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Effect of host age upon interleukin-2-mediated anti-tumor responses in a murine fibrosarcoma model*

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Summary. The age-associated decline in immune function may be an important factor in both the pathogenesis of neoplastic diseases and the response to immunopharmacological therapies. With the increased efforts to develop immunotherapy with such agents as interferon and interleukin-2 (IL-2), the question of the effect of host age upon response is of practical importance. Phase I and phase II clinical trials of IL-2 have included primarily young patients, and toxicity and efficacy have not been reported with specific reference to host age. In this study, we examined young and old mice with regard to in vitro natural killer and lymphokine-activated killer (LAK) cell functions. We also assessed the effects of exogenously administered recombinant human IL-2 in tumor-bearing mice of various ages. We found that natural killer cell function was demonstrably lower in old mice but that LAK cell function was comparable (young versus old). Furthermore, IL-2 treatment was successful in increasing survival time in old mice, similar to results in young mice. Our observations allow the prediction that immune senescence *per se* does not preclude successful anti-neoplastic treatment with IL-2.

Introduction

Interleukin-2 (IL-2) has recently been intensively investigated as an antitumor agent [9, 10, 18, 26]. It is a lymphokine of approximately 15 kDa produced by a subset of T cells. It is speculated that the anticancer activity of IL-2 is mediated by its activation of a specific subset of lymphocytes, lymphokine-activated killer or LAK cells [2, 3, 8]. In vitro, IL-2-stimulated lymphocytes demonstrate cytotoxicity against fresh and cultured tumor cells [16, 30] and, for that reason, IL-2 has been combined with autologous or allogeneic lymphocytes stimulated in vitro in clinical trials [14, 27, 29].

In preclinical studies in mice, the use of IL-2, with or without LAK cells has been successful in reducing tumor burden and experimental metastases [15, 20, 28, 35]. Similarly, in clinical trials, the antitumor activity of IL-2 in conjunction with the infusion of LAK cells has resulted in objective improvement, but this has been associated with substantial toxicity including fluid retention, pulmonary edema and renal failure [1, 31].

In our laboratory, we have been interested in the treatment of mice with low tumor burden with IL-2. We have also had a particular interest in tumor biology and cancer treatment in older hosts [4–7, 34]. Immune senescence involves primarily cellular deficiencies, and immunotherapeutic strategies that rely upon various cellular immune functions may be unsuccessful because of the underlying deficiency. It was the purpose of these studies to estimate the degree to which the age-associated decline in cell-mediated immunity altered tumor cytotoxicity in vitro and in IL-2-treated, tumor-bearing mice.

Materials and methods

Mice

Young female C57BL/6 mice were purchased at 6 weeks of age from Harlan Sprague Dawley (Madison, Wis). Old female mice were obtained at 22 months of age from the NIA aging colony maintained at Charles River Breeding Laboratory (Kingston, NY). Mice were maintained in our animal care facility for at least 1 month prior to use in these experiments. The median survival for mice of this strain is approximately 24–26 months.

Tumor

A fibrosarcoma was induced in the mice by the subcutaneous injection of methylcholanthrene (0.01 mg/0.1 ml sesame oil). The primary tumor

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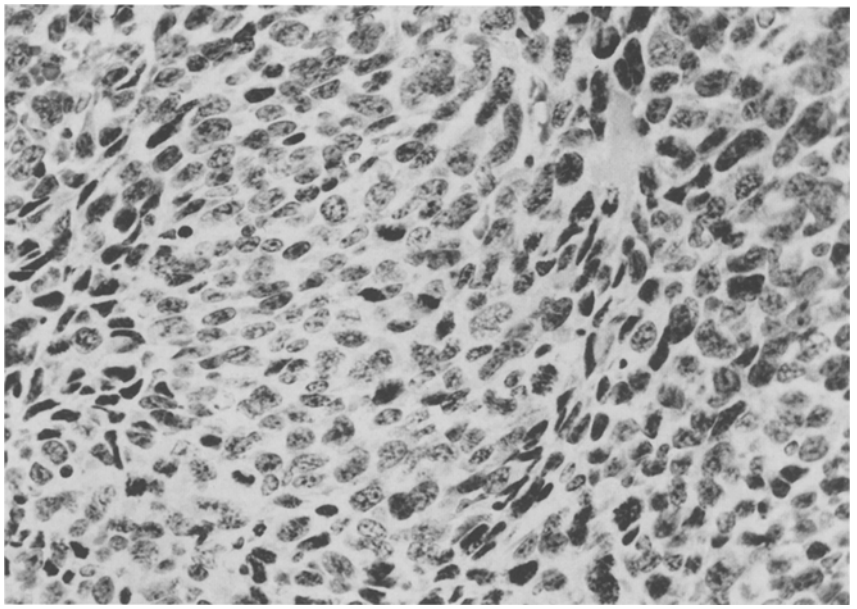

Fig. 1. Histological appearance of fibrosarcoma MV2 from subcutaneous transplantation. Note the spindle cells and fibrous swirls characteristic of tumor of mesenchymal origin. × 400

(Fig. 1) was minced and inoculated sequentially into three mice (subcutaneously). From the tumor in the third mouse a tumor cell suspension was prepared by enzymatic digestion (collagenase, hyaluronidase and DNase) [24]. Clonal growth was established by soft agar technique [25]. A specific clone (MV2) was selected for these studies. This tumor was expanded in culture and aliquots were frozen in liquid nitrogen.

The murine lymphoma YAC-1 was selected for the natural killer cell (NK) assay. This cell line was purchased from American Type Tissue Culture (TIB160) and is stored in liquid nitrogen.

To prepare cells for the LAK cell assay, frozen cells were thawed, cultured, and injected subcutaneously into the flank of a young mouse. When the tumor was between 1 cm and 2 cm in diameter (usually, 14–17 days after inoculation) it was resected and the cells were dispersed into a single-cell suspension by enzymatic digestion as described above.

For the IL-2 treatment protocol (as below), MV2 cells were grown in vitro in media [Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY)] supplemented with 10% fetal calf serum, until confluent. The monolayer was resolubilized with trypsin/EDTA (Gibco), and washed twice in DMEM. Cells (10^3) were inoculated intraperitoneally (i. p.) on day 0.

Cytotoxicity assays

Effector cells. Young or old mice were sacrificed by cervical dislocation and their spleens were removed aseptically and placed on sterile screens in Petri dishes containing phosphate-buffered saline (PBS). The spleen was flushed with sterile PBS and teased apart. The cells were transferred into a 15-ml plastic centrifuge tube (Corning), set at room temperature for 5 min, then transferred and centrifuged for 10 min at 900 rpm. The cells were resuspended and red blood cells were lysed by brief hypotonic exposure. The remaining splenic mononuclear cell fraction was washed twice more in sterile PBS.

Natural killer cell assay. The NK-sensitive murine lymphoma line YAC-1 cells served as target cells. Varying numbers of the fresh splenic mononuclear cells were cultured in triplicate with 10^4 chromium-51 (^{51}Cr)-labeled target cells in round-bottomed 96-well microtiter plates (Corning) in a total volume of 0.25 ml. For the labelling, 5×10^6 tumor (target) cells were incubated for 2 h at 37° C in 0.5 ml medium containing 125 μCi ^{51}Cr. The cells were then washed twice and resuspended to the appropriate concentration in complete medium and incubated for 4 h at 37° C. The supernatants were subsequently removed from the wells using the Skatron harvest system (Sterling, Va), and the radioactivity (cpm) was counted in a ME*plus* automatic gamma counter (Huntsville, Ala). Maximum isotope release was determined by incubating the target cells with the lysing detergent centrimide and assessing ^{51}Cr content in the culture supernatants. Specific cytotoxicity was estimated by the formula:

$$\text{Cytotoxicity (\%)} = \frac{^{51}\text{Cr release (test - spontaneous)}}{^{51}\text{Cr release (maximum - spontaneous)}} \times 100\%$$

Cytotoxicity was also described in lytic units, which are defined as the number of cells or "cytotoxic units" per 10^7 spleen cells that result in 20% killing of 10^4 target cells (as derived by the method described in [23]).

Lymphokine-activated killer cell assay. The cytotoxicity of LAK cells was determined as described above (for NK activity) with the changes confined to the effector and target cells. For LAK activity, IL-2-stimulated splenocytes (as above) served as effector cells. The effector cells were splenocytes that were adjusted to a concentration of 2×10^6/ml in RPMI (Gibco), supplemented with 10% fetal calf serum (Gibco), 50 μM 2-mercaptoethanol (Sigma, St. Louis, Miss), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids (Gibco), 2 mM L-glutamine, antibiotics (penicillin 100 units/ml and streptomycin 100 μg/ml) with or without added rIL-2 at 500 units/ml in 25-cm^2 culture flasks (Corning). Culture flasks were incubated in humidified air at 37° C, 5% CO_2 for 3 days. Target cells were freshly prepared by enzymatic digestion and were labeled with ^{51}Cr by methods described above. For these experiments recombinant human IL-2 (2×10^7 units/mg) in PBS was generously provided by Hoffmann La Roche Inc. (Nutley, NJ) and aliquots were stored at –70° C.

In Vivo IL-2 treatment

After injection of tumor cells (day 0), 5×10^4 U IL-2 in 0.5 ml saline was administered twice daily (9 a.m., 9 p.m.) for 5 days in succession each week for either 2 or 4 weeks. Control mice were injected on the same schedule with PBS alone. In each experiment there were ten mice per group.

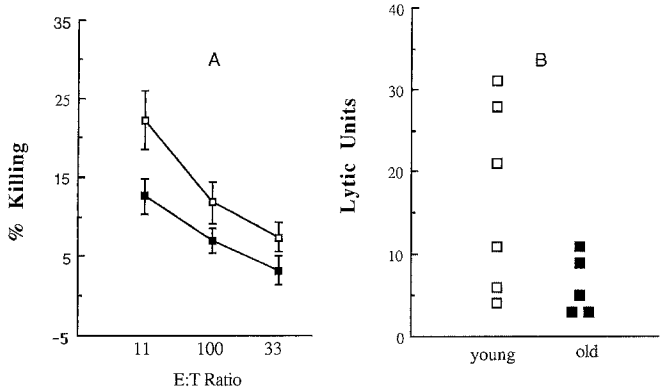

Fig. 2 A, B. Natural killer (NK) cell activity in young and old mice. Splenocytes from individual mice were prepared and cytotoxicity against YAC cells was determined. **A** Specific cytotoxicity calculated by the formula provided in Materials and methods. □, Fresh young mouse splenocytes; ■, fresh old mouse splenocytes. **B** Activity expressed in lytic units at 20% lysis per 10 million cells as described in [23]

Statistical analysis

The determined values for cytotoxicity (either percentage or lytic units: LU) and survival (days) were compared: young versus old and treated versus non-treated. Where appropriate, the statistical significance of the observed differences was estimated by Student's *t*-test.

Results

Cytotoxicity assays

To determine natural killer (NK) cell activity, freshly prepared splenocytes were co-cultured with ^{51}Cr-labelled YAC cells. As can be seen in Fig. 2, splenocytes from old mice were less effective at killing the YAC cell targets than those from young animals. Figure 2A represents the compiled cytotoxicity for the six young and five old mice and Fig. 2B depicts the results of individual mice in (LU). The difference between young and old (LU) was statistically significant ($P = 0.039$).

To determine lymphokine-activated killer (LAK) cell activity, freshly digested fibrosarcoma (MV2) cells were labeled with ^{51}Cr and served as targets. Freshly prepared splenocytes were cultured for 3 days in the presence of IL-2 (500 units/ml) and were then co-cultured with the radiolabeled tumor cell targets. The data from four experiments are depicted in Fig. 3; unlike NK activity there was no observed age difference in the capability of exerting LAK cell killing.

In Vivo IL-2 effects

Preliminary experiments had indicated that an intraperitoneal (i.p.) inoculation of 10^3 MV2 cells would result in lethal tumor in 90% young (3 months) C57BL/6 mice. For

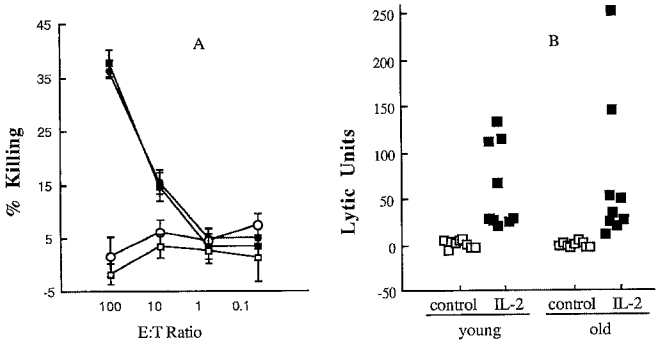

Fig. 3 A, B. LAK activity in young and old mice. Splenocytes from individual mice were cultured at $(1-2) \times 10^6$ cells/ml with 500 U/ml IL-2 for 3 days and cytotoxicity was determined with MV2 cells as the target. **A** Specific cytotoxicity calculated by the formula provided in Materials and methods. ○, Control young mouse splenocytes; □, control old mouse splenocytes; ●, IL-2-stimulated young mouse splenocytes; □, IL-2-stimulated old mouse splenocytes. **B** Activity expressed in lytic units at 20% lysis per 10 million cells as described in [23]

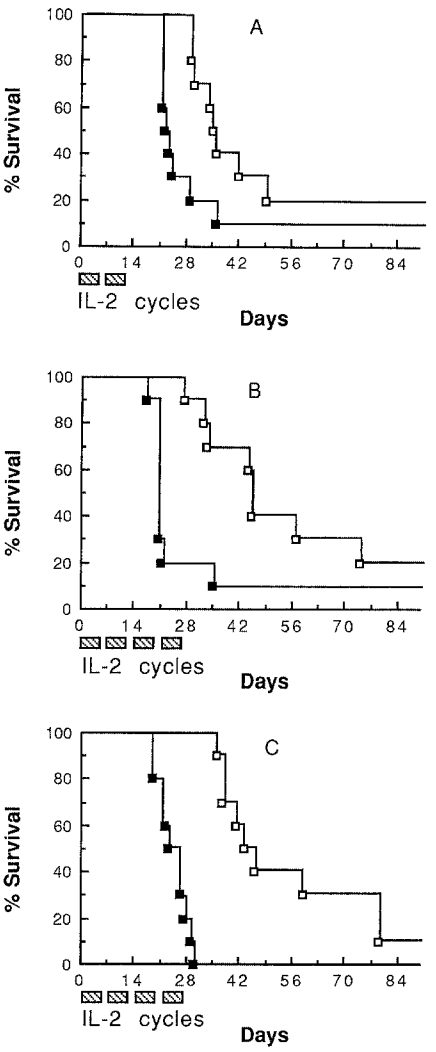

Fig. 4 A–C. Survival curve of mice inoculated with 10^3 MV2 cells intraperitoneally on day 0, and treated with 5×10^4 U of IL-2 i.p. twice daily for 5-day treatment cycles. Ten mice were used in each group. ■, Control; □, IL-2 treated. **A** Young mice treated with two cycles, separated by 2 days. **B** Young mice treated with four cycles, each cycle separated by 2 days. **C** Old mice treated with four cycles each cycle separated by 2 days

the IL-2 experiments, this dose of tumor cells was injected (day 0) and IL-2 was administered i.p. beginning the following day (day 1). As seen in Fig. 4, the survival of young mice that had received either two or four cycles of IL-2 (5×10^4 units twice daily) was greater than saline-injected controls [$P = 0.0008$ for two cycles (Fig. 4A) and $P = 0.0002$ for four cycles (Fig. 4B)]. Old mice (23–24 months) that had received four cycles of IL-2 were also shown to have increased survival when compared to the age-matched, saline-treated animals [$P = 0.0001$ (Fig. 4C)]. There was no difference in the IL-2 enhancement of survival between young and old mice.

Discussion

Immune factors have been shown to contribute to the recognition and destruction of neoplastic disease in various experimental models. It is understandable, therefore, that there has been great effort invested in the better understanding of antitumor immunity and the exploration of potential immunotherapeutic approaches. One such approach that has recently been advanced is adoptive immunotherapy, employing IL-2 with or without coadministration of lymphocytes activated in vitro by IL-2 [14, 17, 27, 29, 32]. In fact, in some systems, IL-2 alone has proven to be of therapeutic benefit (17, 28, 32]. In clinical studies, autologous lymphocytes have been expanded in vitro by incubation with IL-2 and subsequently administered. These cells from a cancer-bearing patient presumably have been exposed to tumor antigen and specific tumor cell killing by the activated "immune" cells might be occurring. Other mechanisms are no doubt involved, however, because in laboratory animals splenocytes from non-tumor-bearing animals activated in vitro by IL-2 are capable of mediating either in vitro or in vivo tumor cell destruction [15, 20, 30]. Whether cytotoxicity is specific or not, the success in preliminary clinical trials has been encouraging, especially in patients with melanoma and renal cell carcinoma [14, 27, 29]. Significant toxicity occurs, however, and many investigators are currently evaluating doses, schedules and combination with other antitumor agents to allow more general usage [12, 21, 33]. These latter efforts are particularly relevant if such therapeutic approaches are to be applied to elderly patients, who comprise the greatest population of those with cancer but who may be more susceptible to life-threatening toxicities.

In addition to the frailty that is observed in older patients, there are age-associated changes in immune function that may diminish the chances for response to immunotherapeutic approaches. Immune senescence is characterized at the cellular level predominantly by T-cell deficiency [19]. Accordingly, cytotoxic T-lymphocyte function is consistently found to be depressed with advanced age. NK cell function, however, has been reported to be less dramatically reduced with age [11, 13] and although we found some reduction in these studies, these must be considered minor when compared to the more profound T-cell alterations observed in old mice of the same strain [19]. There is currently some debate about the specific lineage of LAK cells, with many currently believing the cell to be NK-related [22]. Support might be gained for this contention by our observation that LAK cell function was not reduced in the older animals.

As mentioned above, there are now clinical research efforts designed to reduce the significant toxicities that have been associated with IL-2 and/or LAK cell therapies. As such therapies become more generally available (and less toxic), the question will, no doubt, arise whether it is appropriate for elderly people. It is possible, for example, that immune senescence may preclude the utility of such an approach. In the current experiments we have demonstrated in an experimental model that LAK cell killing in vitro was comparable (young versus old) and that IL-2 administration was successful in achieving an increase in survival that was of similar magnitude in young and old mice. Of course, these experiments reflect a specific treatment schedule and dose in an inbred murine strain, but the findings allow the speculation that LAK cell function is preserved throughout the lifespan, and that IL-2 may be a useful treatment in elderly, cancer-bearing patients.

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References

1. Belldegrun A, Webb DE, Austin III HA, Steinberg SM, White DE, Linehan WM, Rosenberg SA (1987) Effects of interleukin-2 on renal function in patients receiving immunotherapy for advanced cancer. Ann Intern Med 106: 817
2. Chang AE, Hyatt CL, Rosenberg SA (1984) Systemic administration of recombinant human interleukin 2 in mice. J Biol Response Mod 3: 561
3. Cheever MA, Thompson JA, Kern DE, Greenberg PD (1984) Interleukin-2 administered in vivo induces the growth and augments the function of cultured T cells in vivo. J Biol Response Mod 3: 462
4. Ershler WB (1986) Why tumors grow more slowly in older people. J Natl Cancer Inst 77: 837
5. Ershler WB, Moore AL, Shore H, Gamelli RL (1984) Transfer of age-associated restrained tumor growth in mice by old to young bone marrow transplantation. Cancer Res 44: 5677
6. Ershler WB, Stewart JA, Hacker MP, Moore AL, Tindle BH (1984) B16 murine melanoma and aging: slower growth and longer survival in old mice. Natl Cancer Inst 72: 161
7. Ershler WB, Tuck D, Moore AL, Klopp RG, Kramer KE (1988) Immunologic enhancement of B16 melanoma growth. Cancer 61: 1792
8. Ettinghausen SE, Lipford EH, Mule JJ, Rosenberg SA (1985) Systemic administration of recombinant interleukin 2 stimulates in vivo lymphoid cell proliferation in tissues. J Immunol 35: 1488
9. Grimm EA (1986) Human lymphokine-activated killer cells (LAK cells) as a potential immunotherapeutic modality. Biochim Biophys Acta 865: 267
10. Herberman RB (1984) Summary: Potential of interleukin-2 for the therapy of cancer. J Biol Response Mod 3: 527
11. Herberman RB, Nunn ME, Lawrin DH (1975) Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. I. Distribution of reactivity and specificity. Int J Cancer 16: 216
12. Iigo M, Sakurai M, Tamura T, Saijo N, Hoshi A (1988) In vivo antitumor activity of multiple injections of recombinant interleukin 2, alone and in combination with three different types of recombinant interferon, on various syngeneic murine tumors. Cancer Res 48: 260
13. Itoh K, Suzuki Y, Umezu K, Umezu Y, Hanaumi K, Kumagai K (1982) Studies of murine large granular lymphocytes. II. Tissue, strain, and age distributions of LGL and LAL. J Immunol 129: 395

14. Kohler PC, Hank JA, Moore KH, Stortr B, Bechhofer R, Sondel PM (1987) Phase I clinical evaluation of recombinant interleukin-2. Prog Clin Biol Res 244: 161
15. Lafreniere R, Rosenberg SA (1985) Successful immunotherapy of murine experimental hepatic metastases with lymphokine-activated killer cells and recombinant interleukin 2. Cancer Res 45: 3735
16. Lotze MT, Grimm EA, Mazumder A, Strausser JL, Rosenberg SA (1981) In vitro growth of cytotoxic human lymphocytes IV: lysis of fresh and cultured autologous tumor by lymphocytes cultured in T cell growth factor (TCGF). Cancer Res 41: 4420
17. Lotze MT, Chang AE, Seipp CA, Simpson CG, Vetto JT, Rosenberg SA (1986) High-dose recombinant interleukin 2 in treatment of patient with disseminated cancer. J Am Med Assoc 256: 3117
18. Lotzova E (1987) Role of interleukin-2 and interleukin-2-activated killer cells in cancer therapy. Cancer Bull 39: 5
19. Makinodan T, Kay MM (1980) Age influence on the immune system. Adv Immunol 29: 287
20. Papa MZ, Mule JJ, Rosenberg SA (1986) Antitumor efficacy of lymphokine-activated killer cells and recombinant interleukin 2 in vivo: successful immunotherapy of established pulmonary metastases from weakly immunogenic and nonimmunogenic murine tumors of three distinct histological types. Cancer Res 46: 4973
21. Papa MZ, Yang JC, Vetto JT, Shiloni E, Eisnthal A, Rosenberg SA (1988) Combined effects of chemotherapy and interleukin 2 in the therapy of mice with advanced pulmonary tumors. Cancer Res 48: 122
22. Phillips JH, Lanier LL (1986) Dissection of the lymphokine-activated killer phenomenon. J Exp Med 164: 814
23. Pross HF, Baines MG, Rubin P, Shragge P, Patterson MS (1981) Spontaneous human lymphocyte-mediated cytotoxicity against tumor target cells. IX: the quantitation of natural killer cell activity. J Clin Immunol 1: 51
24. Rayner AA, Grimm EA, Lotze MT, Chu EW, Rosenberg SA (1985) Lymphokine-activated killer (LAK) cells: analysis of factor relevant to the immunotherapy of human cancer. Cancer 55: 1327
25. Roberts AB, Frolik CA, Anzano MA, Assoian RK, Sporn MB (1984) Purification of type β transforming growth factors from nonneoplastic tissues. In: Barnes DW, Sirbasku DA, Sato GH (eds) Methods for preparation of media, supplements, and substrata for serum-free animal cell culture. Alan R Liss, Ny, p 181
26. Rosenberg SA (1984) Immunotherapy of cancer by systemic administration of lymphoid cells plus interleukin-2. J Biol Response Mod 3: 501
27. Rosenberg SA, Lotze MT, Muul LM, Leitman S, Chang AE, Ettinghausen SE, Matory YL, Skibber JM, Shiloni E, Vetto JT, Seipp CA, Simpson C, Reichert CM (1985) Observation on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. N Engl J Med 313: 1485
28. Rosenberg SA, Mule JJ, Spiess PJ, Reichert CM, Schwartz SL (1985) Regression of established pulmonary metastases and subcutaneous tumor mediated by the systemic administration of high dose recombinant interleukin-2. J Exp Med 161: 1169
29. Rosenberg SA, Lotze MT, Muul LM (1987) A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. N Engl J Med 316: 889
30. Rosenstein M, Yron I, Kaufmann Y, Rosenberg SA (1984) Lymphokine-activated killer cells: lysis of fresh syngeneic natural killer-resistant murine tumor cells by lymphocytes cultured in interleukin-2. Cancer Res 44: 1946
31. Rosenstein M, Ettinghausen SE, Rosenberg SA (1986) Extravasation of intravascular fluid mediated by the systemic administration of recombinant interleukin 2. J Immunol 137: 1735
32. Sosman JA, Kohler PC, Hank J, Moore KH, Bechhofer R, Storer B, Sondel PM (1988) Repetitive weekly cycles of recombinant human interleukin-2: responses of renal carcinoma with acceptable toxicity. J Natl Cancer Inst 80: 60
33. Talmadge JE, Phillips H, Schindler J, Tribble H, Pennington R (1987) Systematic preclinical study on the therapeutic properties of recombinant human interleukin 2 for the treatment of metastatic disease. Cancer Res 47: 5725
34. Tsuda T, Kim YT, Siskind GW, Deblasio A, Schwab R, Ershler W, Weksler ME (1987) Role of the thymus and T-cells in slow growth of B16 melanoma in old mice. Cancer Res 47: 3097
35. Vaage J (1986) Local and systematic effects during interleukin-2 therapy of mouse mammary tumors. Cancer Res 47: 4296