Combination Treatment of 2-Chlorodeoxyadenosine and Type I Interferon on Hairy Cell Leukemia–like Cells: Cytotoxic Effect and MHC-Unrestricted Killer Cell Regulation

By Zvi Reiter, Sue Tomson, Osman N. Ozes, and Milton W. Taylor

Hairy cell leukemia (HCL) is a lymphoproliferative disorder of B lymphocytes. Interferons (IFNs), especially of the alpha (α) subtype, have shown a significant antitumor effect in HCL patients. However, the therapeutic effect of IFN-α is still rather limited. The purine analogue 2-chlorodeoxyadenosine (2-CdA) was reported recently to be an effective agent in the treatment of HCL. In the present study, we find that the HCL cell lines HS-1 and HS-2 as well as Eskol and its IFN-resistant clone (IRES-4) are sensitive to the cytotoxic activity of 2-CdA. Combination treatment of IFN-Con1 and 2-CdA results in a synergistic effect at low doses but has an additive inhibitory effect at higher concentrations. IRES-4 cells responded only to 2-CdA treatment. All the HCL cell lines are resistant to natural killer (NK) cell–mediated cytotoxicity (CMC) but are relatively sensitive to IFN-Con1–primed or interleukin-2 (IL-2)–primed NK-CMC activities. No inhibition in killing ability was measured when only the effector cells (NK) were treated with 2-CdA. Pretreatment of the HCL target cells with 2-CdA increases their susceptibility to NK-CMC. Pretreatment with IFN-Con1 can reduce the susceptibility of target cells to NK-CMC in HS-1, HS-2, and Eskol cells but not in the IFN-resistant clone IRES-4. 2-CdA abolished this IFN-induced protection against NK-CMC. Normal fibroblasts only responded to treatment with relatively high doses of 2-CdA, and only a moderate additive cell growth inhibitory effect was seen in combination of 2-CdA with IFN-Con1. Only high doses of 2-CdA increased the susceptibility of fibroblast culture to NK-CMC. Thus, combination of IFN-Con1 and 2-CdA results in an in vitro enhancement of the direct antiproliferative/cytotoxic activity of each treatment alone and increases the efficacy of the NK activity against the HCL cell lines.

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of some of the granules or their contents from the effector cell. The cytotoxic molecule (NK cytotoxic factor, NKCF) would then bind to the target cell, presumably at sites separated from those recognized by the NK receptor. IFN plays a central role in the regulation of NK cell activity. On the one hand, IFN increases the lytic activity of NK effector cells against sensitive target cells and induces NK-mediated cytotoxic activity against normally resistant target cells. On the other hand, pretreatment of target cells with IFN decreases their sensitivity to NK cell-mediated cytotoxicity (NK-CMC). Eskol cells were found to be completely resistant to NK-CMC but were relatively sensitive to IFN-α-prime NK cell activity and to lymphokine-activated killer (LAK) cell activity. We reported that the resistance of Eskol cells to NK-CMC is because of the low binding of Eskol to effector cells.

In this article, we report that a combination of IFN-Con1 and 2-CdA resulted in an additive cell growth inhibitory effect on the HCL cell lines. Moreover, at relatively very low doses, those at which the effect of each drug alone is unmeasurable, the combination treatment resulted in a synergistic effect. The IFN-resistant HCL clone (IREs-4) responded only to 2-CdA. All the HCL cell types are NK-CMC-resistant cells but are sensitive to IFN-Con1–primed or interleukin-2 (IL-2)–primed NK cell activities. No inhibition in the NK killing activity was found when only the NK effector cells were treated with 2-CdA. Moreover, there is no stimulatory or inhibitory effects of 2-CdA on IFN-Con1 or IL-2 activation of NK cells. On the other hand, 2-CdA used alone increases the susceptibility of the HCL cell lines to NK-CMC, whereas IFN-Con1 alone protects HS-1, HS-2, and Eskol cells but not the IFN-resistant clone IRES-4 from NK-CMC. The combination of IFN-Con1 and 2-CdA abolishes the protective effect against NK-CMC induced by IFN-α in the IFN-sensitive HCL cell lines. Thus, combination of IFN-Con1 and 2-CdA results in at least an additive in vitro enhancement of the direct cell growth inhibitory activity of each treatment alone and in an increased efficacy of the NK-CMC activity against the IFN-sensitive HCL cell lines.

**MATERIALS AND METHODS**

**Target cells.** The HCL-like cell line, Eskol, IFN-resistant Eskol clone (IREs-4), the HCL cell lines HS-1 and HS-2 obtained from a patient with chronic myelogenous leukemia (FS-4 cells) were used as control. The HCL cell lines 1, 2, 3, 4, and 5 were grown and assayed in RPMI 1640 medium (GIBCO Inc, Grand Island, NY) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a humidified 5% CO2 incubator. FS-4 cells were grown in minimal essential medium supplemented as described.

**Effectors**. Purification of effector cells was done as described. Briefly, nonadherent human leukocytes were purified from freshly collected peripheral blood of healthy donors. The cells were fractionated by sedimentation on ficoll-paque (Pharmacia LKB Biotechnology Inc, Piscataway, NJ) followed by adsorption on nylon-wool columns. The nonadherent cells were used as the effector cell population in the experimental procedures.

**IFN, IL-2, and 2-CdA.** Recombinant consensus IFN-Con1 (specific activity 3 × 10^9 U/mg) and human recombinant IL-2 (“Ala-125,” specific activity >5 × 10^9 U/mg) were provided by L. Blatt (Amgen Inc, Thousand Oaks, CA). 2-CdA was provided by Dr. D.A. Carson (Scripps Clinic, La Jolla, CA)

**Treatment of cells with IFN, IL-2, and 2-CdA.** NK effector cells (5 × 10^6 cells/mL) were treated with medium alone, with IFN-Con1 (300 U/mL), or IL-2 (100 U/mL), or with and 2-CdA for 8 hours at 37°C. The HCL cell lines and K-562 cells (at 1 × 10^5 cells/mL for the antiproliferative assay and 1 × 10^6 cells/mL for the NK assays) were grown in suspension and treated with IFN-Con1, 2-CdA, or combination of IFN-Con1 and 2-CdA for various periods of incubation under the same conditions.

**Measurement of cell proliferation.** The HCL cells (1 × 10^6 cells/mL) were treated as described. Cell samples were harvested at 24-hour intervals and counted following trypan blue staining for cell viability. FS-4 cells (5 × 10^5 cells/mL were used as control. Following incubation, the cells were stained with crystal-violet (8%) in methanol and the dye extracted with sodium citrate (0.1 mol/L) in 50% ethanol (50 μL/well). Absorbance was recorded at 590 nm. Growth inhibition is expressed as the percentage increase in cell number in the treated cultures relative to the increase in cell number in untreated cultures according to the formula: % relative growth = [(I - C)/ (C × C)] × 100, where I is the number of treated cells at day n; C is the number of control cells at day n; and C is the number of control cells at day 0. Each datum point represents an average of triplicate assays.

**^51Cr-release cytotoxicity assay for NK-CMC.** HCL and K-562 cells were labeled with ^51Cr-sodium chromate (specific activity 393.2 mCi/mg; New England Nuclear, Boston, MA) for 1.5 hours. The cells were washed twice with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 media to a density of 1 × 10^6 cells/mL and dispensed into 96-well conical-bottom microtiter plates (5,000 cells/well). The cells were labeled with Cr-sodium chromate for 8 hours. Following labeling, the cells were washing three times with PBS. Various amounts of NK effector cells were then added (final volume 200 μL/well) for 4 hours at 37°C. Aliquots of the supernatants were collected and their radioactivity measured in a γ-counter (Beckman Instruments, Irvine, CA). Total cycles per minute (cpm) was obtained by adding Triton X-100 (2%, 100 μL/well). The cells were washed three times with PBS. Various amounts of NK effector cells were then added (final volume 200 μL/well) for 4 hours at 37°C. Aliquots of the supernatants were collected and their radioactivity measured in a γ-counter (Beckman Instruments, Irvine, CA). Total cycles per minute (cpm) was obtained by adding Triton X-100 (2%, 100 μL/well). The cells were washed three times with PBS. Various amounts of NK effector cells were then added (final volume 200 μL/well) for 4 hours at 37°C. Aliquots of the supernatants were collected and their radioactivity measured in a γ-counter (Beckman Instruments, Irvine, CA). Total cycles per minute (cpm) was obtained by adding Triton X-100 (2%, 100 μL/well). The cells were washed three times with PBS. Various amounts of NK effector cells were then added (final volume 200 μL/well) for 4 hours at 37°C.
stained target cells in conjugates was divided by the total number of conjugates, giving a percentage of dead cells in conjugates. Cultures of target cells alone or effector cells alone were used as controls and treated under the same conditions. No “conjugates” were found in cultures of Eskol cells alone. Also, the viability of cells remained very high (>98%) following this treatment. Following treatment with 2-CdA, a large number of dead cells (trypan blue-positive cells) were present. These cells were excluded from calculation of the conjugate formation assay. Control cultures of effector cells alone did not form conjugates spontaneously or clusters under the conditions used in this assay.

Cold target competition assay. Various amounts of unlabeled K-562 or Eskol cells were used as competitive targets for NK activity. Competitor cells were added with effector cells (10^6 cells/well) and incubated for 15 minutes at 37°C. 51Cr-labeled Eskol cells (2 × 10^6 cells/well) were then added (final volume 150 µL/well), the plates were incubated, and specific released radioactivity was assayed as previously described.

Production of crude NKCF preparations. Cell-free supernatants containing NKCF were generated by incubation of mixed effector and target cells, either K-562 or Eskol (effector:target ratio 50:1) in serum-free medium. Cultures of either effector or target cells alone were used as controls. After 48 hours of incubation (37°C in 5% CO₂), the supernatants were collected, filtered through a 22-µm Millicell filter (Millipore Co, Bedford, MA), and stored at −70°C until further use. Eskol cells were used as NKCF inducers as previously described to determine the effect of IFN-Con1, 2-CdA, or combination on this level of the killing process. K-562 cells were used as NKCF inducers to supply high cytolytic supernatants (containing NKCF) in order to study the effect of IFN-Con1, 2-CdA, or combination on Eskol cell susceptibility to NKCF. Preliminary results revealed that the ability of Eskol cells as NKCF inducers is lower than that of K-562 cells. Supernatants from cultures of effector cells alone showed a low level of cytotoxicity 1.4% ± 0.4% when Eskol cells were used as targets and 2.9% ± 0.8% when K-562 cells were used as targets. Supernatants from cultures of either Eskol or K-562 cells alone had no (<1%) cytotoxic effect over the spontaneous release of 51Cr.

Assay for NKCF activity. 51Cr-labeled target cells, either Eskol or K-562(10^6 cells/well, in 50 µL serum-free media) were sedimented in 96-well plates. Supernatants containing various amounts (10, 50, or 100 µL/well) of crude NKCF preparations were added and the total volume was 150 µL/well. The cultures were incubated for 20 to 24 hours. Aliquots of the supernatants were then collected and specific released radioactivity was assayed as previously described.

Statistical analysis. All the results are expressed as an arithmetic mean ± standard error of the mean (SEM). Comparisons were carried out by a Student’s t-test.

RESULTS

The effect of 2-CdA on growth of HCL cells. Eskol (Fig 1A), IRES-4 (Fig 1B), HS-1 (Fig 1C), and HS-2 (Fig 1D) cells were incubated in increasing concentrations of 2-CdA for various time periods. The various HCL cells exhibited a similar pattern of growth inhibition by 2-CdA, although Eskol and IRES-4 cells (Fig 1A, B) were relatively more sensitive than HS-1 and HS-2 HCL lines (Fig 1C, D). After 24 hours of incubation with 2-CdA, only cells exposed to 1 µg/mL of 2-CdA were significantly (P < .05) inhibited. A significant (P < .05 to P < .005) inhibitory effect of 2-CdA was found after 72 hours of incubation at all concentrations of 2-CdA above 1 ng/mL when compared with untreated cultures. FS-4 cells (Fig 1E) responded only to relatively high doses of 2-CdA (100 ng/mL and 1 µg/mL). A significant (P < .05) growth inhibition of FS-4 cells was seen only after 72 hours of incubation (Fig 1E) at these high concentrations.

A synergistic/additive effect of IFN-Con1 and 2-CdA on HCL cell growth inhibition. HCL cell lines were treated in combination with IFN-Con1 and 2-CdA for various periods of incubation time. After 24 hours of incubation, a significant (P < .05 to P < .01) synergistic effect was found on cell growth when Eskol (Fig 2A), HS-1 (Fig 2C), and HS-2 (Fig 2D) cells were treated with 300 U/mL of IFN-Con1 and either 1, 10, or 100 ng/mL of 2-CdA. Neither IFN-Con1 alone nor 2-CdA (in that concentrations) alone exhibits cell growth inhibitory activity after 24 hours. Combination of IFN-Con1 and relatively high levels of 2-CdA (1 µg/mL) resulted in an additive cell growth inhibitory effect following 24 hours of incubation. A significant (P < .05 to P < .01) additive effect, compared with untreated cells, was also found in Eskol, HS-1, and HS-2 cultures treated with IFN-Con1 and all the tested concentrations of 2-CdA after 3 days of incubation. The cell growth inhibitory effects were found to be both time and dose dependent (Fig 2A, C, D). IRES-4 cells responded only to 2-CdA treatment but not to IFN-α (Fig 2B). Neither additive nor synergistic effects were found following combination treatment of IFN-Con1 and 2-CdA in IRES-4 cells. In FS-4 cells, only an additive cell growth inhibitory effect was seen (Fig 2E) when the cells were treated with high doses of 2-CdA and IFN-Con1 for 3 days.

2-CdA affects neither NK cell activity nor NK cell activation by IFN or IL-2. 2-CdA treatment has no effect on the killing activity of NK effector cells when targets were either the NK-resistant Eskol cells or the NK-sensitive K-562 cells (not shown). Combination treatment (8 h) of IFN-Con1 (300 U/mL) and 2-CdA resulted in similar levels of NK activation as IFN treatment alone. Similarly, 2-CdA did not affect activation of NK cells by IL-2 (100 U/mL). Various doses of 2-CdA (1 ng/mL to 1 µg/mL) were tested in these experiments. As expected, the cytotoxic activity of IFN-primed NK cells was higher than that of unprimed NK cells, and IL-2-primed NK cells exhibited the highest level of cell-mediated cytotoxicity against Eskol and K-562 cells (not shown).

2-CdA increases the sensitivity of HCL cells to NK-CMC. The susceptibility of Eskol cells (Fig 3) and the other HCL cell lines (IRES-4, HS-1, and HS-2; not shown) to NK-CMC was increased significantly (P < .01 to P < .005), compared with untreated cultures, following preincubation of the tumor cells with 2-CdA (1 ng/mL to 1 µg/mL). The effect was found to be dose dependent (Fig 3). The sensitivity of the HCL cells to either IFN-primed NK or IL-2-primed NK cell activities was also increased when the target cells were pretreated with 2-CdA (not shown). Only relatively high doses (100 ng/mL and 1 µg/mL) of 2-CdA increased the sensitivity of FS-4 cells to NK-CMC (not shown).

2-CdA increased sensitivity to NK-CMC can abolish the IFN-induced protective effect against NK-CMC. Treatment of target cells with IFN reduced their susceptibility to NK-CMC. This phenomenon was found in K-562 cells when the effectors were NK, IFN-primed NK, and IL-2-primed NK cells (not shown) and in Eskol, HS-1 and HS-2 cells, regardless of whether the effector cells were IFN-primed NK cells (not shown) or IL-2-primed NK cells (Fig 4A, B, C). Combined treatment of target cells with IFN-Con1 and 2-CdA can abol-
Fig 1. Effect of 2-CdA on relative growth of HCL and fibroblasts cell lines. Eskol (A), IRES-4 (B), HS-1 (C), and HS-2 (D) cells (1 × 10⁶ cells/mL) were grown in suspension and treated with 2-CdA (1 ng/mL, [a]; 10 ng/mL, [■]; 100 ng/mL, [▲]; 1 μg/mL, [□]) for varying periods of time. Cell samples were harvested at 24-hour intervals and counted following trypan blue staining for cell viability. FS-4 cells (E) (5 × 10⁴ cells/well in 200 μL) were similarly treated, stained with crystal-violet (8% in methanol) and the dye extracted with sodium citrate (0.1 mol/L) in 50% ethanol. The cytotoxic effects of 2-CdA are expressed as the percentage increase in the number of viable cells in the treated cultures relative to the increase in the number of viable cells in untreated cultures. The curves represent the data of one of two or three experiments. Standard error of the mean (SEM) of triplicates was 1.5 to 6.0.
Fig 2. Effect of 2-CdA + IFN combination on cell growth. Eskol (A), IREs-4 (B), HS-1 (C), and HS-2 (D) cells (1 × 10^6 cells/mL) were treated with 2-CdA alone ([ ], 1 ng/mL; [ ], 10 ng/mL; [ ], 100 ng/mL; [ ], 1 μg/mL), with IFN-Con1 ([ ], 300 U/mL) alone, or with combinations of IFN-Con1 (300 U/mL) and 2-CdA ([ ], 1 ng/mL; [ ], 10 ng/mL; [ ], 100 ng/mL; [ ], 1 μg/mL) for either 1 or 3 days. Cell samples were harvested and counted following trypan blue staining. FS-4 cells (E) (5 × 10^4 cells/well in 200 μL) were similarly treated, stained with crystal-violet (8% in methanol) and the dye extracted with sodium citrate (0.1 mol/L) in 50% ethanol. The relative growth inhibition is expressed as a percentage from the calculation of the increase in the number of viable treated cells relative to the increase in the number of viable control cells. The histograms represent the data of one of three experiments. SEM of triplicates was 1.3 to 6.9.
Various amounts of NK effector cells were sedimented for 4 hours of incubation. The curves represent the percentage of specific Wr doses (100 ng/mL and 1 pg/mL) of 2-CdA resulted in (significance P < .05) decreased compared to untreated cultures, whereas following 2-CdA treatment, significantly (P < .05) more conjugated dead cells were counted. Combination treatment of IFN and 2-CdA resulted in a similar level of dead target cells in NK-Eskol cell conjugates as untreated cultures (Table 1). Thus, it seems that 2-CdA affects Eskol cells in postbinding phase of the killing process.

IFN reduces the ability of tumor cells to induce activation of effector cells and NKCF release following effector cell–target cell conjugate formation. 2-CdA did not show a significant effect in this step of the killing process (not shown). Eskol cell–effector cell supernatants containing NKCF were assayed for their cytotoxic activity on either K-562 or Eskol target cells. Treatment of Eskol cells with IFN-Con1 (300 U/mL) significantly (P < .05) reduced the NKCF production by effector cells. 2-CdA (1 µg/mL) did not change significantly the level of NKCF release, whereas combination treatment of IFN and 2-CdA resulted in similar NKCF production as seen in IFN treatment alone (not shown). However, 2-CdA treatment did have an effect on the susceptibility of target cells to cytotoxic activity of crude preparations of NKCF. It was found that 2-CdA increased significantly (P < .01 to P < .001) and in a dose-dependent manner the sensitivity of Eskol cells to NKCF activity (Fig 6). In this stage of killing, IFN is not involved. Combination treatment of 2-CdA with IFN had the same effect as 2-CdA treatment alone; ie, enhancement of the susceptibility of the tumor cells to crude preparations of NKCF (Fig 6).

DISCUSSION

IFN-α has an antiproliferative effect on a variety of tumor cells and plays a key role in the regulation of various immunologic functions, including regulation of the natural killing arm of the immune system. The mechanisms responsible for the beneficial action of IFN-α in patients with HCL are unclear, but several hypotheses have been suggested, including the induction of terminal differentiation of hairy cells, a direct antiproliferative effect of IFN, and modulation of the immune response, especially the NK system.

Using an HCL-like cell line, Eskol, we have studied both the direct effect of IFN-α and IFN-Con1 on this cell line as well as the involvement of IFN-α and IL-2 in the regulation of NK-CMC against Eskol cells. We have found that IFN-α and IFN-Con1 have a significant but low growth inhibitory activity on Eskol cells. In addition, it was found that Eskol cells, like fresh HCL cells, are completely resistant to unprimed NK activity but are relatively sensitive to IFN-primed NK cells or IL-2–primed NK cells. Recently, a clone resistant to the direct cell growth inhibition by IFN-α (IRES-4) was established in our laboratory. However, this clone is still sensitive to IFN-activated NK-induced killing. 2-CdA is a congener of deoxyadenosine that is resistant to deoxyadenosine deaminase activity. 2-CdA was found to be very effective drug against HCL. We have found that both Eskol and IRES-4 cells exhibit a similar level of sensitivity to the cytotoxic effect of 2-CdA. IFN-α appears to be capable of inducing a clinical response in about 80% of patients with
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HCL, but the rate of complete remission is low (10% to 15%). Piro et al have reported that 2-CdA administered as a single 7-day infusion produced complete remissions in 11 of 12 patients with HCL, that the twelfth patient obtained a partial remission, that there had been no recurrence of disease in any patient at a median follow-up of over a year, and that, aside from fever without apparent infection, the treatment was without toxicity. Recently, other clinical trials confirmed these findings. It seems that our HCL IFN-sensitive and IFN-resistant cells may reflect (at least) two types of naturally occurring HCL cells. Both types of cells were found to be very sensitive to 2-CdA treatment. The relevance and the possible correlation of these results to the clinical response to IFN-α and IFN-Con1 and 2-CdA in patients with HCL should be studied.

In the last few years, clinical trials of combination of IFN-α with chemotherapeutic drugs in cancer patients have been described. It was suggested that chemoinmunotherapy of tumors may involve first a reduction in the tumor load by chemotherapeutic agents. Surviving tumor cells may then be removed by immune defense cells, such as the NK cell population, the activity of which can be increased by immuno-stimulators, such as IFN-α and IL-2. Indeed, in vitro synergistic or additive interactions of IFN with chemotherapeutic agents such as the pyrimidine analogue 5-FU have been reported. Clinical trials on patients with colon carcinoma, renal cell carcinoma, and other diseases revealed that such a combination is also clinically significant. We have investigated the role of this combination on the in vitro regulation of the natural killing arm of the immune system.

Because both IFN-α and the purine analogue 2-CdA are effective drugs against HCL, we investigated in the present study both the direct cell growth inhibitory effects and the effect on the regulation of the NK system following such as combination treatment. We have found that although 2-CdA and 5-FU belong to different families of nucleotides analogues, they exhibit a similar pattern of effects. Treatment of Eskol cells with each drug in combination with IFN-α or IFN-Con1 resulted in either additive or synergistic cell growth inhibitory effects. Neither 5-FU nor 2-CdA affect the activity of NK cells nor the priming of NK cells by either IFN or IL-2. On the other hand, both drugs increased the sensitivity of the tumor cells to NK-CMC and abolished the protective effect against NK-CMC induced by IFN. The lack of

Fig 4. 2-CdA–induced sensitivity to NK cell activity could counteract the protective effect against NK-CMC induced by IFN. Eskol (A), HS-1 (B), and HS-2 (C) cells were cultured in the presence of medium alone (○), IFN-Con1 alone (□), 300 U/mL, or with combination of IFN-Con1 (300 U/mL) and 2-CdA (△, 1 ng/mL; ▼, 10 ng/mL; ■, 100 ng/mL; △, 1 μg/mL) for 8 hours. IL-2–primed NK effector cells were added for 4 hours of incubation. The curves represent the percentage of specific ⁶⁷Cr release from Eskol cells. Spontaneous ⁶⁷Cr release was 7% to 18% and SEM of triplicates was 1.4 to 4.8. Figure 4 represents the data of one of three experiments using effector cells from three different donors.
IFN-primed NK IFN 4.9
IL-2-primed NK 2-CdA + IFN 5.1
IL-2-primed NK IFN t 5.3
IL-Z'-primed NK - 4.8

cells from three different donors.

IFN-primed NK 2-CdA + IFN 4.9
IFN-primed NK 2-CdAS 5.8 ± 0.4 38.0
mL) for 8 hours.

The NK parameter of the immune system is functional in the suppression of NK activity (which was tested at doses corresponding to the therapeutic levels of the drug) suggests that agents on the immune system is based on drug selectivity. It is possible that the effect of the chemotherapeutic presence of either

In this respect, the large granular lymphocytes associated with NK activity may possess membrane characteristics that do not allow these agents to penetrate into the cytoplasm and

exert an immunosuppressive effect. It seems that at least two different intracellular mechanisms are involved in the effects induced by 2-CdA. Following a short time (8 h) of incubation, no cytotoxic effect of 2-CdA can be measured. However, using the same concentrations of 2-CdA, this time of incubation is sufficient to increase the sensitivity of the target cells to NK-CMC and to abolish the protective effect against NK-CMC induced by IFN.

Fig 5. Competitive ability of IFN-treated and 2-CdA-treated target cells. IL-2–primed NK effector cells were cultured with 51Cr-labeled Eskol cells, at effector to target ratio of 50:1. Unlabeled, untreated K-562 (a) cells, IFN-treated K-562 cells (d), 300 U/mL, 2-CdA–treated K-562 cells (x, 1 μg/mL), IFN plus 2-CdA–treated K-562 cells (l), untreated Eskol cells (o), IFN-treated Eskol cells (+), 2-CdA–treated Eskol cells (m), and IFN plus 2-CdA–treated Eskol cells (o) were assayed for their competitive ability at various competitor to target ratios. The experiments were performed in triplicates for 4 hours when the spontaneous 51Cr release was 4% to 12% and SEM was 1.2 to 4.5. The curves represent the data of one of two experiments using effector cells from two different donors.

Fig 6. 2-CdA increases the susceptibility of target cells to NKCF. Eskol cells were pretreated with the presence of various concentrations of 2-CdA alone (□) or in combination with IFN-Con1 (■) 300 U/mL for 8 hours. Then, the cells were washed, 51Cr-labeled and assayed for their sensitivity to NKCF. The histograms represent the results of experiments when 100 μL/well of crude NKCF preparation was added to 50 μL of target cell suspension. Similar results, with a dose-dependent manner, were obtained when only 10 μL/well or 50 μL/well of crude preparations of NKCF were added to target cell culture. The assay was done in triplicate and the SEM was 3.2 to 6.6. The histograms present data of one of two experiments.

Table 1. Conjugate Formation Between Eskol and Effector Cells

<table>
<thead>
<tr>
<th>Effector Cells</th>
<th>Target Cell Treatment</th>
<th>Conjugate Formation (%)</th>
<th>Conjugated Dead Cells (%) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2*–primed NK</td>
<td>—</td>
<td>4.8 ± 0.4</td>
<td>34.5 ± 4.2</td>
</tr>
<tr>
<td>IL-2–primed NK</td>
<td>IFN†</td>
<td>5.3 ± 0.6</td>
<td>14.6 ± 3.1</td>
</tr>
<tr>
<td>IL-2–primed NK</td>
<td>2-CdA†</td>
<td>4.7 ± 0.5</td>
<td>46.3 ± 5.4</td>
</tr>
<tr>
<td>IL-2–primed NK</td>
<td>2-CdA + IFN</td>
<td>5.1 ± 0.4</td>
<td>32.6 ± 4.0</td>
</tr>
<tr>
<td>IFN†–primed NK</td>
<td>—</td>
<td>5.1 ± 0.3</td>
<td>22.3 ± 3.0</td>
</tr>
<tr>
<td>IFN–primed NK</td>
<td>IFN</td>
<td>4.9 ± 0.5</td>
<td>11.5 ± 1.3</td>
</tr>
<tr>
<td>IFN–primed NK</td>
<td>2-CdA</td>
<td>5.8 ± 0.4</td>
<td>38.0 ± 5.6</td>
</tr>
<tr>
<td>IFN–primed NK</td>
<td>2-CdA + IFN</td>
<td>4.9 ± 0.4</td>
<td>27.5 ± 3.5</td>
</tr>
</tbody>
</table>

The conjugate formation assay was done in duplicate using effector cells from three different donors.

* Effector cells were treated with IL-2 (100 U/mL) for 8 hours.
† Target cells or effector cells were treated with IFN-Con1 (300 U/mL) for 8 hours.
‡ Target cells were treated with 2-CdA (1 μg/mL) for 8 hours.

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cells to activate NK cells (following conjugate formation with target cells) and reduces the induction of NKCF release from the effector cells. We have found that 2-CdA does not affect the conjugation step nor the effector cell induction. However, our results indicate that 2-CdA enhanced the sensitivity of tumor cells to NKCF. Combination treatment of 2-CdA and IFN resulted in increased tumor cell susceptibility to NK-CMC and/or counteracted the protection of target cells against NK cell activity induced by IFN. These effects may reflect the enhancement of target cell sensitivity to NKCF induced by 2-CdA.

We used in our experiments crude preparations of NKCF. Because both TNF and IFN-γ are present in peripheral blood lymphocytes-target cell supernatants, one can argue that these cytokines may be responsible for cytotoxicity, alone or in combination. However, NKCF does differ from tumor necrosis factor (TNF) in number of important aspects, and its lytic activity cannot be substantially attributed to TNF. IFN-γ presents in crude NKCF preparations may exhibit a role in NKCF cytotoxicity. However, we have shown that unlike IFN-α, exogenous IFN-γ did not affect Eskol cell susceptibility to NK-CMC. Exogenous IFN-α induced protection against NK-CMC, but it did not affect the sensitivity of the hairy leukemic cells to NKCF. Thus, although crude preparations of NKCF may contain TNF and IFN-γ, it seems that these cytokines do not play a central role in cell cytotoxicity induced by supernatants containing NKCF.

More studies are needed in order to examine the effect of 2-CdA treatment on the sensitivity of tumor cells to other immune cell–cytotoxic mechanisms, including the cytotoxic T lymphocytes (CTLs), TNF, antibody-dependent cell cytotoxicity (ADCC), and the complement system. In our studies, we have found that 2-CdA is more effective on HCL cells than on normal fibroblast cultures, although relatively high doses of 2-CdA had a direct cytotoxic effect on the fibroblasts and also increased the sensitivity of these cells to NK-CMC. Combination of IFN-Con1 and low doses of 2-CdA resulted in synergistic cell growth inhibitory effects on the HCL cells as well as increased their sensitivity to NK cytotoxicity. These low concentrations of 2-CdA had almost no effect on normal fibroblasts. Thus, it seems that the combination of 2-CdA and IFN-Con1 or IFN-α may be useful in clinical trials in patients with HCL. The relevant doses of 2-CdA and IFN that will affect the tumor cells but not the normal cells under in vivo conditions has to be determined.

Although the molecular mechanisms involved in the interaction of IFN and 2-CdA are not yet identified, we suggest that this combination has clinical potential. A major problem of chemotherapeutic and immunotherapeutic treatment is severe side effects. Treatment with 2-CdA results in fewer side effects than treatments with other useful chemotherapeutic drugs. Combination of IFN and 2-CdA may permit the reduction of the total dose of both drugs.

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