Analysis of Interferon-Inducible Genes in Cells of Chronic Myeloid Leukemia Patients Responsive or Resistant to an Interferon-α Treatment


Recombinant human interferon-α (IFN-α) can induce a hematologic remission in patients with chronic myeloid leukemia. However, some patients are resistant and others develop late resistance to the IFN-α treatment. To understand the molecular mechanism of this resistance, we have analyzed the expression of 10 IFN-inducible genes in the cells of three resistant patients, two responsive patients, and six healthy controls. Northern blot hybridizations showed that all the genes were induced in vitro IFN-α treated peripheral blood cells of the patients and healthy controls. These genes were also inducible in peripheral blood and bone marrow cells of two out of two resistant patients administered an injection of IFN-α. We conclude that the resistance to the IFN-α treatment of the chronic myeloid leukemia patients we studied is not due to (1) the absence of induction of any of the 10 IFN-inducible genes we studied, including the low-molecular-weight 2′-5′oligoadenylate synthetase; (2) the presence of an antagonist of IFN-α in the peripheral blood or bone marrow cells; and (3) the presence of neutralizing anti-IFN-α antibodies.

SEVERAL STUDIES indicate that natural, as well as recombinant human interferon-α (IFN-α) has a therapeutic effect during the chronic phase of chronic myeloid leukemia (CML). In fact, partial or complete hematologic remission is achieved in a large proportion (65% to 90%) of patients.1,2 IFN-α can also lead to a partial or complete disappearance of Philadelphia chromosome (Ph′) positive cells in 50% to 70% of the responding patients, depending on their previous history.3,4 Complete suppression of the Ph′ positive clone, shown by cytogenetic analysis, was confirmed at the molecular level.4 At least 10% of the responding patients develop late resistance to IFN-α,5,6 but this percentage may well be higher because the phenomenon has not been well documented and longer follow-ups are necessary to obtain more accurate data.

The reason for the effectiveness of IFN-α in CML is not known, but the action of this cytokine on cells is well characterized.7 In addition to its antiviral and immunomodulatory activities, IFN-α also has an antiproliferative effect on normal and on CML marrow progenitor cells.8,9 These biologic activities are probably mediated by the genes, at least 30, which are induced by IFN in sensitive cells.10,11 About 20 of them have been cloned, including the genes coding for class I HLAs, the 2′-5′oligoadenylate synthetase (2′-5A synthetase), the metallothionein IIa (MTIIa), and genes that are called IFI-15K, IFI-54K, IFI-56K, IFI-78K, IFI-4, 1,8, and 6-16. Even though the function of the proteins encoded by most of these genes (in particular the last seven genes named) is unknown, the regulation of their expression has been abundantly studied. All IFN-inducible genes are induced transcriptionally, by a factor varying from a few times up to 100 times, and some are probably also post-transcriptionally regulated.12,13

In certain IFN-resistant cell lines, some IFN-inducible genes are not inducible anymore by an IFN-α treatment.14,15 Therefore, we wondered whether the initial or late resistance to IFN-α of some CML patients could be correlated to the absence of induction of genes normally inducible by IFN-α. The expression of 10 IFN-inducible genes was analyzed by Northern blot hybridization in cells of five patients in chronic phase (one resistant, two late resistant, and two responsive patients) and six healthy controls. We observed that all IFN-inducible genes analyzed were induced in the in vitro IFN-α treated peripheral blood cells of the responsive patients and controls, as well as in those of the resistant patients. Moreover, the genes were also expressed in peripheral blood and bone marrow cells of two out of two resistant patients, 4 hours after they had been given an injection of IFN-α. Thus, the resistance to the IFN-α treatment of some CML patients cannot, at least in the three resistant patients we analyzed, be explained by the absence of induction of any of the 10 IFN-inducible genes tested.

MATERIALS AND METHODS

Patients and controls. Five Ph′ positive CML patients, who had or had not received previous chemotherapy, were treated with recombinant human IFN-α2c according to the EORTC protocol 06842. The daily dose of IFN-α ranged from 5 to 10 million units. Details on these patients are given in Table 1.

Response of the patients to the IFN-α treatment was determined by clinical examination, usual blood hematologic parameters, and marrow cytogenetic analysis of at least 15 mitoses. None of these patients showed complete or even partial cytogenetic conversion in response to the IFN-α therapy; 100% of their bone marrow cells were Ph′ positive at the time of the present study.

The five patients and six healthy controls had given their informed consent for the study.

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Submitted March 12, 1990; accepted August 3, 1990.

Supported by ULB-Actions de Recherche Concertées of the Belgian Government; by a grant from the CGER Fonds Cancérologiques to J.C. and G.A.H.; by grants from the Fund for Medical Scientific Research (Belgium), no. 34,560.85 to J.C. and G.A.H. and 34,522.82 to P.S.; and by a grant from the Fund for Joint Basic Research (Belgium) no. 2,9006.79 to J.C. M.G.W. is Senior Research Assistant of the National Fund for Scientific Research (Belgium). I.M.C. is fellow of the Fondation Rose et Jean Huguet (Belgium).

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Blood, Vol 76, No 11 (December 1), 1990: pp 2337-2342

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Peripheral blood and bone marrow cells. Leukocyte counts of the peripheral blood and bone marrow specimens were determined with a Coulter counter. After having diluted the specimens twofold with Hank's Balanced Salt Solution (HBSS), mononuclear and polymorphonuclear (PMN) cells were separated on a Ficoll-Hypaque double gradient (L.M.S., International Medical Product, Belgium) (density 1.077 and 1.12), washed twice with HBSS, and recounted with the Coulter counter.

In vitro studies were performed by incubating separately PMN and mononuclear cells for 4 hours, at 2.10^6 cells/mL in RPMI 1640 containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL fungizone (GIBCO, Belgium) at 37°C in 5% CO₂ in the presence or absence of 20 U/mL recombinant human IFN-α2c (Boehringer Ingelheim).

Cells, incubated in vitro or not, were washed twice with HBSS, pelleted by centrifugation, and stored in liquid nitrogen until RNA extraction.

RNA extraction and Northern blot analysis. Pellets of up to 10^8 cells were thawed in 3.6 mL of 4 mol/L guanidium thiocyanate, 25 mM/L 5-(3-chloro-4-methylphenylsulfonyl)imidazole (CM), 0.5 M/L phenylmethylsulfonyl fluoride (PMSF), 200 mM/L benzamidine, 100 mM/L EDTA pH 8, and 1% sarcosyl. RNA was collected by layering the homogenate on a 1.3-mL cushion of 5.7 mol/L CsCl (prepared in 100 mM/L EDTA pH 6.5) and centrifugation for at least 12 hours at 42,000 rpm in an SW50.1 rotor at 20°C. The RNA pellet was dissolved in 300 μL Tris.Cl 10 mM/L pH 9, and ethanol precipitated in the presence of 0.2 mol/L NaCl. RNA was dissolved in water and its concentration determined by measuring the optical density at 260 nm.

Total RNA was denatured with glyoxal and dimethylsulfoxide, electrophoresed through a 1% agarose gel, and transferred to nylon membranes.

Probes. 32P-labeled antisense RNA probes were synthesized with the T3, T7, or SP6 RNA polymerase according to the manufacturer's instructions (Amersham for SP6 transcription and Vector Cloning Systems for T3 and T7 transcriptions). Unincorporated nucleotides were eliminated by Sephadex G-50 spun-column chromatography. The cDNA fragments used for the synthesis of the probes are: 2-5A synthetase, a 320-bp PstI/Pro I fragment (nucleotides 32 to 360); IFI-56K, a 412-bp PstI-HindIII fragment (nucleotides –31 to 368); IFI-54K, a 297-bp EcoRI fragment (nucleotides 2063 to 2360); IFI-15K, a 525-bp PstI fragment; MT IIa, a 174-bp BamHI/PvuII fragment (nucleotides 3 to 177); rat GAPDH, a 1.28-kb fragment (entire cDNA).

RESULTS

Analysis of the expression of IFN-inducible genes in in vitro IFN-α treated cells from CML patients. Four chronic-phase CML patients (patient 1, who responded to IFN-α; patients 3 and 4, who developed a late resistance; and patient 5, who was de novo resistant) and six healthy controls were analyzed for the expression of IFN-inducible genes in in vitro IFN-α treated cells.

Peripheral blood was separated in mononuclear and PMN cells, which were then incubated separately for 4 hours in vitro in the presence or absence of 20 U/mL of human IFN-α. PMN and mononuclear cells were separated separately because the PMN isolated by this method constitute a relatively pure population of leukemic cells. The mononuclear cells, a heterogenous cell population (B and T lymphocytes, monocytes, different precursors, etc) was examined for comparison in case an anomaly in the expression of IFN-inducible genes would have been present in PMN only.

The dose of IFN used for the in vitro induction (20 U/mL) was chosen to be as close as possible to the average in vivo situation. Indeed, a few hours after an injection of 5 × 10^8 U of IFN-α to a patient, the serum concentration of IFN-α is approximately 20 U/mL.

After the in vitro incubation, total RNA was extracted and 1 μg of each RNA sample was submitted to Northern blot hybridization. Such small quantities of RNA were analyzed because extremely small amounts of total RNA were obtained from the cells. The cells from which we obtained the smallest quantity of RNA were the PMN of the controls; only about 13 μg of total RNA was obtained from 10^8 cells, as compared with 1 μg of RNA obtained from 10^6 lymphoblastoid cells in culture.

Northern blots were hybridized with radiolabeled probes corresponding to the following IFN-inducible genes: a class I HLA gene; the metallothionein IIA gene; the gene coding for the low-molecular-weight 2-5A synthetase; the genes IFI-78K, IFI-56K, IFI-54K, IFI-15K, IFI-4, 1-8, and 6-16. Hybridization with a probe corresponding to the rat glyceroldehyde-3-phosphate dehydrogenase (GAPDH) gene served as an internal control to evaluate the amount of RNA submitted to electrophoresis and successfully transferred to the nylon membranes.

Figure 1 shows the Northern blots of cells from a represen-
In this last patient, the expression of only four genes could be analyzed in the PMN cells, because only a few micrograms of total RNA were obtained. The results obtained with the five other controls (data not shown) were very similar to those of the control shown in Fig 1, and the Northern blots of patient 4 were identical to those of patient 3, and are shown in Fig 2. It is apparent from these Northern blots that the 10 IFN-inducible genes analyzed were induced to the same extent in the cells of the resistant patients (nos. 3, 4, and 5), as in the cells of the responsive patient (no. 1) and controls. It should be noted that the small quantitative differences that were observed for some genes between samples from patients and from the control shown in Fig 1 were in the range of the variation observed among the samples of the six different controls tested (data not shown).

Some genes were more inducible in the PMN than in the mononuclear cells: it was the case of the genes I-8, IFI-15K, IFI-54K, and IFI-56K, but this peculiarity was seen in the controls as well as in the patients.

Expression of the HLA gene was only slightly increased in both the patients and the controls. This observation is in agreement with previous studies on cell lines, which indicate that the increase in expression of HLA class I genes is only moderate at 4 hours, because the maximum level of its messenger RNA (mRNA) (a 10-fold increase) is reached 12 hours after the beginning of the IFN-α treatment. Longer incubation times are thus necessary to observe a higher induction level.

A certain rate of expression of the gene coding for the 2-5A synthetase was present in unstimulated mononuclear cells of the six controls and of two (of four) patients: patients 1 and 5 (Fig 1). Because patient 1 was responsive and patient 5 was resistant to the IFN treatment, no correlation can be drawn between the presence of an uninduced level of expression of the 2-5A synthetase gene and the responsiveness of a patient to the IFN treatment.

Two mRNAs of different sizes can be transcribed from the gene encoding the low-molecular-weight 2-5A synthetase. These 1.6- and 1.8-kb mRNAs are generated by differential splicing of the same gene and they code for a 42- and a 46-Kd protein, respectively. We found that IFN induced either the 1.6- or the 1.8-kb mRNA or both, but no correlation could be drawn between the type of mRNA expressed and the disease or the responsiveness of a patient to the IFN-α treatment. The reason for this peculiarity is unknown, but it is known that different cell lines express different 2-5A synthetase messengers.

Although small differences in the induced and noninduced level of expression of some IFN-inducible genes were found, no fundamental differences in the expression of the 10 IFN-inducible genes studied were found between resistant patients on one side and responsive patients and controls on the other.

Comparison of in vitro to in vivo induction of IFN-inducible genes in cells of CML patients. To analyze the expression of IFN-inducible genes in more physiologic conditions, we compared the expression of the 10 IFN-inducible genes in cells taken after an injection of IFN-α with their

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**Fig 1.** Northern blot analysis of 10 IFN-inducible genes in in vitro IFN-α treated mononuclear (M) and PMN (P) cells of three CML patients and one control. Patient 1 was responsive to the IFN-α therapy, patient 3 developed a late resistance, and patient 5 was de novo resistant. -, cells incubated 4 hours in the absence of IFN.

**Fig 2.** Northern blot analysis of 10 IFN-inducible genes in in vivo compared with in vitro IFN-α treated mononuclear and PMN cells of a resistant patient (no. 4). The definitions of M, P, +, and − are indicated in the legend to Fig 1.
expression in cells taken before the injection and incubated in vitro with IFN-α.

Peripheral blood samples from two resistant patients (nos. 3 and 4) were taken for analysis before and 4 hours after a subcutaneous injection of 5 x 10^6 U IFN-α (in vivo study). Bone marrow was also taken 4 hours after the injection. A fraction of the peripheral blood samples taken before the injection was separated in mononuclear and PMN cells, which were then incubated separately in vitro in the presence or absence of 20 U/mL IFN-α (in vitro study). Total RNA was extracted and submitted to Northern blot analysis as described in the previous section.

For ethical reasons, this experience could not be performed with healthy controls. However, we analyzed in vivo IFN-α induced peripheral blood cells of a responsive patient (no. 2). No in vitro incubation of his peripheral blood cells was performed.

Northern blot analysis showed that the 10 IFN-inducible genes analyzed were all induced by IFN-α in the mononuclear and PMN blood cells of the two resistant and the sensitive patients given an injection of IFN-α. In addition, the level of induction of the genes was the same as in the cells incubated in vitro with IFN-α (Fig 2). Results obtained with the cells of only one patient (no. 4) are shown in Fig 2, since the same results were obtained with the other resistant patient (no. 3).

A certain level of expression of the 6-16 gene was present in mononuclear cells of patient 4 taken before the injection, and it disappeared completely after 4 hours of in vitro incubation (Fig 2). We have no explanation for this observation, but it is not a characteristic of resistant patients because it was not seen in the case of patient 3.

The level of expression of the genes in the bone marrow of the two resistant patients was as high as in the in vitro IFN-α treated peripheral blood cells and is shown in Fig 3 for patient 3.

In conclusion, we have shown that the level of induction of the 10 IFN-inducible genes did not depend on the in vivo or in vitro nature of the IFN-α treatment in one responsive and two resistant patients. Thus, in vitro as in vivo, in healthy controls as in CML patients, peripheral blood and bone marrow cells showed a similar response to IFN-α.

**DISCUSSION**

In order to understand why some CML patients are resistant to the IFN-α treatment, we compared the induction by IFN-α of 10 IFN-inducible genes in the cells of IFN-α resistant CML patients, responsive patients, and healthy controls. Indeed, the resistance could be due to the absence of inducibility of one or more genes normally inducible by IFN-α and necessary for its biologic activities. By Northern blot analysis, we showed that an in vitro incubation of the peripheral blood PMN and mononuclear cells in the presence of IFN-α resulted in the induction of all 10 IFN-inducible genes studied in 6 out of 6 controls, 1 out of 1 responsive patient, as well as in 3 out of 3 resistant patients. The genes were also induced in the peripheral blood cells of 2 out of 2 resistant patients and in 1 of 1 responsive patient given an injection of IFN-α (in vivo induction). The bone marrow cells of the same two resistant patients also showed an expression of all 10 IFN-inducible genes after an injection of IFN-α.

The results from the in vitro studies showed that the cells of the resistant patients (inherent and late resistances) were able to respond to IFN. This means that they possessed IFN-α receptors. Thus, our results are in agreement with previous observations of Maxwell et al. and Rosenblum et al., indicating that the cells of both responders and nonresponders bind IFN-α to the same extent. Maxwell et al also reported that type I IFN receptors are downregulated after an injection of IFN-α and that their recovery took 2 to 7 days. As indicated in Table 1, we analyzed the cells 3 or 4 days after the last administration of IFN to induce cells that had recovered their IFN receptors. Because the level of induction of the genes was as high in these patients as in the controls and other patients, it seems that the cells had already recovered their receptors only 3 days after the last IFN dose.

The comparison of in vivo with in vitro inductions of peripheral blood cells of two resistant patients showed that the genes were induced similarly in both cases. This observation rules out the possibility that resistance to IFN in these patients was due to the presence of an antagonist of IFN-α or of neutralizing IFN-α antibodies, because these would have
decreased the response of the cells to IFN-α. However, we cannot rule out the possibility that the level of induction of the genes is higher in the in vivo than in the in vitro IFN treated cells of healthy controls, because IFN-α was not administered in vivo in the healthy controls.

Rosenblum et al. reported that in 67% of the responding patients, the 2-5A synthetase activity is increased up to 100-fold in their peripheral blood cells after an injection of IFN-α, but that in 70% of the nonresponders, no 2-5A synthetase activity was detected. According to their data, two of the three resistant patients we studied should not have any detectable 2-5A synthetase activity after an IFN-induction. The fact that we observe a normal 2-5A synthetase mRNA accumulation after an IFN-induction in the three resistant patients is, however, not necessarily in contradiction with their results. Three explanations can be proposed for this apparent discrepancy: (1) The gene could be transcribed normally, but the translation of its mRNA could be defective in the cells of the resistant patients. (2) It is possible that Rosenblum et al. measured principally the activity of the high-molecular-weight forms (69 to 100 Kd) of this enzyme, while we measured the mRNA level of the gene coding for the low-molecular-weight 2-5A synthetases (either 42 or 46 Kd). It could be that the high-molecular-weight forms of the enzyme are predominant over the low-molecular-weight forms in blood cells, or more active, or even that the conditions of the 2-5A synthetase activity test of Rosenblum et al. favored the activity of the high-molecular-weight forms of the enzyme. If any of these hypotheses are correct it would mean that the nonresponsive patients are deficient in the high-molecular-weight forms of the 2-5A synthetase, but that the low-molecular-weight 2-5A synthetase gene is normally inducible by IFN-α. (3) The difference between our results and those of Rosenblum et al could simply be due to the fact that none of the three resistant patients we studied belong to the 70% of the nonresponding patients, in whom the 2-5A synthetase is not induced by IFN. More responding and nonresponding patients have to be analyzed to clarify this point.

The conclusion that can be drawn from the present study is that the nonresponsiveness of CML patients to the IFN-α treatment, at least in the case of the three patients we analyzed, is not due to the absence of induction by IFN-α of any of the 10 IFN-inducible genes we studied, including the low-molecular-weight 2-5A synthetase. We cannot exclude the possibility that another IFN-inducible gene, not included in these 10 genes, could be noninducible by IFN in the cells of the resistant patients and be the cause of the resistance. However, the genes we studied here are among the most inducible by IFN and thus are probably important for the biologic activities of IFN. It is also possible that the response to IFN is unaltered in the leukemic cells of resistant patients. This would mean that IFN exerts its inhibitory effect on leukemic cells indirectly by acting on other cells, such as those involved in immune surveillance, and that IFN-α resistant CML patients could have a defect in the response of these cells to IFN-α.

ACKNOWLEDGMENT

We thank Drs A. Hovanessian, M. Karin, and J. Cornelius for providing us with the HLA class I gene, the metallothionein gene, and the rat glyceraldehyde-3-phosphate dehydrogenase gene, respectively. We also thank Boehringer Ingelheim for the recIFN-α2Arg.

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Analysis of interferon-inducible genes in cells of chronic myeloid leukemia patients responsive or resistant to an interferon-alpha treatment

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