Modulation of the Expression of Major Histocompatibility Antigens on Splenic Hairy Cells—Differential Effect Upon in vitro Treatment With Alpha-2b-Interferon, Gamma-Interferon, and Interleukin-2

By Volker H. Gressler, Regine E. Weinkauff, Wilbur A. Franklin, and Harvey M. Golomb

The mechanism of action responsible for the beneficial effect of alpha-interferon (α-IFN) in patients with hairy cell leukemia (HCL) is still unknown. Direct antineoplastic and immunomodulating effects on both the host immune system and the hairy cells themselves have been implicated. To evaluate whether lymphokines have any regulatory effect on antigens of the major histocompatibility complex (MHC) on hairy cells and whether this corresponds to prognostic clinical parameters, we studied splenic hairy cells from ten previously untreated patients. The samples were incubated with recombinant human α-IFN, gamma-IFN (γ-IFN), and interleukin-2 (IL-2). In an indirect staining procedure, cells were labeled with monoclonal antibodies (MoAb) to HLA ABC and HLA DR surface structures and subjected to cytofluorimetric analysis. Results concerning the expression of MHC class I and HLA DR antigens were mixed for incubation of hairy cells with γ-IFN and IL-2, whereas α-IFN had a distinct effect on HLA DR antigen expression. α-IFN strongly enhanced the intensity of staining with HLA DR MoAb in six patients, and it increased the percentage of MoAb-positive cells in five of these samples. In contrast, the staining intensity in samples from four patients was reduced considerably on α-IFN treatment. In this group, two samples showed a sharp α-IFN–induced decrease in the number of HLA DR MoAb-positive cells from originally high values, and in one sample the very low percentage of positive cells was unaffected by α-IFN exposure. These two groups of patients whose hairy cells displayed contrasting HLA DR expression on incubation with α-IFN in vitro, were found to differ in their subsequent clinical course.

The availability of recombinant interferons (IFN) has led to a number of clinical studies on their efficacy in the treatment of a variety of neoplastic disorders. Among lymphoproliferative malignancies, the impressive clinical response of patients with hairy cell leukemia (HCL) to low-dose alpha-2b-interferon (α-2b-IFN) could be confirmed unequivocally. However, the mechanism of this response remains obscure. IFNs and interleukin-2 (IL-2) are known to mediate the interaction of T cells and thus to regulate the generation and activity of natural killer (NK) and cytotoxic T cells. Consequently, one hypothesis among several, ascribed the therapeutic effect of IFN to reconstitution of the impaired NK-cell activity observed in patients with HCL. Stimulation of previously low NK-cell activity in HCL samples in vitro has also been postulated for IL-2. This hypothesis was left unexplained, however, why gamma-interferon (γ-IFN), clearly a more potent enhancer of NK cell activity than α-2b-IFN and IL-2, proved to be ineffective as a therapeutic agent in HCL. In addition, NK cells from patients have been shown to lack cytolytic effects on hairy cells, and IFNs evidently exert antitumor effects without requiring a functioning immune system. Recent studies revealed that lymphokines may have a direct impact on normal and tumor cells by changing the expression of surface structures, particularly of products of the major histocompatibility complex (MHC). HLA ABC antigens may serve as target determinants in cell-mediated lympholysis; MHC class II antigens are crucially involved in the process of presentation and recognition between target cells and effect T cells. However, the role of MHC structures on tumor cells, especially in the context of tumor therapy with lymphokines, remains poorly understood.

We undertook the present study in an attempt to shed light on the role of HLA ABC and HLA DR antigens in the clinical course of patients with HCL. The striking clinical effect of IFN in HCL, the availability of splenic hairy cell samples from ten patients, and detailed follow-up data seemed to give us a unique opportunity to address the following questions: (1) To what extent do hairy cells express class I and HLA DR antigens on their surface? (2) Are interferons and IL-2 capable of modulating the expression of these antigens, and do different IFN subtypes induce differential effects? (3) Did the samples from six patients who were clinically responsive to splenectomy either alone or in combination with subsequent α-2b-IFN therapy show a pattern of antigen expression that differed from that in cells from four patients with a less favorable clinical course?

For this study, cultured splenic hairy cells from ten patients were treated with α-2b-IFN, γ-IFN, or IL-2 for five days or received no treatment (controls). Indirect immunofluorescence assays on samples labeled with monoclonal antibodies (MoAbs) to HLA ABC and HLA DR determinants were followed by cytofluorimetric analysis.

MATERIALS AND METHODS

Patients

The study comprised ten patients treated at the University of Chicago, Department of Medicine, Joint Section of Hematology/Oncology, between 1983 and 1987 for HCL. Clinical data on these...
patients are summarized in Table 1. Based on the clinical course, two prognostically distinct groups, designated “responders” and “non-responders,” were distinguished. Response was defined as complete disappearance of hairy cells in the differential blood count, >50% reduction of leukemic cells in the bone marrow, and normalization of peripheral blood counts, all lasting at least 6 months. Patients classified as responders were those who met these criteria after splenectomy and required no further treatment, or patients who relapsed at 6 months or more after splenectomy, but who again fulfilled the response criteria after subsequent α-IFN therapy. Non-responders were those patients who did not meet these criteria, either after splenectomy or after subsequent α-IFN therapy. Responders. Six patients were doing well after splenectomy. Two of these patients (no. 5 and 6, Table 1) relapsed (at 24 and 27 months, respectively, after splenectomy), but responded to subsequent α-2b-IFN treatment consisting of 2 x 10^6 IU α-2b-IFN/m² administered subcutaneously three times per week for 1 year. The mean follow-up time for this prognostically favorable group was 39.7 months.

Non-responders. One patient (no. 9, Table 1) entered a leukemic phase 2 months after splenectomy. This patient was diagnosed 6 months later as having Hodgkin’s disease concurrent with HCL and was treated with radiotherapy. In three patients (no. 7, 8, and 10, Table 1), splenectomy resulted in only temporary (mean, 5.7 months) improvement of peripheral blood counts, and they received subsequent α-2b-IFN therapy. These patients did not meet the response criteria either after splenectomy or subsequent α-IFN therapy (mean follow-up time, 44 months).

**Splenic Hairy Cells**

Hairy cells were obtained from the spleens of the ten previously untreated patients immediately after splenectomy. The spleen tissue was sieved through a stainless-steel mesh. A single-cell suspension was layered on a Ficoll-Hypaque gradient, and mononuclear cells were separated by centrifugation.²¹ Cytocentrifuge preparations (four minutes at 250 g) were used for Wright’s stain and tartrate-resistant acid phosphatase staining. The samples from the ten patients studied were comprised of >90% hairy cells. Labeling experiments with anti-kappa and anti-lambda MoAb were performed before cryopreservation and at the time of class I/HLA DR analysis, and it was shown that >95% of cells were monoclonal.

**Cryopreservation.** All samples of splenic hairy cells were cryopreserved in RPMI 1640, 20% fetal calf serum (FCS; GIBCO, Grand Island, NY), and 10% dimethyl sulfoxide (Alfa Products, Danvers, MA). The cells were kept at −80°C for 12 hours and then stored in liquid nitrogen.

**Culture and lymphokine treatment in vitro.** On use, cells were rapidly thawed and washed twice in RPMI 1640 at 4°C. Cells were resuspended in RPMI containing 10% FCS and 50 μg/mL gentamycin. The cells were then counted, and viability was determined with the Trypan blue dye exclusion test. The cell concentration was adjusted to 2 x 10^6 cells/mL. Flasks, each containing 4 mL of cell suspension, were placed in an incubator (37°C, 3.6% CO₂). Twenty-four hours later, one of the following was added to each flask: 100 IU/mL α-2b-IFN, 1,000 IU/mL α-2b-IFN, 100 IU/mL γ-IFN, 1,000 IU/mL γ-IFN, or 1,000 IU/mL IL-2. One flask without added IFN or IL-2 was used as a negative control. Recombinant α-2b-IFN was kindly provided by the Schering Corporation (Bloomfield, NJ); recombinant γ-IFN was a gift from Biogen (Cambridge, MA); and IL-2 was obtained from Hoffman-La Roche, Nutley, NJ. The reagents were used as freshly prepared sterile solutions in RPMI 1640 medium at 10^4 IU/mL (stock solution, 10^1 IU/mL).

**Monoclonal Antibodies**

Mouse anti-human HLA ABC IgG₂ and mouse anti-human HLA DR IgG₁ (protein concentrations, 25.23 mg/mL and 10 mg/mL, respectively) were obtained from Dako Corp. Santa Barbara, CA. The working dilution used for both MoAbs was 1:10 in phosphate buffered saline (PBS). Fluorescein-conjugated F(ab')₂, goat antimouse IgG (protein concentration, 0.98 mg/mL) was purchased from Tago Inc, Burlingame, CA, and was used at a dilution of 1:40 (in PBS).
Indirect Immunofluorescence Assay

After five days' incubation, cells were harvested, washed twice in RPMI 1640, and pre-incubated in 3% pooled, heat-inactivated human AB serum. Cells were then washed, transferred to microtiter plates (Nunc, Denmark) at a concentration of 4 × 10^6 cells per well, and subjected to immunofluorescence (IF) analysis as follows. The cells were spun down, supernatants were removed, and the cells were resuspended in 50 μL of diluted anti-HLA ABC or anti-HLA DR MoAb. Fifty microliters of mouse Ig (protein concentration, 12.56 mg/mL with an IgG portion >90%) instead of MoAb was added to some wells used as controls. After incubation at 4°C for 30 minutes, cells were washed three times in PBS and resuspended in 50 μL fluorescein-labeled goat anti-mouse MoAb. The cells were again incubated at 4°C for 30 minutes, washed three times in RPMI, and analyzed on an EPICS V flowcytometer (Coulter Electronics, Inc, Hialeah, FL). A minimum of 10,000 cells were evaluated, and the absolute number of positive cells was normalized against the reactivity of the controls that had been incubated with mouse Ig (first step) and with the fluorescein-labeled secondary antibody. Staining intensity (channels) of positive cells was normalized against the reactivity of the controls and proved to be statistically significant at <.05 for 100 IU/mL and <.05 for 1,000 IU/mL of α-2b-IFN. The alpha values were <.005 for 100 IU/mL and <.05 for 1,000 IU/mL of α-2b-IFN. At 100 and 1,000 IU/mL, γ-IFN increased the percentage of MoAb-positive cells in five patients. Treatment with IL-2 resulted in downregulation of HLA DR expression in six of the ten patients. However, the consistent difference in the effect on the prognostically distinct groups of patients that was found for α-IFN could not be demonstrated for IL-2.

RESULTS

HLA ABC

Splenic hairy cells obtained from the ten patients displayed a wide range of positivity for HLA ABC MoAb (12% to 92% of cells were positive). Samples from patients (no. 2, 4, 5, and 6), who had an initially rather low percentage of HLA ABC expression, showed an increase in the number of positive cells on treatment in vitro with either α-2b-IFN (100 IU/mL, 1,000 IU/mL) or IL-2 (1,000 IU/mL). The staining intensity was increased in two of these patients (no. 2, 6). Incubation of cells from patient no. 7, who had an initially high percentage of HLA ABC-positive cells, resulted in a stable, unchanged number of positive cells—a pattern also seen in patient no. 1. The results for four patients (no. 3, 8, 9, and 10) revealed a marked decrease in class I antigen-positive cells and in staining intensity after treatment with α-2b-IFN.

Comparison of changes observed in samples from patients no. 1 through 6 (responders) to those from patients no. 7 through 10 confirmed this differential effect for α-2b-IFN; the alpha values for α-2b-IFN were <.05 and <.10 for 100 IU/mL and 1,000 IU/mL, respectively. All samples incubated with γ-IFN displayed a rather heterogeneous pattern of HLA ABC expression, with no significant difference between the two prognostic groups was detectable (Table 2).

HLA DR

Table 2 shows the results obtained after labeling of hairy cells with the anti-HLA DR MoAb. Again, the samples from the ten patients tested revealed a broad reactivity pattern in untreated cells (0% to 86%). The results for patients no. 1 through 6 showed a striking difference from those obtained for the remaining four patients. α-2b-IFN incubation of samples from patients no. 8, 9, and 10 resulted in a considerable reduction in the HLA DR antigen expression (ie, in three of ten patients), whereas an increment was detected in five patients (no. 1, 2, 3, 5, and 6). These α-IFN induced changes in the percentage of HLA DR MoAb-positive cells were evaluated and proved to be statistically significant at α <.01 (100 and 1,000 IU/mL). This result was also observed with respect to changes in staining intensity (Fig 1); binding to anti-HLA DR MoAb was decreased in all samples from the nonresponder group, but was increased in the prognostically favorable group, after incubation with α-2b-IFN. The alpha values were <.005 for 100 IU/mL and <.05 for 1,000 IU/mL of α-2b-IFN. At 100 and 1,000 IU/mL, γ-IFN increased the percentage of MoAb-positive cells in five patients. Treatment with IL-2 resulted in downregulation of HLA DR expression in six of the ten patients.

DISCUSSION

Besides their antiviral activity, interferons are acknowledged to have distinct antiproliferative, immunomodulating, and differentiation-inducing effects on tumor cells. Current concepts in tumor immunology using lymphokines as therapeutic agents are based on two major approaches: (1) enhancement of the cytotoxic activity of the effector compo-

| Table 2. HLA Expression on Untreated Hairy Cells and on Cell Samples Incubated With Various Lymphokines |
|-------------------------------------------------------|-------------------------------------------------------|
| Patient No. | Control (100 IU) | α-2b-IFN (100 IU) | γ-IFN (100 IU) | γ-IFN (1,000 IU) | IL-2 (1,000 IU) |
|-------------------------------------------------------|-------------------------------------------------------|
| Percentage of HLA ABC-positive cells | | | | | |
| 1 | 47 | 57 | 30 | 37 | 34 | 45 |
| 2 | 12 | 45 | 36 | 69 | 71 | 74 |
| 3 | 37 | 0 | 18 | 50 | 0 | 28 |
| 4 | 39 | 79 | 79 | 79 | 73 | 71 |
| 5 | 19 | 14 | 37 | 0 | 0 | 48 |
| 6 | 28 | 60 | 82 | 8 | 9 | 41 |
| 7 | 93 | 95 | 93 | 92 | 93 | 94 |
| 8 | 79 | 41 | 67 | 44 | 67 | 43 |
| 9 | 83 | 15 | 13 | 66 | 75 | 79 |
| 10 | 21 | 1 | 8 | 5 | 7 | 16 |

The percentages of positive cells represent the mean of three tests (SD <4.5).

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IFN and IL-2 on MHC-expression in HCL

Fig 1. Cytofluorimetric analysis of splenic hairy cells from ten patients, cultured with α-2b-IFN, γ-IFN, or IL-2. Two sets of samples from each patient were labeled with anti-HLA ABC and anti-HLA DR MoAb, respectively. Triplicate tests per sample were carried out (standard deviation <12) and the means recorded. Bars represent difference in staining intensity between cells treated in vitro and untreated controls. Empty bars, HLA ABC staining; filled bars, HLA DR staining. The differences in staining intensity between treated cells and controls are expressed as channel numbers.

We studied the effect of α-2b-IFN, γ-IFN, and IL-2 on samples of splenic hairy cells from ten patients. Indirect IF tests were performed with MoAbs to polymorphic class I determinants and products of the HLA DR locus. In previous experiments without added lymphokines and in the presence of α-IFN, γ-IFN, or IL-2 we had found that hairy cells survive in FCS-supplemented RPMI medium for 13 days and longer (unpublished data). During this period, no significant change in the ratio of leukemic to normal cells could be observed.

As a major factor, nonspecific reactions of hairy cells with MoAbs have to be taken into account; indirect staining procedures especially are prone to give false-positive fluorescence reactions via avid receptors for the Fc portion of IgG (FcIgG). Pre-incubation with IgG (as contained in heat inactivated human serum), however, has been shown to avoid unspecific binding. In addition, the use of F(ab')2 fragments as second-step MoAb proved, in our experience, to yield reliable results, with minimal background staining. We evaluated both the percentages of MoAb-positive cells and the cytofluorimetric staining patterns; this approach had already been proved to be feasible in studies on IFN-induced changes in MHC expression. Changes in the degree of HLA ABC expression varied considerably in each of the ten samples after incubation with IFN or IL-2. In a comparison of the two prognostic groups of patients by statistical analysis, we found that only treatment with α-2b-IFN resulted in enhanced class I antigen, and that this increment was seen only on cells obtained from responders.

Our finding is in agreement with a number of reports describing the inducibility of class I determinants on a variety of normal and malignant cells. This effect has been demonstrated for both α- and γ-IFN and is thought to be related mainly to the cell type tested. Both the metastatic properties and the immunogenicity in vivo of certain mouse tumors were found to be correlated with the expression of class I MHC antigens. Experiments with leukemia cells revealed a correlation between HLA ABC expression and reactivity in mixed-lymphocyte cultures. In mice, it could be shown that cytotoxic T cells recognize neoplastically transformed cells only in association with H 2, an MHC-class I antigen. Antibodies to HLA ABC antigens have been shown to inhibit specific cell-mediated lysis of tumor cells. The role and biological significance of class I antigens in human tumors, however, are poorly understood.

When splenic hairy cells from patients with a favorable clinical course were tested for reactivity with anti-HLA DR MoAb, a dramatic increase in antigen expression after incubation with 100 IU and/or 1,000 IU/mL α-2b-IFN was noted. This effect was reflected both in an increased number of MoAb-stained cells and in enhancement of the staining intensity. This reaction pattern was strikingly different from that found on hairy cells from patients who had failed to respond to splenectomy and subsequent α-2b-IFN therapy. In these patients, following incubation of cells in vitro with α-2b-IFN, HLA DR expression was found to be markedly down-regulated.

These results confirm data published by Baldini et al, who found an increase in HLA DR antigen expression after incubation of samples from three HCL patients with α-IFN in vitro. Interestingly, both induction and down-regulation of class II antigens had already been observed previously in TPA-stimulated hairy cells. To our knowledge, however, none of the investigators in studies published thus far have looked at the expression of MHC antigens on tumor cells obtained from patients with a prognostically different clinical course.

MHC class II determinants, in particular products of the HLA DR locus, are considered to play a key role in the regulation of cellular immune reactions; their presence can be demonstrated on a variety of normal and malignant cells. T cells interact with these surface structures via specific receptors. The generation and activation of immune-competent effector cells seem to be closely related to the expression of class II antigens on tumor cells. Blocking studies showing that anti-HLA DR antibodies prevent the acquisition of IL-2 sensitivity in lymphocytes lend further support to this view.

α- and γ-IFN are known to enhance the expression of MHC antigens on neoplastic cells; the effect depends on the
MHC determinants and the type of tumor studied. Detailed information, including data on regulatory events at the molecular-genetics level, is available from experiments on breast cancer and melanoma cell lines.26,29,30 One of the conclusions drawn from these studies was that γ-IFN generally acts as a far more potent stimulator of products of the MHC class II locus than does α-IFN. Although type II receptors have not been demonstrated on hairy cells thus far, γ-IFN has been shown to induce characteristic proteins in these cells, a process that requires a direct interaction of hairy cells with γ-IFN.34 Therefore, it is not surprising that our experiments revealed changes in HLA ABC and/or HLA DR antigen expression, in agreement with similar effects of γ-IFN on other tumor entities. Our data, however, show that α-IFN, an established agent for treatment of HCL, but not γ-IFN exerts a differential effect on the expression of HLA DR in two prognostically different groups of patients with HCL. Because the number of splenic hairy cells available for our experiments was limited, we could not study the time course of HLA expression on IFN exposure. Future experiments may show that our data did not reflect the maximal effect of α-IFN on HLA expression in HCL.

The effect of α-IFN in HCL is generally considered to be a multifactorial process. Our results do not answer the question as to the mechanism of action underlying the therapeutic efficacy of α-IFN for HCL patients. However, our findings are consistent with the concept that the maintenance of a certain level of MHC antigen expression may be required for efficient host immune control. One could hypothesize that the inducibility of MHC antigens as seen in our experiments, identifies a group of patients in whom splenectomy (and thus reduction of tumor mass) and endogenous or exogenous IFN render the hairy cells susceptible to host immune control. Therefore, the stimulatory effect of α-IFN on the expression of these antigens may be an important factor contributing to the beneficial effect of α-IFN for some patients with HCL.

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Modulation of the expression of major histocompatibility antigens on splenic hairy cells--differential effect upon in vitro treatment with alpha-2b-interferon, gamma-interferon, and interleukin-2

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