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# Chronic myeloid leukemia cells resistant to interferon-α lack STAT1 expression

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***Introduction***: Interferon-α (IFN) plays a role in the management of different neoplasias, particularly those of hematological origin. The mechanisms of action of IFN are still poorly understood and the individual response is unpredictable. In the present study, the pattern of intracellular gene expression following *in vitro* and *in vivo* exposure of chronic myeloid leukemia (CML) cells to IFN was evaluated and correlated with the response to *in vivo* treatment with IFN.

***Materials and methods***: CML patients in different phases of the disease were studied. The pattern of expression of two IFN-inducible proteins involved in IFN-mediated biological activities, the p91 and p84 proteins (STAT1α and STAT1β), components of the IFN-stimulated gene factor 3 (ISGF3) complex and the enzyme 2′-5′ oligoadenylate synthetase (2′-5′ OASE) were investigated by Western blot in peripheral blood mononuclear cells stimulated or not *in vitro* by IFN.

***Results and conclusions***: In 6/9 patients evaluated before starting treatment, STAT1 was expressed either constitutively or after *in vitro* stimulation by IFN. In three cases, STAT1 remained negative even after *in vitro* activation. The pattern of protein expression correlated with the subsequent hematological response to prolonged *in vivo* IFN administration: the presence of STAT1 being associated with the clinical response to IFN and the absence and non-inducibility of STAT1 with resistance to IFN. This was further substantiated by studies carried out in ten patients analyzed at the time of a documented clinico-hematological response or resistance to the *in vivo* administration of IFN. Finally, in order to establish whether the pattern of response to IFN treatment could be predicted at diagnosis, cells cyropreserved at diagnosis from patients with a documented complete response, confirmed also by cytogenetic negativity, or resistance, were studied. While complete responders proved STAT1 positive, none of the four resistant cases ever expressed STAT1. The expression of 2′-5′ OASE did not correlate with the clinical response to IFN. This study documents the pivotal role of STAT1 in the *in vitro* and *in vivo* responses of CML cells to IFN. The constitutive or induced presence or absence of STAT1 shows a predictive correlation with the response or resistance to treatment with IFN and could be utilized to identify, at diagnosis, resistant patients who may be spared an expensive and unnecessary prolonged IFN administration.
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***Keywords:*** CML; IFNα; STAT1; 2′-5′ OASE

## Introduction

Interferons are a family of proteins produced by eukaryotic cells infected by viruses or by other agents which have the capacity to interfere with viral reproduction and with other cell functions, such as cell growth, differentiation and immune response.[1] The two types of interferons use distinct cell surface receptors, designated a type I receptor for IFNα/β and type II receptor for IFNγ. Signal transduction from the cell surface receptor to the nucleus is then mediated by receptor-association Janus family tyrosine kinases (JAK) and STAT (signal transducer and activator of transcription) proteins. Briefly, IFNα/β receptor-associated JAK1 and Tyk2 tyrosine phosphor-

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ylate STAT1α and STAT1β proteins. Activated STAT1α and β bind to each other and, in combination with the p48 DNA-binding protein, form a complex called IFN-stimulated gene factor 3 (ISGF3). After translocation into the cell nucleus, this complex initiates transcription by binding to conserved IFN-stimulated response element sequence (ISRE) located within the promoters of IFN responsive genes.[2,3]

Interferon-α treatment induces a set of genes whose products are considered to be responsible for its biological actions. Among the IFN-inducible enzymes, 2′-5′ oligoadenylate synthetase (2′-5′ OASE) is composed of four isoforms with molecular weights ranging from 40 kDa to 100 kDa.[4] These enzymes catalyze the conversion of ATP into 2′-5′ A oligomers, which activate a latent ribonuclease, designated Rnase L. Although the antiviral activity of the 2′-5′ OASE/Rnase L system against a variety of RNA and DNA viruses is now well documented, a role in the control of cell proliferation and differentiation of hematopoietic cells has also been proposed.[4–7]

Interferon-α (IFN) has a well established role in the management of different tumors, particularly those of oncohematological origin. The mechanism(s) by which IFN exerts its anti-tumor activity and the reasons for the heterogeneous and unpredictable clinical responses remain poorly understood. Attempts have been made to correlate the pattern of receptor expression and intracellular gene activation (or non-activation) with the *in vitro* or *in vivo* responsiveness to IFN. However, no clear-cut correlation between susceptibility of cancer cell populations to IFN and a given transcriptional activation pattern has so far been reported. In the present study, we have utilized as a model chronic myeloid leukemia (CML), a disease in which IFN is capable of inducing hematological remissions[8,9] and, in a proportion of patients, also sustained partial and even complete cytogenetic responses.[10–12] Multicenter randomized studies have demonstrated that prolonged treatment with IFN induces more karyotypical responses than conventional chemotherapy, delays disease progression and prolongs the overall survival.[8,9,11] The administration of IFN is associated with three notable limitations: duration of treatment, individual toxicity and high costs. The latter have been estimated at about 200 times those of conventional chemotherapy.[11] Preclinical susceptibility tests aimed at recognizing patients likely to respond or to prove resistant to IFN would, thus, be of primary importance. By Western blot analysis we have investigated the pattern of expression of two IFN-inducible proteins involved in IFN-mediated biological activities – the p91 and p84 proteins (STAT1α and STAT1β), components of the ISGF3 complex, and the enzyme 2′-5′ OASE – in the peripheral mononuclear cells of a series of CML patients in different phases of their disease, in an attempt to answer the following questions: (1) Are the above markers activated following *in vitro* exposure of CML to IFN? (2) Does the activation occur *in vivo* following administration of IFN? (3) Is there a correlation between *in vivo* response or resistance to IFN and modulation of the expression of these IFN-induced proteins? (4) Can a documented clinical response or resistance to IFN be predicted by studying cells collected at diagnosis?

## Materials and methods

### *CML sample collection*

Peripheral blood samples were collected in preservative-free heparin from CML patients in different phases of their disease before and after treatment with IFN. Patients were considered as 'responders' in the presence of a complete hematological response, on the basis of a normal blood count (hemoglobin $>11$ g/l, platelets $<500\times10^9$/l, WBC $<10\times10^9$/l), of a differential leukocyte count devoid of immature cells and of a non-palpable spleen.[8] The cytogenetic response was defined as complete when no abnormal karyotypes were found in at least ten bone marrow metaphases analyzed.[8] Patients were defined as 'resistant' when no response was observed after six months of IFN administration at the maximum dose of $5\times10^6$/m$^2$.

Except for cases in which the study was performed on mononuclear cells cryopreserved at presentation (see below), all analyses were carried out on freshly drawn samples. Peripheral blood mononuclear cells, recovered following a Lymphoprep (Nycomed AS, Oslo, Norway) gradient separation, were washed twice in phosphate-buffered saline (PBS), resuspended in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS; HyClone, Cramlington, NE, USA), counted and further incubated, as detailed below.

### *Cell lines*

The human fibroblast cell line 2fTGH, which expresses ISGF3 and responds to IFN, and its mutant U3A, defective in the synthesis or activation of the α subunit of ISGF3,[13] were obtained from the laboratory of Sandra Pellegrini, INSERM U276, Institut Pasteur, Paris, France.

### *Culture conditions*

CML mononuclear cells and cell lines were incubated at 37°C in a 5% $CO_2$ humidified atmosphere with $1\times10^3$ U/ml of either natural (Wellferon, Glaxo Wellcome Spa, Verona, Italy) or recombinant (Intron, Shering-Plough Corp, Milano, Italy) IFN for 16 h. Control cultures without IFN were always set up and incubated for the same time period. Cultured cells were then washed twice in PBS, counted and pelletted for subsequent protein extraction (see below). Since no difference was observed in the degree of signal transduction activation induced *in vitro* by natural or recombinant IFN, the data

reported hereafter refer to results obtained with Wellferon (Glaxo Wellcome).

Retrospective studies on patients with a documented complete response or resistance to IFN were carried out by incubating, under the same culture conditions, peripheral blood mononuclear cells cryopreserved at diagnosis, thawed, washed and checked for viability.

### *Preparation of whole cell extracts*

Untreated and IFN-treated cells were collected following centrifugation and washed with cold PBS. Total cell extracts were prepared by lysis of cells in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS and 50 mM Tris (pH 8.0), supplemented with 1 mM phenyl methyl sulphonyl fluoride (PMSF) and 50 $\mu$g/ml of leupeptin), briefly sonicated and the cell debris pelletted.

### *Western blot analysis*

For Western blotting, 50 $\mu$g of proteins were separated on a 8.5% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) filter (Amersham International, Buckinghamshire, UK). The filter was then placed in a blocking buffer (5% low fat dry milk, 0.1% Tween 20 in PBS) overnight at 4°C. For STAT1 detection, filters were incubated with a monoclonal antibody (MoAb) (Transduction Laboratories, Lexington, Kentucky, USA) which recognizes both STAT1$\alpha$ and STAT1$\beta$ (p91 and p84 kDa proteins) diluted 1 : 2000 for 1 h at room temperature. After washing, a sheep anti-(mouse Ig)-horseradish-peroxidase conjugate was used as second antibody at a dilution of 1 : 3000 and immunocomplexes were then detected by enhanced chemiluminescence (ECL, Amersham).

For 2′-5′ OASE detection, a polyclonal antibody capable of recognizing all four human 2′-5′ OASE isoforms (p100, p69, p46, p40) was used.[14] This antibody was raised in sheep with a 20 aminoacid synthetic peptide corresponding to the position 284-303 of E16 and E18 cDNA.[4] The immunopurified sheep antibody was then used at 10 $\mu$g/ml in 1% bovine serum albumin-tris buffered saline-Tween (BSA-TBS-T) at 4°C for 18 h. Thereafter, filters were washed five times in TBS-T for 5 min and incubated with a peroxidase conjugate donkey anti-sheep Ig (Sigma Chemical Co, St Louis, MO, USA) diluted in 5% non-fat milk TBS-T for 1 h at room temperature. Filters were then washed five times in TBS-T and developed with the ECL system (Amersham).

### *Immunocytochemical method for morphological cell observation*

Cytospins from the 2fTGH cell line (and from the U3A mutant) and from CML samples were prepared with a concentration of $5 \times 10^4$ cells per slide, air dried overnight, wrapped in aluminum foil and stored at −20°C until immunostaining. The anti-STAT1 MoAb described above (Transduction Laboratories) was used at a final concentration of 5 $\mu$g/ml. The immunocytochemical reaction was performed with the APAAP technique (DAKO reagents, Glaustrup, Denmark). The 2fGTH and U3A cell lines were used as positive and negative controls, respectively. Five hundred cells were observed by light microscopy by oil immersion (magnification × 1000).

## Results

### *Case population groups*

Overall, 22 CML patients in different phases of their disease entered the study. The first group comprised patients who were studied prior to starting IFN and who were subsequently evaluated for response to the *in vivo* administration of IFN. In a proportion of cases, the *in vitro* analysis was also repeated after 15 days of IFN treatment. The second group was represented by patients who were studied during IFN administration at a time when a documented response to, or resistance to, IFN had been recorded. The third group was composed of patients with either a documented complete response, confirmed also by the negativity of the Ph chromosome, or with resistance to IFN from whom pathologic cells had been collected and cryopreserved at diagnosis. This allowed testing of whether the complete response or resistance to the *in vivo* administration of IFN could be predicted *in vitro* on pre-treatment material. In all cases, the population studied was represented by at least 70% leukemic cells.

### *Pre-treatment group*

The mononuclear cells of nine previously untreated CML patients were tested for STAT1 and 2′-5′ OASE expression prior to starting IFN. The results obtained are shown in Table 1. STAT1 was constitutively expressed in 4/9 samples tested: following 16 h *in vitro* exposure to IFN, the expression of STAT1 increased further in three of the four constitutively expressing cases (Nos 1–3, Table 1) and became positive in an additional case (No 4). In a further case (No 5) a weak p91 signal, not associated with a concomitant p84 isoform signal, was recorded after pre-incubation with IFN. Three cases, remained negative, but in one (No 7) the p84 isoform showed a weak expression. In two of the latter cases, a 2–48 h time course experiment was carried out in the presence of IFN and STAT1 always remained negative. The enzyme 2′-5′ OASE was constitutively expressed in two of the six samples tested; following incubation with IFN, 2′-5′ OASE expression increased in these two samples and two further cases not expressing basal 2′-5′ OASE levels (Nos 1 and 2) became positive. Representative cases are illustrated in Figure 1.

As planned, all nine patients were treated with IFN. Four patients were re-tested after 15 days of treatment

**Table 1** CML patients studied prior to starting IFN *in vivo*

| Patient | Initials | Age/Sex | WBC count at study ($\times 10^6$/ml) | p91/84 Control | p91/84 +IFN | 2′-5′ OASE Control | 2′-5′ OASE +IFN | Subsequent response to IFN treatment |
|---|---|---|---|---|---|---|---|---|
| 1 | SF | 58/F | 16.4 | + | ++ | – | + (p100) | Responder |
| 2 | OE | 65/F | 16.4 | + | ++ | – | + (p69) | Responder |
| 3 | CM | 27/M | 6.6 | + | ++ | + | ++ (p100, p69) | Stopped IFN for toxicity |
| | | | | | | – | + (p46) | |
| 4 | CD | 20/M | 30.0 | – | + | nd | nd | Responder |
| 5 | CG | 61/M | 24.2 | – | ± (p91) | + | ++ (p69, p40) | Stopped IFN for toxicity |
| 6 | GD | 64/F | 15.9 | – | – | – | – | Resistant |
| 7 | VA | 51/F | 170.0 | – | – (p91) ± (p84) | nd | nd | Resistant |
| 8 | BA | 50/F | 8.9 | – | – | – | – | Resistant |
| 9 | SM | 38/M | 4.4 | + | + | nd | nd | Responder |

with IFN. As shown in Table 2, cases Nos 1 and 2 did not modify their pattern of activation, while in case No 4 the *in vivo* administration of IFN induced the expression of STAT1. In all cases, *in vitro* incubation with IFN was still capable of increasing further, or of promoting (in the case of 2′-5′ OASE), the expression of STAT1 and of 2′-5′ OASE. Case No 8 remained persistently negative for STAT1 and 2′-5′ OASE, both constitutively and after *in vitro* incubation with IFN.

The nine patients treated with IFN were subsequently evaluated for response: four showed a complete hematological response, two had to stop IFN early due to toxicity and three were resistant (Table 1). When the pattern of expression of the two IFN-inducible proteins observed prior to starting IFN was correlated with the clinico-hematological response to treatment, STAT1 was expressed constitutively in three of the four responsive patients (Nos 1, 2, 4 and 9) and in all four after *in vitro* incubation with IFN. In both cases tested, 2′-5′ OASE was expressed in IFN stimulated cells. In none of the three patients resistant to IFN (Nos 6, 7 and 8), was STAT1 found to be constitutively expressed. Following *in vitro* stimulation with IFN, p91 remained negative in all three cases. The p84 isoform was absent in two cases, while a weak signal was seen in one (No 7). In two of the three cases tested, 2′-5′ OASE was also negative and non-inducible. Case No 8 was re-tested after 15 days of treatment with IFN and still proved completely negative (Table 2).

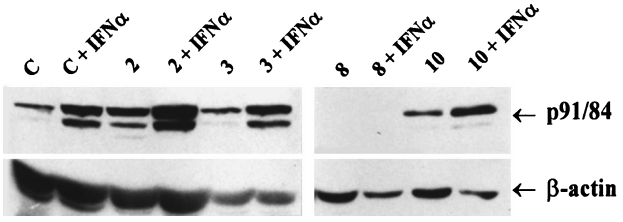


**Figure 1** Western blot analysis of STAT1 expression in peripheral blood mononuclear cells from representative CML patients without or with *in vitro* stimulation with IFN. C = normal peripheral blood mononuclear cells. Numbers represent individual cases, as detailed in Tables 1 and 3. The upper band refers to p91 and the lower band to p84

**Table 2** CML patients studied after 15 days of *in vivo* administration of IFN

| Patient | p91/84 Control | p91/84 +IFN | 2′-5′ OASE Control | 2′-5′ OASE +IFN | Subsequent response to IFN treatment |
|---|---|---|---|---|---|
| 1 | + | ++ | – | + | Responder |
| 2 | + | ++ | – | + | Responder |
| 4 | + | ++ | + | ++ | Responder |
| 8 | – | – | – | – | Resistant |

## *Treated group*

Ten CML patients still on treatment with IFN and with a documented response or resistance to IFN were studied (Table 3). One (No 8) had also been tested prior to starting treatment, as well as after 15 days of IFN. Eight patients were responsive to IFN and in all of them the prolonged administration of IFN had induced the expression of STAT1. By contrast, in both resistant cases (Nos 8 and 11), STAT1 was neither present nor inducible upon further *in vitro* stimulation with IFN. The expression of 2′-5′ OASE correlated less with the response to IFN, being expressed or induced in 7/8 responsive cases and in 1/2 resistant cases. Representative cases are shown in Figure 1.

## *Retrospective analysis according to response to treatment*

Six patients with a well documented response or resistance to IFN could be studied retrospectively on cells cryopreserved at diagnosis (Table 4 and Figure 2). This allowed us to evaluate whether an analysis carried out at diagnosis could bear predictive relevance. For this purpose, and according to stored cell availability, two patients who had shown a complete response to IFN, confirmed also by the Ph negativity, and four patients resistant to prolonged treatment were investi-

**Table 3** CML patients studied during treatment with IFN and correlated with response to therapy

| *Patient* | *Initials* | *Age/Sex* | *WBC count at study* | *Months of IFN* | *Dose of IFN at study (MU)* | *p91/84* | | *2′-5′ OASE* | | *Response to IFN treatment* |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *Control* | *+IFN* | *Control* | *+IFN* | |
| 8 | BA | 50/F | 12.1 | 2.5 | 5 | – | – | – | – | Resistant |
| 10 | DME | 61/F | 5.8 | 8 | 6 | ++ | ++ | ++ | ++ (p69,p40) | Responder |
| 11 | PB | 56/F | 17.2 | 8 | 9 | – | – | + | + (69) | Resistant |
| | | | | | | | | + | ++ (p40) | |
| 12 | TCA | 47/M | 3.5 | 16 | 1.5 | ++ | ++ | ++ | ++ (p69) | Responder |
| 13 | CM | 26/M | 3.1 | 12 | 1.5 | ± | ± | – | + (p69) | Responder |
| 14 | DIG | 40/M | 3.7 | 33 | 4.5 | + | ++ | ± | + (p69) | Responder |
| 15 | CA | 26/M | 5.0 | 11 | 12 | ± | ± | – | – | Responder |
| 16 | CM | 48/M | 5.5 | 18 | 3 | + | nd | + | nd | Responder |
| 17 | DSA | 34/F | 3.6 | 34 | 5 | + | ++ | + | ++ | Responder |
| 18 | LP | 39/M | 4.8 | 21 | 5 | ++ | ++ | ++ | ++ | Responder |

**Table 4** Retrospective study on CML cells cryopreserved at diagnosis in six patients with documented complete response or resistance to IFN treatment

| *Patient* | *Initials* | *Age/Sex* | *p91/84* | | *Response to IFN treatment* |
|---|---|---|---|---|---|
| | | | *Control* | *+IFN* | |
| 16 | CM | 48/M | + | ++ | Complete (Ph-) |
| 18 | LP | 39/M | ++ | ++ | Complete (Ph-) |
| 19 | DMA | 28/M | – | – | Resistant |
| 20 | SA | 38/M | – | – | Resistant |
| 21 | EK | 25/F | – | – | Resistant |
| 22 | MD | 43/M | – | – | Resistant |

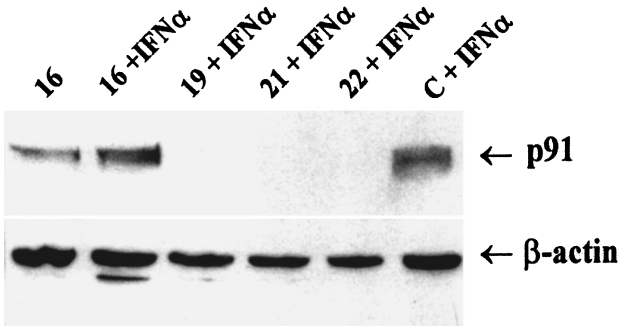


**Figure 2** Western blot analysis of STAT1 expression in peripheral blood mononuclear cells cryopreserved at diagnosis from CML patients responsive (No 16) or resistant (Nos 19, 21, 22) to the *in vivo* administration of IFN. C = normal peripheral blood mononuclear cells. The single band refers to p91 only

gated for the expression of STAT1. As shown in Table 4, cells from both complete responders expressed STAT1 constitutively and upon *in vitro* activation with IFN. By contrast, in none of the four resistant cases was STAT1 ever expressed, either constitutively or upon *in vitro* stimulation with IFN.

### *Clinical follow-up of the patients studied*

When the overall clinical course was evaluated, it was found that within the eight IFN resistant patients – all of whom were p91 negative, while one showed a weak p84 signal upon *in vitro* IFN activation – four subsequently responded to hydroxyurea, two entered an accelerated phase and two a blast crisis. Of the 12 responders, all of whom expressed STAT1, only one has so far proceeded to an accelerated phase. The clinical follow-up of the remaining two patients could not be correlated with the response to IFN since the latter had to be discontinued early due to toxicity.

### *Morphological cell observation*

Upon anti-STAT1 immunostaining, 2fTGH cells demonstrated an intracytoplasmic positivity for STAT1, while the mutant U3A cells were completely negative (Figure 3a,b). In 2/3 cases studied, primary CML cells showed an anti-STAT1 positivity that increased further following *in vitro* stimulation with IFN (Figure 3c,d). Leukemic cells from the last patient were negative both constitutively, as well as after *in vitro* IFN stimulation. These findings correlated well with the Western blot data and with the clinical response to the *in vivo* administration of IFN.

## Discussion

The possibility of correlating the clinical response to IFN of patients with various neoplastic conditions to *in vitro* tests with potential predictive value has been a long-sought goal. This is of particular relevance in view of the heterogeneous response to IFN and of the knowledge that in all patients the responses are slow, requiring a prolonged administration period which often is in excess of 1 year. This limiting factor is associated with the evidence that treatment with IFN is not devoid of individual toxicity and that the costs for an adequate IFN-based protocol are extremely high. For these reasons the possibility of identifying, prior to starting treatment, potential responsive or resistant patients would be of utmost relevance. Despite a progressively more refined understanding of the mechanisms of action of IFN, particularly at the gene regulation level, so far no conclusive correlation has been established between *in vitro* data and *in vivo* responses. It has been suggested that, in patients with

CML, resistance to IFN treatment may be associated with a defective induction of 2′-5′ OASE activity.[5] Some degree of correlation between *in vitro* 2′-5′ OASE activity and clinical response to IFN has been reported for hairy cell leukemia patients.[6] It has also been shown that B-cell chronic lymphocytic leukemia (B-CLL) cells resistant *in vitro* to IFN display deficient levels of 2′-5′ OASE.[7] On the other hand, the expression of the 2′-5′ OASE gene, as well as of the other nine IFN-inducible genes, studied by Northern blot did not correlate with the clinical response to IFN.[15] Over the past few years, transcriptional activation signals induced by interferons have been analyzed. In CML, it has been shown that cellular protein extracts can alter the electrophoretic mobility of complexes formed between nuclear proteins and IFN-inducible transcriptional enhancers, and that this effect can be reduced following exposure of CML cells to IFN.[16] The sensitivity of cells to the *in vitro* IFN-induced changes was observed in virtually all responders to the *in vivo* administration of IFN and not in IFN-resistant patients. B-CLL clones resistant *in vitro* to IFN have a defective activation of ISGF3.[17] Utilizing as a model the B lymphoma cell line Daudi, it could be shown that, at variance from the parental cells, no ISGF3 could be detected in an IFN-resistant clone of Daudi cells and that only low levels could be induced upon incubation with IFN.[18] More recently, it has been reported that human solid tumor cell lines of different origin may be defective in the IFN-induced formation of ISGF3.[19] Non-responsiveness was correlated with an inhibitory activity characterized as a 19 kDa protein capable of preventing binding of ISGF3 to DNA and thus of enabling IFN to exert its antiproliferative effect. Finally, Fisher *et al.*[20] have demonstrated by Northern blot analysis that the *in vitro* and *in vivo* treatment of CML cells with IFN leads to an increased expression of different IFN-dependent genes, including 2′-5′ OASE, but that this appears to be independent from the response to IFN treatment.

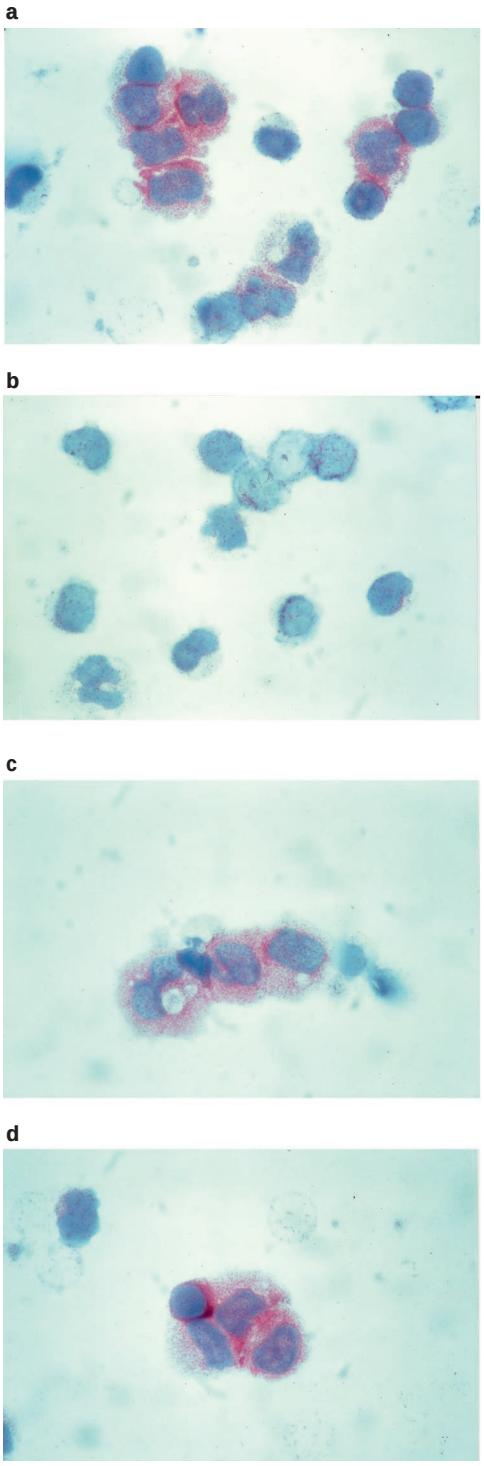


**Figure 3** Immunostaining of intracytoplasmic STAT1 in the 2fTGH (**a**) and U3A (**b**) cells lines, and in CML cells before (**c**) and after (**d**) treatment *in vitro* by IFN

In the present study, the expression pattern of STAT1 and 2′-5′ OASE was investigated by Western blot analysis after *in vitro* and *in vivo* exposure to IFN. The results obtained allow a further understanding of the intracellular signal transduction pathways which occur in CML cells and enable investigators to draw a relationship between the pattern of activation and the response to IFN treatment. First of all, the STAT$\alpha/\beta$ protein – which is rapidly activated by a post-transcriptional mechanism upon IFN stimulation of target cells,[2] though its expression can be also stimulated transcriptionally by IFN leading to an increase in protein levels – appears to play a pivotal role in the process of IFN-induced gene activation in CML cells, as in normal cells.[21,22] The pattern of expression in untreated cases is heterogeneous. Constitutively, CML cells either can or cannot express STAT1; upon *in vitro* activation with IFN, positive cases usually increase STAT1 expression, while STAT1 negative cases either become positive or remain persistently negative. When the pattern of expression was correlated with the *in vivo* response to IFN, it was found that all evaluable patients in whom STAT1 was expressed either constitutively or upon *in vitro* activation responded to IFN treatment. On the other hand, all patients in

whom STAT1 remained consistently negative were resistant to the *in vivo* administration of IFN.

These findings were further confirmed when the analysis was carried out in patients who were on treatment with IFN and in whom a documented response or resistant to IFN had already been documented. All responsive cases expressed STAT1, while the two resistant patients were negative both constitutively and upon further *in vitro* incubation with IFN. It should be noted that case No 8 was tested repeatedly and always proved STAT1 negative. The role played by the STAT1 complex gains further indirect confirmation by the evidence that STAT1 could be induced in all responsive patients treated *in vivo* with IFN. The lack of STAT1 induction accompanied by the lack of response to IFN treatment can be due either to the absence of the IFN receptors on the leukemic cells, thus preventing IFN binding, or to a mutation in the STAT1 complex that impairs its induction upon IFN treatment. Though at present we cannot fully clarify the underlying defect occurring in a proportion of CML patients, it should, however, be recalled that the absence of p91 and p84, the two proteins that constitute STAT1, in human fibroblast mutant cell lines prevents *in vitro* gene induction and responsiveness to interferons.[13,21] Complementation of such mutants with cDNA constructs expressing the p91 or the p84 kDa product restored the response to interferons.[21] Furthermore, it has been documented that the mutant U3A cells, which are defective in STAT1 and do not respond to interferons, become susceptible to the inhibitory action of IFN following reintroduction of STAT1$\alpha$.[21] The results of our study represent the first demonstration that a defective expression of STAT1 can take place in human primary neoplastic cells and that a strict correlation exists between STAT1 expression and response to IFN.

Unlike STAT1, the role of 2′-5′ OASE appears to be less closely correlated with the response of CML patients to IFN treatment, as already suggested by Northern blot analysis.[20] In fact, in both the responsive and resistant subgroups cases with a constitutive or stimulated presence or absence of 2′-5′ OASE were found.

Taken together, these results indicated a stringent association between expression of STAT1 and susceptibility or resistance to IFN treatment in CML. This was, however, based on analyses carried out pre-treatment and correlated with the subsequent clinico-hematological response or on patients studied whilst on treatment. In order to define the pre-clinical predictive value of STAT1 expression, we evaluated retrospectively cells cryopreserved at diagnosis, prior to starting treatment, from two patients who had shown a complete response to IFN, documented also by the cytogenetic disappearance of the Ph chromosome, and from four patients with a complete resistance to IFN. The results obtained showed a total concordance between response to treatment and pre-clinical *in vitro* studies, since STAT1 was present in both responsive patients and consistently absent, both constitutively and upon *in vitro* activation, in all four resistant patients.

By using the known positive and negative cell lines 2fGTH and U3A,[13] we showed that an immunocytochemical technique can be used to evaluate the presence of STAT1 in the cells. This method allows the localization of the protein and the morphological identification of the positive and negative cells. In the three CML samples studied at presentation, two positive and one negative for STAT1 expression, we found a concordance between Western blot and immunostaining analyses. This finding confirmed that STAT1 expression revealed by Western blot was of leukemic origin. In prospect, the immunocytochemical method could prove valuable for a more refined monitoring of CML patients by clarifying whether, at diagnosis, there is a mosaic of STAT1 positivity that could help to explain the heterogeneous response to IFN and whether the prolonged administration of IFN may generate resistance through a selection of STAT1 negative CML cells.

In order to be a reliable marker of response to IFN, a cellular protein should have the following properties: (i) be the main transducer of the IFN signaling pathway and be constantly expressed upon IFN treatment; (ii) be quickly induced following IFN binding to the cognate receptor, and (iii) be easily detectable in treated cells. Our studies demonstrate that STAT1, rather than 2′-5′ OASE, can be exploited as a suitable marker to monitor and predict response to IFN. The predictive value of the positive or negative STAT1 expression by CML cells treated with IFN needs to be conclusively validated in a larger prospective study.

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