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ERADICATION OF MOUSE MELANOMA BY COMBINED TREATMENT WITH RECOMBINANT HUMAN INTERLEUKIN 2 AND RECOMBINANT MURINE INTERFERON-GAMMA

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Successful immunotherapy of early s.c. or i.p. (B16) melanoma in syngeneic C57BL/6 (B6) mice was achieved with s.c. peri-lesional injections (for s.c. tumors) or i.p. injections (for i.p. tumors) of recombinant human interleukin 2 (rIL-2) and recombinant murine interferon-gamma (rIFN-γ). Over a 28-day period, rIL-2 and rIFN-γ were injected 14 times. Results with this combination were additive with s.c. tumors and synergistic with i.p. tumors. Treatment with 6,250 U–25,000 U of rIL-2 and 2 μg of rIFN-γ began 1–3 days after s.c. inoculation of melanoma. On day 50, 87% (72/83) of mice thus treated were completely free of tumor. None of the 78 control mice (tumor + buffer) survived. Of mice receiving either rIL-2 or rIFN-γ alone, 59% (47/79) and 53% (44/83), respectively, were tumor-free. I.p. tumors were also eradicated by i.p. injections of rIL-2 (50,000 U) with rIFN-γ (5, 10, and 15 μg) as judged by absence of tumor in 81% (21/26) of mice autopsied between days 45 and 65. No control mice survived, and only 17% (2/12) and 20% (6/30) given either rIL-2 or rIFN-γ separately (i.p.) were tumor-free. Doses of rIFN-γ from 1–4 μg were more beneficial in eliminating 1-day s.c. melanomas than were higher doses, and local s.c. treatment was far superior to distant or systemic treatment. Non-adherent peritoneal or splenic cells from mice bearing 6-day-old i.p. melanomas and treated with one or both lymphokines on days 3 and 4 were used in cytotoxicity assays *in vitro*. Significant cytotoxicity against cultured melanoma cells was displayed by cells harvested from lymphokine-treated mice, but there was no evidence of the synergism observed with combination treatment of i.p. tumors *in vivo*. rIFN-γ inhibited proliferation of melanoma cells *in vitro*, whereas rIL-2 stimulated proliferation at 1,000 U/ml. Plating efficiency was increased by at least 30% by culture with 100 U or 1,000 U of rIL-2/ml and both concentrations neutralized the inhibitory effect of 0.05 ng/ml of IFN-γ, but not of 0.5 or 5.0 ng/ml.

Our objective was to achieve successful immunotherapy of mouse melanoma with decreased concentrations of recombinant human interleukin 2 (rIL-2) in combination with other biological response modifiers. We previously reported that the combined use of 50,000 U of rIL-2 per injection and 50 mg/kg of cyclophosphamide (CY) constituted highly effective therapy for B6 mice bearing early B16 melanoma (clone $B_5$59) or Meth 4 sarcoma, curing close to 100% when the tumor was subcutaneous (s.c.) and when rIL-2 was injected s.c. close to the tumor several times weekly for 5 weeks (Silagi and Schaefer, 1986). Since the high doses of rIL-2 needed to cure mice are toxic for humans (Rosenberg *et al.*, 1985; Rosenstein *et al.*, 1986; Lotze *et al.*, 1986), we examined the possibility of combining lower doses of rIL-2 with another immunomodulator to achieve effective therapy in the mouse melanoma model. The properties of interferon as both a potent immunostimulator and enhancer of macrophage and NK activity (Goldstein and Lazlo, 1986; Borden and Ball, 1981; Bonnem and Oldham, 1987) make it a good choice for investigation.

We report here the existence of an additive or synergistic effect between mouse recombinant interferon-gamma (rIFN-γ) and human rIL-2 in the treatment of a highly malignant clone ($B_5$59) of the spontaneously-arising B16 melanoma, which had previously been shown to be non-immunogenic and not to elicit concomitant immunity (Freedman *et al.*, 1980).

MATERIAL AND METHODS

Mice

Female C57BL/6 mice were purchased from the Animal Genetics and Production Branch of the National Cancer Institute (Frederick, MD) and were 7–10 weeks old when used for experiments.

Cells

B16-melanoma-derived clone $B_5$59 (Silagi, 1969, 1976) was maintained as a monolayer in Eagle's minimum essential medium (MEM, GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated serum (5% fetal bovine serum [FBS] and 5% calf serum, Hyclone, Logan, UT), and 50 μg/ml of Gentamycin (Sigma, St. Louis, MO). Cells were tested periodically (Russell *et al.*, 1975) and found to be Mycoplasma-free.

Lymphokines

The highly purified recombinant human interleukin 2 (rIL-2, specific activity = 5×10^6 U/mg protein) used in this study has been described (Silagi and Schaefer, 1986) and was generously provided by Cetus, Emeryville, CA. Lyophilized rIL-2 was reconstituted with 1.2 ml sterile distilled water (7.6×10^5 U/ml), and immediately prior to use diluted to the desired concentrations with sterile phosphate-buffered saline (PBS) (Dulbecco's, without Ca or Mg, GIBCO). Purified (≥95% pure by SDS-polyacrylamide gel electrophoresis) recombinant murine rIFN-γ-A was kindly provided by Schering, Kenilworth, NJ, as a solution of 0.7–0.8 mg/ml of protein with a specific activity of $1–2 \times 10^6$ IU/mg in 50% glycerol as assayed on L929 cells with encephalomyocarditis virus. The r-IFN-γ was diluted with sterile PBS immediately before use.

Tumor inoculation

Clone $B_5$59 of the murine B16 melanoma was inoculated s.c. or i.p. into syngeneic B6 mice at 2.2×10^5 viable cells/mouse in 0.2 ml of Hanks' Balanced Salt Solution (HBSS, GIBCO). Mice were palpated and observed 3 times weekly for tumor formation. At the end of the observation period, usually 50 days, mice were killed and autopsied to determine whether non-palpable tumors were present.

In vivo *tumor treatment*

In most experiments treatment began one day after s.c. or i.p. inoculation of tumor cells. Either rIL-2 or rIFN-γ or both were injected either s.c. peri-lesionally, or i.p. Lymphokines

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were injected once daily on 4 consecutive days during the first 2 weeks, and then 3 times weekly for a total of 14 injections over a 4-week period. Total dosage of IL-2 was approximately 10%–45% of that previously reported (50,000 U/injection for 17 injections over a 5-week period). Control animals received either PBS, diluted excipient buffer (provided by Cetus for rIL-2 controls) and/or glycerol diluted in PBS for rIFN controls.

Preparation of mononuclear lymphoid cells

Melanoma or buffer was inoculated i.p. into at least 6–10 mice per experimental group on day 0. On days 3 and 4, appropriate groups were injected i.p. with buffer, rIFN-γ, rIL-2, or both lymphokines. On day 6 each experimental group of mice was killed separately, then peritoneal cells (PC) were collected by lavage of the peritoneal cavity (Calvelli *et al.*, 1982) and pooled by group, and finally erythrocytes were lysed using hypotonic salt solutions. After centrifugation, the PC were separated into glass-adherent and nonadherent populations (Calvelli *et al.*, 1982). Splenic leukocytes were obtained by mechanical dissociation of the spleens between ground-glass slides; larger pieces were strained and processed further as described for PC. Nonadherent mononuclear cells were used in the functional assays. At each stage of separation, samples were cytofuged onto glass slides and stained, then the percentage of lymphocytes was estimated.

Assay for cytotoxicity

B_559 target cells were labelled by overnight incubation with 5 μCi of ^{3}H-dT (NET 027, methyl-^{3}H, 6.7 Ci/mM, 1 mCi/ml, New England Nuclear, Boston, MA) per 3 × 10^5 cells in 10 ml of MEM in 100-mm Petri dishes. Labelled cells were harvested in the usual manner, washed twice and resuspended at 10^5 cells/ml in complete medium. Aliquots (0.1 ml) of the suspension were placed in wells of a flat-bottomed Microtest III plate (Falcon 3072). Effector cells were added in 0.1 ml medium per well to achieve the appropriate effector:target cell ratios. The plates were incubated overnight and the contents of the wells harvested on filters using a mini-Mash (M.A. Bioproducts, Walkersville, MD). After filters were dried and placed in vials containing Econofluor (New England Nuclear) routine scintillation counting was performed. The percentage lysis was calculated from the mean cpm for triplicate or quadruplicate experimental wells, as compared with control wells.

Titration of rIL-2 and rIFN-γ directly on melanoma cells in culture

The direct effects of rIL-2 and rIFN-γ on cell proliferation *in vitro* were tested on exponentially growing B_559 melanoma cells. Cells were seeded on day 0 at 7.5 × 10^4 melanoma cells per 2 ml of medium per 60-mm culture dish and incubated at 37°C with 95% air/5% CO_2. Various concentrations of rIL-2 and/or rIFN-γ in another 2 ml of medium were added 4 hr after plating. The cells were cultured for 72 hr, after which cell number and viability in duplicate cultures were determined. Results are expressed as percentage of control cultures.

In addition, plating efficiencies of replicate control and treated cells were compared by seeding 100 and 200 viable cells per 60-mm dish in 2 ml of MEM on day 0. Four hours later we added 2 ml of MEM containing rIL-2 and/or rIFN-γ at the same concentrations as used for determination of cell number and viability. Colonies were stained and counted after 8–11 days of culture at 37°C in a CO_2 incubator, and relative plating efficiencies were expressed as percentage of the number of colonies in control, untreated cultures.

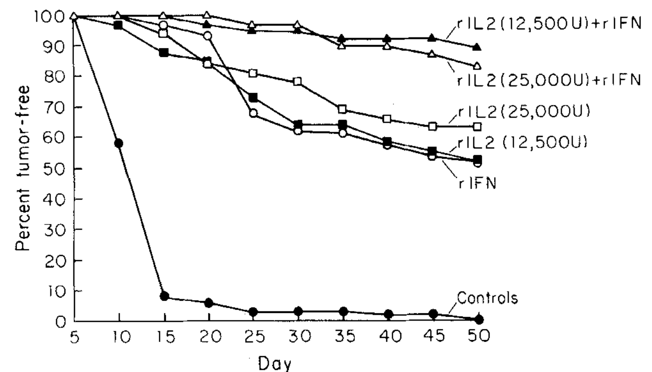

FIGURE 1 – Combined immunotherapy with rIL-2 and rIFN-γ in B6 mice. Treatment began 1 day after s.c. injection of mice with B_559 melanoma. Animals were injected 14 times s.c. in the peri-lesional area with either one or both lymphokines. On day 50, there were 64 tumor-free mice out of 67 treated with both lymphokines, compared to 37/65 treated with either dose of rIL-2 and 36/69 treated with 2 μg of rIFN-γ per injection. All of the control animals had succumbed to their tumors. No significant difference existed between 12,500 U and 25,000 U of rIL-2 whether used alone or with rIFN-γ. The difference between combined lymphokine therapy and single lymphokine therapy was highly significant ($p \leqslant 0.001$), as was the difference between control mice and any lymphokine therapy ($p \leqslant 0.001$). These data are taken from 8 separate experiments.

Statistical analysis

Fisher's exact test (2-tailed) was used to analyze the data in Figures 1 to 4 and Table I. Statistical analysis for Figures 5 and 6 was done using a 4-factor analysis of variance with interactions. Multiple comparisons for ratios were done using Duncan's multiple range test. (For further details, see "Results", under "Cytotoxic activity *in vitro*".)

RESULTS

Treatment using rIFN-γ and rIL-2

We had previously succeeded in eradicating melanomas and sarcomas in almost 100% of B6 mice when treatment was begun at 1 or 3 days post inoculation using 50,000 U of rIL-2/injection with cyclophosphamide (Silagi and Schaefer, 1986). However, since toxic effects of high doses of the same Cetus rIL-2 had been reported in clinical trials (Rosenberg *et al.*, 1985), our aim now was to determine whether we could succeed in immunotherapy of melanoma-bearing B6 mice by treatment with rIFN-γ and rIL-2, using low doses which were non-toxic in mice and separately could cure 1-day-old melanomas in 40%–60% of the mice. Figure 1 shows that, by day 35, one week after lymphokine treatment ceased, there were no palpable tumors in 91% (61/67) of the mice given the combination of rIFN-γ (2 μg/injection) with either 12,500 U or 25,000 U of rIL-2 per injection. All but 3 of these mice remained tumor-free up to day 50, when autopsy was performed. At this time, 37/65 mice treated with either dose of rIL-2 and 36/69 treated with 2 μg/injection of rIFN-γ were tumor-free. All treatments gave significantly different results from controls ($p \leqslant 0.001$), and treatments combining rIL-2 and rIFN-γ were also highly significant ($p \leqslant 0.001$) when compared with either lymphokine alone.

Similar results were obtained when the dosage of rIL-2 was brought down to 6,250 U/injection in mice bearing 1-day melanomas s.c. All 6 control mice developed tumors with a mean latency of 16 days. During the 50-day observation pe-

TABLE I - COMBINATION LYMPHOKINE TREATMENT BEGUN 3 DAYS POST INOCULATION

Lymphokine treatment	Percent tumor-free	MLP ± SD
rIL-2 + rIFN-γ	75 (6/8)	43 ± 4
rIL-2 (25,000 U)	67 (4/6)	32 ± 21
rIFN-γ (2 μg)	33 (2/6)	20 ± 3
None	0 (5/5)	12 ± 2

Mice bearing 3-day melanomas were injected s.c. in the peri-lesional region 14 times over a 28-day period, with the following per injection: rIL-2 + rIFN-γ at 25,000 U and 2 μg, respectively, or rIL-2 (25,000 U), or rIFN-γ (2 μg), or buffer, with the results on day 50 as given in column 2. Fractions in parentheses = number of mice tumor-free/total number of mice. MLP ± SD = mean latent period ± standard deviation.

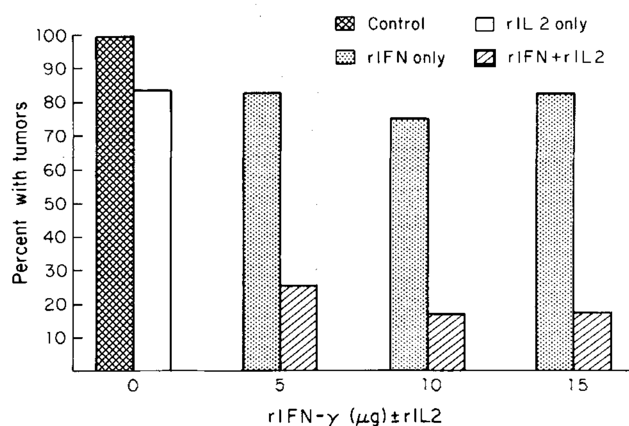

FIGURE 2 - Percentage of animals with i.p. tumors following single or combination i.p. lymphokine treatment. Treatment began 1 day after i.p. injection of mice with B_559 melanoma. Animals were injected 14 times i.p. with 50,000 U rIL-2 either singly or in combination with 0, 5, 10, or 15 μg of rIFN-γ per injection. At autopsy, 21/26 mice receiving both lymphokines were tumor-free as compared to 2/12 receiving only rIL-2 or 6/30 receiving only rIFN-γ. None of the control mice was tumor-free. These data reflect the results of 2 independent experiments. (See text.)

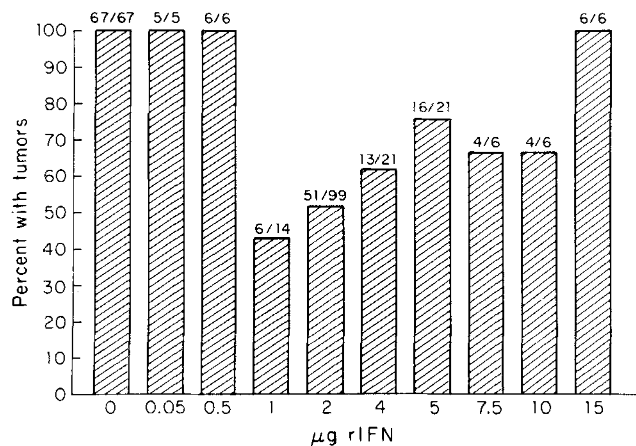

FIGURE 3 - Titration of rIFN-γ (s.c.) in mice bearing 1-day s.c. melanoma. Results given are percentage of mice with tumors on day 50 after beginning treatment. Data are cumulative from 3-12 experiments for controls, 1, 2, 4 and 5 μg/mouse. The remaining data are the results of a single titration.

riod, no palpable tumors developed in the 8 mice injected with the combination of 6,250 U of rIL-2 and 2 μg of rIFN-γ. Tumors did develop on days 24 and 30 in 2/8 mice injected with rIL-2 (6,250 U); and on days 42 and 49 in 2/8 mice injected with rIFN-γ (2μg). Similar tumor suppression occurred when mice bearing 1-day melanomas were treated with 5 μg of rIFN-γ combined with rIL-2 at 12,500 U or 25,000 U per injection. By day 50, tumors remained in 4/11 mice.

Previous data (Silagi and Schaefer, 1986) had indicated that there was no significant difference (p = 0.17) between mice bearing 1-day and 3-day tumors in response to rIL-2 at 50,000 U/injection. The response of bearers of 3-day melanomas to single and combination lymphokines was therefore also tested. As can be seen in Table I, the combination per injection of rIFN-γ (2 μg) and rIL-2 (25,000 U) was more efficient in eradicating tumor than was either lymphokine alone. Comparison of fractions of tumor-free mice on day 50 post-inoculation of melanoma cells in Table I with those in Figure 1 showed that, for each type of treatment, results with 3-day s.c. tumors were not significantly different from results obtained with 1-day tumors.

Synergism between rIFN-γ and rIL-2 in treating i.p. tumors

Early results which showed that i.p. melanomas could be successfully treated by i.p. injection of rIL-2 (Silagi and Schaefer, 1986) have not been consistently repeatable. Most mice thus treated have developed tumors (as in Fig. 2), although with increased latency and decreased tumor volumes as compared to controls (data not shown).

This, however, was not the case when both rIL-2 and rIFN-γ were injected i.p. into B6 mice with 1-day i.p. melanomas. Figure 2 depicts the results of 2 consecutive experiments in which B6 mice were inoculated i.p. with 2.2×10^5 melanoma cells and treated i.p. with, per injection, 50,000 U of rIL-2 or 5-15 μg of rIFN-γ or both. There was no significant difference between groups receiving combined treatment, but results were significantly different from those in controls and groups receiving single lymphokine treatment ($p<0.0001$). In these experiments, 21/26 of the mice receiving both lymphokines showed no signs of tumor when autopsy was performed on day 45 in one experiment and day 65 in the other (Fig. 2). In contrast, only 2/12 mice receiving only rIL-2 remained tumor-free, and when rIFN-γ (5-15 μg) was the single lymphokine injected, only 6/30 mice remained tumor-free. By day 35, all 11 control mice had either succumbed to their tumors or were moribund and had to be killed for humanitarian reasons. Survival was greatly prolonged by treatment. On the day of autopsy 25/26 of the mice receiving both lymphokines, and 28/42 mice receiving either lymphokine singly, were alive.

Titration of recombinant murine IFN-γ in melanoma-bearing mice

Before doing the experiments shown in Figures 1 and 2, we titrated rIFN-γ *in vivo*. Figure 3 combines the results of several titration experiments showing the effect of graded doses of recombinant murine IFN-γ on the growth of B_559 melanoma which had been inoculated s.c. into B6 mice 1 day prior to beginning of treatment. At this time, many mitoses were seen in histological preparations of the tumor site. Tumors grew in all mice injected s.c. peri-lesionally with buffer or 0.05 to 0.5 μg of rIFN-γ/injection, but latency was doubled in the rIFN-γ-injected mice as compared with controls (22 *vs.* 10 days). In contrast, fewer than 53% (70/134) of the mice similarly injected with 1, 2 or 4 μg of rIFN-γ developed progressively growing tumors. They were treated as a single group because there was no statistically significant difference for treatment with 1-4 μg. These doses were more effective in

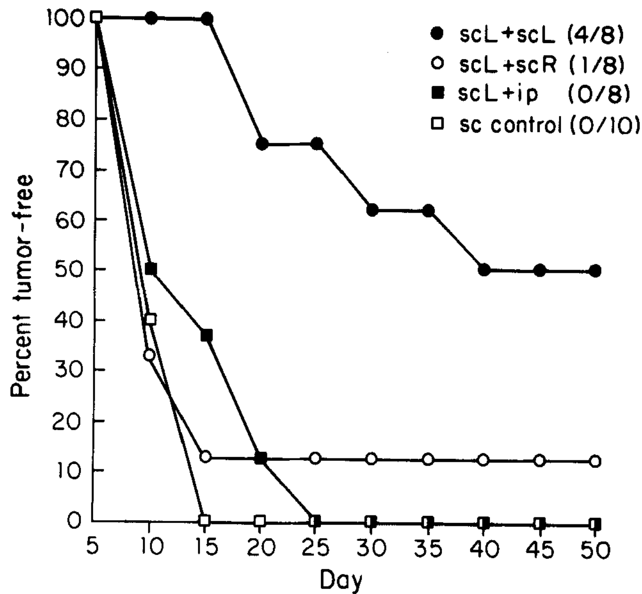

FIGURE 4 – Effect of route of injection of rIFN-γ (4 μg/injection) on 1-day s.c. melanoma. All animals were injected in left flank: scL + scL = melanoma injected in left flank and rIFN-γ injected in left flank in peri-lesional area; scL + scR = melanoma injected as above and rIFN-γ injected in right flank; scL + ip = s.c. left-flank injection of melanoma and i.p. injection of rIFN-γ. Control animals received tumor scL and were injected with buffer in the peri-lesional area.

preventing tumor growth than were 5–10 μg, whereas 15 and 50 μg did not prevent tumor growth, but did increase the latency 3-fold as compared with control mice (latter not shown). Treatment with 50 μg/injection had to be stopped on day 14 instead of day 28 because that dosage proved cachectic. Lower doses did not noticeably affect weight or liveliness of the animals.

Effect of route of injection of rIFN-γ in treating s.c. tumors

Subcutaneous tumors (1 day old) respond best to localized injection of rIFN-γ, as seen in Figure 4. Four groups of mice were inoculated s.c. in the left flank with melanoma. One day later 3 of the groups were injected with 4 μg of rIFN-γ either around the tumor site s.c. in the left flank; or far from the tumor s.c. in the contralateral (right) flank; or i.p. Controls were injected s.c. in the left flank with buffer and glycerol, and all developed tumors (Fig. 4). Only half of the mice treated locally developed tumors, whose latency was greatly increased; 7/8 treated on the contralateral side developed tumors with latency essentially equal to that seen in control mice; and 100% of the i.p. treated mice developed tumors with somewhat increased latency (Fig. 4). These results from a single experiment are very much like results obtained earlier in a similar experiment with rIL-2 (Silagi and Schaefer, 1986). Differences between localized s.c. injections and results from other groups were significant ($p = 0.023$–0.038). In the same experiment (data not shown in Fig. 4), 7/8 of 1-day-old i.p. melanomas grew progressively when treated i.p. with 4 μg of rIFN-γ, with a mean latency of 39 days compared with 23 days for the i.p. control mice, all of which developed tumors.

Cytotoxic activity in vitro *against melanoma cells by peritoneal and splenic mononuclear leukocytes from mice treated with rIL-2, rIFN-γ or both lymphokines*

This report demonstrates that treatment using lower doses per injection of rIL-2 (6,250 U, 12,500 U, 25,000 U) combined with rIFN-γ (2 μg) is highly effective therapeutically, considerably more so than either lymphokine alone at the same dose level. To examine whether correlations exist between *in vivo* suppression of tumors and *in vitro* cytotoxic activity, we studied the lytic effects on melanoma cells of non-adherent leukocytes from spleen and peritoneal cavity of treated and untreated B6 mice with and without tumor. The mice had been inoculated i.p. on day 0 with melanoma cells or buffer and treated by i.p. injection with either rIL-2, rIFN-γ or both on days 3 and 4 post inoculation. Control mice received buffer. On day 6 after tumor inoculation, spleens and PC were harvested and prepared as described in "Material and Methods". Target cells *in vitro* were ^{3}H-dT-labelled B_559 melanoma cells.

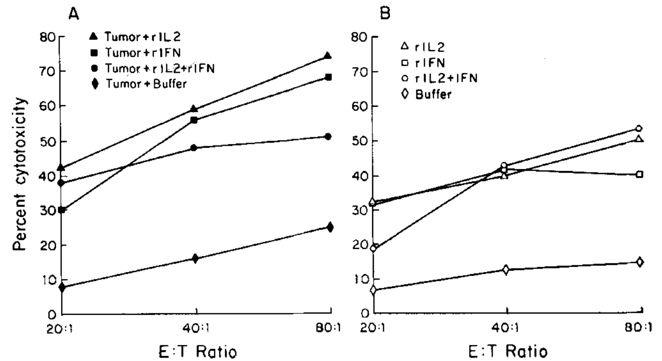

FIGURE 5 – Results of *in vitro* cytotoxicity tests against ^{3}H-dT-labelled-B_559 melanoma cells using non-adherent leukocytes from peritoneal cavities of mice with (*a*) or without tumors (*b*). B_559 melanoma was injected i.p. on day 0 and rIL-2, rIFN-γ or both, or buffer (as shown) was administered i.p. on days 3 and 4. On day 6, peritoneal cells were removed and incubated with ^{3}H-dT-labelled B_559 cells in 96-well plates overnight, as described in "Material and Methods". Each point represents the mean c.p.m. remaining on filters after mini-Mash harvesting of triplicate or quadruplicate wells. Data were pooled from 3 experiments, except in the case of rIFN alone, where only one experiment was done.

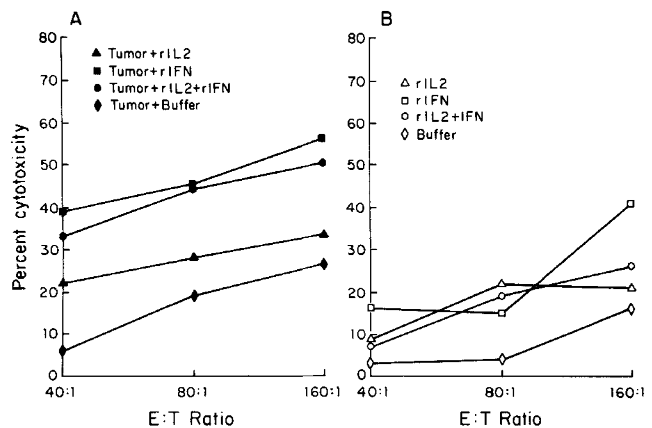

FIGURE 6 – Results of *in vitro* cytotoxicity tests against ^{3}H-dT-labelled B_559 melanoma cells using non-adherent leukocytes from spleens of the mice used for experiments shown in Figure 5. Other details are the same as stated for Figure 5 and in "Material and Methods".

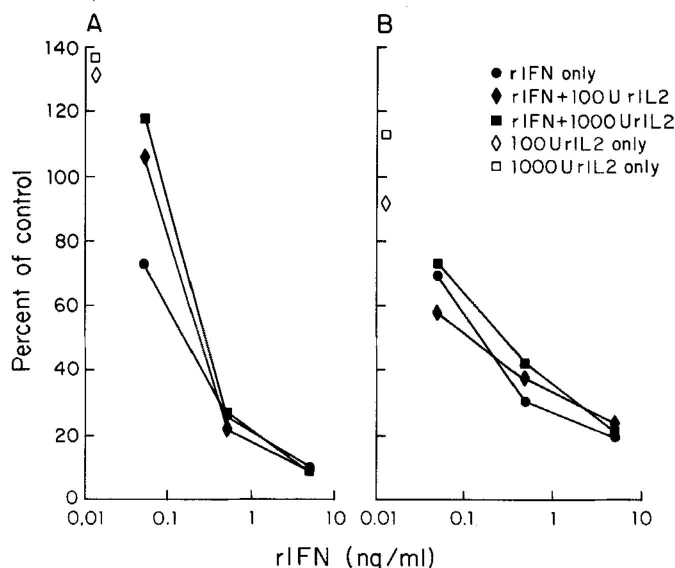

FIGURE 7 – Relative plating efficiency (*a*) and cell counts (*b*) of B_559 melanoma cells cultured in normal medium with rIFN-γ alone at 0.05, 0.5 and 5 ng/ml, or combined with rIL-2 at 100 U/ml or 1,000 U/ml. The symbols next to the Y-axis represent percentage of control plating efficiency or cell counts for cells cultured with rIL-2 at 100 U (◇) or 1,000 U (□) per ml. Cells were plated on day 0 and lymphokines added several hours later. For plating efficiency they were plated at 100 and 200 cells/60-mm culture dish and for counting at 7.5×10^4 cells/dish. Cells were counted on day 3 and percentage of control cells plated without lymphokine was calculated. For plating efficiency, cells were grown for 8–11 days and stained, then colonies were counted to give the percentage of control colonies. The results given are calculated from the means of 3 consecutive experiments, in each of which duplicate cultures were counted. (See "Material and Methods".)

As can be seen in Figure 5*a*,*b*, cytotoxic activity of non-adherent peritoneal cells (NA-PC, 90% of which were lymphocytes as judged by stained cytofuge preparations) against melanoma cells was significantly augmented at all E:T ratios with both lymphokines, whether injected singly or together, and whether in tumor-bearers or non-tumor-bearers, when compared with buffer-injected controls (tumor- or non-tumor-bearers). Furthermore, the percentage of cytotoxicity was proportional to the number of effector cells present in all cases but one (IFN 80:1, Fig. 5*b*). In addition, this activity was consistently and significantly higher in NA-PC from tumor-bearing mice as compared with non-tumor-bearers.

Although *in vitro* cytotoxic activity was high in NA-PC from all treated mice, treatment with the combination of both rIL-2 and rIFN-γ did not give rise to mononuclear cells with additive or synergistically higher cytotoxicity than did treatment with either lymphokine singly.

Statistical analysis was done using a 4-factor analysis of variance with interactions between rIL-2 and rIFN-γ as described in "Material and Methods". The factors analyzed were: (1) inoculation of melanoma or buffer; (2) rIL-2 or no rIL-2; (3) rIFN-γ or no rIFN-γ; and (4) E:T ratio and interaction for IFN-γ and IL-2. All 4 factors were significantly related to percentage of cytotoxicity compared with controls, as were interactions between IL-2 and IFN-γ (*p* values ranged from 0.0001 to 0.002). Results with E:T ratios of 20:1 were significantly different from 40:1 and 80:1, but these last two ratios were not different from each other.

Figure 6 shows similar relationships using non-adherent splenocytes although with lower percentages of cytotoxicity against melanoma cells than were seen with non-adherent PC. Cells from tumor-bearers were more efficient killers than those from non-tumor-bearers, and all cells from lymphokine-treated mice were much more efficient killers than those from controls. Statistical analysis of these data was carried out as described above. All factors except rIL-2 were significantly related to percentage of cytotoxicity, as was interaction between rIFN-γ and rIL-2, and all ratios were significantly different from one another (*p* values ranged from 0.008 to 0.0001).

Direct effects of rIFN-γ and rIL-2 on proliferation and plating efficiency of B_559 melanoma cells in culture

Inhibition of proliferation has been reported extensively for many cultured cell types with various interferons (Borden and Ball, 1981; Bonnem and Oldham, 1987). It was therefore of interest to determine whether rIFN-γ ± rIL-2 would affect proliferation of B_559 melanoma cells in culture. As expected, rIFN-γ inhibits proliferation of melanoma cells. This was true whether plating efficiency or direct cell counting was measured (Fig. 7). The concentration of rIFN-γ that inhibited melanoma cells by 50% compared to controls (I_{50}) by either measurement was 0.18 ng/ml. On the other hand, rIL-2 by itself generally stimulated proliferation. At 100 U/ml, it stimulated plating efficiency (PE) to 131% as compared to 100% for control cells grown without addition of rIL-2; and had no effect on the relative number of viable cells present after 3 days of growth in the presence of the lymphokine. When cells were plated in the presence of 1,000 U/ml of rIL-2, the PE was almost 40% higher than that of control melanoma cells, and the cell count at 3 days was 13% higher than that of control cells.

Cells plated in the presence of 0.05 ng/ml of rIFN-γ and 100 U/ml or 1,000 U/ml of rIL-2 had higher PEs than control melanoma cells plated without lymphokine additives, but cell counts at 3 days were lower than controls. rIL-2 could not neutralize the anti-proliferative effects of rIFN-γ at 0.5 or 5

ng/ml. The I_{50} for rIFN-γ combined with 100 U/ml of rIL-2 was 0.24 ng/ml for relative PE and 0.13 ng/ml for relative cell counts at 3 days. The I_{50} for rIFN-γ combined with 1,000 U/ml of rIL-2 was 0.31 ng/ml for relative PE and 0.30 ng/ml for cell counts at 3 days (Fig. 7).

DISCUSSION

Tumor eradication is significantly enhanced by the combined use of rIFN-γ and rIL-2 to treat B6 mice bearing early s.c. or i.p. melanomas. On day 50 after beginning peri-lesional s.c. treatment of 1- to 3-day s.c. tumor-bearers with 2 μg of rIFN-γ and 6,250–25,000 U of rIL-2 per injection for 14 injections over a 28-day period, 72/83 (87%) mice were tumor-free. This compared with 44/83 (53%) treated with rIFN-γ, 47/79 (59%) treated with rIL-2, and 0/80 buffer-injected controls. Even more impressive, and of possible clinical relevance to certain tumors (*e.g.*, ovarian), was the synergism resulting from combination therapy given i.p. to mice with 1-day i.p. melanomas. Autopsy on days 45–65 revealed that 21/26 (81%) of mice given both rIFN-γ and rIL-2 were tumor-free as compared with 2/12 (17%) given rIL-2, and 6/30 (20%) given rIL-2 and 0/11 control mice.

Thus, administration of a combination of these 2 lymphokines provides an effective approach to cancer therapy. Lower dosages need to be explored in mice and other mouse tumors need to be tested, as do larger and metastatic tumors. The B16 melanoma, which arose spontaneously in a B6 mouse, has been in passage in B6 mice since 1954 and its cells have been passed in culture for about 20 years. The B_559 clone was obtained as described (Silagi, 1969, 1976) and remains predictably malignant, so that 2.2×10^5 viable cells inoculated s.c. or i.p. in a naive B6 mouse grow rapidly and progressively, inevitably leading to death. Although it is possible to immunize mice using 5-bromodeoxyuridine-modified melanoma cells (but not with irradiated cells) (Silagi, 1976; Calvelli *et al.*, 1982), numerous attempts to interfere with tumor progression after inoculation of B_559 into an unimmunized mouse were unsuccessful until we began to use lymphokines (Silagi and Schaefer, 1986). Thus, as an aggressive, spontaneously-arisen, non-immunogenic tumor cell, the B_559 clone of the B16 melanoma remains a useful model system.

We previously described the successful use of recombinant human IL-2 and low-dose cyclophosphamide (CY) in treatment of early mouse melanoma and sarcoma (Silagi and Schaefer, 1986). However, high doses of rIL-2 (50,000 U/injection for 17 injections over 35 days) were necessary to eradicate the tumors. In humans, similar doses/kg induce toxic side effects, including vascular leakage syndrome (VLS) described by Rosenstein *et al.* (1986). The mice in our experiments appeared healthy, with sleek coats and with no aberrant weight gain or loss. It is possible that use of a less intensive protocol, extending over 4 weeks with rest periods between injections, contributed to the absence of VLS.

In earlier experiments mice were injected with CY i.p. (50 mg/kg, 4 × at weekly intervals) along with rIL-2 (Silagi and Schaefer, 1986). CY significantly facilitated the anti-tumor effect of rIL-2 (North, 1982; Greenberg *et al.*, 1985; Rosenberg *et al.*, 1986). Preliminary experiments to determine whether CY would also enhance the anti-tumor effect of rIFN-γ, alone or in combination with rIL-2, indicated that no significant difference in tumor incidence ensued. For example, when the combination of 2 μg of rIFN-γ with 1.25 or 2.5×10^4 U of rIL-2 ± CY was used, 0/16 mice with CY, and 1/16 without CY developed tumors. CY was therefore not used in ensuing experiments. However, the effect of CY needs further exploration.

Most mice "cured" following use of IL-2 remained tumor-free until at least day 90 (Silagi and Schaefer, 1986). The data in Figure 2 show that mice with i.p. melanomas could remain "cured" at least until day 65. More work is needed to determine longevity of mice with "cured" tumors.

Augmentation of cytotoxicity by combined *vs.* single lymphokine treatment in mice was not reflected in *in vitro* cytotoxicity assays using non-adherent PC or spleen cells against cultured melanoma target cells. However, mononuclear leukocytes (90% lymphocytes) from lymphokine-treated mice were significantly more cytotoxic to melanoma cells than were parallel effector cells from buffer-treated control mice. This was true whether or not the mice were tumor-bearers. Leukocytes from tumor-bearers were always more cytotoxic than those from non-tumor-bearers.

The lack of parallelism between *in vivo* and *in vitro* results casts doubt on the relevance of results from *in vitro* assays to the situation in the whole organism. Recently, Papa *et al.* (1986) found a similar lack of correlation between *in vivo* anti-tumor efficacy and *in vitro* cytolytic activity of lymphokine-activated killer (LAK) cells against a variety of mouse tumors.

In these experiments, *in vitro* cytotoxic activity was measured at a definite time (6 days) after tumor-cell inoculation and 3 days after treatment began. In the *in vivo* experiments, mice received many more treatments over 28 days and were autopsied at 50 days. Many presently undefined interactions probably took place within the animals to result in regression of tumors. Interferons and IL-2, while acting cooperatively in many situations (Kuribayashi *et al.*, 1981; Kawase *et al.*, 1983; Weigent *et al.*, 1983; Ortaldo *et al.*, 1984; Shalaby *et al.*, 1985; Itoh *et al.*, 1985; Brunda *et al.*, 1986; Riccardi *et al.*, 1986), appear to follow different pathways in enhancing cytotoxicity (van de Griend *et al.*, 1986). There may be a cascade of separate yet cooperative *in vivo* interactions that culminate in the lysis of tumor cells in many more of the mice that receive both lymphokines than in mice receiving either lymphokine alone.

Doses of rIFN-γ as low as 1 μg and 2 μg per injection are more effective in tumor therapy than higher doses. Although the differences between adjacent concentrations (1 μg–5 μg) shown in Figure 3 were not statistically significant (Fisher's exact test) and the difference between the results with 2 μg and 5 μg were barely significant ($p = 0.05$), the differences between 1 or 2 μg and 15 μg were highly significant ($p = 0.003$ and 0.002 respectively). Similarly, in phase-I trials with cancer patients using recombinant human IFN-γ, Kleinerman *et al.* (1986) reported that high doses administered systemically did not activate blood monocytes whereas lower doses resulted in activation of monocytes to become cytotoxic to their tumors. The authors concluded that "for biologicals with immunostimulatory activity the concept that 'more drug is better' may not be operative". This may also be applicable to our results with combinations of rIL-2 and rIFN-γ in mice. These showed no statistically significant differences for concentrations of rIL-2 from 6,250 U to 25,000 U per injection combined with 2 μg of rIFN-γ (Fig. 1 and text). It may be possible to decrease the dose of rIL-2 further while retaining therapeutic benefits.

IFN-γ, like rIL-2 (Silagi and Schaefer, 1986; Bubeník *et al.*, 1985; Kedar *et al.*, 1984; Vaage *et al.*, 1987), is most effective in reducing tumor incidence when administered close to the tumor site (Fig. 4). This was also true for therapy combining both lymphokines (Figs. 1 and 2), and most relevant to i.p. tumors, which, in the case of humans, would

include mucinous adenocarcinoma and ovarian cancers (Ottow *et al.*, 1987).

The mechanisms underlying the anti-tumor effects of these lymphokines, singly or in combination, are being studied. We have been attempting to discern them through the use of antisera against surface markers of mononuclear leukocytes (not shown).

The stimulatory effect of 100 U and 1,000 U of rIL-2 on growth and plating efficiency of the melanoma cells is an unexpected observation. The use of monoclonal antibodies to the mouse IL-2 receptor should help determine whether presence of IL-2 receptor mediates this effect. However, it was not surprising that doses as low as 0.05 ng/ml (0.05 U/ml) inhibited proliferation and plating efficiency since these effects of IFN's have been well documented (Borden and Ball, 1981; Bonnem and Oldham, 1987). The ability of rIL-2 to neutralize the loss of plating efficiency in the presence of 0.05 ng/ml of rIFN-γ but not of 0.5 or 5 ng/ml is notable but not readily explainable.

In summary, the most significant finding from this work is the great increase of s.c. and i.p. tumor elimination in B6 mice by combined localized treatment with relatively low doses of rIL-2 and rIFN-γ. Similar protocols using early and late stages of i.p. tumors, *e.g.*, ovarian, and spontaneously metastasizing tumors would help determine the applicability to human cancer treatment.

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