Interferons (IFN) and retinoids failed to inhibit the growth of a number of breast tumor cell lines. However, a combination of these two biological response modifiers significantly suppressed the cell growth at pharmacologically achievable doses. The molecular basis for such enhancement was investigated in MCF-7, a breast tumor cell line resistant to growth inhibition by IFN-β. Pretreatment of cells with retinoic acid (RA) for 16 h followed by IFN-β, but not the converse, induced cytotoxic effects. Continuous presence of RA was not necessary, although it enhanced the degree of cell death when present. Further analyses revealed that IFN-β failed to activate IFN-stimulated gene transcription. However, IFN-β strongly up-regulated the gene expression in RA-pretreated cells. Both IFN-β- and IFN-γ-inducible gene expression were enhanced via a modulation of the transcriptional factor IFN-stimulated gene factors-3 and GAF binding to respective cognate regulatory elements. STAT1 was undetectable in these cells prior to RA treatment. RA increased the levels of this crucial regulator, thereby restoring IFN responses. Thus, RA augmentation of STAT1 may be an early step in the cooperative anti-tumor effects of IFN and RA.

Interferons are a group of multifunctional cytokines that regulate cellular antiviral, anti-tumor, and immunological responses (1, 2). Transcription of IFN-α-stimulated genes (ISGs) is up-regulated in a transient manner upon treatment of cells with IFNs (1, 2). Products of ISGs carry out the biological effects of IFNs (2). Type I (α, β) and type II (γ) IFNs employ distinct receptors for eliciting cellular responses (2). Genetic and biochemical approaches identified molecular pathways that regulate ISG expression (Ref. 3 and references therein). IFN-α/β-regulated genes are regulated through a promoter motif called the IFN-stimulated response element (ISRE) (3).

IFN-γ stimulates ISGs using a wide variety of elements (2). Foremost of these is the γ-IFN-activated site (GAS). A primary regulatory factor, ISGF-3 (IFN-stimulated gene factor-3), is essential for gene induction by IFN-α/β. ISGF-3 is a hetero-meric factor consisting of four proteins: p48, p84, p91, and p113. Cells lacking this factor or its components fail to respond to IFNs (3). Binding of IFN-α/β to their receptor(s) activates two nonreceptor protein tyrosine kinases, JAK1 and TYK2, that rapidly phosphorylate cytoplasmic p84, p91, and p113, also known as STAT (signal transducing activators of transcription) proteins, at specific tyrosine residues (3). These phosphoproteins migrate to the nucleus and associate with p48, the major DNA binding protein, to induce gene expression. p91, p84, and p113 are known as STAT1α, STAT1β, and STAT2, respectively (3). Ligand-bound IFN-γ receptor associates with two protein tyrosine kinases, JAK1 and JAK2, that subsequently phosphorylate STAT1α. This protein migrates to the nucleus, binds to GAS or GAS-like elements, and stimulates transcription (3). Studies with mutant cells have shown that in a cell mutant lacking STAT1, expression of STAT1α alone restores normal IFN-α/β and IFN-γ responses. Thus, JAK1 and STAT1 are shared components for all IFN signaling pathways (3).

Retinoids are a group of vitamin A-related compounds with profound influences on cell growth and differentiation (4). A prototype of these is all-trans-retinoic acid (RA), a natural metabolite. Upon entry into cells, RA binds to nuclear retinoid acid receptors (RAR), which then associate with RA response elements (RARE) to stimulate target gene expression (5). Multiple RAR isotypes such as α, β, and γ and corresponding subtypes are present in the cells that create a complex pattern of gene regulation. A novel receptor, retinoid X receptor (RXR), is preferentially activated by 9-cis-RA. Similar to the RARs, three isotypes of RXRs and corresponding subtypes have been described (5). RXRs heteromerize to serve as auxiliary activators for RARs, vitamin D3 receptor, and thyroid hormone receptor and regulate ligand-specific gene expression (5). Retinoids suppress the growth of several tumor cells in vitro and in vivo (5, 6). However, mechanisms of growth suppression are not clearly understood.

Although IFNs and retinoids have been shown to suppress the growth of certain tumor cells, a number of tumor cells are not inhibited by these single agents (6, 7). However, a combination of these agents produces significant additive or synergistic anti-tumor activity (7, 8). It is not clear how these two different ligands, of which one uses cytoplasmic STATs (3) and the other which employs nuclear receptor-transcriptional factors (5), cross-talk in the regulation of cellular anti-tumor responses. In an effort toward this direction, we analyzed the
Retinoid Enhancement of IFN Action

In this investigation, using pharmacologically achievable doses of IFN-β and RA, we show that growth of an IFN-unresponsive breast tumor cell is significantly suppressed by such combination. Growth suppression is correlated with an enhanced transcriptional induction of ISGs in the presence of RA. Transcriptional enhancement is due to an increase in the STAT1 levels in RA-treated cells.}

**EXPERIMENTAL PROCEDURES**

Reagents—Human IFN-β was a gift of Berlex Inc. Murine IFN-β was from Lee Biomolecule Inc. All-trans-retinoic acid (RA) was purchased from Sigma. Rabbit anti-STAT1 and anti-STAT2 sera were provided by Chris Schindler, Columbia University, New York. Rabbit anti-48 serum was a gift from David Levy, New York University, New York. Mouse anti-JAK2 antibody (Transduction Laboratories, Lexington, KY) was a gift from Ricardo Feldman, University of Maryland, Baltimore. pRM-Luciferase (9) and other ISGs were described in our previous studies (10). Tamoxifen-resistant MCF-7 cell line and polyoma virus-transformed murine breast tumor cells were provided by Joseph Fontana and Robert Freund of University of Maryland at Baltimore, respectively. STAT1 DNA-plasmid was used under the control of chicken β-actin promoter of the mammalian expression vector pCXN2 (11) was a gift from Keiko Ozato, National Institutes of Health. This vector also carried a neomycin resistance marker for the selection of stably transfected cells.

Cell Culture and Stable Transfection—Cells were cultured in Eagle's minimal essential medium supplemented with 5% charcoal-stripped calf serum. Experiments with MCF-7 breast tumor cells were performed in phenol red free media containing 10−11 M estradiol. MCF-7 cells were transfected either with the vector alone or with the STAT1 expression construct by electroporation. Selection was initiated with 1 mg/ml G418 (Life Technologies, Inc.) on the second day and continued for 25 days until drug-resistant colonies appeared. The resultant colonies were then pooled, grown further, and used in the experiments (12). Expression of STAT1 was confirmed by Western blot analysis, and its functional activity was assessed by IFN-inducible expression of a reporter gene (see below).

Growth Inhibition Assays—Cells (4 × 10⁴) were cultured in phenol red free media containing 5% charcoal-stripped calf serum in 96-well plates as indicated above. Various concentrations of IFN-β and RA were added to the cultures and incubated for 4–5 days. Cells were washed and fixed with 10% trichloroacetic acid for 1 h and then stained with sulforhodamine B in 1% acetic acid for 1 h. After washing, the dye bound to the cells was released with 100 μl of 10 μM Tris-HCl, pH 10, and absorbance at 570 nm was measured. A control plate to determine the initial seeding intensity was also included except that the cells were fixed with trichloroacetic acid immediately. Absorbance obtained with this method directly correlated with cell counts obtained with a Coulter counter.

Transcriptional Analyses—Northern blot, nuclear run-off transcription, and cell transfection assays were performed as described previously (10). Cells were transfected with 10 μg of plasmids as described earlier. After 40 h, they were treated with RA (1 μM) for 12 h where indicated and then with IFN-β (100 units/ml) for 16 h. Cells extracts were prepared, and luciferase assays (12) were performed. Transfection efficiency among different samples was monitored by assaying for galactosidase activity. Chloramphenicol acetyltransferase (CAT) assays were performed as described earlier (10).

Western Blot Analysis—Total protein (70 μg), after various treatments, was analyzed in Western blots (12). Blots were incubated with the indicated primary antibody. After stringent washing, they were incubated with appropriate secondary antibody and developed using an ECL kit (Amersham).

Electrophoretic Mobility Shift Assays (EMSA)—These assays were carried out as described earlier (10). To confirm the specificity and identity of the complexes, competition with specific unlabeled oligonucleotides and supershifting of the complexes with specific antibodies to ISGF-3 proteins were performed. Synthetic double-stranded oligonucleotides of GAS and pRE (from ICSBP gene) were a gift from Keiko Ozato, NIH. 32P-Labeled probes of these oligonucleotides were prepared essentially as described earlier (14).

**RESULTS**

**Retinoid Acid and Interferon-β Combination Inhibits Cell Growth and Causes Cell Death in Several Breast Tumor Cell Lines**—To study the effect of IFN, MCF-7 (a breast tumor cell line) was incubated with varying amounts of IFN-β and scored for growth using sulforhodamine staining (13). Various concentrations of IFN-β were added to the cells and incubated for 5 days. IFN-β failed to inhibit the growth of these cells (Fig. 1A, bars 2-4). Similarly, RA had very little growth inhibitory effect on these cells, although at 1 μM it inhibited growth by only 15% (data not presented). To study whether combination of RA/IFN-β would have any effect on these cells, we incubated these cells with either IFN-β, RA, or a combination of both. Fig. 1B shows the data obtained in such experiments. IFN-β (50 units/ml) or RA (0.1 μM) had little effect on the growth of the cells. In the presence of these single agents only 5% of growth was suppressed (bars 2 and 3). These results are consistent with previous observations from MCF-7 and other tumor cell lines (15) that demonstrated failure of IFN-β or RA to inhibit growth. However, upon combination of these drugs, >80% of the growth was inhibited (Fig. 1A, bar 4). We tested a series of RA and IFN-β concentrations and found that at every concentration tested the combination had significant growth inhibitory activity compared with individual agents (data not presented). Median effect analysis (16) of these data revealed a synergistic growth suppressive activity of IFN-β/RA. Furthermore, we observed cell death at high concentrations within 70–80 h post-treatment (see below). These results were generally consistent with other studies in MCF-7 cells. Our results differ from the previous study (15) in the following respects. 1) Low doses of IFN-β/RA were sufficient to inhibit the growth. 2) Cell death was consistently observed in 3–5 days at pharmacological doses. Although a different study has shown growth inhibitory action of RA alone in MCF-7 cells (17), we observed only marginal effects on cell growth by RA. These differences could be due to use of different methods, duration of drug exposures, fetal bovine sera, types of IFN, and the types of media containing phenol red, which is known to function as an estrogen (18), in the previous studies.

We next examined whether continuous presence of RA was required for growth inhibition. In these experiments, we incubated cells with 1 μM RA for 16 h and then removed it. Cells were washed with serum-free medium and incubated in the
presence of various doses of IFN-β. As shown in Fig. 2, RA pretreatment followed by IFN-β significantly suppressed the growth (Fig. 2, bars 2-4). As little as 10 units/ml IFN-β was sufficient to inhibit growth in these cells by 70% (bar 2). At 100 units, cell death was noticed (bar 3). These data on the negative scale indicate the loss of originally plated cells. A higher extent of cell death was noticed upon treatment with 1000 units of IFN-β (bar 4). Continuous presence of RA during IFN treatment had pronounced effects on the cells compared with pretreatment and its withdrawal during IFN treatment (bars 5-7). Under these conditions, 10 units of IFN-β, 1 μM RA caused cell death (bar 5). At higher doses of IFN-β with a similar dose of RA, more cell death was observed as compared with pretreatment controls (compare bars 2 and 3 to bars 5 and 6). Thus, continuous presence of RA was not necessary; however, it enhanced the cytotoxic effects when present. We performed a similar experiment in which cells were pretreated with 500 units of IFN-β for 16 h, washed, and incubated with 1 and 4 μM RA for the same length as in bars 2-7. No significant growth inhibition was noticed (see Fig. 2, bars 8 and 9). While RA pretreatment makes cells conducive to the growth inhibition of IFN, IFN pretreatment followed by RA did not. Thus, a specific interaction between IFN and RA mediates the arrest of cellular growth.

It was also of interest to determine whether growth inhibition could also be exerted in estrogen receptor negative breast tumor cells treated with IFN/RA combination. In these experiments, we employed the BT-20 breast tumor cell line that lacks estrogen receptor. IFN-β alone inhibited the growth in these cells by 35% (Fig. 3). RA by itself failed to inhibit the growth even at higher doses (data not presented). However, upon cotreatment with these agents, cell death occurred (Fig. 3, bar 4). These doses were identical to the ones described under Fig. 1A. Since these continuous cell lines may have genetically or epigenetically drifted from primary tumors, we tested the effects of the drug combination in several polyoma virus-induced primary murine breast tumor cells (Fig. 4). As observed with human cell lines, growth of these cells was not suppressed by murine IFN-β ranging from 10 to 1000 units/ml (data not presented). RA itself had little effect on these cells. However, the combination treatment of 10 units of IFN-β and 0.1 μM RA suppressed >80% of cell growth (Fig. 4, bar 4). Cell death was noticed upon increment of either IFN-β or RA doses in combination or prolonging the time of treatment (data not shown). Furthermore, we also noted synergistic growth inhibition by RA/IFN in an MCF-7 cell line resistant to tamoxifen (~10 μM) (Fig. 5). As observed with parent MCF-7 cells (Fig. 1, A and B), neither IFN (Fig. 5, bars 2 and 3) nor RA (Fig. 5, bars 4 and 5) were effective inhibitors, but the combination strongly suppressed its growth (bars 6 and 7).

RA Pretreatment Enhances the Gene Induction by IFN-β—Using the above described assays, we determined that RA (1 μM) pretreatment for 12 h was optimal for growth inhibition. Since IFN failed to inhibit cell growth significantly and RA pretreatment enhanced the growth inhibitory action of IFN-β, we examined whether IFN-inducible gene expression was modulated by RA in these cells. Although low dose of RA (0.1 μM) could enhance the expression, longer treatment time was required for induction. We observed that 1 μM RA provided optimal conditions for ISG expression. Therefore, these conditions were employed for the rest of the study. Northern blot analyses were performed to detect IFN-induced gene expression. Two IFN-inducible gene products, 2'-5' oligoadenylate synthetase and protein kinase R, which are implicated in growth inhibition (1, 2), were chosen. A low dose of IFN was chosen because we intended to work with pharmacologically achievable doses. In IFN-β-treated cells (Fig. 6 lane 2), no significant expression of these mRNAs was observed. RA alone did not induce these genes (Fig. 6, lane 3). However, pretreatment of cells with RA
did not induce the gene expression (protein and a luciferase gene driven by the multimerized CAT vector driven by the promoter of guanylate binding protein) better than the single agents (15). Two reporter plasmids, a

followed by IFN resulted in a strong induction of ISGs (Fig. 6, lane 4).

We next tested whether the enhanced expression of ISGs was due to an increase in the stability of mRNA or induction of transcription in these cells, because a previous study in neuroblastoma cells indicated a posttranscriptional effect of RA (19). Nuclear run-off transcription assays were performed to identify the level of regulation (Fig. 7). MCF-7 cells were treated with indicated agents as described above, except that IFN-β treatment was for 45 min. Nuclei were isolated, and run-off transcription assays were performed as described earlier (10). As shown in Fig. 7, no transcription of three ISGs was detected in control as well as IFN-β-treated cells (lanes 1 and 2). RA alone did not induce the gene expression (lane 3). However, a dramatic induction of ISG transcription by IFN-β was noted in the RA-pretreated cells (Fig. 7, lane 4). Lack of gene expression in these cells was not global because a housekeeping gene, β-actin, was transcribed normally under all these conditions. Thus, transcriptional up-regulation by IFN-β was stronger in RA-pre-treated cells.

RA Pretreatment Up-regulates the Expression of Transfected Genes—Transcriptional up-regulation was also assessed by transiently transfecting MCF-7 cells with IFN-stimulated reporter genes. As shown in Fig. 8A, expression of pRM-luciferase, containing promoter elements of ISG-561, was strongly induced by IFN-β only in the cells pretreated with RA (Fig. 8A, bar 4). Neither IFN nor RA by themselves was capable of inducing the transfected gene (bars 2 and 3). A similar experiment was performed using a CAT reporter, in which promoter elements from ISG-6–16 direct the gene expression (Fig. 8B). Consistent with data in Fig. 8A, ISG-6–16 CAT gene induction by IFN-β was also noted only in the cells pretreated with RA (see Fig. 8B, lane 4). Thus, RA modulation of IFN-inducible gene expression occurs at the transcriptional level. Similar augmentation of the reporter constructs was noted in a murine breast tumor cell line (data not presented).

RA Augments IFN-γ-inducible Gene Expression—Since the IFN-α/β and IFN-γ gene regulatory pathways have a significant overlap, we next examined whether RA pretreatment also modulated the IFN-γ-inducible gene expression. Furthermore, IFN-γ and RA combination inhibited certain breast tumor cells better than the single agents (15). Two reporter plasmids, a CAT vector driven by the promoter of guanylate binding protein and a luciferase gene driven by the multimerized (4 ×) pRE element of ICSBP IFN consensus sequence binding pro-

protein (14), were employed for this purpose. Similar to IFN-α/β-regulated reporters, no expression of luciferase (Fig. 9A) and CAT (Fig. 9B) gene was observed in MCF-7 cells upon IFN-γ treatment (lanes 2). However, upon RA treatment followed by IFN-γ, a significant induction of luciferase and CAT activities were observed (lanes 4). Thus, RA also modulated IFN-γ-inducible gene expression.

RA Enhances the Activation of Transcriptional Factors by IFNs—Since the foregoing experiments indicated that transcriptional activation of ISGs was modulated by RA treatment of cells, mechanisms of such augmentation were investigated. IFNs regulate gene expression via ISRE, GAS, and pRE elements (3). Therefore, we examined the binding of transacting factors to these elements. Fig. 10 shows the binding of transacting factors to ISRE. Cells were pretreated with RA as described above and then with IFN-β for 30 min. Equal amount of nuclear extracts from different treatment groups was incu-
control, IFN-γ-acting factor binding to GAS or pIRE element was observed in GAS and pIRE elements. Similar to ISGF-3 binding, no trans-acting factor binding to these elements was observed in untreated cells. However, in RA-pretreated cells, IFN-γ efficiently activated the binding of factors to these elements (see lanes 4 in Fig. 11, A and B). In RA-treated cells a slightly enhanced binding of STAT1α to pIRE element, but not to GAS element, was observed. These complexes were supershifted with an anti-STAT1α antibody indicating their authenticity (data not presented). Thus, transcriptional activation by IFNs was augmented by RA at the level of trans-acting factor binding. Mixing of RA/IFN-treated extracts with control cell extracts in vitro did not alter binding of ISGF-3 or STAT1 to their cognate elements (data not shown). Thus a direct inhibitor of ISRE/GAS binding is absent in the untreated cell extracts.

RA Augments the Levels of STAT1 Protein in MCF-7 Cells—Because IFN-γ and IFN-β pathways were operated in an inefficient manner in MCF-7 cells and RA augmented the IFN-inducibility of the cellular genes, we then investigated the reasons for cellular unresponsiveness to IFN. It is important to note here that most cells have a constitutive level of STAT proteins. (3). Western blot analyses were performed to determine which component of the IFN signaling pathway was deficient in these cells and how RA affected them. First, the levels of ISGF-3 components were investigated because these factors were important for IFN-α/β signaling, at least, was inefficient in these cells upon IFN treatment (Fig. 10, lane 2). RA did not induce any novel ISRE binding factors (Fig. 10, lane 3). In contrast, IFN-β activated ISGF-3 binding to ISRE in RA-pretreated cells (Fig. 10, lane 4). There was also slight enhancement of ISGF-2 binding in this treatment group. The identity of ISGF-2 was established by preincubating the extracts with anti-p48, anti-STAT1, and anti-STAT2 antibodies and supershift of these complexes in EMSA (data not presented). Thus RA pretreatment augments the activation of ISGF-3 by IFN-β.

We next determined the binding of transacting factors to GAS and pRE elements. Similar to ISGF-3 binding, no trans-acting factor binding to GAS or pRE element was observed in control, IFN-γ, or RA-treated cells (Fig. 11, A and B, lanes 1-3). However, in RA-pretreated cells, IFN-γ efficiently activated the binding of factors to these elements (see lanes 4 in Fig. 11, A and B). Cells were treated with RA as described under Fig. 10. They were treated with IFN-γ (100 units/ml) for 30 min. Nuclear extracts were prepared and the EMSA was performed using 6 μg (A) and 3 μg (B) of total nuclear extract from each sample. EMSA was performed as described under Fig. 10, except that either a 32P-labeled GAS (A) probe (80,000 cpm) or pRE (B) probe (80,000 cpm) were employed. Arrow indicates the STAT1 (GAF) complex.
Fig. 13. A, over-expression of STAT1 gene promotes the growth inhibition by IFN-β. MCF-7 cells, stably transfected with either a pCXN2 control vector (bars 1-3) or the same vector that carries the STAT1 cDNA (bars 4-6), were assayed for growth inhibition by IFN-β for 4 days. Treatments were as follows: none (bars 1 and 4), 10 units/ml IFN-β (bars 2 and 5), and 100 units/ml IFN-β (bars 3 and 6). B shows the over-expression of STAT1 protein in the transfected cells. Cell extracts (50 µg) were Western-blotted as described under “Experimental Procedures.” Rabbit anti-STAT1 was used as a primary antibody. Lane 1, cells transfected with vector alone; lane 2, cells transfected with expression vector carrying the STAT1 cDNA. C shows the functional activity of the transfected STAT1 protein. Cells were transfected with ISG 6–16-CAT (as in Fig. 8). They were either untreated or treated with IFN-β and the CAT activity was assayed. Lanes 1 and 2, cells that transfected with STAT1 gene. Lanes 3 and 4, cell that carried the expression vector alone. Treatments were as follows: none (lanes 1 and 3), 100 units/ml IFN-β (lanes 2 and 4).

mained. No change in JAK1 levels was observed under the conditions of all treatments (Fig. 12D). However, STAT1 protein was not detected prior to IFN treatment (see Fig. 12C, lanes 1). IFN-β treatment by itself did not increase its levels (lane 2). RA treatment clearly enhanced the STAT1 protein levels (lane 3). Immunoprecipitation (data not shown) and Western blotting yielded similar results. A 10-fold increase in the levels of STAT1 was seen. We observed a variable enhancement of STAT1 levels upon IFN treatment in RA-pretreated cells. Prolonged exposure of these blot showed a faint STAT1 band in control and IFN-treated lanes, suggesting a substantially depressed level of this protein in the cells (data not shown). Thus, enhancement of STAT1 levels by RA appears to be an early step in the synergy between IFN/RA.

Transfection of STAT1 Gene Restores Growth Inhibition by IFN-β—Because STAT1 levels are undetectable in MCF-7 cells, we next examined whether over-expression of STAT1 under the control of a heterologous promoter could restore the growth inhibitory action of IFN-β. Two MCF-7 cell-stable transfectants that carried 1) an expression vector pCXN2 (11) and 2) the same vector expressing the STAT1 cDNA were employed for this purpose. The effect of IFN-β in these cells was examined in the absence of RA treatment. As expected IFN-β failed to inhibit the growth significantly in the cell line that was transfected with control vector alone (Fig. 13A, bars 1-3). However, in STAT1-transfected cells, IFN inhibited the cell growth in a dose-dependent manner (Fig. 13A, bars 4-6). Expression of STAT1 in the transfected cells was confirmed by Western blot analysis (Fig. 13B). In the transfected cells STAT1 protein was readily detectable in high levels (Fig. 13B, lane 2), whereas no such expression was seen in the cells transfected with pCXN2 vector alone (lane 1). Functional activity of the over-expressed STAT1 protein was determined by transfection of ISG 6–16-CAT. In the STAT1 expressing cells 6–16-CAT was highly induced by IFN-β (Fig. 13C, lane 2) but not in control cells that carried the vector alone (Fig. 13, lane 4). Therefore, defective expression of STAT1 could be relieved either by RA treatment or by over-expression of STAT1 gene.

DISCUSSION

Our studies have identified an early molecular event that results in enhanced growth suppression. In IFN-β-resistant MCF-7 cells (Fig. 1), pretreatment of cells with RA was necessary to mediate the growth inhibitory action of IFN (Fig. 2). RA alone could not mediate growth-suppressive actions in IFN-pretreated cells. These results indicate that RA modulates IFN effects rather than the converse. Continuous presence of RA during IFN treatment induced cytostatic action. These data indicate an additional level of RA/IFN interaction. This is evident from the data obtained with STAT1-transfected cells where IFN inhibited the growth but failed to induce cytoxicity. In addition, cytotoxic and cytostatic effects may be mediated by different gene products. Based on these observations, we suggest that conversion to an IFN-responsive state is an early event in IFN/RA synergy. Consistent with this notion, IFN-β readily induced ISG transcription in RA-pretreated cells. Specifically, RA modulates the activity of transcriptional factor ISGF-3 which in turn activates ISGs. RA also enhances IFN-γ-regulated gene expression. Therefore, RA modulates a common step in the regulatory cascade induced by these ligands. STAT1, a shared component of IFN-α/β and IFN-γ signaling pathways (3), was undetectable in these cells. RA treatment increased the level of this crucial molecule thus restoring cellular IFN responses.

A number of extracellular ligands that include cytokines and growth factors use the JAK-STAT pathway for transducing signals that regulate cell growth (3). Several of these ligands activate STAT1, in addition to other specific STAT proteins (3). Unlike these ligands, RA does not affect the functional activity of these STAT1 proteins, rather it appears to increase its physical levels. With the availability of STAT1, IFN could efficiently activate the signal transduction cascade that leads to growth inhibition. Subversion of normal growth regulatory pathways either by pathogenic agents due to mutations in the signaling components or over-expression of oncogenes may lead to sustenance of cell growth in the absence of ligands. Examples for these mechanisms are presented by recent studies that indicated that in both human T-cell leukemia virus-1 and Abelson virus-transformed cells (20, 21) and in v-srctransformed cells (22), the JAK-STAT pathway is operated in a ligand-independent manner. Defective IFN signaling has been documented in oncogene-transfected cells. For example, the adenoviral oncogene E1A and hepatitis B viral terminal protein shut off IFN-induced gene expression by interfering with the formation of ISGF-3 (11, 23–25). Inhibition of anticellular activity of IFN-α was also observed in Epstein-Barr viral nuclear antigen-2 transfected cells (26). A protein factor that interferes with binding of IRF-1 and p48 to ISRE in certain human tumor cells has also been reported (27). Mutations in JAK gene homologue of Droso phila resulted in leukemia-like disease (28). Thus, either through an interference of their activation or by expression of inhibitors of signal transduction components of IFN pathway, certain tumor cells may evade the action of cytokines that mediate growth arrest.
Down-regulation of signaling component(s) in some tumor cells may be a novel mechanism of resistance to the growth inhibition by IFNs. Consistent with such a notion, in normal breast epithelium and cell lines STAT proteins are not down-regulated (data not shown). At a supra-pharmacological dose of IFN-α (1500 units/ml), we observed a faint ISGF-3 band formation in MCF-7 cells (data not presented) which indicates a weaker activation of STAT1. RA may restore growth control in association with IFNs, by increasing the STAT1 levels in IFN-resistant cells. The mechanism of such augmentation is being investigated. Lastly, augmentation of IFN action by RA may not be due to enhancement of IFN receptors because both in breast tumor cells and embryonic tumor cells, which differentiate in response to RA, no increase in the affinity or number of IFN receptors was noted in RA-treated cells (29, 30).

Our investigations have identified a mechanism of RA/IFN synergy and illustrate how disparate extracellular growth regulatory molecules cross-talk and cooperate in mediating these effects. To our knowledge this is the first report on the defective STAT activation in tumor cells and its correction by a simple molecule such as RA. It will be interesting to examine whether other tumor cell types also have reduced STAT levels and what other small molecules can induce their levels. Interestingly, in a melanoma (31) and an acute promyelocytic leukemia cell line (32), STAT expression was undetectable. In the case of acute promyelocytic leukemia cell line, RA treatment enhanced STAT activation in response to RA, n-increase in the affinity or number of IFN receptors was noted in RA-treated cells (29, 30).

An important finding that emerges from our studies is that RA/IFN combination effectively suppresses the growth of breast and other tumors in vitro and in vivo (this paper) in a manner independent of their type, etiology, species, and estrogen receptor status. Potent antiproliferative activity, irrespective of their estrogen receptor status, of the IFN/RA combination makes it an attractive regimen for the therapy of human breast cancer. Furthermore, this combination also inhibits the growth of drug-resistant cells. Recent studies have shown that IFN-α and 13-cis-RA produced substantial therapeutic benefits in patients with advanced squamous cell carcinomas of the cervix and skin (7). It remains to be seen if this combination provides a new strategy for breast cancer therapy where IFNs were marginally effective as single agents (35).

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Modulation of Interferon (IFN)-inducible Gene Expression by Retinoic Acid:
UP-REGULATION OF STAT1 PROTEIN IN IFN-UNRESPONSIVE CELLS
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