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INTERFERON-INDUCED SURFACE ALTERATIONS IN HAIRY CELLS. A REVIEW

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Abstract

Hairy cells (HCs), derived from the peripheral blood and spleen of hairy cell leukemia (HCL) patients, constantly displayed both ruffles and microvilli. HCs which were kept in culture for up to three days exhibited extremely polarized and active surfaces with elongated microvilli and exaggerated "spiked" ruffles. Cells derived from 11 cases of HCL were treated with alpha-interferon (IFN) in-vitro and examined by immuno - scanning electron microscopy (immuno-SEM). In 8 cases, up to one-third of the IFN-treated hairy cells displayed deformed surfaces with "bubbling" membrane and markedly villous bud-like formations. Monoclonal antibodies (MoAb), used in conjunction with immuno-gold labeling, facilitated better correlation between these morphological changes and the immunological profiles of the cells before and after interferon treatment in-vitro. Immuno-SEM analyses revealed no remarkable changes in the labeling of HCs with Leu-14 and Leu-M5 MoAbs before and after IFN treatment, even in cases showing membrane changes. However, a significant icrease in the labeling intensity for HLA-DR and HLA-DQ was noticed in HCs from cases where IFN-induced membrane changes were evident. A review of the literature on membrane changes in IFN-treated cells proposes that such immuno-ultrastructural alterations might reflect unique interferon-induced membrane reorganization in the target malignant cells.

Key words: Hairy cell, leukemia, interferon, monoclonal antibodies, immunogold, immunolabeling, ruffles, microvilli.

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Introduction

For the first twenty years following its discovery by Isaacs and Lindenmann [1957], interferon (IFN) was studied as an antiviral agent. Currently, the inhibition of virus multiplication ('antiviral effect'), the inhibition of cell proliferation ('antimitogenic effect'), and cell regulatory activities, such as effects on cell differentiation and immune functions, are the most important effects of IFN. On the other hand, several groups of proteins were found to be induced by IFN, namely, translation-regulatory enzymes, cell surface antigens and intracellular proteins detected by gel electrophoresis [Taylor-Papadimitriou, 1980; Van Damme and Billiau, 1981; Vilcek et al., 1982: Friedman and Vogel, 1983]. Besides intracellular and surface proteins, IFN treatment may also affect the activity of genes for cytoskeletal proteins [Tamm et al., 1982; Wang et al., 1984; discussed by Revel, 1984]. The resulting changes in the cytoskeleton are numerous and could lead to abortive mitosis as well as to abnormal control of cell size, shape, and function.

Human interferons were successfully harnessed in the treatment of various viral diseases [Merigan, 1981]. Effective therapy with IFNs, derived from cell culture or cloning technology, was also reported in hairy cell leukemia (HCL), chronic myelocytic leukemia, IgA myelomas, renal cancer, skin cancers due to papillomavirus, melanomas, and metastatic breast cancer [Strander, 1983; Bonnem and Spiegel, 1984]. Studies have shown that IFN lead to the elimination of cancer cells in the blood or the tissues of the treated patients, but the actual mechanism of its action is still unknown.

HCL is characterized by pancytopenia, splenomegaly, and infiltration of the bone marrow and spleen by distinct abnormal mononuclear cells [Golomb et al., 1983]. Since HCL might be confused with other malignant lymphomas or chronic lymphocytic leukemia, which are treated in an utterly different manner, accurate diagnosis of HCL is important. It is preferable initially to advise only splenectomy, or no treatment, rather than radiotherapy or chemotherapy [Golomb and Vardiman, 1983]. However, recent clinical studies indicated that most HCL patients treated with

alpha-IFN showed a striking clinical response to the drug [Quesada et al., 1984].

Most scanning electron microscopy (SEM) studies on hairy cells (HCs) initially aimed to determine whether these cells were of lymphocytic or monocytic origin [references cited in Gamliel et al., 1985]. Generally, these SEM studies revealed cells displaying ruffles (monocytoid features) mixed with microvilli (lymphocytoid features), making it impossible to favor a lymphocytic or monocytic nature of the HC under the SEM. However, recent multidisciplinary studies have proven that HCL cells are malignant B-lymphocytes which display some monocyte-like characteristics [Reiber et al., 1977; Burns and Lawley, 1979; Yanovich et al., 1979; Golomb et al., 1982; Jansen et al., 1982].

Based on the surface morphology of HCs from 18 patients, Gamliel et al. [1985] identified two major types of hairy cells: (i) cells showing areas of ruffles alongside areas of clustered microvilli, and (ii) cells displaying microvilli scattered among ruffles.

The clinical efficacy of alpha-IFN in HCL led us to utilize the SEM for the study of the effect of IFN on HCs in-vitro. In a previous paper [Gamliel et al., 1987], we reported for the first time that HCs treated with alpha-IFN in-vitro displayed unique alterations in their surface morphology. The present report extends on seven more HCL cases treated with IFN in-vitro, and reviews other studies on IFN-induced membranal alterations.

Materials and Methods

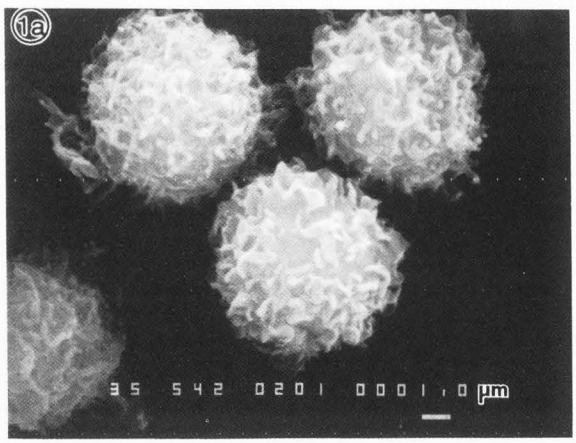
The main guidelines for including HCL cases in this study were: patients with the leukemic form of their disease, i.e., those who had greater than 60% abnormal cells in the differential counts and greater than 10,000 white blood cells/mm^3. These criteria were set as it is relatively easy to score the HCs in such cases in comparison to most HCL patients who are leukopenic with less than 10% HCs. Eleven such patients were selected for evaluation of cell surface features before and after IFN treatment. HCL was diagnosed by the following tests: the presence of 'hairy' cells in the peripheral blood (PB) and/or bone marrow and spleen; the existence of tartrate-resistant acid phosphatase isoenzyme in the malignant cells; the demonstration by transmission electron microscopy (TEM) of a ribosome-lamella complex in some cells; the presence of splenomegaly and pancytopenia, and a chronic clinical course.

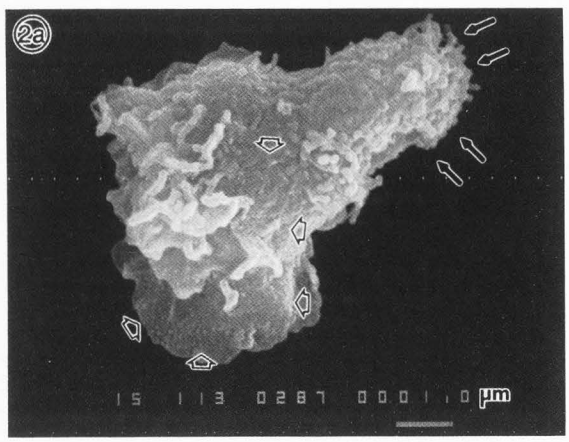
For the isolation of PB-HCs, samples of venous blood were collected in sterile evacuated tubes (Vacutainer Systems, Rutherford, NJ) containing heparin as an anticoagulant, and layered on a Ficoll-Paque gradient (see below). For the isolation of spleen HCs, 2.5 centimeter sections of fresh spleen tissue were kept in cold Hank's balanced salt solution (HBSS) with 1% heparin while they were gently teased across a sterile fine mesh screen, producing a liquid homogenate. Mononuclear cells were isolated from the peripheral blood and/or the spleen homogenate by the Ficoll-Paque density gradient method. Cells were removed from the mononuclear band of the gradient, and washed three times in HBSS or Dulbecco's phosphate buffered saline (DPBS). Using this procedure, more than 80% of the mononuclear splenic cells were HCs, while the number of monocytes never exceeded 5% in all PB and spleen samples. PB cells from 6 HCL patients and splenic cells from 5 other cases were treated with IFN in-vitro. Cell suspensions (10^6 cells), in 2.5 ml RPMI medium 1640 and 10% fetal calf serum, were incubated in tissue culture flasks, at 37°C in a 5% CO_2 humidified atmosphere. After 24 h of incubation, RDα_2-IFN (recombinant human leukocyte interferon; Schering Corp., Bloomfield, NJ) in a 0.9% NaCl solution was added at a concentration of 1000 international units per 1 ml. Control cultures received NaCl solution without IFN, or IFN in the presence of neutralizing concentration (50 µl/1000 IU IFN) of rabbit anti-IFN reagent (Interferon Sciences, Inc., New Brunswick, NJ). Cultures were harvested on the first, second, and third days of incubation for viability and differential cell counts, and for SEM analyses. In some cases, usually those with high blood counts, cells were also harvested after 2, 4, and 6 h. Viable cell counts were performed using trypan blue dye exclusion. For routine SEM examinations, cells were fixed with 2% glutaraldehyde in DPBS for 1 h.

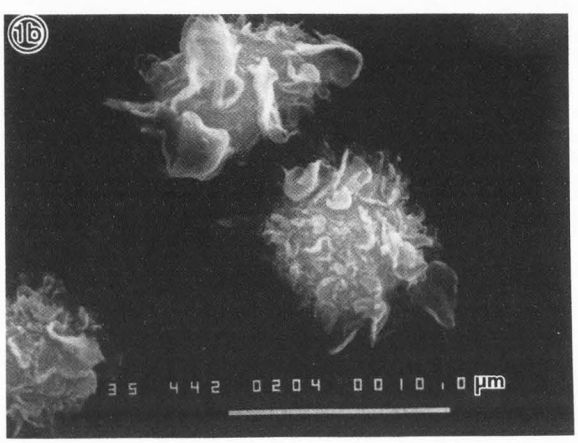
The following monoclonal antibodies (Becton Dickinson, Mountain View, CA) were used to study control and treated cells: Leu-4 (specific for T cells), Leu-14 (for B cells), Leu-M3 (for monocytes/macrophages), Leu-M5 (for hairy cells and monocytes), anti-HLA-DR (for B-cells and monocytes), and anti-HLA-DQ (Leu-10; for B cells and monocytes).

For immuno-gold labeling, cells were fixed in 0.2% glutaraldehyde for 10 min, washed with DPBS, and treated with 0.1M glycine for 5 min. Cells were then washed with DPBS, loaded on poly-L-lysine treated glass coverslips for 1 h, and washed with an 'immuno' buffer (DPBS containing 1% bovine serum albumin and 1% decomplemented human AB serum). Subsequently, cells were reacted with 50 µl of diluted monoclonal antibody for 30 min, washed 3 times with the 'immuno' buffer, and incubated with 50 µl of 40 nm colloidal gold particles tagged with goat anti-mouse MoAb (GAMIgG+IgM-G40; Janssen Pharmaceutica, Piscataway, NJ). After 30 min, cells were rinsed with DPBS and postfixed in 2% glutaraldehyde until processed for SEM.

SEM preparation of cells was carried out using either conventional critical point drying (CPD), or the newly introduced GTGO - air drying (GTGO-AD) procedure which minimizes cell shrinkage due to improved fixation routine [Gamliel et al., 1983; Gamliel, 1985]. Briefly, after fixation with glutaraldehyde, coverslips with a monolayer of attached cells were either processed immediately for CPD [Gamliel, 1985], or incubated in DPBS containing 2% tannic acid and 2% guanidine-HCl, for 1 h, as part of the GTGO-AD procedure. Thereafter, cells were thoroughly washed for 15 min with DPBS and subsequently immersed in DPBS containing 2% osmium tetroxide for another hour. Dehydration was carried out through a graded series of ethanol in distilled water (25%, 50%,







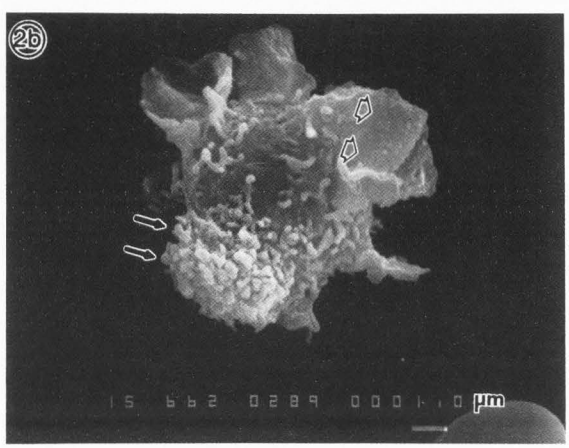


Fig. 1a: Control, untreated HCL cells showing clustered microvilli beside prominent ruffled membrane.
Fig. 1b: Control HCL cells after one day of cultivation without interferon, displaying exaggerated ruffles and microvilli.

Fig. 2a: IFN-treated spleen hairy cell showing a bud-like formation (arrows) and bubbling membrane on the cell body and ruffles (wide arrows).
Fig. 2b: IFN-treated peripheral blood HCL cell displaying a villous bud-like formation (arrows) and areas of bubbling membrane (wide arrows).

75% and three times 100%) followed by a graded series of Freon 113 (TF) in absolute ethanol (25%, 50%, 75% and three times 100%), for 10 min in each solution. Samples fully immersed in Freon 113 were picked out one by one with delicate forceps and vigorously shaken in the air for about 10s. After drying, specimens were mounted on Cambridge aluminum stubs using double-sided sticky tape or a gluey silver paint. Coverslips with the cell monolayers were coated with a thin layer of carbon using a sputter coater apparatus (SPI Supplies, West Chester, PA). While JEOL JSM-35 SEM was used in the preliminary studies, the JEOL 840A, equipped with highly-sensitive annular type backscattered electron image (BEI) detector, was used in recent studies. Attempts to use the GTGO-AD technique for preparing gold-labeled cells resulted in the inability to get BEI of the gold particles because of the high background signal contributed by the heavy metal solution (OsO_4).

Results

Control PB-HCs were similar to those isolated from the spleen, showing variable numbers of microvilli, and a wide spectrum of ruffles (Fig. 1a) with minute spikes outlining their uppermost profile ("spiked-ruffles"). Cultured HCs exhibited large ruffles as well as clusters of microvilli, and consistent polarity of these surface features (Fig. 1b). Many of these cultured HCs displayed exaggerated, undulating, broad-based, "spiked" ruffles, whereas clusters of elongated microvilli were often encountered.

The majority of IFN-treated HCs displayed the same surface features characterizing their cultured, untreated equivalents. However, distinct surface alterations were found in about one-third (19 to 36%) of the HCs from the PB and spleen specimens in 8 out of the 11 cases treated with IFN in-vitro. These changes comprised the appearance of a "bubbling" membrane on the cell body and/or on the ruffles (Figs. 2a,b), while the microvilli became much shorter compared to the microvilli of the cultured, untreated cells. The majority of these cells also showed a bud-like formation at their extremity (Figs. 2a,b) which was markedly villous and frequently was the only villous part of the cell. The same type of surface changes were found in the parallel samples which underwent CPD after GA fixation. None of the above described surface alterations were encountered in control specimens treated with IFN-free NaCl solution, nor in cultures that were treated with IFN and a neutralizing concentration of an anti-IFN reagent.

Some of the above described changes were also found in the cultures after 2, or 4 h of incubation in the presence of IFN. These cells primarily showed bud-like structures whereas 'bubbling' membranes were rarely seen. However, these early changes were found in less than 15% of the HCs.

Correlative immuno-SEM studies were performed to determine the immunological profiles of the control untreated cells, and the cultured cells with and without IFN treatment. Highly specific monoclonal antibodies were used to label the cells for B (Leu-14) and T (Leu-4) antigens, and for antigens presented by monocytes (Leu-M3, Leu-M5), and hairy cells (Leu-M5) . In addition, we used anti-HLA-DR and anti-HLA-DQ (Leu-10) reagents to assess the expression of class II HLA antigens on the treated cells. These immuno-gold labeling studies were done on 7 out of the 11 IFN-treated cases. All of these cases were positive (higher than 60% labeled cells) for Leu-14, Leu-M5, HLA-DR and HLA-DQ, and negative (less than 5% labeled cells) for Leu-4 and Leu-M3. In 2 of these cases neither morphological nor immunophenotypical changes were noted. The other 5 cases showed IFN-induced membrane changes, but displayed no significant alterations in labeling pattern with Leu-14 and Leu-M5 (Fig. 3). However, all these 5 cases showed a significant increase in the labeling pattern for HLA-DQ and HLA-DR (Fig. 4).

Diverse types of normal and leukemic, circulating and cultured leukocytes were used as controls in this study. Normal mononuclear cells, chronic lymphocytic leukemia and myeloid leukemia cells, all did not show any noticeable immuno-ultrastructural changes when treated with IFN in-vitro. On the other hand, Daudi (B-lymphoma) cultured cells did display a change in their villousity. These alterations, which were quite different from those exerted by HCs, were accompanied by an increase in the expression of class II HLA antigens. The above changes were abrogated when these cultures were treated with a neutralizing concentration of anti-IFN.

Trypan blue test and careful SEM examinations were used to test changes in the viability rates of control vs. IFN-treated HCs. In all studied cases, no significant differences in the numbers of viable cells were noted, and the viability rates were always over 75% of the total number of the cells.

Discussion

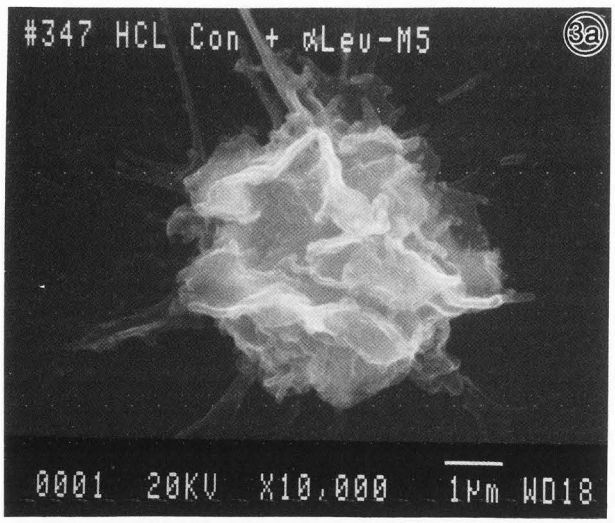
Interferons are reported to directly stimulate cell killing by enhancing the cytotoxicity of T-lymphocytes [Sethi and Brandis, 1978; Zarling et al., 1978], of macrophages [Schultz and Chirigos, 1979; Stanwick et al., 1980], of monocytes [Jett et al., 1980], and of NK cells [Welsh, 1981; Herberman et al., 1982] against viral-infected cells or tumor cells. The mechanism is unknown but it may be related to increased synthesis and expression of membrane antigens, or may reflect differentiation of the immune cells under the influence of IFN.

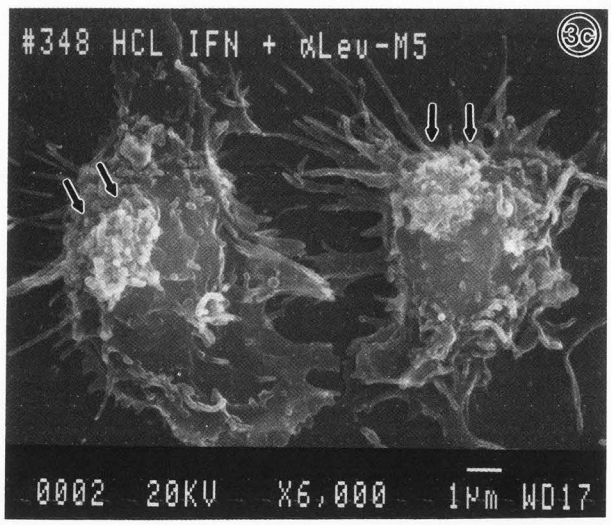
Since alpha-, beta-, and gamma-IFN also stimulate HLA class I antigen synthesis and the expression on the cell surface of antigen-HLA complexes, it is possible that this enhances the ability of the immune cells to recognize cells presenting non-self antigens and destroy them [Revel, 1984]. This effect of IFN on recognition of non-self antigens on target cells has not been demonstrated in-vitro. However, when immune deficient patients with low HLA-A or B expression are treated with IFN, their cells show an increased ability to be recognized by specific cytotoxic T cells [Durandy et al., 1983].

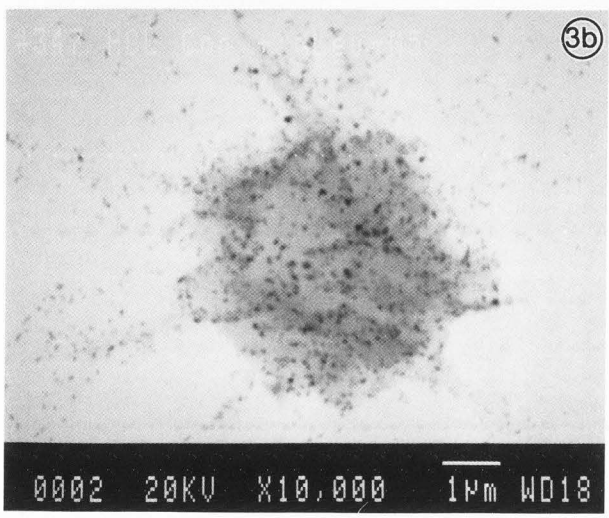
Studies have suggested that alpha-, beta-, and gamma-IFNs first bind to cell surface receptors and subsequently induce an intracellular response [reviewed by Revel, 1984]. After a rapid initial binding of IFN, clustering and activation of the IFN receptors can take place [Shulman and Revel, 1980], and the interaction of IFN with cell membranes seems needed to induce the biological response.

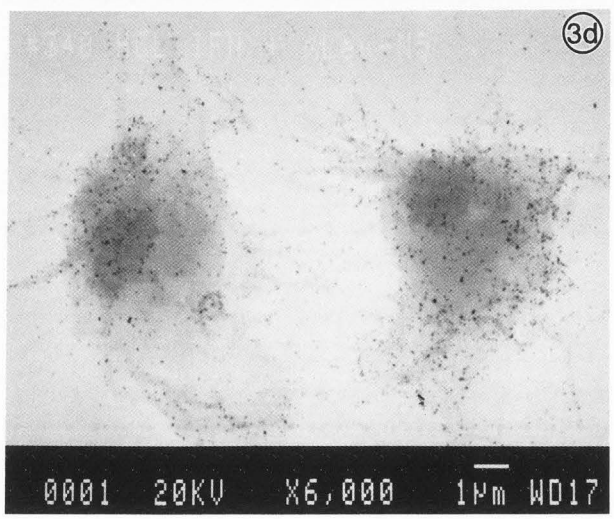
In a preliminary study we have shown that when HCs are treated in-vitro with alpha-IFN, dramatic changes in surface architecture occur in up to 32% of the HCs from 3 out of 4 HCL patients [Gamliel et al., 1987]. We have also shown that the IFN-induced surface alterations were dose and time dependent, however, the most dramatic changes were encountered in cells treated with 1000 IU IFN for 3 days. No significant reduction in the viability of cells was noted after IFN treatment, suggesting that IFN have had no direct cytotoxic effect on in-vitro treated hairy cells. In a correlative study on these cases, Samuels et al. [1986] have shown that the exposure to IFN also caused the induction of specific proteins in the treated HCs. The present study demonstrates that in 8 out of 11 HCL cases the exposure to IFN resulted in specific surface membrane changes, in 19 to 36% of IFN-treated HCs. The main changes were the appearance of small bubbles in the cell membrane and the smoothening of otherwise very active surface areas which is often accompanied with the formation of a markedly villous bud-like process at the cell extremity.

An increase in HLA-A, B, C, and DR antigens as well as beta$_2$ microglobulin, after IFN treatment, was observed on various human cells in-vitro and in-vivo [Lucero et al., 1982; Basham and









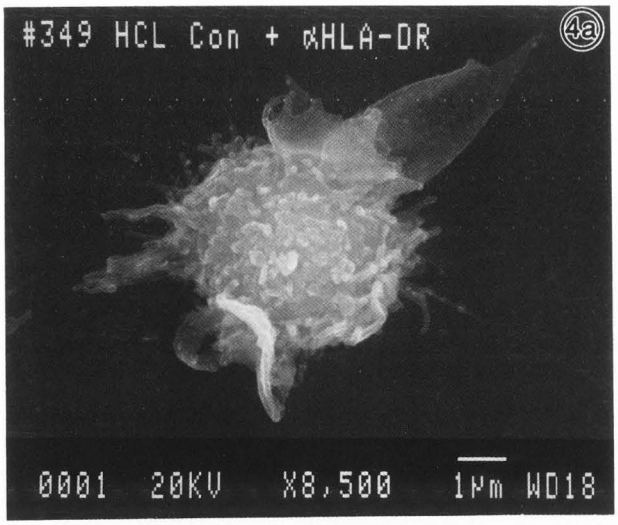
Figs. 3a,b: Control hairy cells labeled for Leu-M5 by colloidal gold. a. SE image of a control cell. b. BE image (inverted) of the same cell (the black dots represent the marker).

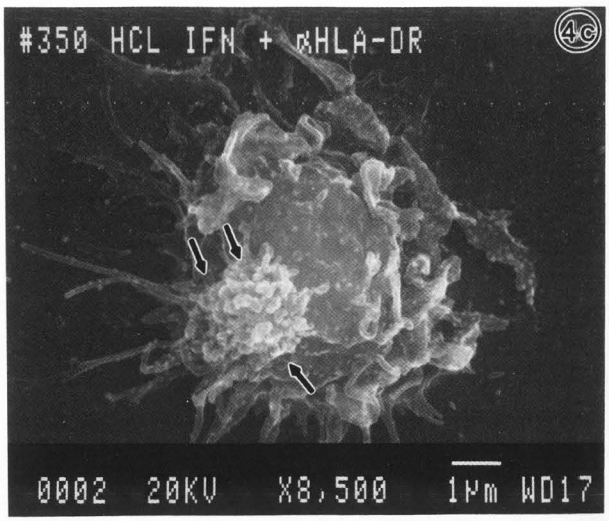
Figs. 3c,d: IFN-treated hairy cells labeled for Leu-M5 by colloidal gold. c. SEI of two cells treated with IFN in-vitro (arrows point to a bud-like formation). d. BE image (inverted) of same cells.

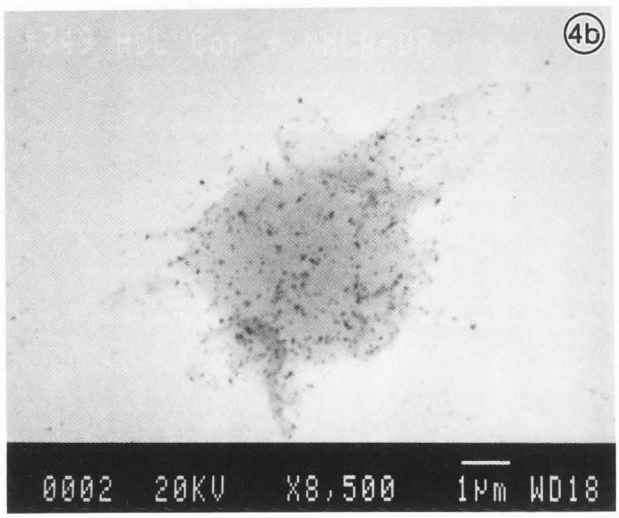
Merigan, 1983; Rosa et al., 1983]. Burrone and Milstein [1982] have described a protein of the cell surface of T-leukemic cells (Molt 4) and B-lymphoblastoid cells (Raji or Daudi), which is strongly increased after treatment by alpha-IFN. This protein becomes the major surface antigen of the IFN-treated cells, but its function is yet unknown. On the other hand, human fibroblasts treated with IFN showed an increase in the level of prostaglandin E [Yaron et al., 1977]. This enhanced prostaglandin synthesis was believed to be a feed-back mechanism which protects the cells against excessive membrane damage by IFN [Fitzpatrick and Stringfellow, 1980; Gurari-Rotman, 1982].

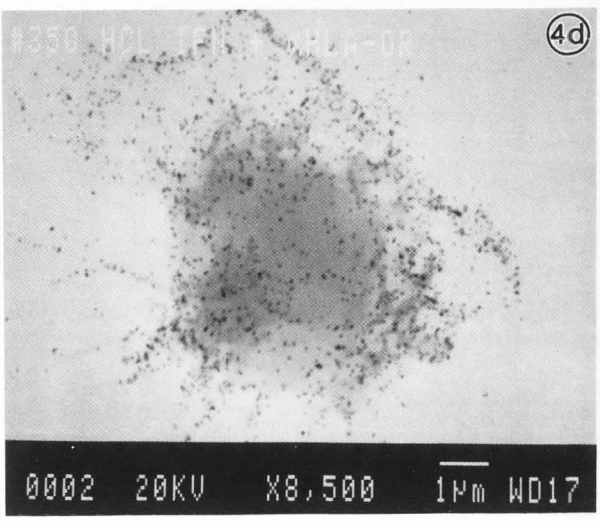
IFN was also shown to induce a reduction in the fluidity of the cell membrane, which is closely related to major cytoskeletal modifications. Actin containing microfilaments were reported to become more dense and form meshworks under the membrane of IFN treated HeLa or L-929 cells [Tamm et al., 1982; Wang et al., 1981]. IFN-induced changes in actin microfilaments were also reported to impair cell surface receptor movements and to inhibit capping of Concanavalin A receptors [Pfeffer et al., 1980a,b].

The unique IFN-induced morphological changes, delineated by SEM in the present and previous studies, and the increased expression of class II HLA antigens demonstrated in the present study, most likely reflect alterations in membranal and/or sub-membranal elements of the target cell. Membranal reorganization probably plays a major role in the response of the cells to IFN, and reflects a distinct change in the HCs that might initiate their disappearance in HCL patients treated with IFN in-vivo.









Figs. 4a,b: Control hairy cells labeled for HLA-DR by colloidal gold. a. SEI of a control cell. b. BEI (inverted) of the same cell.

Figs. 4c,d: IFN-treated hairy cells labeled for HLA-DR by colloidal gold. c. SEI of a cell treated with IFN in-vitro (arrows point to a bud-like formation). d. BEI (inverted) of the same cell.

Acknowledgments

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Discussion With Reviewers

M. Revel: This work described very briefly observations made after incubating 1 sample of spleen cells and 1 sample of peripheral blood cells from hairy cell leukemia patients, with 1000 U/ml rIFN-alpha for 24 hours. Changes in morphology were observed by SEM on 20-30% of the cells in these two cases, while no changes seem to have been seen in a third case.
Authors: The aim of this work was to review for SEM people the literature on the ability of IFN to induce changes in target cells. Beside that goal, it brought our results regarding a total number of 7 cases, of which 3 were new ones. As was stressed in the original paper, the changes in morphology were seen in 5 out of the 7 studied cases, which indicated a unique phenomenon. In this revised manuscript we added 4 more cases, and reviewed the results from a total of 11 cases treated with IFN in-vitro in our lab. These unique alterations were seen in 8 out of the 11 cases.

M. Revel: Leaving aside the fact that the changes illustrated only by a couple of micrographs in the unique figure, are difficult to see for a non-expert microscopist, the question is whether these 20-30% of cells are all the hairy cells in the mononuclear cell fraction or whether only 20-30% of the hairy cells are modified? The number of hairy cells (tartrate-resistant phosphatase positive, or stained with specific antibodies) should be given for each sample examined.
Authors: Having in mind the non-expert microscopists, we have replaced the original photographs and added others that clearly demonstrate the differences. Regarding the numbers of altered cells, we clearly noted that the changes were seen "in 22-31% of IFN-treated HCs", which refers to the absolute number of HCs, not to the mononuclears. Therefore, the actual number of hairy cells in each case is not important, but was always more than 60% in the PB and more than 80% in the spleen, as was noted in the Materials and Methods section.

M. Revel: If only a small proportion of hairy cells is affected by IFN, is it possible that these changes just reflect the position of the cells in the cell-cycle, which could be affected by IFN? This seems to me a most critical point.
Authors: We do agree that this is a most important topic. This whole issue is now under intensive study in our laboratory, especially, by the use of synchronized cell samples. We do not agree with the use of the word 'just' in this context since our aim is to answer the question as to why IFN is so effective in some leukemias, and is not effective in others. If a proper position in the cell cycle enables IFN to react effectively through the changes that we described, this might be 'just' what we are trying to clarify.

M. Revel: Furthermore, if changes were not seen in 1 out of 3 samples is there any relation to response to treatment in-vivo?
Authors: All 11 patients in this study were treated with IFN in-vivo. Except in one case, they all reacted well to this treatment, as indicated by a decrease in the number of malignant cells in their blood. The exceptional case also did not show morphological changes in-vitro, but on the other hand, the cells from two other cases did not display membrane alterations under the SEM, but reacted well to the drug in-vivo. Thus, further studies are necessary to correctly relate the response in-vivo to that demonstrated in-vitro by ultrastructure.

M. Revel: Another unanswered question is whether such "changes" are seen in other lymphoid cells than those of hairy cell leukemia?
Authors: We are currently studying various types of normal and leukemic leukocytes treated with IFN in-vitro. So far, none of these cell types did show the same changes as did hairy cells. Daudi (B-type lymphoma cells) do show some surface membrane alterations, mainly, with regard to the distribution of surface microvilli, but they do not show extensive bubbling of the membrane like the IFN-treated hairy cells, nor the same bud-like formation.

Reviewer III: Other questions that are unresolved are, for example, whether similar changes can occur upon exposure of hairy cell leukemia to other biologic response modifiers, such as interleukin-2 or BCGF, factors that have produced biologic response in-vitro. It is not known whether an antibody to interferon alpha will abrogate the observed results (which is a final test to derive the conclusion that these changes are related to interferon).
Authors: Hairy cells treated with either BCGF or interleukin-2 did not show, in our laboratory, same surface alterations as did interferon-treated hairy cells. On the other hand, when an adequate neutralizing concentration of anti-interferon antiserum was added to the interferon treated cultures, it totally inhibited the induction of alterations by interferon. These facts prove that the initially described SEM surface changes are specific to interferon.

Reviewer IV: Did you observe bud-like formation only in IFN-treated HCs, and did you find the microvilli of the treated cells of Fig. 2b much shorter than those of untreated cells of Fig. 1a?
Authors: The answer to both questions is - yes.