Brief Definitive Report

PASSIVE IMMUNIZATION AGAINST TUMOR NECROSIS FACTOR PARTIALLY ABROGATES INTERLEUKIN 2 TOXICITY

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IL-2 alone or in combination with the adoptive transfer of either lymphocyte-activated killer cells (LAK) or tumor-infiltrating lymphocytes (TIL) has shown efficacy in the treatment of certain human malignancies (1, 2). However, IL-2-based immunotherapy protocols are associated with considerable systemic toxicity (1, 3). The pathophysiologic consequences of high dose IL-2 administration in man are variably composed of a "vascular leak syndrome," marked fluid retention and weight gain, decreased systemic vascular resistance, increased cardiac output, hypotension, elevated creatinine, and elevated bilirubin (4–7). This constellation of changes is remarkably similar to the alterations seen in septic shock. A series of recent investigations have identified the monocyte/macrophage product, TNF, as an important mediator of toxicity in endotoxemia and Gram-negative sepsis (8, 9). The clinical similarities between IL-2 toxicity and sepsis suggest a potential role for TNF as a mediator of the toxic effects of IL-2 (3). Evidence to support this hypothesis is that IL-2 induces transcription and translation of TNF in LAK cells and macrophages (10–12), as well as recent studies that measure circulating TNF in humans receiving IL-2 alone or IL-2 and LAK (13, 14).

The present study investigates the role of TNF in IL-2 toxicity by administering high levels of neutralizing anti-TNF antibody concurrently with high dose IL-2 treatment in mice. The effects of TNF antibody on IL-2 toxicity as well as the antitumor efficacy of IL-2 are evaluated.

Materials and Methods

Mice. Female C57BL/6 mice and C3H/HEN mice 12–16 wk old were obtained from the small animals section, Veterinary Resources Branch, NIH, Bethesda, MD.

muTNF Antibody Preparation. New Zealand white rabbits 3–4 kg were immunized to recombinant murine TNF-α (rmuTNF) (Genentech, South San Francisco, CA) or to recombinant rat IFN-γ (Amgen Corp., Thousand Oaks, CA) and bled in a standard fashion. Sera were passed over a recombinant protein A column (10 ml bed volume), washed, eluted, and desalted per manufacturer's recommendations (Beckman Instruments, Inc., Fullerton, CA). Column aliquots were screened for TNF antibody as described below and positive aliquots were pooled to obtain the TNF antibody solution used in all experiments.

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Characterization of rmuTNF Antibody. The neutralizing activity of the TNF antibody solution was quantitated by serial dilution using the L929 cytolytic assay (15). The greatest dilution of antibody solution that led to 100% survival of L929 cells was used to quantitate neutralizing activity. IgG concentration of the antibody solution was measured by radial immunodiffusion (ICN Immunobiologicals, Irvine, CA).

Pulmonary Metastases Tumor Model. A murine pulmonary metastases model using a weakly immunogenic methylcholanthrene-induced tumor (MCA-106) was used. Tumor preparation and analysis were done as previously described (16); metastases were counted in a blinded fashion (17). By prior convention, if there were >250 metastases that lung was scored as >250 as higher numbers of lesions could not be counted (17). Mice were treated with recombinant human IL-2 (Cetus Corp., Emeryville, CA) 100,000 U i.p. every 8 h or HBSS control.

Statistics. Survival curves were analyzed by the Breslow modification of the Kruskal-Wallis test. Metastases number are shown as mean ± SEM and compared by the Wilcoxon Rank Sum test corrected for multiple comparisons.

Results and Discussion

The polyvalent antisera to recombinant murine TNF used in all experiments neutralized 5×10^6 U of TNF per milliliter of antibody solution in a standard L929 cytolytic assay. The specificity of the antisera was evaluated by bioassay and immunoblot techniques. The TNF antibody had no neutralizing activity against rIL-2 in a CTLL proliferation assay (data not shown). To evaluate the efficacy of the TNF antibody in vivo as well as to establish an appropriate in vivo dose, we repeated the experiment of Beutler et al. (8) using polyvalent TNF antibody to protect against a lethal dose of endotoxin. Pretreatment of mice with 5×10^5 U i.p. of neutralizing activity 6 h before challenge with an LD_{100} dose of endotoxin gave complete protection from death in all mice; whereas pretreatment with 2.5×10^5 U of neutralizing activity afforded no protection.

To determine the effects of TNF antibody on IL-2 toxicity, mice were injected with 100,000 U i.p. of IL-2 every 8 h and the number of doses of IL-2 received before death was recorded. C3H/HEN mice receiving control rabbit IgG died reproducibly after receiving 10–12 doses of IL-2 (mean dose, 11.1). Mice that were injected with varying doses of TNF antibody ranging from 0.5 to 2.0×10^6 U i.p. of neutralizing activity once daily during IL-2 treatment survived significantly more doses of IL-2 than control mice treated with nonspecific antibody ($p < 0.005$ vs. control for all TNF antibody groups; Fig. 1 *A*). The protective effect of TNF antibody against IL-2 lethality was observed as a dose-response. Another control using polyvalent rabbit antisera against rat IFN was no different than control rabbit IgG, indicating that the TNF protective effect was not due to administration of a hyperimmune sera (Fig. 1 *A*).

TNF antibody afforded a similar protective effect against IL-2 toxicity in C57BL/6 mice with microscopic (3 day) and macroscopic (10 day) pulmonary sarcoma metastases (Fig. 1 *B*). The mean dose of IL-2 plus control IgG that resulted in death was 14.5 for mice with 3-d pulmonary metastases and 14 for mice with 10-d pulmonary metastases, but 14/15 mice with 3-d pulmonary metastases and 12/12 mice with 10-d metastases treated with antibody to TNF tolerated 20 doses of IL-2 without mortality ($p < 0.001$).

The effect of TNF antibody on the antitumor activity of IL-2 can be evaluated by comparing the treatment response of mice receiving equal doses of IL-2 with or without the TNF antibody. Mice given IL-2 with TNF antibody have significantly

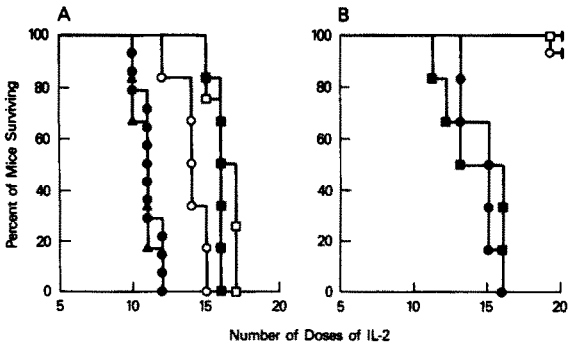

FIGURE 1. Survival curves for mice receiving IL-2 and TNF antibody or control antibody. (*A*) Non-tumor-bearing C3H/HEN mice were injected with 100,000 U i.p. of IL-2 in 0.5 ml HBSS every 8 h and the number of doses until death was recorded. Mice received daily injections of 0.4 ml of either control rabbit IgG, varying quantities of anti-murine TNF antibodies, or anti rat IFN-γ antibodies. Control rabbit IgG (●; n = 14); 0.5 × 10^6 neutralizing units TNF Ab once daily (○; n = 6); 1.25 × 10^6 neutralizing units TNF Ab (■; n = 6); 2.0 × 10^6 neutralizing units TNF Ab (□; n = 4); anti rat IFN-γ Ab (▲; n = 6). $p < 0.005$ for all TNF Ab groups vs. control Ab or IFN-γ Ab. $p < 0.05$ for 1.25 × 10^6 or 2.0 × 10^6 U TNF Ab vs. 0.5 × 10^6 U TNF Ab. (*B*) C57BL/6 mice were injected via tail vein with either 3 × 10^5 or 1 × 10^6 MCA-106 sarcoma cells and 3–10 d later treatment with 100,00 U IL-2 in 0.5 ml HBSS intraperitoneally every 8 h was initiated. Mice received once daily intraperitoneal injections of either control rabbit IgG or 2 × 10^6 neutralizing units of TNF Ab and survival was followed. 3-d Metastases, control Ab (●; n = 12); 3-d metastases, TNF Ab (○; n = 12); 10-d metastases, control Ab (■; n = 12); 10-d metastases, TNF Ab (□; n = 15). $p < 0.001$ for TNF Ab vs. control Ab.

fewer metastases than control mice receiving the vehicle (HBSS) alone or HBSS and TNF antibody ($p < 0.001$ vs. both HBSS controls in all experiments; Table I). TNF antibody–treated mice have a consistent but not statistically significant increase in pulmonary metastases compared with mice receiving an equal amount of IL-2 with control antibody in three experiments.

Whether additional administration of IL-2 made possible by the protective effect of TNF antibody improves the therapeutic response can be evaluated by comparing mice receiving equitoxic amounts of IL-2 with or without the TNF antibody. The maximal amount of IL-2 tolerated by mice receiving control antibody was given in the experiments shown in Table I, as evidenced by the occurrence of IL-2 treatment deaths. One subgroup of mice given TNF antibody received additional IL-2 until obvious toxicity and imminent mortality was observed, and these mice can be considered to have received equitoxic doses of IL-2 compared with mice given control antibody and IL-2. In the 3-d pulmonary metastases model, IL-2 alone is so effective, 96 and 89% reduction in metastases number in Exps. 1 and 2, respectively (Table I), that it is difficult to demonstrate an improved therapeutic effect with additional doses of IL-2 made possible by TNF antibody in this micrometastases model.

In mice given 3 × 10^5 MCA-106 cells and treated at 10 d after tumor injection with 20 doses of IL-2 plus TNF antibody (Exp. 3) there was a modest but insignificant decrease in pulmonary metastases compared with mice given 12 doses of IL-2 and control antibody (24 vs. 53 metastases, $p = 0.06$; Table I). In a similar 10-d metastases experiment in which mice were injected with 10^6 MCA-106, an improved response after additional IL-2 administration was more apparent. In this experiment control mice given HBSS with control or TNF antibody had >250 metastases (Fig. 2, *A* and *B*). Mice given 12 doses of IL-2 and daily control antibody had an IL-2 treatment mortality of 25% (3/12) and the remaining nine mice had a mean of 230 pulmonary metastases (Fig. 2 *C*). Again, mice given an equal number of doses of IL-2

TABLE I

Effect of IL-2 and TNF Antibody on Microscopic (3-d) and Macroscopic (10-d) Pulmonary Metastases

Exp.	*n*	TNF Ab	Tx	No. of IL-2 doses	IL-2 tx deaths	No. of metastases
1	12	–	HBSS	–	–	168 ± 17
	6	+	HBSS	–	–	167 ± 12
	12	–	IL-2	12	3/12 (25%)	7 ± 4*
	6	+	IL-2	12	0/6 (0%)	32 ± 14*
	6	+	IL-2	20	0/6 (0%)	5 ± 2*
2	12	–	HBSS	–	–	>250
	6	+	HBSS	–	–	>250
	24	–	IL-2	14	5/24 (21%)	28 ± 8*
	6	+	IL-2	14	0/6 (0%)	47 ± 14*
	9	+	IL-2	20	1/9 (11%)	23 ± 6*
3	12	–	HBSS	–	–	171 ± 15
	6	+	HBSS	–	–	173 ± 24
	24	–	IL-2	12	2/24 (8%)	53 ± 8*
	6	+	IL-2	12	0/6 (0%)	83 ± 9*
	6	+	IL-2	21	0/6 (0%)	24 ± 8*

Exp. 1 is the treatment of 3-d pulmonary metastases after intravenous injection of 3×10^5 MCA-106 cells. Exp. 2 is the treatment of 3-d pulmonary metastases after intravenous injection of 10^6 MCA-106 cells. Exp. 3 is the treatment of 10-d pulmonary metastases after intravenous injection of 3×10^5 MCA-106 cells.

Treatment consisted of 100,000 U i.p. of IL-2 every 8 h in 0.5 ml HBSS or HBSS alone. TNF antibody (Ab)-treated mice received 2×10^6 U i.p. of Ab once daily or the equivalent rabbit IgG control. Treatment with both agents was begun 3 d or 10 d after MCA-106 tumor cell injection. Number of metastases are shown as mean ± SEM. Tx, treatment.

* $p < 0.001$ vs. HBSS groups.

with TNF antibody had a similar IL-2 treatment reponse (mean number of metastases, 228; Fig. 2 *D*). A second subgroup of mice given daily TNF antibody in this experiment tolerated 20 doses of IL-2 without treatment mortality and the number of metatases was 173 ($p < 0.02$ vs. IL-2 plus control antibody). Although the apparent de-

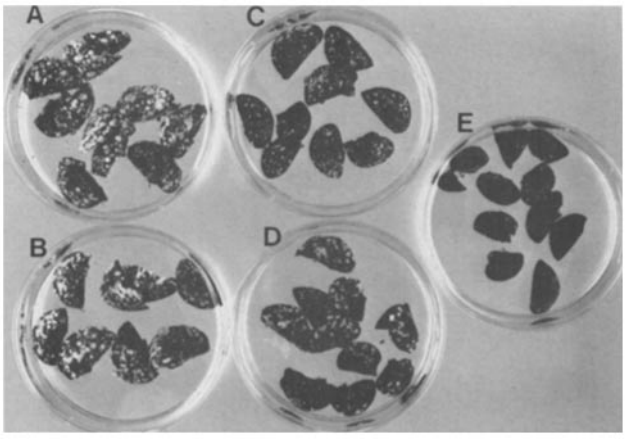

FIGURE 2. Lungs from mice injected with 1×10^6 MCA-106 cells 10 d before treatment with thrice daily injection of HBSS or 100,000 U of IL-2 intraperitoneally and daily injections of rabbit IgG or 2×10^6 U neutralizing activity of TNF antibody. Lungs were treated with India ink and Fekete's such that tumor metastases appear white on a black background of normal lung. (*A*) HBSS and control rabbit IgG; (*B*) HBSS and TNF antibody; (*C*) 12 doses of IL-2 and control rabbit IgG; (*D*) 12 doses of IL-2 and TNF antibody; (*E*) 20 doses of IL-2 and TNF antibody.

crease in tumor number is relatively small, the actual decrease in tumor burden by the additional eight doses of IL-2 made possible by concurrent TNF antibody is greater as the size of the metastases in this group also appears to be substantially decreased (Fig. 2 *E*).

The results in both non-tumor-bearing and tumor-bearing mice indicate that endogenous TNF production is a mediator of systemic IL-2 toxicity. Mice treated with TNF antibody tolerate between 30 and 60% more IL-2 than control mice before IL-2 death. Since TNF antibody-treated mice eventually die secondary to IL-2 toxicity despite continued antibody injection, then either other mechanisms of IL-2 toxicity exist or inadequate amounts of TNF antibody were given. The former explanation is most likely. IL-2 administration induces other potentially toxic host factors such as lymphotoxin, IFN-γ, and IL-1 (10, 11, 17), as well as having pleotropic immune effects that would be unaffected by TNF antibody.

Passive immunization against TNF may dissociate the toxic effects of IL-2 from the antineoplastic activity of IL-2. In consecutive experiments where equal doses of IL-2 were given, the addition of TNF antibody did not significantly alter the antineoplastic effect of IL-2. Lessening IL-2 toxicity while maintaining treatment efficacy is a potentially clinically relevant observation. In one experiment additional IL-2 administration made possible by TNF antibody effected a modest decrease in tumor number but a dramatic decrease in tumor burden compared with equitoxic doses of IL-2 plus control antibody (Fig. 2). Further experiments using different tumor histologies at different anatomic sites are necessary to delineate the utility of TNF antisera as a strategy to improve the therapeutic index in IL-2 immunotherapy.

Summary

Passive immunization against TNF allowed non-tumor-bearing C3H/HEN mice and tumor-bearing C57BL/6 mice to tolerate significantly more doses of IL-2 before death ($p < 0.005$ and $p < 0.001$, respectively). The antitumor effect of IL-2 against both 3-d and 10-d pulmonary metastases was maintained in mice treated concurrently with neutralizing antibodies to TNF. In one experiment with 10-d pulmonary metastases, increased administration of IL-2 made possible by passive immunization against TNF significantly improved the antitumor response compared to equitoxic doses of IL-2 and control antibody. The results indicate that TNF is a mediator of IL-2 toxicity but contributes minimally to the antitumor effects of IL-2. Strategies to inhibit TNF may improve the therapeutic index of IL-2 as a neoplastic agent.

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References

1. Rosenberg, S. A., M. T. Lotze, L. M. Muul, A. E. Chang, F. P. Avis, S. Leitman, W. M. Linehan, C. N. Robertson, R. E. Lee, J. T. Rubin, C. A. Siepp, C. G. Simpson, and D. E. White. 1987. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activiated killer cells and interleukin-2 or high dose interleukin-2 alone. *N. Engl. J. Med.* 316:889.
2. Rosenberg, S. A., B. S. Packard, P. M. Aebersold, D. Solomon, S. L. Topalian, S. T. Toy, P. Simon, M. T. Lotze, J. C. Yang, C. A. Siepp, C. Simpson, C. Carter, S. Bock, D. Schwartzentruber, J. P. Wei, and D. E. White. 1988. Use of tumor infiltrating lym-

phocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. *N. Engl. J. Med.* 319:1676.

3. Herberman, R. A. 1989. Interleukin-2 therapy of human cancer: potential benefits versus toxicity. *J. Clin. Oncol.* 7:1.
4. Rosenstein, M., S. E. Ettinghausen, and S. A. Rosenberg. 1986. Extravasation of intravascular fluid mediated by the systemic administration of interleukin-2. *J. Immunol.* 137: 1735.
5. Lee, R. E., M. T. Lotze, J. M. Skibber, E. Tucker, R. O. Bonow, F. P. Ognibene, J. A. Carrasquillo, J. H. Shelhamer, J. E. Parillo, and S. A. Rosenberg. 1989. Cardiorespiratory effects of immunotherapy with interleukin-2. *J. Clin. Oncol.* 7:7.
6. Gaynor, E. R., L. Vitek, L. Sticklin, S. P. Creekmore, M. E. Ferraro, J. X. Thomas, S. G. Fisher, and R. I. Fisher. 1988. The hemodynamic effects of treatment with interleukin-2 and lymphokine-activated killer cells. *Ann. Intern. Med.* 109:953.
7. Belldegrun, A., D. E. Webb, H. A. Austin, S. Steinberg, D. E. White, W. M. Linehan, and S. A. Rosenberg. 1987. Effects of interleukin-2 on renal function in patients receiving immunotherapy for advanced cancer. *Ann. Intern. Med.* 106:817.
8. Beutler, B., I. W. Milsark, and A. C. Cerami. 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science (Wash. DC).* 229:869.
9. Tracey, K. J., B. Beutler, S. F. Lowry, J. Merryweather, S. Wolpe, I. W. Milsark, R. J. Hariri, T. J. Fahey, A. Zentella, J. D. Albert, G. T. Shires, and A. Cerami. 1986. Shock and tissue injury induced by recombinant human cachectin. *Science (Wash. DC).* 234:470.
10. Nedwin, G. E., L. P. Svedersky, T. S. Bringman, M. A. Palladino, and D. V. Goeddel. 1985. Effect of interleukin-2, interferon-γ, and mitogens on the production of tumor necrosis factors α and β. *J. Immunol.* 135:2492.
11. Kovacs, E. J., S.K. Beckner, D. Longo, L. Varesio, and H. A. Young. 1987. Lymphokine gene expression during the generation of LAK cells. *Fed. Proc.* 46:925.
12. Economu, J. S., R. Essner, K. Rhoades, W. H. McBride, S. H. Golub, E. C. Holmes, and D. L. Morton. 1988. Molecular mechanisms of interleukin-2 induction of tumor necrosis factor gene expression in macrophages. *Surg. Forum.* 37:426.
13. Mier, J. W., G. Vachino, J. W. M. VanderMeer, R. P. Numerof, S. Adams, J. G. Cannon, H. A. Bernheim, M. B. Atkins, D. R. Parkinson, and C. A. Dinarello. 1988. Induction of circulating tumor necrosis factor (TNF-α) as the mechanism for the febrile response to interleukin-2 (IL-2) in cancer patients. *J. Clin. Immunol.* 8:426.
14. Gemlo, B. T., M. A. Palladino, H. S. Jaffe, T. P. Espevik, and A. A. Raynor. 1988. Circulating cytokines in patients with metastic cancer treated with recombinant interleukin-2 and lymphokine activated killer cells. *Cancer Res.* 48:5864.
15. Fraker, D. L., M. C. Stovroff, M. J. Merino, and J. A. Norton. 1988. Tolerance to tumor necrosis factor in rats and the relationship to endotoxin tolerance and toxicity. *J. Exp. Med.* 168:95.
16. Mulé, J. J., S. Shu, S. L. Schwarz, and S. A. Rosenberg. 1984. Successful adoptive immunotherapy of established pulmonary metastases of multiple sarcomas with lymphokine-activated killer cells and recombinant interleukin-2. *Science (Wash. DC).* 225:1487.
17. Lotze, M. T., Y. L. Matory, S. E. Ettinghauser, A. A. Raynor, S. O. Sharrow, C. A. Siepp, M. C. Custer, and S. A. Rosenberg. 1985. *In vivo* administration of purified human interleukin 2 II. Half life, immunologic effects, and expansion of peripheral lymphoid cells *in vivo* with recombinant IL-2. *J. Immunol.* 135:2865.