**Interferon-α Alters Spectrin Organization in Normal and Leukemic Human B Lymphocytes**

By Sharon S. Evans, Wan-Chao Wang, Carol C. Gregorio, Tin Han, and Elizabeth A. Repasky

Interferon-α (IFN-α) regulates the growth, differentiation, and recirculation of normal and malignant B lymphocytes. In this report we examine the effects of IFN-α on the distribution of the cytoskeletal protein spectrin in peripheral blood B lymphocytes from normal donors and patients diagnosed with chronic lymphocytic leukemia (CLL) and hairy cell leukemia (HCL). Exposure of normal and leukemic B cells to IFN-α in vitro was shown by immunofluorescence microscopy to cause a dose-dependent increase in the percentage of cells containing discrete focal accumulations of spectrin, i.e., a single large aggregate or cap-like structure near the plasma membrane. Although the magnitude of this effect was variable among individual patient samples, in some experiments IFN-α induced a fourfold increase in the percentage of leukemic B cells exhibiting focal accumulations of spectrin. Spectrin reorganization induced by IFN-α was abrogated by the protein synthesis inhibitor cycloheximide. In addition, IFN-α increased the total cellular content of spectrin in B-CLL cells by approximately twofold to fourfold. Finally, a role for protein kinase C in mediating the effects of IFN-α on spectrin’s organization is implicated by studies in which calphostin C inhibited the IFN-induced focal accumulation of spectrin. Taken together, these studies suggest that the immunomodulatory activities of IFN-α in normal and malignant B cells involve a change in the organization of the spectrin-based cytoskeleton.

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**MATERIALS AND METHODS**

Cytokines and reagents. Recombinant human IFN-α was kindly provided by Dr P. Trotta (Schering Corp, Bloomfield, NJ). Recombinant human interferon-γ (IFN-γ), interferon-β (IFN-β), and interferon-γ (IFN-γ), and type II (IFN-γ), bind to distinct cell surface receptors and elicit common and unique biologic effects. IFN-α has been shown in clinical trials to be highly effective in the treatment of B-cell lymphoproliferative diseases including hairy cell leukemia (HCL) and early stage chronic lymphocytic leukemia (CLL). These diseases are characterized by abnormalities of spectrin distribution in lymphocytes. The clonal expansion of B-HCL cells in vitro consistently resulted in the formation of discrete focal accumulations of spectrin, as well as an increase in the total cellular content of spectrin. These results are discussed in view of the known functional aspects of spectrin distribution in lymphocytes.

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binant human IFN-γ was purchased from Genzyme Corp (Cambridge, MA). The specific activities of the IFN-α and IFN-γ reagents were 2.2 × 10^8 IU/mg and 2.5 × 10^8 IU/mg, respectively. The PKC inhibitor calphostin C was obtained commercially (Kamiya Biomedical Co, Thousand Oaks, CA).

**Antibodies.** The rabbit anti-chicken erythrocyte α spectrin anti-serum has been previously characterized. Fluorescein isothiocyanate (FITC)-conjugated murine monoclonal antibodies (MoAbs) specific for human Ig (Boehringer Mannheim Corp, Indianapolis, IN) and CD3 (Becton Dickinson, Immunocytometry Systems, Mountain View, CA) were obtained commercially. FITC- and TRITC-conjugated goat anti-rabbit IgG reagents were purchased from Southern Biotechnology Association (Birmingham, AL).

**Separation of mononuclear cells.** HCL and CLL patients selected for this study had not been treated with chemotherapy regimens for 4 months and had peripheral white blood cell counts >20 × 10^9/m^3. Peripheral blood mononuclear cells (PBMC) were isolated from the venous blood of normal donors and patients by Ficoll-Hypaque density centrifugation, as previously described.

**RESULTS**

IFN-α alters the subcellular distribution of spectrin in human leukemic and normal B cells. Mononuclear cells from B-cell leukemia patients and normal donors were immunostained for spectrin immediately after their isolation from the peripheral blood or following incubation in the presence or absence of recombinant human IFN-α. Indirect immunofluorescence analysis of neoplastic cells from two CLL patients (Fig 1, a and d) indicated that greater than 90% of peripheral blood B-CLL cells constitutively exhibit a diffuse fluorescence pattern of spectrin. Culture of B-CLL cells for 24 hours in medium containing 10% FCS resulted in a slight increase in heterogeneity among cells with respect to spectrin distribution (Fig 1, b and e). However, a marked change in the subcellular distribution of spectrin was observed in B-CLL cells after incubation with IFN-α at 1,000 IU/mL such that there was a loss of the diffuse staining pattern and a concomitant increase in the number of cells exhibiting distinct focal accumulations of spectrin (Fig 1, c and f). Notable differences were observed in the type of focal accumulation that occurred in B-CLL cells from these two patients after exposure to IFN-α. Specifically, IFN-α induced the formation of either aggregate or caplike configurations near the plasma membrane in ~45% of B-CLL cells from one patient (Fig 1c), whereas spectrin was found predominantly in discrete aggregates in ~77% of B-CLL cells from a second patient (Fig 1f).

FRESHLY isolated leukemia B cells from the peripheral blood of an HCL patient were also found to exhibit a diffuse intracellular localization of spectrin (Fig 2a), comparable with the staining pattern observed in B-CLL cells. Incubation for 24 hours in culture medium containing 10% FCS alone had negligible effects on the distribution of spectrin in B-HCL cells from this patient (Fig 2b), whereas incubation in the presence of 1,000 IU/mL of IFN-α resulted in the formation of both aggregates or caplike structures of spectrin in approximately 60% of B-HCL cells (Fig 2c). In the studies shown in Figs 1 and 2, lymphocytes were immunostained for spectrin after their attachment to Alcian blue-coated glass coverslips, as previously described. Identical results were obtained when lymphocytes were immunostained for spectrin in suspension (data not shown), indicating that focal accumulations of spectrin in IFN-treated cells were not formed as a result of adhesion to a glass substrate.
IFN-α ALTERS SPECTRIN DISTRIBUTION IN B CELLS

To further characterize the effect of IFN-α on spectrin organization in neoplastic B-cell populations, we examined the localization of spectrin in response to IFN-α in B-CLL cells isolated from 26 stage 0-III B-CLL patients. The results shown in Fig 3 indicate that spectrin was uniformly distributed in the subplasma membrane region in peripheral blood leukemic B cells isolated from the majority of CLL patients studied. Furthermore, incubation with 1,000 IU/mL of IFN-α consistently increased the percentage of B-CLL cells expressing focal accumulations of spectrin (ie, both aggregates and plasma membrane associated caplike structures), although the magnitude of the response to IFN-α was highly variable among individual patient samples.

The effect of IFN-α on the distribution of spectrin in normal peripheral blood B and T lymphocytes was determined by double-immunostaining peripheral blood mononuclear cells with anti-spectrin antibodies and either anti-CD3 or anti-Ig monoclonal reagents. Cells were fixed in formaldehyde before staining with anti-Ig or anti-CD3 MoAbs because previous reports have shown that antibody-mediated cross-linking of cell surface antigen receptors causes a coincident redistribution of spectrin. The results shown in Table I
indicate that a majority of normal peripheral blood B and T lymphocytes exhibited a diffuse spectrin staining pattern while incubation of lymphocytes for 24 hours in culture medium alone increased the number of cells exhibiting caplike accumulations of spectrin to a variable extent. IFN-α at a concentration of 200 IU/mL markedly increased the presence of focal accumulations of spectrin in the B-cell populations of four normal donors, while IFN-α had only a moderate effect on spectrin organization in T cells. Taken together, these results indicate that IFN-α alters the intracellular distribution of spectrin in both normal and neoplastic B lymphocytes.

**Dose dependence and kinetics of IFN-α-induced reorganization of spectrin.** To determine the effect of IFN-α concentration on the intracellular localization of spectrin, B-CLL cells from two CLL patients were incubated with increasing concentrations of IFN-α for 24 hours and then immunostained with anti-spectrin antibodies. The results shown in Fig 4 show that IFN-α acted in a dose-dependent manner to cause the formation of discrete focal accumulations of spectrin. Moreover, low IFN-α concentrations (ie, 10 to 100 IU/mL) increased the focal accumulations of spectrin, suggesting that spectrin reorganization in leukemic B cells occurs at both physiologic and pharmacologic concentrations of IFN-α. It is of interest that similar results were obtained in repeat experiments performed at a 1-month interval using freshly isolated malignant B lymphocytes from the same CLL patient (Fig 4).

B-CLL cells from two patients were incubated with 200 IU/mL IFN-α for various periods of time to determine the kinetics of IFN-α-induced spectrin reorganization (Fig 5). A slight change in spectrin distribution could first be observed 6 hours following the addition of IFN-α while more marked effects on spectrin distribution were evident after incubation for greater than 18 hours. The presence of spectrin in a concentrated region of the cell is maintained for up to 48 hours during continuous exposure to IFN-α.

**Effect of protein synthesis and PKC inhibitors on IFN-induced spectrin reorganization.** Results demonstrating that maximal spectrin reorganization occurs after exposure to

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Cell Population</th>
<th>% Cells With Focal Accumulations of Spectrin</th>
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<tbody>
<tr>
<td></td>
<td></td>
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<td>4</td>
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<td>14.5</td>
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<tr>
<td></td>
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</table>

* The subcellular organization of spectrin was determined by immunofluorescence microscopy immediately after isolation of lymphocytes from the peripheral blood (0 hours) or after incubation for 24 hours in the absence or presence of 200 IU/mL of IFN-α.
IFN-α ALTERS SPECTRIN DISTRIBUTION IN B CELLS

Changes in the organization and synthesis of cytoskeletal proteins have been associated with fundamental cellular responses to a variety of external and internal signals. We have shown here that IFN-α induces changes in the subcellular organization of the cytoskeletal protein spectrin in peripheral blood B lymphocytes from normal individuals and in leukemic B cells from CLL and HCL patients. Incubation of both normal and malignant B cells with IFN-α resulted in a marked increase in the number of cells in which spectrin was distributed in a concentrated loci instead of the typical diffuse pattern. The effect of IFN-α on spectrin distribution in B cells occurred in a time and dose-dependent manner and focal accumulations of spectrin were maintained for periods up to 48 hours during continuous exposure to IFN-α.

The biologic or therapeutic result of these effects of IFN-α on spectrin distribution in normal and leukemic B cells remains to be determined. One possibility is that a cytoskeletal reorganization relates to the recirculation or extravasation potential of B lymphocytes. In previous studies, IFN-α and IFN-γ have been shown to alter the recirculation of B and T lymphocytes in murine and sheep models. In these studies, marked lymphopenia was observed within 24 hours of systemic administration of IFN-α and was associated with the increased uptake and retention of lymphocytes in lymph nodes. IFN-α has also been shown to cause a rapid decrease in the number of leukemic B-CLL and B-HCL cells in the circulation during therapy. In erythrocytes, spectrin is thought to play a major role in red blood cell recirculation because of its ability to regulate plasma membrane fluidity and deformability. A similar relationship between the or-

IFN-α for greater than 6 hours suggested that new protein synthesis may be involved in this process. To determine the requirement for new protein synthesis during IFN-α-induced reorganization of spectrin, B-CLL cells from six patients were incubated for 1 hour with 15 μg/mL of cycloheximide before the addition of 200 IU/mL IFN-α and the intracellular organization of spectrin was determined 24 hours later (Fig 6). After exposure to IFN-α alone, approximately 35% of the total B-CLL cell population exhibited focal accumulations of spectrin in these patient samples. Cycloheximide consistently abrogated the capacity of IFN-α to increase the focal distribution of spectrin in leukemic B cells, indicating that spectrin reorganization triggered by IFN-α was dependent on new protein synthesis. Calphostin C, a specific inhibitor of PKC activity, also inhibited the IFN-induced formation of spectrin aggregates in B-CLL cells (Fig 6). Similar results were obtained using the PKC and PKA inhibitor H-7 (data not shown). The results shown in Fig 6 further demonstrate that IFN-γ was as effective as IFN-α in altering the intracellular localization of spectrin in leukemic B cells.

IFN-α increases spectrin content in B-CLL cells. The total cellular content of spectrin in B-CLL cells was determined by Western blot analysis immediately after cells were isolated from the peripheral blood or following culture for 24 hours in the absence or presence of 200 IU/mL of recombinant IFN-α (Fig 7). In this experiment, equivalent amounts of protein were loaded on a 10% polyacrylamide gel, as confirmed by Coomassie blue patterns of duplicate samples (Fig 7a), and then transferred to nitrocellulose and probed with anti-spectrin antiserum (Fig 7b). Scanning densitometry analysis indicated that exposure to IFN-α in vitro increased the total amount of the 240-Kd α spectrin band in leukemic B cells by approximately twofold compared with untreated cells. Immunofluorescence analysis of identical samples indicated that focal accumulations of spectrin were observed in 2.0% of freshly isolated cells, 7.0% of cells cultured in medium alone, and 27.2% of cells exposed to IFN-α (Table 2, patient 1). Analysis of the intracellular spectrin content in two additional CLL patients after incubation for 24 hours in the presence or absence of 200 IU/mL of IFN-α further indicated that IFN-α increased the total cellular content of spectrin by greater than twofold in these B-CLL cells (Table 2). Because the data shown in Figs 1 through 6 suggest that IFN-α does not affect the organization of spectrin in every cell within an individual patient sample, the measurement of the total cellular content of spectrin may in fact underestimate the effect of IFN-α on spectrin content. These results indicate that the reorganization of spectrin detected by immunofluorescence in B-CLL cells correlates directly with an increased cellular content of this cytoskeletal protein.

DISCUSSION

Changes in the intracellular or-
organizational states of lymphocyte spectrin and of plasma membrane lipids has been reported. Lymphocytes expressing a spectrin aggregate exhibit tightly organized membrane lipids, whereas lymphocytes with a uniform, plasma membrane-associated distribution of spectrin exhibit loosely organized lipids and a more fluid plasma membrane.29,30 These studies suggest that an alteration in the subcellular distribution of α-spectrin in lymphocytes may be one mechanism by which IFN-α influences membrane fluidity31,32 and possibly alters the recirculation of normal and malignant B cells.

The molecular mechanism or cellular pathway by which IFN-α acts to cause distinctive changes in the subcellular organization of spectrin is unknown; however, several possibilities seem tenable. Studies in which cycloheximide abrogated the IFN-induced focal accumulation of spectrin suggest that this process involves new synthesis of spectrin or some other protein, possibly a chaperone protein, which may be required for the movement of spectrin within a cell. It is noteworthy in this regard that the 70-Kd heat shock protein that functions as a molecular chaperone in some systems33 has been found in spectrin aggregates in murine lymphocytes.34 Evidence that IFN-α increased the total spectrin content in B-CLL cells by greater than twofold suggests that localization of spectrin immunofluorescence in concentrated regions within B lymphocytes may represent sites of ongoing spectrin synthesis. However, this is unlikely to be the sole explanation for the observed effect of IFN-α on spectrin distribution because focal accumulations of spectrin are found in regions not normally associated with new protein synthesis (eg, plasma membrane associated caplike structures). Moreover, incubation of B lymphocytes with IFN-α was found to result in a sustained loss of the ring-type plasma membrane-associated spectrin staining pattern, as well as a decrease in the diffuse staining pattern, suggesting that mobilization of pre-existing spectrin from cytoplasmic and plasma membrane sites also occurs in response to IFN-α.

Another possible mechanism by which IFN-α may affect spectrin is suggested by the data showing that the PKC inhibitor calphostin C blocked the IFN-α-induced formation of focal accumulations of spectrin. These data imply that the effects of IFN-α on spectrin distribution may involve changes in PKC activity. Work by others has already shown that IFN-α activates specific PKC isoenzymes in B lymphoblastoid and epithelioid cell lines.35,36 Specifically, IFN-α causes the rapid translocation of specific PKC isoforms from the soluble
to the particulate, detergent-insoluble subcellular fraction in both the Daudi B lymphoid and the HeLa epithelioid cell lines.\textsuperscript{25,26} Several previous reports from our group have shown that lymphocyte cell surface events that affect PKC activation can rapidly and significantly alter the subcellular localization of spectrin.\textsuperscript{20,21} Moreover, in murine T cells and B cells we have observed a close positioning between the PKC $\beta$1 isoform and focal accumulations of spectrin that occur naturally among tissue lymphocytes and that treatments (such as activation via the antigen receptor or phorbol esters) that cause a change in the distribution and solubility of spectrin cause identical changes in PKC $\beta$1.\textsuperscript{21} From these data, we speculate that the changes in the distribution of spectrin induced by IFN-\(\alpha\) could reflect changes in the activation state or solubility of PKC, although the delayed kinetics of the spectrin response to IFN-\(\alpha\) described in this report compared with our earlier work suggest that it is unlikely to be an immediate result of the engagement of the IFN-\(\alpha\) cell surface receptor.

Future studies that define the specific molecular mechanisms by which IFN-\(\alpha\) regulates spectrin synthesis and organization in normal and malignant B lymphocytes are expected to provide important insight into the biologic mechanism of action of IFN-\(\alpha\). Moreover, recent studies have determined that the erythroid spectrin gene is tightly linked to a gene cluster consisting of IFN-activated genes on distal mouse chromosome 1, suggesting the possibility that coordinated transcriptional regulation of these genes may be mediated by a common enhancer sequence.\textsuperscript{27,28} Therefore, it will also be of interest to determine if the gene for lymphocyte spectrin is similarly linked to IFN-stimulated genes.

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**REFERENCES**


**Table 2. IFN-\(\alpha\) Causes Spectrin Reorganization and Increases Spectrin Content in Leukemic B Cells**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>IFN-(\alpha)</th>
<th>% Focal Accumulation of Spectrin$^*$</th>
<th>Relative-Fold Increase of $\alpha$ Spectrin$^\dagger$</th>
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<tr>
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<td>+</td>
<td>27.2</td>
<td>2.19</td>
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<tr>
<td>3</td>
<td>Stage IV CLL</td>
<td>+</td>
<td>52.4</td>
<td>4.28</td>
</tr>
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</table>

$^*$ The intracellular spectrin organization was determined by indirect immunofluorescence microscopy after incubation of B-CLL cells for 24 hours in the presence or absence of 200 IU/mL of IFN-\(\alpha\).

$^\dagger$ The relative-fold increase in intracellular spectrin concentration was determined by scanning densitometry of Western immunoblots.

**Fig 7.** IFN-\(\alpha\) increases the intracellular content of spectrin in B-CLL cells from a stage IV patient as determined by Western blot analysis of cytoplasmic extracts. Proteins were separated by SDS-PAGE and either (a) stained with Coomassie brilliant blue or (b) transferred onto nitrocellulose paper and subsequently probed with anti-$\alpha$ spectrin antiserum. Lanes 1 and 4, freshly isolated cells; lanes 2 and 5, cells cultured in medium alone for 24 h; lanes 3 and 6, cells cultured in the presence of 200 IU/mL of IFN-\(\alpha\) for 24 hours.


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