Primary Leukemia Cells Resistant to α-Interferon In Vitro Are Defective in the Activation of the DNA-Binding Factor Interferon-Stimulated Gene Factor 3

By Bo Xu, Dan Grandér, Olle Sangfelt, and Stefan Einhorn

Cells from one-third of chronic lymphocytic leukemia (CLL) patients are resistant to α-interferon (α-IFN) as measured by induction of blast transformation. We have previously shown that all CLL clones express α/β-IFN receptors, but that the resistant cells are defective in the induction of the enzyme 2',5'−oligoadenylate synthetase (2',5'-A synthetase). Thus, the deficiency in IFN sensitivity is localized somewhere between the interaction of the IFN molecule with its receptor and induction of 2',5'-A synthetase. We have now further characterized the resistance of CLL clones to IFN by investigating whether it is associated with a defect in the activation of IFN-stimulated gene factor 3 (ISGF3), which is involved in the activation of α-IFN−stimulated genes (ISGs). A defect in induction of ISGF3 after α-IFN treatment was found in 4 of 12 CLL patients. There was a close correlation between defective induction of ISGF3 and a lack of enhancement of 2',5'-A synthetase as well as induction of blast transformation. Pretreatment with γ-IFN and mixing experiments with extracts from IFN-sensitive cells indicate that a lack of the γ-component of ISGF3 was the reason for defect in activation in 2 of the patients. We conclude that a defect in activation of ISGF3 is a possible cause for resistance in CLL cells to IFN-induced blast transformation in vitro.

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IMTERFERONS (IFNs) are a family of proteins that are produced by eukaryotic cells when challenged by viruses and other agents. Binding of IFNs to specific cell surface receptors elicits several physiologic responses in cells, including inhibition of viral replication, induction of differentiation and inhibition of cell proliferation.1

IFNs can also exert antitumor activity in a variety of tumors.2 Among the hematologic malignancies that have been shown to respond to IFN therapy are hairy cell leukemia,3 chronic myelogenous leukemia (CML),4 malignant lymphoma,5 myeloma,6 and chronic lymphocytic leukemia (CLL).7 Tumors from different patients vary greatly in their sensitivity to IFN therapy, with some being more or less susceptible and others being completely resistant. The reason behind the variation in IFN sensitivity is not known. We have previously shown that induction of blast transformation in CLL cells in vitro correlates with increased levels of intracellular 2',5'-oligoadenylate synthetase (2',5'-A synthetase) and that a low sensitivity is not caused by a lack of IFN receptors.8,9 A correlation between in vivo antitumor effects of IFN and in vitro induction of 2',5'-A synthetase has been shown in mid-gut carcinoid,10 CML,11 and lymphoma.12 These findings indicate that the reason why some tumor cells do not respond to IFN may be a defect in the signal transduction pathway between the occupation of IFN receptors and induction of α-IFN−stimulated genes (ISGs).

Activation of ISGs by IFN initiates multiple physiologic effects on cells.1 Transcription of ISGs is strongly associated with the activation of an IFN-stimulated DNA-binding protein complex, the IFN-stimulated gene factor 3 (ISGF3),13 which is activated through phosphorylation by the protein tyrosine kinases TYK2 and JAK1.14 After activation, ISGF3 translocates to the cell nucleus and binds to conserved regulatory sequences, the α−IFN−stimulated response elements (ISREs), present in all ISGs characterized to date, including 2',5'-A synthetase.15 Consequently, it triggers the transcription of the ISGs. Three established IFN-resistant cell lines have been shown to have a defect in the activation of ISGF3.16 However, IFN-resistant Burkitt's lymphoma cell lines that express EBNA2 have been shown to maintain an intact activation of ISGF3, but show a deficient induction of ISGs.17

In this study, we investigate whether IFN resistance in vitro of primary leukemic cells from patients with CLL can be caused by a deficiency in the activation of these DNA-binding proteins.

MATERIALS AND METHODS

Patients. Twelve patients with a diagnosis of chronic type B-lymphocytic leukemia were investigated. Diagnosis was established by morphologic and immunologic studies on peripheral blood, bone marrow, and lymph node samples according to the Kiel Lymphoma Study Group.18

IFN preparations. Recombinant α2−IFN (from Schering, Kenilworth, NJ) was derived from Escherichia coli. The specific activity of this preparation was 2.0 × 10^11 U/mg protein, and the purity was greater than 99%. E. coli−derived recombinant human γ−IFN (from Ernst-Boehringer-Institute für Arzneimittel Forschung, Vienna, Austria) had a specific activity of 2 × 10^7 U/mg protein and a purity of greater than 99%.

Cells and culture conditions. Heparinized peripheral blood from patients were centrifuged on a layer of Lymphoprep (Nycomed, Oslo, Norway). The interface was collected and washed. Cells from CLL patients with a relatively high proportion of nonmalignant cells after lymphoprep separation were depleted of monocytes and T cells. Monocytes were removed by adherence to plastic plates and by magnetic exclusion of iron powder phagocytizing cells. Erythrocyte rosetting T cells were depleted by incubation with neuraminidasedreated sheep red blood cells.19 After the various procedures, the B-cell populations were ≥95% pure, as tested by staining with the monoclonal antibodies B1-fluorescent isothiocyanate (FITC) (Coulter Immunology, Hialeah, FL) and/or Leu 4−FITC (Becton

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Dickinson, Immunocytometry Systems, Mountain View, CA). In control experiments, ≥5% contaminating nonmalignant cells did not influence the results from any of the parameters studied.

The cells were cultured at 37°C in a humidified 5% CO₂-air atmosphere with Eagle’s modified minimal essential medium supplemented with 10% heat-inactivated human AB+ serum, 1% L-glutamine, and antibiotics (50 μg/mL penicillin and 50 μg/mL streptomycin).

For experiments measuring blast transformation and 2',5'-A synthetase induction, the cells were, with few exceptions, used immediately after washing, whereas for the study of ISGF3, the cells were in most cases frozen in liquid nitrogen and used at a later stage. In repeated tests, the fresh and the frozen/thawed cells gave similar results in assays for blast transformation, 2',5'-A synthetase induction, and activation of ISGF3.

For the blast transformation test, 2 × 10⁵ cells were cultured in a volume of 1 mL in 5-mL round-bottomed tubes (Falcon 2058; Lincoln Park, NJ) in medium with or without IFN (5,000 U/mL) for 4 days. For the 2',5'-A synthetase test, 3 × 10⁵ cells in a volume of 0.2 mL were cultured in 96-well U-bottom tissue culture plates (Falcon 3077) for 24 hours in the absence and presence of IFN (5,000 U/mL).

For studies on ISGF3 activation, 50 to 100 × 10⁵ cells were cultured in 15 mL of medium in the absence or presence of 500 or 5,000 U/mL of α-IFN for 30 minutes (if not stated otherwise). In experiments testing the combined effect of γ-IFN and α-IFN, γ-IFN (100 U/mL) was added to the culture medium 16 hours before the addition of α-IFN and the cultures continued in the presence of both IFNs. In some experiments, cells were cultured in the presence of γ-IFN only. The ISGF3 complex was quantified visually as well as using scanning densitometry by comparison of the ISGF3 band and one of the constant bands.

The cell viability after 1 and 4 days of culture was determined by trypan blue exclusion and was found to be in the range of 77%

**Table 1. Blast Transformation, 2',5'-A Synthetase Levels and Activation of ISGF3 in Malignant Cells from 12 CLL Patients**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Day 0*</th>
<th>Day 4</th>
<th>2',5'-A Synthetase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No IFN*</td>
<td>IFN†</td>
<td>No IFN‡</td>
</tr>
<tr>
<td>1</td>
<td>ND</td>
<td>118 ± 6.6</td>
<td>1.16</td>
</tr>
<tr>
<td>3</td>
<td>82 ± 3.9</td>
<td>81 ± 4.0</td>
<td>1.22</td>
</tr>
<tr>
<td>5</td>
<td>68 ± 2.1</td>
<td>66 ± 2.5</td>
<td>1.21</td>
</tr>
<tr>
<td>6</td>
<td>98 ± 5.1</td>
<td>82 ± 4.3</td>
<td>1.29</td>
</tr>
<tr>
<td>7</td>
<td>91 ± 9.9</td>
<td>116 ± 9.3</td>
<td>1.06</td>
</tr>
<tr>
<td>8</td>
<td>97 ± 5.8</td>
<td>97 ± 6.1</td>
<td>1.18</td>
</tr>
<tr>
<td>11</td>
<td>81 ± 2.8</td>
<td>78 ± 2.2</td>
<td>1.19</td>
</tr>
<tr>
<td>12</td>
<td>78 ± 3.4</td>
<td>89 ± 3.2</td>
<td>1.04</td>
</tr>
<tr>
<td>2</td>
<td>82 ± 3.7</td>
<td>98 ± 3.5</td>
<td>0.90</td>
</tr>
<tr>
<td>4</td>
<td>90 ± 3.9</td>
<td>118 ± 3.5</td>
<td>1.03</td>
</tr>
<tr>
<td>9</td>
<td>82 ± 4.3</td>
<td>84 ± 3.0</td>
<td>0.99</td>
</tr>
<tr>
<td>10</td>
<td>105 ± 3.4</td>
<td>125 ± 8.6</td>
<td>0.91</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>87 ± 3</td>
<td>96 ± 6</td>
<td>1.10 ± 0.04</td>
</tr>
</tbody>
</table>

**Abbreviation:** ND, not done.

* Relative induction of blast transformation expressed as mean area of treated cells divided by mean area of untreated cells.

† Nanomoles of ATP incorporated per 5 × 10⁵ cells during 24 hours.

§ Relative 2',5'-A synthetase concentrations (nanomoles of ATP incorporated in cells incubated with IFN divided by nanomoles of ATP incorporated in cells incubated in medium alone).
to 100% (mean, 95%) after 1 day of culture in medium alone. After 4 days of culture, the cell viability was 60% to 100% (mean, 88%) for cells cultured in medium alone and 62% to 100% (mean, 93%) for cells cultured with α-IFN.

HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum and used as a positive control for ISGF3 in the protein-DNA binding assay.

Measurement of blast transformation. We have previously shown that IFN induces blast transformation in a proportion of CLL clones as measured by an increase in cell diameter, nuclear size, and cytoplasm/nuclear ratio as well as by the appearance of intracellular Ig followed by secretion of Ig.20,21 It was also found that measurement of cell size as described below correlates closely to the parameters above.22 For the assay, cells from duplicate cultures were centrifuged onto glass slides, stained with May-Grünwald-Giemsa and mounted with a coverglass. Codified samples were examined using the Videoplan image analysis system (Kontron, Munich, Germany) as previously described.22 The area of a cell was measured by image analysis at a magnification of 400×. The analysis system was calibrated by a micrometer scale. The microscopic fields were selected at random, and the contour of the whole cell was manually drawn. At least 100 cells/slide were randomly counted. Mean values of duplicates with standard errors of cell sizes were calculated.

Assay for 2',5'-A synthetase. The cytoplasmic concentrations of 2',5'-A synthetase were determined as described previously.8,9 Relative 2',5'-A synthetase induction was calculated as nanomoles of ATP per 6 × 10⁶ cells with IFN divided by nanomoles of ATP per 6 × 10⁶ cells without IFN.

Preparation of nuclear and cytoplasmic extracts. Nuclear and cytoplasmic extracts were prepared by a modification of the procedure of Dignam et al.23 The cells were collected by centrifugation, washed with cold phosphate-buffered saline (PBS), and swelled in RSB buffer (10 mmol/L Tris [pH 7.4], 10 mmol/L NaCl, 3 mmol/L MgCl₂, supplemented with 0.5 mmol/L DTT and 0.5 mmol/L phenylmethylsulfonyl fluoride [PMSF]) on ice for 15 minutes. The cells were then homogenized by sucking up and down in a 27 G syringe. Supernatants were obtained from cell homogenates by centrifugation at 15,000g for 10 seconds and then clarified at the same speed for 10 minutes. The resultant was a cytoplasmic extract. For nuclear extracts, homogenized cells were resuspended at 2 × 10⁷ nuclei per milliliter in buffer C (20 mmol/L HEPES [pH 7.9], 0.4%
RESULTS

α-IFN-induced blast transformation. Blast transformation, measured as mean cell area after 4 days of culture in the absence or presence of α-IFN (5,000 U/mL), was studied in 12 CLL patients. The cells showed a variable sensitivity to α-IFN-induced blast transformation (Table 1 and Fig 1). There was some variability in cell viability after 4 days of culture in medium alone, ranging from 60% to 100%. A slight correlation was shown between the IFN-induced blast transformation and the viability of cells cultured in medium alone (r = 0.66, P < 0.05). No significant correlation was observed between IFN-induced blast transformation and the viability of cells cultured with α-IFN.

Induction of 2′,5′-A synthetase. The intracellular levels of 2′,5′-A synthetase were measured in cells from all 12 CLL patients. There was a large variation between clones in sensitivity to IFN-induced enhancement of 2′,5′-A synthetase from a factor of 0.6 to a factor of 10.3 (Table 1). Baseline concentrations of 2′,5′-A synthetase also showed a wide range, from 11 to 588 nmol ATP per 6×10⁶ cells. Linear regression analysis showed no significant correlation between baseline 2′,5′-A synthetase levels and relative induction by IFN.

Cell viability after a 24 hours of incubation in vitro was greater than 90% in all patients except one (patient no. 4, 77%). Linear regression showed no correlation between induction of 2′,5′-A synthetase and cell viability.
treatment with IFN. In contrast to this, blast transformation was usually induced in samples with a high induction of 2',5'-A synthetase. However, linear regression analysis showed no statistically significant correlation between these parameters.

Activation of ISGF3. The activation of ISGF3 was studied in all 12 patients. In ISGF3-inducible CLL clones, ISGF3 appeared in both nuclear and cytoplasmic extracts, although the level of ISGF3 activity in the cytoplasm was lower than that in the nucleus (Fig 2A). Prolonged exposure of the autoradiograph was usually required to obtain distinguishable bands corresponding to the ISGF3 in cytoplasmic extracts.

In competition experiments using a mutated ISRE (see Materials and Methods), it was found that the top bands, presumed to be ISGF3 in the CLL extracts, were not competed by the mutated oligo, indicating that these bands are indeed ISGF3, whereas the band in the ISGF2 region was competed to some extent, indicating its identity as ISGF2 (Fig 2B).

To detect differences in the induction of ISGF3 by α-IFN at different concentrations, cells from 2 patients were treated by 50 to 5,000 U/mL of α-IFN. It was found that a maximal ISGF3 activity was found with 500 to 5,000 U/mL of IFN (Fig 3).

Kinetics of induction of ISGF3 by α-IFN was studied in 1 patient. ISGF3 was induced 30 minutes after treatment and a longer incubation time did not give a further increase in signal intensity (Fig 3).

Leukemic B cells from 8 of the 12 patients could be induced to generate ISGF3 activity after α-IFN treatment, whereas this was not seen in the 4 other patients (Table 1 and Fig 4). To ascertain whether the lack of ISGF3 activation in the negative clones was not caused by proteolytically cleaved protein extracts, 3 of the negative patients were run with a labeled oct-1 probe. All samples gave rise to clear shifts using this probe, showing the protein extracts to be intact in these patients (Fig 5).

The induction of ISGF3 was correlated to the induction of blast transformation and 2',5'-A synthetase. Clones in which ISGF3 activation was observed after α-IFN treatment showed a significantly higher induction of blast transformation (median 1.17 times increase) than did clones that showed no activation of ISGF3 (median 0.95 times increase) (P < .01, Fig 6).
The same was also true for a comparison between ISGF3 activation and enhancement of 2',5'-A synthetase, in which clones functional in ISGF3 activation showed a significantly higher induction (median 5.6 times increase) of 2',5'-A enzyme than did clones in which ISGF3 activation could not be detected (median 1.6 times increase) ($P < .05$, Fig 7).

**Effects of γ-IFN.** Because the level of the ISGF3 γ-component of the ISGF3 complex is a rate-limiting factor for its assembly, the possibility of a lack/downregulation of this factor in some clones was investigated. In initial experiments the combined treatment of γ-IFN and α-IFN was studied in the CLL cells. In α-IFN-sensitive clones, stronger ISGF3 bands appeared with the combined treatment than with α-IFN alone in most cases. In the clones in which α-IFN alone could not induce ISGF3 activity, γ-IFN pretreatment followed by α-IFN induced a clear induction of ISGF3 in the nuclear extract of 1 patient (patient no. 2, Fig 4). Faint ISGF3 bands could be seen in the cytoplasm as well (data not shown). CLL cells from patients no. 4, 9, and 10, showing no activation of ISGF3 by treatment with α-IFN alone, did not show any induction of ISGF3 with the combination of γ-IFN and α-IFN.

Mixing experiments using cytoplasmic extracts from α-IFN-sensitive cells treated separately with γ-IFN alone (as a source of the ISGF3 γ-component) were performed in the 4 patients in which ISGF3 was not activated when the cells were treated by α-IFN only. After mixing, a clear activation of ISGF3 was observed in patient no. 9 (Fig 8) and a weak activation was seen in patient no. 2. No activation was seen in patients no. 4 and 10 (data not shown).

**DISCUSSION**

α-IFN can induce blast transformation, differentiation, and proliferation of malignant B cells from patients with CLL.\(^8,9,20,21^\) However, there is an in vitro variability of IFN sensitivity in primary CLL cells from different patients. Whereas IFN induces blast transformation in approximately two-thirds of the CLL clones, no major effect is observed in the remaining one-third.
The major aim of this project is to establish the reason for IFN-resistance to blast transformation in CLL clones. In previous studies, the correlation between 2',5'-A synthetase induction and induction of blast transformation was investigated. It was observed that, although the cells expressed receptors for IFN, the clones resistant to IFN-induced blast transformation did not induce the enzyme 2',5'-A synthetase, an enzyme that has been implicated in the antiviral and cell multiplication inhibitory effects of IFN. We concluded that IFN-resistance in CLL clones was caused by a defect somewhere between binding to receptor and induction of 2',5'-A synthetase.

One of the primary responses of cells to IFN treatment is the transcriptional activation of a set of normally quiescent genes (ISGs), of which the 2',5'-A synthetase gene is one. The transcriptional activation of ISGs is preceded by the induction of the IFN-induced DNA-binding factor ISGF3. ISGF3 and the concomitant transcription of ISGs are induced by α-IFN within minutes of IFN-receptor binding without requirement of new protein synthesis. It has been shown that IFN-resistant established cell lines have a defect in the activation of ISGF3. However, resistance to IFN in EBNA2-expressing Burkitt’s lymphoma cells, coupled with an impaired induction of ISGs, has recently been shown to occur without impairment of ISGF3 activation. In this study, we raised the question of whether IFN-resistance in primary malignant cells also can be caused by a defect in ISGF3 activation. To evaluate this possibility, we used an in vitro model system with primary malignant cells from patients with CLL.

Primary leukemic cells from 8 of 12 patients with B-cell CLL were able to activate ISGF3 after α-IFN induction. An incapacity of α-IFN to induce activated ISGF3 was observed in four clones with a low or absent sensitivity to the actions of IFN with regard to 2',5'-A synthetase induction and blast transformation (Table 1).

These results suggest that activation of the transcription factor ISGF3 is important in the sensitivity of primary malignant B cells to IFN’s cellular effects. Defects in its activation, leading to resistance of malignant cells to IFN, seem to be a common event in primary CLL clones. In two of the CLL clones, clear induction of ISGF3 as well as blast transformation were observed concomitant with a relatively low induction of 2',5'-A synthetase (patients no. 1 and 3). The reason for this discrepancy is not known. Cells from these patients are no longer available, which makes it impossible to exclude the possibility of technical error and, if that can be excluded, to evaluate the mechanism behind this discordant finding.

Activated ISGF3 is a complex of four proteins (113, 91, 84, and 48 kD). The 113-, 91-, and 84-kD proteins comprise the ISGF3 α-component, whereas the 48-kD protein is termed the ISGF3 γ-component because of its induction by γ-IFN. ISGF3α, a preexisting cytoplasmic component, is activated through tyrosine phosphorylation by two α-IFN–activated tyrosine kinases, TYK2 and JAK1. Phosphorylated ISGF3α then associates with ISGF3γ to form a complex capable of high-affinity binding to the ISRE sequence and activation of ISG transcription. There might be several ways to explain the defect in ISGF3 activation, such as a decreased expression/defect of one or several of the subunits of the ISGF3 complex or blockage in the process of tyrosine phosphorylation of ISGF3α. The findings that pretreatment with γ-IFN as well as addition of extract from γ-IFN–treated cells, reconstituted the ISGF3 activity in 2 patients, indicate that the reason for resistance in a fraction of the resistant clones may downregulation or lack of a normal ISGF3 γ-component.

In addition to ISGF3, α-IFN induces another transcription factor termed ISGF2 or IRF-1. This factor shares the same binding sequence with ISGF3 and has been implicated in sustaining transcription or negative regulation of ISGs. Unlike ISGF3, induction and maintenance of this factor at a high level require new protein synthesis. Because the time of incubation used in this study was designed to detect activity of ISGF3, the conditions were not optimal to detect differences in levels of ISGF2 induction. Moreover, the bands seemingly corresponding to ISGF2 and the constitutively expressed ISGF1 migrated closely together under our experimental conditions (Fig 2). These facts are the reasons why we have not evaluated correlations between ISGF1 and ISGF2 levels to α-IFN sensitivity in the primary CLL cells.

In summary, our previous studies have shown that CLL cells that are resistant to the cellular effects of IFN, as measured by blast transformation, have a defect somewhere from the receptor-ligand interaction to the induction of the ISGs. In the present study, we show that clones that are resistant to IFN have a defective activation of ISGF3, a defect occurring in approximately 30% of the cases. We are currently further characterizing this defect.

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Primary leukemia cells resistant to alpha-interferon in vitro are defective in the activation of the DNA-binding factor interferon-stimulated gene factor 3

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