was adjusted to approximately 200,000 cpm/well.

### IFN assay

Interferon activity was assayed by inhibition of the cytopathic effect of vesicular stomatitis virus (VSV) on bovine MDBK cells (Flow Laboratories, Bethesda, Md). Briefly, confluent monolayer cultures of MDBK cells were incubated for 24 hours at 37°C with interferon samples of twofold dilution series. Subsequently the cell monolayers were challenged with 1 TCID50/100 μL VSV. Inhibition of cytopathic effect was determined after 24 hours' incubation with the challenge virus. Human recombinant DNA alpha 2 IFN served as a reference with a specific activity of 2 x 10^6 U/mg protein. Titers were standardized to the NIH human leukocyte interferon No. GA 902-530 and are expressed in international units (U/mL).
RESULTS

High-affinity binding of 125I-labeled alpha 2 IFN to specific cell surface receptors of hairy cells was measured immediately before and at various intervals after onset of recombinant alpha IFN therapy. The proportion of hairy cells in peripheral blood is listed in Table 1 for each determination of receptor number. Figure 1 shows the evolution of the number of high-affinity alpha 2 IFN receptors in the first 4 weeks of IFN therapy. Initial numbers of high-affinity alpha 2 IFN receptor sites per cell ranged from ISO affinity cells in peripheral blood is listed in Table 1 for each specific cell surface receptors of hairy cells was measured was also observed in patient 2, who did not respond consistently below 25% of the pretreatment level. Weekly intervals after onset of therapy revealed a binding was after the first recombinant alpha IFN injection. Twenty-four hours after the first injection, the binding capacity was restored partially in most patients, while it remained at less than 20% of the pretreatment level in two cases (Fig 1). This recovery was only temporary, since binding experiments performed at weekly intervals after onset of therapy revealed a binding capacity consistently below 25% of the pretreatment level. After the second week of treatment, the binding capacity remained virtually unchanged before and six hours after recombinant alpha IFN injections. A decreased binding capacity was also observed in patient 2, who did not respond clinically to IFN therapy. Serum IFN was assayed at each point of determination of binding capacity. While no IFN activity was detectable before onset of therapy, recombinant alpha IFN treatment yielded serum levels between 0 and 40 U/mL. Titers did not correlate significantly with the decreased binding capacity (data not shown).

In contrast to the number of binding sites, binding constants (Kd) determined before IFN treatment did not differ significantly and ranged from 1.4 to 6.7 x 10^10 mol/L. As exemplified for one case in Fig 2, the affinity of the residual binding sites remained unchanged after IFN injection as compared to the binding properties before treatment.

To distinguish between blocking of receptors by circulating recombinant alpha 2 IFN and decreased expression of high-affinity receptors, the degree of receptor occupation before and six hours after IFN injection was studied in an indirect binding assay with a monoclonal antibody (I/11) directed against alpha 2 IFN bound to its receptor or a control antibody (I/O). Binding of these antibodies was revealed by use of an 125I-labeled rabbit antimouse IgG antibody. As shown in Fig 3, virtually the same amount of radioactivity was bound to hairy cells immediately before and six hours after the first alpha 2 IFN injection with both specific and control antibody. These results suggest that the decreased binding capacity after IFN injection as revealed in the direct binding assay (Fig 1) was not due to occupied receptors. As a control, the IFN binding capacity before and six hours after IFN injection was also determined in the indirect assay. For this purpose the cells were preincubated with a saturating amount of alpha 2 IFN (10,000 U/mL) prior to incubation with the monoclonal antibodies I/11 or I/O, respectively. The results (Fig 3) are consistent with those obtained in direct binding experiments depicted in Fig 1. Thus six hours after the first injection, the IFN binding capacity of hairy cells from patients 4 and 25 was decreased to the same extent in both assays (Fig 3A and B), while the binding capacity of cells from patient 29 remained unchanged before and six hours after the first IFN injection (Fig 3C). As exemplified with patient 2, the IFN binding capacity of peripheral hairy cells remained low, and no bound IFN was detectable after 10 weeks of IFN therapy.
The present study was carried out to investigate the initial steps of direct IFN action of hairy cells, which are responsive to alpha IFN treatment in vivo. High-affinity cell surface receptors with binding properties identical to those described for normal peripheral lymphoid cells were found on circulating hairy cells of all ten patients included in the present study. Although pretreatment numbers of detectable binding sites showed up to sevenfold variations (Fig 1), they were of the same order of magnitude as on normal peripheral lymphocytes. After onset of recombinant alpha IFN treatment (see treatment protocol in Materials and Methods), all patients showed a marked decrease of detectable alpha IFN receptors on circulating tumor cells (Fig 1). To distinguish between specific blocking and a decreased number of receptors, bound alpha 2 IFN was revealed indirectly by use of a monoclonal antibody against alpha 2 IFN, which recognizes an epitope still accessible upon receptor binding. Tumor cells from different patients were investigated using this approach immediately before and six hours after the first recombinant alpha IFN injection, as well as upon prolonged IFN therapy (Fig 3). Clearly, the results indicate that the loss of binding sites as early as six hours after onset of therapy was not due to receptor occupation but rather to a decreased receptor expression, called “down-regulation.” Alternatively, a pronounced drop in binding affinity may have also accounted for decreased binding of labeled alpha 2 IFN and could not have been distinguished from decreased receptor expression. As shown in Fig 2, the binding affinity of the residual binding sites was unchanged as compared to the data before IFN therapy. Prolonged IFN treatment of cells in vitro may lead to selection of resistant cells. Since a decreased IFN binding capacity was observed rapidly after onset of IFN treatment, selection of tumor cells lacking IFN receptors seems unlikely.

Binding-induced down-regulation of receptor expression was observed in all patients over the entire period of IFN therapy, resulting in marked tumor regression in 9 of 10 patients. Although in most patients receptor expression recovered to a variable proportion within 24 to 48 hours after the first recombinant alpha IFN injection, a permanent “down-regulated” state was established at the latest after the second week of IFN treatment (Fig 1). Down-regulation of alpha IFN receptors, therefore, seems to reflect a continuous response to IFN at the receptor level, indicating that cells remain responsive to IFN despite a low degree of high-affinity receptor expression. After 3 to 4 weeks of treatment, the number of hairy cells in peripheral blood was markedly decreased in most patients (see part 1). However, decreased expression of high-affinity receptors was observed independently of the percentage of tumor cells in the mononuclear cell-enriched fraction (Fig 1, Table 1), suggesting that receptor down-regulation can occur in the majority of circulating leukocytes. Since in most cases the proportion of hairy cells did not vary significantly within the first 2 weeks of treatment (Table 1), receptor down-regulation can hardly be attributed to shifts in cell populations.

Down-regulation might reflect a shift in the dynamic equilibrium between receptor expression and receptor processing. The fraction of occupied receptors at a given...
concentration of free ligand depends proportionally on the number of expressed receptors. Therefore, receptor down-regulation could result in a decreased sensitivity of the target cell. However, a maximum biological response might require occupation of only a small fraction of accessible receptors, the majority of expressed receptors being "spare." Down-regulation of insulin receptors seems to correlate with insulin resistance in some forms of type II diabetes, but it is not clear whether this mechanism could be more generally responsible for decreasing target cell sensitivity for polypeptide ligands. Recently, human Daudi cells rendered resistant to human alpha IFN by prolonged incubation with IFN were shown to "down-regulate" their receptors in the same way as sensitive Daudi cells.

Preliminary results seem to indicate that expression of gamma IFN receptors in hairy cells remains unaffected by alpha IFN treatment (data not shown), in agreement with previous results. Down-regulation occurs rapidly at interferon doses that yield barely detectable serum levels. Thus down-regulation of alpha/beta IFN receptors in vivo could provide a sensitive early response marker, not only for monitoring alpha IFN administration but also to reveal endogenous IFN production in untreated patients with the possibility of distinguishing between alpha and beta IFNs and gamma IFN in situations of low or undetectable serum IFN.

Degree and time course of alpha IFN receptor down-regulation were similar in all patients, independent of clinical response to the treatment. The results presented here seem to indicate, therefore, that neither initial IFN receptor levels nor down-regulation is of predictive importance in IFN therapy of HCL. Still, to evaluate the role of receptor down-regulation and its relevance as a response marker for IFN therapy in vivo, it seems important to reevaluate this parameter after prolonged IFN therapy and to extend similar studies to additional cases of IFN-resistant HCL and also to IFN trials in other leukemic diseases.

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