Human Recombinant Interferon α-2C Enhances the Expression of Class II HLA Antigens on Hairy Cells

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Isolated splenic hairy cells from three untreated patients were cultured in presence of recombinant human interferon alpha-2C (IFNα). Ultrastructural cytochemistry and immunophenotype analysis with a large panel of monoclonal antibodies were performed to study cellular modification induced by IFNα. Hairy cells showed a typical phenotype: SmIg$^+$, B1$^+$, BA1$^\pm$, anti-Tac$^+$, OKDR$^+$, Leu-M5$^+$, HC2$^+$, TRAP$^+$, myeloperoxidase$^-$. Under our experimental conditions, we found no direct cytotoxic effects or significant variations in morphology, cytochemistry, and percentage of reactivity with the tested monoclonal antibodies. After culturing in the presence of different doses of IFNα, we observed a significant enhancement of the expression of class II HLA antigens as demonstrated by increased fluorescence for OKDR, OKIa, Leu-10 at fluorescence-activated cell sorting analysis. In agreement with this finding IFNα-treated hairy cells showed an increased stimulatory capacity *v* allogeneic T cells in one-way mixed lymphocyte reaction. To our knowledge this is the first report describing the induction of class II HLA antigens on hairy cells by IFNα.

THE MECHANISMS OF antitumor action of the interferons (IFNs) have not yet been clearly established. They are postulated to have a direct effect on tumor cell growth and to enhance the host's immune responses, properties that could be of great importance in inhibiting tumor proliferation.[1-3] Recently, moreover, it has been demonstrated that IFN may act by promoting cell differentiation in tumor cell systems.[4,5] Next to the morphological and immunophenotypic differentiating effect, their most striking characteristic is an ability to enhance in vitro the expression of class I and II HLA antigens.[6-8] In particular, IFNγ has been shown to induce Ia antigens on B lymphomas and macrophagic cell lines.[9] Considering their natural origin, it would be interesting if IFNs had a "physiologic" antitumor effect due to their differentiating and modulating properties.

Hairy cell leukemia (HCL) is a B cell tumor[10] with a slow proliferative rate in which IFNα has recently been shown to have a clear antitumor effect.[11] With the aim of clarifying the action of IFN in this malignancy, we studied isolated splenic hairy cells in liquid phase cultures in presence of recombinant human IFN type alpha-2C.

MATERIALS AND METHODS

Patients. Three untreated patients with HCL were studied. Diagnosis was based on the clinical picture, light microscopy, and transmission electron microscopy (TEM) of bone marrow biopsies or isolated peripheral blood mononuclear cells, and the presence of tartrate-resistant acid phosphatase activity. Each patient underwent splenectomy because of enlarging spleen and/or severe cytopenia.

Isolation of hairy cells. Splenic specimens were teased through a stainless steel mesh sieve to obtain a single cell suspension, which was centrifuged on a Ficoll-Hypaque gradient (MSL, Eurobio, Paris). The mononuclear cells recovered included more than 80% hairy cells at ultrastructural analysis. Except for the ultrastructural studies, all marker tests and function assays were performed more than once on fresh and cryopreserved cells.

Cell cultures. Cells were cultured at a concentration of 1.5×10^6 in RPMI 1640 medium (Flow Laboratories, Irvine, UK) supplemented with 20% heat-inactivated fetal calf serum (FCS, GIBCO, Grand Island, NY) and antibiotics with or without IFNα (recombinant human interferon type alpha-2C, Boehringer, Ingelheim, FRG) at concentrations of 20, 100, and 500 IU/mL. Cultures were done in plastic Petri dishes (Sterilin Ltd, Teddington, UK) and incubated for 48 or 96 hours at 37 °C in a 5% CO_2 humidified incubator. In case 2 only, an additional set of cultures was carried out in which 20 IU/mL IFNα was added every 24 hours for four days of culture. Viability tests with the trypan blue exclusion method and all of the following tests were performed at the beginning and end of cultures.

Immunophenotype determination. We studied surface membrane immunoglobulins (SmIg) by direct immunofluorescence using fluorescein isothiocyanate-conjugated (FITC) polyclonal rabbit antibodies to human heavy and light chains (Dakopatts, Glostrup, Denmark) as previously described.[12]

Cell staining by monoclonal antibodies (mAb, listed in Table 1, references 13–16) was done by direct or indirect immunofluorescence methods using as second-step reaction FITC-labeled rabbit antimouse F(ab)$'_2$ fragments (Nordic, Immunological Laboratories, The Netherlands).

Before staining, the cells were incubated at 37 °C for ten minutes with acetate buffer, then for two hours in medium, and, subsequently, with rabbit nonimmune serum to eliminate cytophilic Ig and nonspecific Fc fragment binding. Samples were analyzed with a Leitz Dialux 20EB fluorescence microscope (Leitz, Wetzlar, Germany); fluorescence intensity of the cells was determined by flow cytometry on FACS 420 (Becton Dickinson, Mountain View, Calif) and on Spectrum III (Ortho Diagnostic Systems, Westwood, Mass). Cells (10,000) were scored, and the number of positive cells was calculated after subtracting the reactivity of cells stained with FITC rabbit antimouse antiserum alone.

Mouse rosettes. Mouse (M) rosettes were tested as previously described.[12]

Cytoplasmic Ig. Cytoplasmic immunoglobulins (CyIg) were evaluated with the method described by Vogler et al[17] using FITC polyclonal rabbit antibody to heavy and light chains in appropriate dilutions (Dakopatts, Glostrup, Denmark). A Leitz Dialux 20EB fluorescence microscope was used to observe the slides.

Ultrastructural study. In addition to conventional analysis, splenic hairy cells were examined with TEM. Routine morphology was carried out on the whole series of samples as described elsewhere.[18] The Graham and Karnovsky technique was used to detect the presence of myeloperoxidase reaction[19] in hairy cells before and after culture.

The immunogold staining reaction[20] was also performed on some

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Table 1. Antibodies and Their Specificity

Antibody	Specificity	Source
OKIa1	Human Ia antigens, B lymphocytes, monocytes, activated T lymphocytes	Ortho Pharmaceutical Corp, Raritan, NJ
OKDR	HLA-DR monomorphic antigens on B lymphocytes, monocytes, activated T lymphocytes	Ortho Pharmaceutical Corp, Raritan, NJ
OKM1	Monocytes, granulocytes, natural killer cells	Ortho Pharmaceutical Corp, Raritan, NJ
BA1	B lymphocytes, non-T/non-B ALL, pre-B ALL, CLL, malignant lymphoma, granulocytes	Hybritech Inc, San Diego
B1	B lymphocytes	Coulter Immunology, Hialeah, Fla
Leu-1	Peripheral T cells, thymocytes, B CLL	Becton Dickinson, Sunnyvale, Calif
Leu-2a	T suppressor/cytotoxic cells	Becton Dickinson, Sunnyvale, Calif
Leu-3a	T helper/inducer cells	Becton Dickinson, Sunnyvale, Calif
Leu-7	NK and K cells	Becton Dickinson, Sunnyvale, Calif
Leu-10	HLA-DC/DS antigens on B lymphocytes, B cell lines	Becton Dickinson, Sunnyvale, Calif
Leu-11a	NK and K cells, neutrophils	Becton Dickinson, Sunnyvale, Calif
FMC32	Monocytes, macrophages[13]	Gift of H. Zola, Bedford Park, Australia
αHC2	Hairy cells, leukemic myeloblasts, some lymphomas[14]	Gift of D. Posnett, Rockefeller University, New York
Leu-M5	Hairy cells, monocytes[15]	Gift of H. Stein, Freie Universität, Berlin
Anti-Tac	Membrane receptor for interleukin 2 on malignant and activated T cells[16]	Gift of T.A. Waldmann, National Cancer Institute, Bethesda, MD

samples selected for immunoultrastructural investigations because previous immunofluorescence screening had presented problems of interpretation; nonhomogeneous expression of a surface antigen with a homogeneous cell population, unexpected positivity for an mAb usually nonreactive with hairy cells, and a large increase or decrease of expression of an mAb were the most frequent reasons why immunogold staining at ultrastructural level was performed. Ultrathin sections were examined before and after staining with a Philips 410 TEM.

Mixed lymphocyte reaction (MLR). T lymphocytes from four healthy donors (T_1 through T_4) were purified as previously described[21] and used as responder cells. Purified spleen hairy cells from cases 2 and 3, cultured for 48 hours with or without 100 IU IFNα, as described previously, and subsequently irradiated with 3,000 rad, were used as stimulator cells (T_1 and T_2 cells *v* hairy cells from case 2, T_3 and T_4 cells *v* hairy cells from case 3). Cocultures were established in quintuplicate, incubating 10^5 responder cells with 2.5, 5.0, and 10.0 × 10^4 stimulator cells in RMPI 1640 medium supplemented with 20% heat-inactivated FCS and antibiotics in round-bottomed wells of Microtest plates (Sterilin) at 37 °C in a 5% CO_2 humidified incubator for seven days. To evaluate cell proliferation, 1 μCi ^{3}H-thymidine (2 Ci/mmol, Radiochemical Centre, Amersham, UK) was added to the cultures 18 hours before harvesting. ^{3}H-Thymidine uptake was determined in a Packard tri-carb liquid scintillation counter. Conventional mixed lymphocyte cultures (MLC) were carried out in the same way, culturing 10^5 purified peripheral T_1 through T_4 cells with 10^5 allogeneic peripheral non-T cells treated with 5,000 rad.[22] Controls consisted of responder and stimulator cells cultured alone.

Statistical analysis. The statistical significance of data was evaluated with Student's *t* test.

RESULTS

Changes in morphology, cytochemistry, and immunophenotype in splenic hairy cells cultured with IFNα. After separation, the recovered cell fraction consisted mainly of mononuclear cells in which typical ultrastructural features of hairy cells could be found. At TEM, more than 80% of the cells had oval and deeply indented nuclei, numerous cytoplasmic vesicles, mitochondria, and characteristic narrow-based long projections at the periphery (Fig 1A). Graham and Karnovsky's myeloperoxidase reaction was negative.[19] The cell surface antigen expression detected by mAb in fresh spleen cells from our patients is reported in Table 2. All three cases displayed a B cell phenotype; a high percentage of cells expressed monoclonal-type SmIg, B1 antigen, and class II HLA antigens. Intensity of staining with Leu-10 was weaker than that with OKDR and OKIa1 but present on most cells. Positivity with Leu-10 in hairy cells was also less intense than that usually observed in normal circulating B lymphocytes. Leu-M5 staining was strongly positive in most hairy cells in contrast to BA1 antigen expression, which varied in the three cases. Positivity to anti-HC2 and anti-Tac was weak but detected in a high percentage of cells in all the cases, whereas OKM1 positivity was observed only in case 3.

No significant variations in viability, cell number, morphology at light microscopy, and cytochemcial pattern were observed at any culture time point, irrespective of the presence or amount of IFNα. No CyIg or M rosettes were detected either before or after culture.

After culture with or without IFNα, ultrastructural morphological changes appeared in the cells, indicating increased intracellular protein synthesis. More than 50% of the cell population had a large Golgi apparatus, numerous short strands of endoplasmic reticulum, and a very high number of cytoplasmic vesicles and pale granules. The Graham and Karnovsky myeloperoxidase reaction continued to be negative (Fig 1B through D).

We did not find any significant variation in percentage positivity for the various surface antigens investigated except in case 2. In all cultures of cells from this patient, we observed increased positivity to OKM1, which went from a low basal level (6%) to 50% and 60%, respectively, for cells exposed and unexposed to IFNα. On the other hand, at ultrastructural analysis, we observed persistent myeloperoxidase negativity as well as low expression (<5%) of other mAbs with known macrophage–monocyte specificity, such as Leu-M1 and FMC32 (Fig 1C).

The only IFN-induced variation we observed was in intensity of expression of class II HLA antigens. In all three cases studied, incubation of spleen hairy cells with IFNα clearly enhanced the intensity of surface expression antigens detected by OKDR, OKIa1, and Leu-10. This effect was

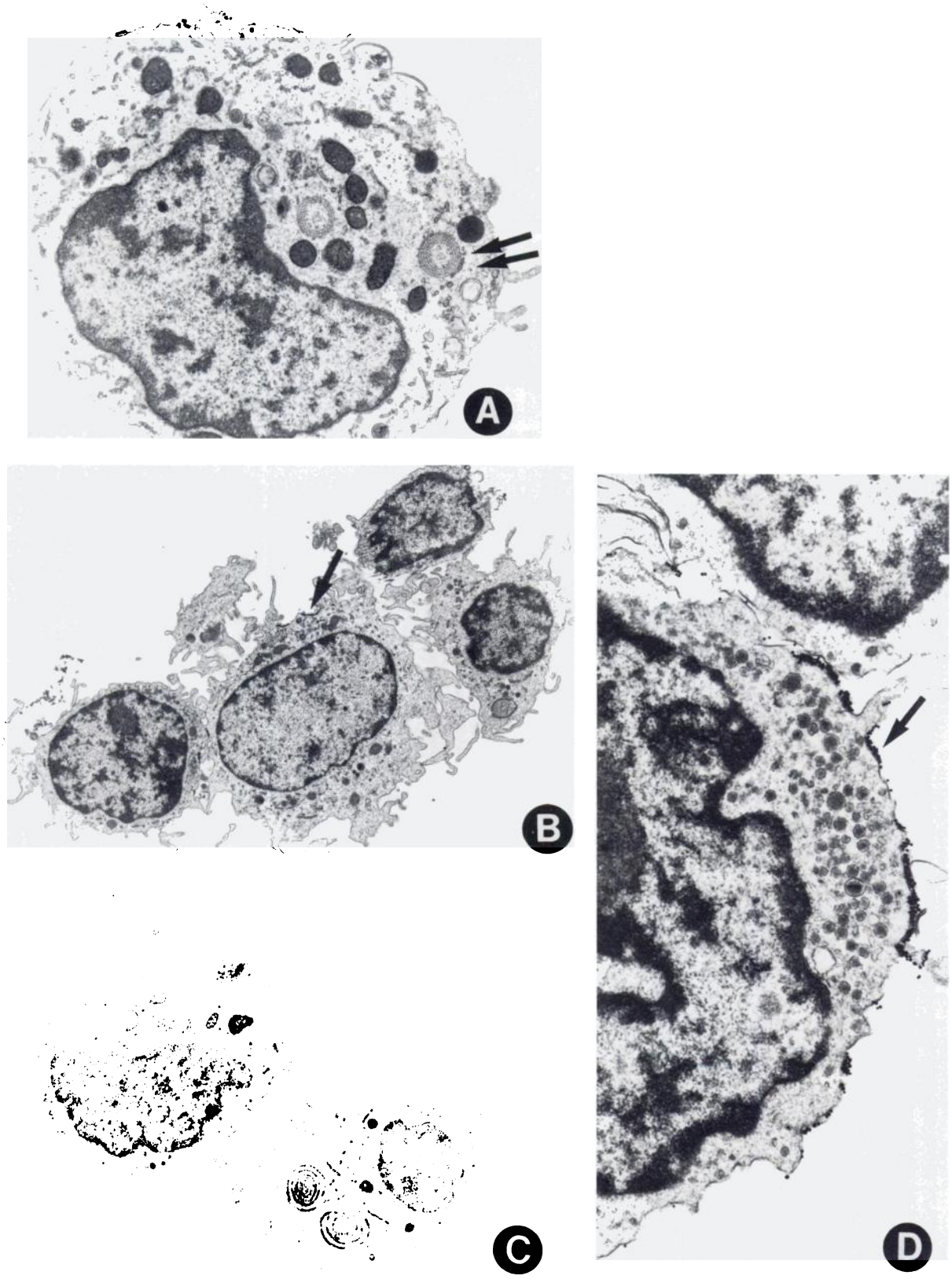

Fig 1. Splenic hairy cell suspension. (A) Oval and indented nuclei, cytoplasmic vesicles, and ribosome–lamella complexes are typical ultrastructural features of hairy cells (PbUa; original magnification ×10,000; current magnification ×7,700). (B) After culture in presence of IFN. The cells are positive for Leu-M5 (single arrow) at immunogold staining (PbUa; original magnification ×10,000; current magnification × 8,100). (C) After culture in presence of IFN. The cells are myeloperoxidase negative with Graham and Karnovsky's method (unstained; original magnification ×7,000; current magnification ×5,390). (D) Detail of cultured hairy cells strongly positive for Leu-M5 (single arrow). The increased number of cytoplasmic vesicles suggests active cell synthesis (PbUa; original magnification ×26,000; current magnification ×20,800).

Table 2. Phenotype of Fresh Mononuclear Cells From Spleen of Patients With HCL

	Positive Cells (%)		
	Patient 1	Patient 2	Patient 3
SmIg	60δλ	80γκ	70μδλ
OKIa1	83	75	80
OKDR	82	84	82
Leu-10	81	78	75
B1	75	85	84
BA1	5	25	50
Anti-Tac	70	60	80
Leu-M5	83	74	85
αHC2	76	84	78
OKM1	10	6	75
Leu-M1	5	3	5
FMC32	6	1	3
Leu-1, -2, -3, -7, -11	<5	<5	<5

shown by fluorescence microscopy, and even more clearly by cytofluorimetric analysis, and was detectable already at 48 hours' culture and with IFN concentrations as low as 20 IU/mL. Possibly due to some degree of activation induced by FCS, intensity of expression of these surface antigens was slightly increased in hairy cells cultured in the absence of IFN compared with freshly stained cells. However, this enhancement was significantly lower than that observed in the presence of IFN. FACS histograms derived from case 2 hairy cell analysis (Fig 2) show the characteristics of enhancement of class II HLA antigen expression, which were almost identical to those of cases 1 and 3 (not shown). The addition of a neutralizing concentration (1 μg/10 IU IFN) of an anti-IFNα mAb (EBI1, kindly provided by Dr G.R. Adolf, Ernst Boehringer Institut, Vienna) at the start of cultures of case 2 hairy cells with 100 IU/mL IFN was found to inhibit the enhancement of class II HLA antigen expression significantly compared with hairy cells cultured with IFNα and without EBI1. This enhancement was not significantly modified when hairy cells from case 2 were preincubated with anti-Tac for one hour at 4 °C.

Stimulatory capacity in MLR of spleen hairy cells cultured with IFNα. The increase in class II HLA antigens expression after IFN treatment in vitro was accompanied by enhancement of the stimulatory activity of spleen hairy cells against allogeneic purified peripheral T lymphocytes in one-way MLC. The responsiveness of these T cells was previously shown to be in the normal range (mean ± SD of ^{3}H-thymidine incorporation: 22 ± 3 × 10^3 cpm) in conventional MLC with T-depleted allogeneic peripheral mononuclear cells from normal subjects.

After 48 hours' culture in presence of 100 IU/mL IFNα, the stimulatory capacity of the hairy cells of both the cases studied was markedly increased (Fig 3), the difference being statistically significant at the lowest cell concentrations. At the highest cell concentrations, the MLR was no longer proportional to the number of stimulator cells added. As shown in Fig 3, at the highest cell concentration, the increase of stimulatory capability after exposure to IFNα was significant only in case 3 hairy cells v T_4 cells.

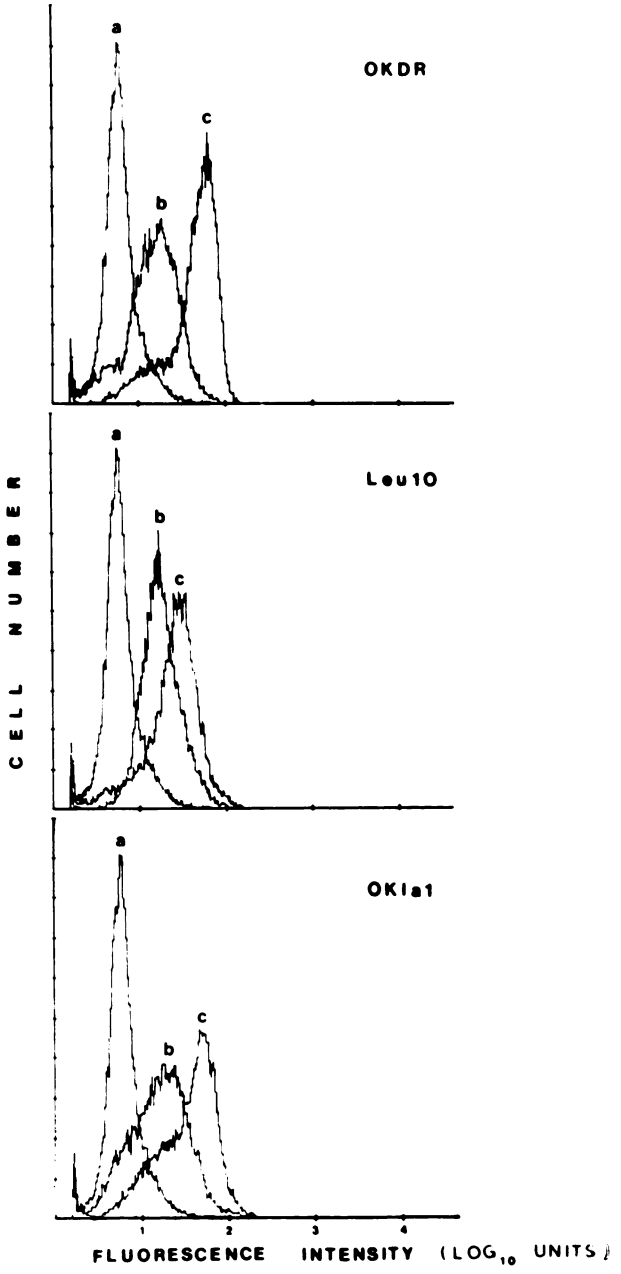

Fig 2. FACS analysis of splenic hairy cells from case 2 with OKDR, OKIa1, and Leu-10. The cells were cultured with or without 100 IU/mL of recombinant IFNα for 48 hours. (A) Control of IFN-treated cells. (B) Non-IFN-treated cells. (C) IFN-treated cells. Settings for FACS 420 were photomultiplier 700 mV, laser 350 mW at 488 nm.

DISCUSSION

We studied the effects in vitro of recombinant IFNα on homogeneous spleen hairy cell populations. For this purpose, immunophenotype analysis was carried out with a large panel of mAbs: the cytofluorimetric pattern of positivities and ultrastructural cell features was then evaluated. In addition, functional behavior in MLC was investigated. In agreement with a recent report by Kosmeyer et al,[10] fresh

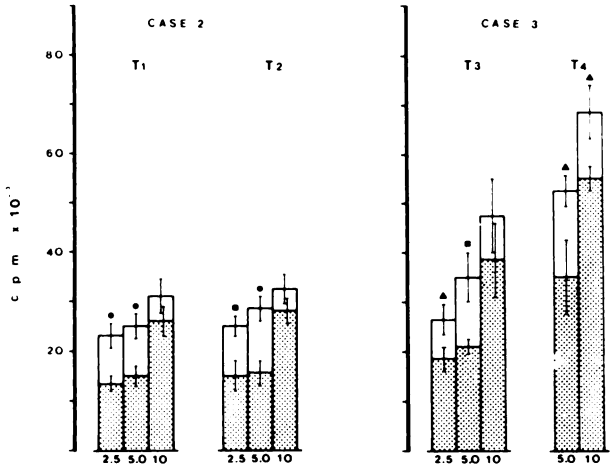

Fig 3. Increase of stimulatory capacity of IFN-treated splenic hairy cells in MLC. Splenic hairy cells were cultured with (□) or without (▨) 100 IU/mL IFN for 48 hours, irradiated with 3,000 rad, and then cocultured at three concentrations (2.5, 5.0, and 10 × 10^4 cells/200 μL as shown on the abscissa) with 10^5 purified T cells from four healthy donors (T_1 through T_4). The mean ± SD of ^{3}H-thymidine uptake of purified T_1 through T_4 cells in conventional MLC with non-T peripheral mononuclear cells from allogeneic donors was 22 ± 3 × 10^3 cpm. Cultures were done in quintuplicate and results are expressed as cpm ± SD. (▲, $P < .05$; ■, $P < .02$; ●, $P < .01$).

hairy cells from the three cases we studied all expressed a typical immunophenotype consistent with their proposed B cell origin ($SmIg^+$, $B1^+$, $BA1^+$, $OKIa1^+$, $OKDR^+$). Positivity for anti-Tac, which binds interleukin 2 (IL 2) receptor, was initially reported in activated and malignant T cells.[16] Recently, however, IL 2 receptor has also been identified on activated B cells,[23] and our samples showed positivity for this mAb. Furthermore, our study confirmed that Leu-M5 and HC2 are useful in identifying cells positive for a B cell-specific antibody (ie, B1) as hairy cells.[14,15]

The expression of class II HLA antigens on the surface of hairy cells was studied with both OKDR (specific for DR monomorphic antigens) and Leu-10 (specific for DR-related DC/DS molecules) in view of the observation that they are expressed differentially on normal hemopoietic and leukemic human cells according to their degree of differentiation.[24] Although the fluorescence intensity for Leu-10 was weaker than in normal peripheral B lymphocytes, we detected both antigens in all the cases, as previously described in Meijer et al's immunohistochemical study.[25] On the contrary, Faille et al[26] were not able to demonstrate DC/DS molecules on isolated cells in one case of HCL using immunoprecipitation techniques; however, they did report the expression of these antigens after exposure of hairy cells to chemical inducers.

In our study, viability, morphology, and cytochemistry of hairy cells were unaffected by culturing. We only observed constant ultrastructural features of increased protein synthesis. As this finding was detected in presence or absence of IFN, we consider it attributable to an aspecific effect of FCS.

Also the immunophenotype was unaffected by culturing apart from the appearance of OKM1 positivity in case 2 irrespective of the presence of IFN. The unchanged morphological and cytochemical features of cultured cells allowed us to exclude a relative increase of monocytes in the culture system. Moreover, OKM1 is not strictly specific for the monocytic lineage,[27,28] and it has already been shown by others to react with both fresh and cultured hairy cells.[29,30]

It is interesting that in this study, human recombinant IFN alpha-2C enhanced the expression of class II HLA antigens in hairy cells. This phenomenon was accompanied by increased stimulatory activity of hairy cells in MLC, functional behavior well known to be closely HLA-DR–linked.[31] This effect was more evident at the lowest hairy cell concentrations, probably due to a functional limit of cultured T cells' responder activity. The increased expression of class II HLA antigens can be attributed to a direct action of IFN, since it was neutralized by the addition of anti-IFNα mAb at the start of culture. No differences were seen when hairy cells were pretreated with anti-Tac; this supports the hypothesis that the IL 2 receptor is not involved. IFNα was active in vitro at a low concentration (20 IU/mL), comparable to that reached in vivo with the administration of therapeutic doses (5×10^6 IU/d).

It is known that IFNs can modify the membrane behavior of cells exposed to their action and, particularly, HLA antigen and Fc receptor expressions.[1] In fact, enhancement of class II HLA antigens has been shown on human monocyte cell lines[6] and on various murine cell lines of hemopoietic, lymphoid, macrophage, fibroblast, and neuronal origin[7,9] in presence of recombinant or highly purified IFNγ. An increase of mRNAs codifying these HLA antigens has been demonstrated by other authors in IFN-treated human lymphoblastoid and melanoma cell lines.[32]

The biological meaning of these effects is still discussed. The induction of Ia antigens, particularly on B lymphoma and macrophage cell lines, may result in a functional enhancement of antigen-presenting capability.[9] Another hypothesis is that hemopoiesis may be modulated by IFN through Ia-restricted communication with regulatory T cells.[33,34] Finally, it is conceivable that this class II HLA antigen modulation is an expression of differentiation in normal hemopoietic cells and their neoplastic counterparts. The fact that IFNα and β are less effective in inducing class II HLA antigens[6] than IFNγ, and that the latter is normally released by activated T cells, seems to indicate that IFNγ plays a more important role in the physiologic regulation of these antigens.

Hokland et al,[35] using partially purified IFNα, demonstrated an increase of HLA antigen expression detected by β_2-microglobulin on peripheral blood mononuclear cells and in various human non-T lymphoblastoid cell lines. Nevertheless, in the same experimental conditions, the HLA-DR–related Ia antigens were unaffected. Our study shows that hairy cells are sensitive to IFNα, as indicated by the increase of HLA-DR and DR-linked antigen expression. This sensitivity was particularly notable, since we failed to observe such a phenomenon on cells from four cases of B-CLL. Moreover, in $OKDR^+$ $Leu\text{-}10^-$ cells from ALL (two cases of

n-ALL, one case of c-ALL), IFNα treatment did not induce either DR antigen expression enhancement or positivity to Leu-10 (data not shown).

IFNα did not seem to have a differentiating action on hairy cells, since we did not observe any other immunocytomorphological modification, indicating cellular maturation. On the other hand, HLA antigens are known to be involved in cell-mediated cytotoxicity *v* virus-infected and possibly tumor cells. In particular, class II HLA antigens have been implicated in the sensitization phase of cell-mediated cytolysis and as restriction antigens in cytotoxicity mediated by $T4^+$ clones.[36] This cytotoxicity mechanism has been demonstrated also in interactions between cytotoxic effector lymphocyte clones and autologous B lymphoblastoid lines transformed by Epstein-Barr virus.[37] It may be speculated that the selective enhancement of class II HLA antigens shown in our study, by potentiating a cytotoxic mechanism, contributes to the antileukemic activity IFNα exerts in vivo. Our data may therefore help to elucidate the multifactorial mechanism of therapeutic effect of IFNα in HCL.

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