Immunotherapeutic Approaches to Leukemia: The Use of the Friend Virus-induced Erythroleukemia Model System

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Abstract

We have developed a model system to study immunologically mediated regression of leukemia based on Friend virus-induced erythroleukemias. This system has been used to evaluate the immunotherapeutic activity of macrophages, specifically reactive T-cells (CTL/RFB), lymphokine-activated killer cells and interleukin 2, and tumor necrosis factor α and interferon-γ. In the present studies, CTL/RFB were evaluated for their ability to prevent disease recurrence. Animals with the regressing strain of Friend virus at Day 39 post virus were treated with either one or two injections of 5 × 10⁶ CTL/RFB. Animals given one or two injections of CTL/RFB had a significantly lower rate of recurrence than did untreated animals. The helper T-cell component of CTL/RFB was implicated in causing leukemia regression. Interleukin 1α and tumor necrosis factor α, multifunctional cytokines with similar biological activities, were evaluated for their ability to suppress leukemic erythroid colony-forming cells and induce regression. Interleukin 1α suppressed the conventional strain of, but not the polycythemia-inducing strain of, Friend virus-erythemic late erythroid colony-forming units and caused only a temporary regression of disease, while tumor necrosis factor α suppressed both forms of the disease and with multiple inoculations could cause permanent disease regressions. This system provides an excellent model for examining the efficacy of immunotherapy of leukemias with various mediators and effector mechanisms.

Introduction

The FV4 erythroleukemia model system has been used extensively to elucidate the mechanisms involved in the host's response to malignancy. FV leukemia is characterized by massive proliferation of erythroid progenitor cells in the splenic red pulp, viremia, immunosuppression, and death (1). The FVA and CFV strains both induce a lethal erythroleukemia associated with mild anemia and are characterized by CFU-E that retain their dependence on EPO for growth and differentiation (2, 3). The FVP strain, which induces a lethal leukemia with mild anemia and are characterized by CFU-E that are also capable of causing temporary regressions of CFV (FVA) erythroleukemia. The mechanisms involved in regression appear to involve interactions between T-cells, macrophages, and immunomodulatory factors or cytokines. We report here that CTL/RFB can prevent leukemia recurrence in FV-regressed mice and that IL-1α was capable of suppressing CFV-leukemic CFU-E and causing temporary regressions of the disease. In contrast to TNF-α, IL-1α did not suppress EPO-independent CFU-E in FVP-leukemic mice, nor did IL-1α induce even temporary remissions of this leukemia. These results demonstrate that the FV erythroleukemia model is an excellent system with which to evaluate immunotherapeutic approaches to leukemias and related diseases and provides a basis for analyzing the mechanisms of these effects in vitro and in vivo.

Materials and Methods

Virus. The N-tropic FVA and FVP were originally obtained from Dr. C. Friend and Dr. E. Mirand, respectively. FVA which was passaged in NIH/Swiss mice (12) was termed CFV. Viruses were maintained by serial passage of cell-free virus stocks prepared from spleens of leukemic mice (20% w/v, in phosphate-buffered saline), as previously described (12), and stored in sealed ampuls at -70°C. Mice were inoculated i.p. with 0.5 ml of phosphate-buffered saline containing approximately 10⁵ ID₅₀ of virus.

Mice. These experiments were performed in inbred Swiss mice, NIH/PLCR, that were originally obtained from the Veterinary Research Branch, NIH, and inbred in our laboratories by brother-sister mating. The colonies were regularly monitored for the absence of adventitious viruses. Mice were age and sex matched for experimental use and were assessed for the percentage of regression by spleen palpation biweekly in a “blinded” fashion. At the termination of experiments, disease status was confirmed by spleen weight, virus assays, and/or histopathology (5, 7). For hematopoietic colony assays, spleens or femurs were removed from three to five animals per treatment group and assayed individually. All experiments were replicated at least 3 times.

Cells. Fibroblasts infected with the FV complex were prepared as previously described (13, 14) using cell lines derived in our laboratory from the NIH/PLCR mouse strain. The cell line NIH/RFB23 was routinely used as stimulator in T-cell culture procedures. These are
NIH/PLCR fibroblasts that are infected with FV and express gp70 and other viral proteins on their surface (13). NIH/PLCR fibroblast cell lines that are productively infected with the MuLV component of FV, PLCR/110R2, or nonproductively infected with the SFFV component of FV, PLCR/110, were also used.

IL-1. Recombinant murine IL-1α was generously provided by Dr. Peter Lomedico, Hoffmann-LaRoche, Nutley, NJ. The specific activity of the IL-1α was 1.3 x 10^8 units/mg of protein, as determined using the D10.G4.1 assay (15). All solutions were made in pyrogen-free reagents; the endotoxin content of the IL-1α was 0.1 endotoxin unit/mg.

T-Cells. T-cells were cultured as described previously (10). Briefly, spleen cells (5 x 10^6 cells/ml) from immunized animals were put into culture with mitomycin C-treated stimulator cells (1.25 x 10^5 cells/ml) and IL-2 33% using MLA 144-derived IL-2; see Ref. 16. The cultures were fed weekly with fresh IL-2 and stimulator cells. After 14 days in culture, >95% were lysed by anti-Thy-1.2 plus complement.

Hematopoietic Progenitor Assays. The plasma clot method of McLeod et al. (17) was used for the culture of mature erythroid progenitors (CFU-E) as previously described (18). A modification of the methylcellulose method of Iscove (19) was adopted to agar for the culture of BFU-E progenitors (9). Agar cultures for CFU-M progenitors were performed as described previously using L-cell-conditioned medium as a source for colony-stimulating factor (20).

Results

In Vitro and In Vivo Activity of CTL/RFB and Recurrence of Leukemia. We have shown that FVA progressor leukemic mice can be induced to regress by the passive transfer of T-cells specifically reactive against virus and leukemia cell antigens, CTL/RFB (10). Spleen cells or lymph node cells are isolated from normal animals immunized with irradiated NIH/RFB23 (fibroblast cell line productively infected with virus and expressing viral antigens) and are grown and expanded in vitro in the presence of IL-2 and irradiated NIH/RFB23 cells. After 2 wk in culture, CTL/RFB demonstrate high levels of in vitro specific cytotoxicity against FV antigens and no lysis of natural killer-sensitive targets. A single treatment with CTL/RFB of 14-day FVA leukemic mice results in a significant percentage of permanent leukemia regressions within 2 to 3 wk (10).

The recurrence rate for CTL/RFB or TNF-α ± IFN-γ-induced regression of CFV leukemia is low, 5 to 15% (9, 10), whereas 60 to 80% of RFV-regressed mice recur, and nearly 100% of macrophage (3, 11)-regressed mice recur. To determine whether recurrence rates could be decreased by immunotherapy, RFV-regressed animals were treated with CTL/RFB at various time post virus. CTL/RFB were generated as described previously (10) and, as shown in Table 1, were specifically cytotoxic to cells infected with the whole FV complex (NIH/RFB23) and the MuLV isolated from RFV (PLCR/110R2). It is of interest that no cytotoxicity was detected against PLCR/110 which are normal PLCR/3T3 cells nonproductively infected with the SFFV component of RFV.

At 39 days post virus, groups of RFV spontaneously regress animals were randomized and treated with either one or two injections, a week apart, of 5 x 10^6 CTL/RFB. As shown in Fig. 1, the incidence of disease recurrence (spleen weight, >0.5 g, hepatomegaly, viremia) was significantly lower in animals given one injection of CTL/RFB when compared with untreated controls. The incidence of recurrence in regressed animals treated with two injections (Days 38 and 46) of CTL/RFB was very low (<20%), and most of the animals remained disease free through at least Day 145 post virus.

In Vivo Effects of IL-1α on Leukemic Hematopoiesis. Helper T-cells have been implicated in CTL/RFB-induced permanent leukemia regressions (10). One mechanism by which helper CTL/RFB may cause leukemia regression is through the production of soluble mediators or cytokines that either directly or indirectly cause the response. Culture supernatants from CTL/RFB may cause leukemia regression is through the production of soluble mediators or cytokines that either directly or indirectly cause the response. Culture supernatants from CTL/RFB may cause leukemia regression is through the production of soluble mediators or cytokines that either directly or indirectly cause the response.
mice were treated with a single i.p. dose of $1.25 \times 10^6$ units of recombinant murine IL-1$\alpha$ per mouse and assayed for hematopoietic effects 48 h later. As shown in Table 2, IL-1$\alpha$ significantly suppressed CFU-E in both the spleen and bone marrow of CFV-leukemic mice. In contrast, no significant suppressive effect was observed on the largely EPO-independent CFU-E from FVP-leukemic mice in either the bone marrow or spleen. Treatment with IL-1$\alpha$ caused a significant decrease in the spleen weight in CFV- but not FVP-leukemic mice. As demonstrated previously (23), IL-1$\alpha$ significantly suppressed normal CFU-E in both the bone marrow and spleen.

In contrast, the BFU-E compartments in the spleen and bone marrow of normal animals were stimulated by IL-1$\alpha$ treatment (Table 3). Spleen BFU-E in both FVP- and CFV-leukemic mice were decreased in animals treated with IL-1$\alpha$, but were increased by IL-1$\beta$ in normal animals.

As demonstrated previously (23), IL-1$\alpha$ stimulates CFU-M in normal animals. CFU-M were significantly stimulated in the spleens of both CFV- and FVP-leukemic mice (Table 4). In the bone marrow, CFU-M was stimulated only in CFV- and not FVP-leukemic mice.

Therapeutic Effects of IL-1$\alpha$ on FV Leukemia. To determine whether IL-1$\alpha$ induced suppression of FV-leukemic CFU-E results in regression of leukemia, progressive CFV-leukemic mice were treated with various doses of IL-1$\alpha$. Animals were treated at 14 days post virus, a time when the spleen weight is greater than 0.5 g and the animal is acutely viremic and leukemic (5, 7, 12). As noted above, a single dose of $1.25 \times 10^6$ units of IL-1$\alpha$ resulted in a rapid and significant decrease in spleen weight in CFV leukemias (clinical remission). However, within 7 days after the peak of leukemia regression, almost all the animals treated with a single dose of IL-1$\alpha$ experienced recurrence of leukemia. To determine whether repeated injections of IL-1$\alpha$ would result in a sustained regression, groups of leukemic mice were treated with $1.25 \times 10^6$ units of recombinant murine IL-1$\alpha$ daily for 5 days. As shown in Fig. 2, 75% of the animals were induced to regress within 2 wk following the last IL-1$\alpha$ injection. The recurrence rate, however, was extremely high and, after 98 days post virus, only 20% of the treated animals remained regressed.

Treatment of FVP-leukemic animals with various doses of IL-1$\alpha$ had no effect on spleen weight or leukemic status regardless of time, dose, or frequency of inoculation in accord with the findings that IL-1$\alpha$ had no in vivo suppressive effect on FVP CFU-E.

**Table 2. Effect of IL-1$\alpha$ on CFU-E from CFV- and FVP-leukemic mice**

<table>
<thead>
<tr>
<th>Source</th>
<th>Treatment</th>
<th>Spleen wt (g)</th>
<th>EPO (0.25 units/ml)</th>
<th>No. of colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVP</td>
<td>None</td>
<td>$1.25 \pm 0.30^b$</td>
<td>+</td>
<td>90.0 $\pm$ 15.0 $\pm 1234.7 \pm 103.7$</td>
</tr>
<tr>
<td></td>
<td>IL-1$\alpha$</td>
<td>1.60 $\pm$ 0.44</td>
<td>–</td>
<td>82.8 $\pm$ 9.5 $\pm 1334.0 \pm 70.7$</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>–</td>
<td>–</td>
<td>80.8 $\pm$ 14.5 $\pm 1046.0 \pm 85.9$</td>
</tr>
<tr>
<td></td>
<td>IL-1$\beta$</td>
<td>97.8 $\pm$ 7.4$^*$</td>
<td>+21</td>
<td>97.9 $\pm$ 15.6 $\pm 9$</td>
</tr>
<tr>
<td>CFV</td>
<td>None</td>
<td>0.59 $\pm$ 0.07</td>
<td>+</td>
<td>152.5 $\pm$ 27.7 $\pm 1421.0 \pm 68.1$</td>
</tr>
<tr>
<td></td>
<td>IL-1$\alpha$</td>
<td>0.28 $\pm$ 0.02$^d$</td>
<td>$\pm 34$</td>
<td>100.7 $\pm 10.1^b$ $\pm 921.0 \pm 102.0^b$</td>
</tr>
<tr>
<td>Normal</td>
<td>None</td>
<td>0.18 $\pm$ 0.04</td>
<td>+</td>
<td>104.9 $\pm$ 16.9 $\pm 460.8 \pm 51.4$</td>
</tr>
<tr>
<td></td>
<td>IL-1$\alpha$</td>
<td>0.17 $\pm$ 0.05</td>
<td>$\pm 52$</td>
<td>49.9 $\pm$ 7.2$^b$ $\pm 259.0 \pm 40.7^b$</td>
</tr>
</tbody>
</table>

* Determined in 3 to 5 animals that were assayed individually (3 cultures counted/animal) 3 days after i.p. injection of $1.25 \times 10^6$ units of IL-1$\alpha$.

* Mean ± SD.

* Significantly different from control, no treatment (individual animals), $P < 0.05$ (Student’s t test).

$^b$ $P < 0.001$. 

**Discussion**

The erythroleukemia induced in susceptible mice by FV is a progressive, lethal disease. While several lines of hematopoietic differentiation are involved in the disease, the most prominent pathological expression of virus infection is seen in the erythrocytic lineage (1, 2, 4). Previous studies in our laboratory using this system have emphasized the identification and analysis of the immunological (3, 6, 8-11, 13, 20), viral (5, 7, 12, 14, 24), and host genetic (25, 26) factors responsible for leukemia regression.

Intact macrophage (6, 20) and T-cell function (27, 28) are required for leukemia regression. Suppression or elimination of macrophages in RFV-leukemic animals with silica, carrageenan, trypan blue, or antimacrophage sera abrogates regression (6). In FVA, CFV, FVP, or RFV progressor leukemic (i.e., those mice that will not regress) macrophages are infected with the virus and have decreased phagocytic activity, adherence, and mobility of their Fe receptor. In RFV regressor leukemic (i.e., those mice that will regress) macrophages remain uninfected and retain their function. Macrophage infection occurs at the level of the CFU-M (20). The progenitors become infected in all mice inoculated with virus, but in mice that are going to regress, the infected CFU-M are eliminated and replaced with normal progenitors. The leukemia in CFV or FVA progressor mice can be made to regress temporarily by replacement therapy with purified, normal peritoneal macrophages.

Immunosuppression by any of several methods, including thymectomy, anti-thymocyte serum treatment, irradiation, or treatment with $^3$Hr, inhibits regression (27). Conversely, specific and nonspecific immunostimulation of RFV-infected mice enhances the incidence of regression and prevents leukemia recurrence in regressed mice. All RFV regressors and all regressed mice have detectable T-cell-mediated immunity against Friend virus antigens (28). In contrast, humoral immunity to virus-directed antigens does not correlate with regression, but may relate to prevention of disease recurrence in regressed mice (29).

We determined that FVA progressor leukemic mice can be induced to regress by passive transfer of specifically reactive T-cells, CTL/RFB (10). This immunotherapy was effective even in fully leukemic animals and required no concurrent or prior adjunctive treatment, such as irradiation or cytotoxic drugs. To determine the role of particular T-subsets in immunotherapeutic leukemia regression, in vitro depletion studies were carried out. We found that Lyt 1$^+$ cells caused cures, while Lyt 2$^+$ cells induced only temporary leukemia remissions (10). These results suggest that helper T-cells are required for permanent regression of leukemia. To further evaluate helper T-cell function, we examined the role of L3T4$^+$ cells in immunotherapeutic regression in vivo. Preliminary studies have demonstrated that, when CTL/RFB are specifi-
that has recurred is often more severe than the primary disease, and prognosis is usually poorer. Elucidation of the mechanisms involved in recurrence and the development of methods for its prevention have been difficult to achieve in part due to the lack of suitable in vivo experimental systems for analysis. Using the FV model system, the transfer of CTL/RFB was able to significantly prevent the recurrence in RFV-regressed mice. Recurrence could be the result of the appearance of antigenic variants of the virus that are less susceptible to the immune reactivity in regressed mice but more responsive to the activity of CTL/ RFB. We have demonstrated that the virus, as determined by virus neutralization kinetics assays, is antigenically different in the primary and recurrent disease states in the same animal (30). Studies are currently in progress to examine the mechanisms involved in the use of CTL/RFB or the cytokines, IL-1α and TNF-α, in preventing recurrence.

The mechanism by which macrophages regulate in vivo erythropoiesis and are responsible for spontaneous regression of RFV-induced leukemias has been examined (3, 11). Macrophages constitutively produce IL-1α which suppresses CFU-E, a countervailing negative element to the positive stimulus provided by EPO (23, 31). IL-1α appears not to act directly on CFU-E, but induces production of TNF-α which, either directly or indirectly, suppresses the growth or development of erythroid progenitors.

We have found that CFV (EPO-dependent) erythroleukemias are reversed by treatment with macrophages (3), macrophage supernatants (11), IL-1α, or TNF-α (9). In contrast, FVP (EPO-independent) leukemias are not responsive to macrophages, macrophage supernatant, or IL-1α, but do undergo regression by treatment with TNF-α. A direct interpretation of these results is that CFV-infected macrophages fail to produce (or secrete) sufficient IL-1α to complete the normal regulatory circuit by induction of TNF-α (and any more proximal mediators). Restoration of this regulatory axis, either by infusion of macrophages or by provision of the mediators IL-1α or TNF-α, causes disease regression. In contrast, FVP-infected animals may be unable to produce TNF-α in response to IL-1α. Thus, FVP leukemias do not regress following IL-1α treatment, but do so, albeit temporarily, with TNF-α. Production of these mediators by FV-infected cells is currently in progress. These results are in accord with our hypothesis that a driving force behind the erythroid hyperplasia characteristic of FV leukemias is loss of the normal regulatory constraints on CFU-E (i.e., macrophage production of IL-1α and TNF-α). This lack of regulation occurs because macrophages become infected with virus. Restoration of this regulatory network by provision of normal, functional macrophages, their products, or elimination of infected macrophages through immunological reactivity against virus and leukemia cell antigens causes regression of the disease. This system provides the opportunity to elucidate the mechanisms involved in these therapeutic interventions, as well as the delineation of the regulatory mechanisms operative in normal erythropoiesis.

**Acknowledgments**

We thank Linda Koch for preparation of the manuscript.

**References**

IMMUNOTHERAPY OF LEUKEMIA


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