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Cancer vaccines: The interleukin 2 dosage effect

(granulocyte–macrophage colony-stimulating factor/tumor vaccine/murine melanoma)

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ABSTRACT **Cancer vaccines genetically engineered to produce interleukin 2 have been investigated intensively in a series of animal models and are at the point of entering into clinical trials. In this study we demonstrate a strong correlation between the rate of interleukin 2 production and the protection efficiency of murine S91 melanoma cell (clone M-3) vaccines. Best immunization is achieved with vaccines producing medium interleukin 2 levels of 1000–3000 units per 10^5 cells per day. Reduced interleukin 2 production evokes a corresponding decline in the number of successfully treated animals. Unexpectedly, when interleukin 2 expression is raised to high levels of 5000–7500 units per 10^5 cells per day, protection is completely absent because of impaired generation of tumor-specific cytotoxic T lymphocytes. In comparison, granulocyte–macrophage colony-stimulating factor as immunomodulator induces substantial immunization even at a moderate level of secretion and protects all animals at the maximal obtainable level of secretion. Our findings demonstrate the importance of the interleukin 2 level produced by genetically modified tumor cells and may have substantial impact for the clinical application of cancer vaccines.**

Tumor cells genetically engineered to produce interleukin 2 (IL-2) have been intensively studied as immunomodulators of cancer vaccines in a series of animal models (1, 2) and are at the point of entering into clinical trials (3). It has been demonstrated that the presence of IL-2 affects nonimmunogenic or moderately immunogenic tumor cells in such a manner that they become a target for rapid destruction by the immune system. Injection of viable, IL-2-secreting tumor cells into syngeneic hosts evokes a T-cell-dependent immune response that is capable of rejecting the tumor inoculum (4) or at least of delaying tumor growth compared with the injection of unmodified cells (5). Macrophages, granulocytes, natural killer (NK), and lymphokine-activated killer (LAK) cells have been identified as the major components of the primary infiltrate (6). It also has been demonstrated that IL-2-secreting tumor cells can induce a long-lasting antitumor response by mechanisms only partially understood at present. For different tumor models, it has been shown that systemic immunity is substantially connected to the clonal selection of tumor-specific $CD8^+$ (7) or $CD8^+/CD4^+$ (8) T cells. Based on this cellular immune response, animals are able to reject parental tumor inocula when vaccinated with IL-2-releasing tumor cells prior to challenge.

For the generation of IL-2-secreting cancer cells as vaccines, several different strategies have been applied. Most of them used established tumor cell lines that constitutively express an IL-2 transgene. This has usually been achieved by stable transfection protocols like calcium phosphate precipitation (7, 9) or liposome-mediated DNA transfer (lipofection) or retroviral transduction (4, 10), followed by a selection procedure for single clones that express the desired gene construct. At the end of a laborious and time-consuming process, clones are identified that express the IL-2 transgene. Dependent upon the gene construct delivered and the respective target cell line, the described expression levels have been quite low (1) with one exception (11).

We have used receptor-mediated transport of DNA in which polylysine is covalently linked to transferrin and complexed ionically to DNA in a method called "adenovirus-enhanced transferrinfection" (AVET) to transfect IL-2 expression vectors into M-3 melanoma cells. After transfection with this method, up to 2×10^5 units of IL-2 can be produced by 10^6 cells in 24 hr *in vitro* (unpublished results) without the necessity of further manipulations like the generation of stable clones. As previously reported (8), we have vaccinated DBA/2 mice in the M-3 melanoma model with 10^5 irradiated tumor cells producing 33,000 units of IL-2 per 10^6 cells. Systemic immunity in the M-3 model was found to be dependent upon specific $CD8^+$ and $CD4^+$ cells (8). As a result, animals are protected against subsequent challenge with high dosages of parental tumor cells.

Recently, the C57BL/6 mice B16-F10 melanoma cells were retrovirally transduced to produce high amounts of IL-2 (11). When applied as vaccine, these cells did not protect animals from subsequent challenge with viable tumor cells, whereas vaccines producing granulocyte–macrophage colony-stimulated factor (GM-CSF) were found to work efficiently. Based on the above inconsistency, we have analyzed the vaccination efficiencies of irradiated M-3 melanoma cells whose relative IL-2 expression levels ranged over 6 orders of magnitude and compared them to GM-CSF-producing vaccines. This study shows that the level of IL-2 production is the key feature for the successful generation of antitumor immunity by IL-2-releasing cancer vaccines. We find that tumor cells generated to produce IL-2 at a medium level of 1000–3000 units per 10^5 cells per day efficiently immunize mice against challenge with parental M-3 cells. When the amount of IL-2 released by the vaccine was lower than *ca.* 100 units per 10^5 cells per day, the protection rate in the challenged animals was imperfect or not improved compared with control vaccines of irradiated M-3 or irradiated mock-DNA-transfected cells. On the other hand, when we approached high levels of IL-2 (5000 or more units per 10^5 cells), generation of tumor-specific cytotoxic T lymphocytes (CTLs) is completely disrupted. As a consequence, animals are not protected against tumor challenge. These data highlight the importance of the IL-2 dosage produced by tumor cells used in a vaccination protocol and may help to explain the different findings in distinct tumor models.

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Abbreviations: IL-2, interleukin 2; GM-CSF, granulocyte–macrophage colony-stimulating factor; CTLs, cytotoxic T lymphocytes.

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MATERIALS AND METHODS

Mouse IL-2 and GM-CSF Expression Vectors. The IL-2 expression vector pWS2m has been described (8). For pWS2ms, the murine IL-2 cDNA was excised as a *Sal* I/*Bam*HI fragment from the vector BMG-neo-IL-2 (12) together with the upstream rabbit β-globin intron and the downstream rabbit β-globin polyadenylylation signal. The human IL-2 expression vector pWS2 (W.S., unpublished data) was digested with *Sal* I and *Bam*HI, resulting in a human IL-2 cDNA fragment and the vector backbone. The vector backbone containing the cytomegalovirus promoter/enhancer element, the simian virus 40 polyadenylylation signal, and the pBR322 background for plasmid maintenance in *Escherichia coli* was purified by agarose gel electrophoresis and ligated with the murine IL-2 DNA fragment to obtain pWS2ms. The murine GM-CSF expression vector pWS-GM-CSF contains the same functional elements as pWS2. The GM-CSF cDNA was cloned by shotgun ligation (13) as a fully synthetic gene from 12 oligonucleotides. Missing nucleotides at four different positions of the synthetic gene were found after sequencing and were repaired by the phosphorothioate method of site-directed mutagenesis (14).

Plasmid Preparation. Plasmid DNA was isolated by a Triton X-100 lysis procedure followed by two CsCl-gradient centrifugations as described (15). The second centrifugation step was extended from 4 hr to an overnight run, which significantly reduced lipopolysaccharide (LPS) contamination. Endotoxin content was measured by the limulus amebocyte lysate assay (BioWhittaker). LPS-contaminated DNA preparations were further purified by Amicon 30 centrifugation or polymyxin B treatment as described (16). LPS content of plasmids used in transfection experiments was <0.05 units/μg of DNA.

Preparation of the Tumor Cells Used for Vaccination. The Cloudman S91 melanoma cells (clone M-3) were purchased from the American Type Culture Collection. M-3 cells (10^6) were transfected by the adenovirus-enhanced transferrinfection method using the plasmid pSP65 (Boehringer Mannheim) for "mock-DNA-transfected" control groups and pWS2m or pWS2ms to achieve IL-2 expression. For high levels of IL-2 expression (7500, 5850, or 5050 units per 10^5 cells per day), transfection complexes were formed by mixing 1.22×10^{10} particles of *E4*-defective, biotinylated, and psoralen/UV-inactivated adenovirus type 5 (Ad5-dl1040) with 1 μg of streptavidin/polylysine, 12 μg of pWS2m, and 13 μg of transferrin/polylysine as described (8, 17). Medium levels of 2500, 2000, and 1000 units of IL-2 per 10^5 cells per day were obtained by mixing transfected M-3 cells expressing high levels of IL-2 with nontransfected irradiated M-3 cells at the appropriate ratio—e.g., 27% of transfected cells secreting 7500 units of IL-2 per 10^5 cells per day with 63% of nontransfected irradiated cells to adjust the level of IL-2 to 2000 units per 10^5 cells per day. For IL-2 expression of 500 units per 10^5 cells per day, complexes were formed by mixing 0.95×10^{10} virus particles with 0.5 μg of streptavidin/polylysine, 2 μg of pWS2ms, and 6.25 μg of transferrin/polylysine. To obtain expression levels of 250 and 130 units of IL-2 per 10^5 cells, mixing of transfected and nontransfected cells was performed as described above. Transfection complexes consisting of 0.64×10^{10} virus particles, 0.8 μg of streptavidin/polylysine, 6 μg of pWS2ms, and 6 μg of transferrin/polylysine were prepared to obtain IL-2 expression of 65 units per 10^5 cells per day. IL-2 levels beyond that were again adjusted by mixing with nontransfected tumor cells.

GM-CSF expression levels were regulated in a similar way by using 2 μg of plasmid DNA and different amounts of virus particles in the formation of the transfection complexes. Mock-DNA-transfection complexes contained 0.95×10^{10} virus particles, 6 μg of pSP65, 0.8 μg of streptavidin/polylysine, and 6 μg of transferrin/polylysine. M-3 cells ($0.4–1 \times 10^6$) were exposed to the transfection complexes for 4 hr at 37°C in culture medium containing 10% fetal calf serum. Cells were then washed twice with phosphate-buffered saline, and fresh medium was added. Irradiation with 20 Gy was performed at a cesium source (Nordion, Kanata, ON Canada), and cells were incubated at 37°C overnight prior to injection. Supernatant was removed to measure the secreted IL-2 or GM-CSF by using commercially available ELISAs (IL-2, Becton Dickinson; GM-CSF, Endogen, Cambridge, MA). Cells were then trypsinized, washed three times with EBSS (GIBCO/BRL), and adjusted to a density of 10^6 per ml. IL-2 production between transfection and cell harvest was taken as a basis for the calculation of the value—i.e., IL-2 units per 10^5 cells per day.

Animal Experiments. DBA/2 (*H-2*^{d}) mice (6 to 8 weeks old) were obtained from Charles River Wiga (Sulzfeld, Germany). Mice were immunized twice in a weekly interval under halothane anesthesia by subcutaneous (s.c.) injection. Groups of 8–10 animals received 10^5 transfected or nontransfected/irradiated M-3 tumor cells. One week after the second vaccination, a challenge of 3×10^5 viable M-3 cells was applied s.c. into the back. Animals were inspected daily. Tumor development was scored in weekly intervals and followed up for at least 8 weeks.

Determination of IL-2 Serum Levels. Systemic serum levels of IL-2 were detected by ELISA (Intertest-2, Genzyme) according to the manufacturer's directions.

CTL Assay. Spleen cells of five animals, immunized with M-3 cells expressing IL-2 at a medium level (2000 units per 10^5 cells per day *in vitro*) or a high level (12,000 units per 10^5 cells per day *in vitro*) were isolated on day 9 after vaccination. Spleen cells were restimulated for 5 days with M-3 cells that had been cultured in the presence of γ interferon for 18 hr and subsequently fixed with paraformaldehyde. Lytic activity was determined by using the CytoTox96 assay (Promega). A 4-hr incubation period of restimulated spleen cells with viable M-3 cells resulted in specific lysis. The same incubation with KLN 205 cells instead of M-3 cells determines the nonspecific lysis capacity of the spleen cell isolates.

RESULTS AND DISCUSSION

IL-2 Expression Level Is the Critical Parameter for Successful Treatment with Cancer Vaccines. To systematically analyze the impact of the IL-2 dosage on the efficacy of the cancer vaccine, M-3 tumor cells have been engineered to secrete 21 different levels of IL-2. The *in vitro* IL-2 expression was varied from 0.01 to 7500 units per 10^5 cells per day, a range that covered the values investigated in other tumor models so far (4, 6, 11), and 10^5 of these transfected irradiated cells were applied per animal. Control groups received the same number of either irradiated or mock-DNA-transfected and irradiated tumor cells, which allowed us to distinguish between antitumor effects of the tumor cells alone and the enhancement mediated by the presence of the respective IL-2 dosage. We found that the vaccination efficiency is significantly dependent upon the IL-2 dosage produced by the vaccine (Fig. 1). The best protection against subsequent challenge was achieved when animals were immunized with vaccines producing medium IL-2 levels of 1000–3000 units per 10^5 cells; seven of eight animals were protected against tumor challenge. A stepwise decrease of IL-2 production was accompanied by a corresponding decline in the number of protected animals, indicating imperfect antitumor immunity. Below the expression of 10 units of IL-2 per 10^5 cells per day, the efficiency approached the background protection of control groups, being ≈15% when mock-DNA-transfected irradiated cells were used (22 of 26 animals developed a tumor) or 11% with irradiated cells alone (46 of 52 animals were tumor positive). Unexpectedly, in the high IL-2 production range (5000 units per 10^5 cells per day or more), no significant systemic immunity could be generated.

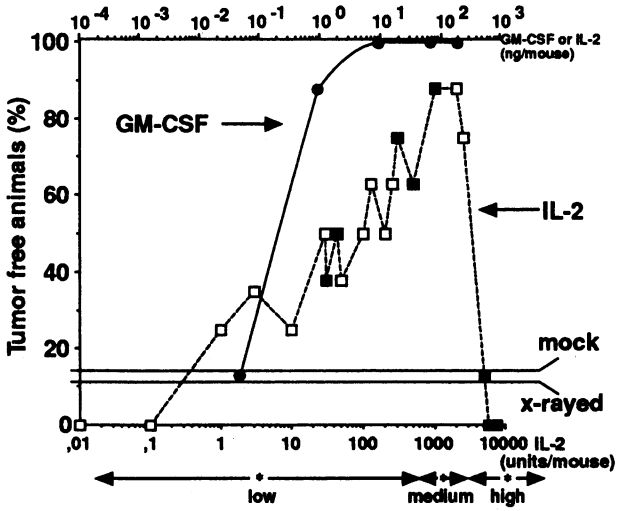

FIG. 1. IL-2 and GM-CSF dosage curves. The broken line shows the protection efficiency of 21 different IL-2-secreting vaccines as a percentage of tumor-free animals (eight animals treated per group); the solid curve depicts the response to GM-CSF secretion. Solid symbols show the protection level derived from transfected irradiated cells alone, whereas open squares show values from mixtures of irradiated transfected M-3 cells with irradiated nontransfected M-3 cells. "x-rayed" refers to the control group receiving irradiated M-3 cells as vaccine (46 of 52 animals were tumor positive). "mock" refers to the control group receiving mock-DNA (pSP65, Boehringer Mannheim)-transfected M-3 cells as vaccines (22 of 26 animals were tumor positive).

Application of 5000 units of IL-2 per 10^5 IL-2-producing cells allowed the growth of parental M-3 cells in seven of eight challenged animals, and higher dosages allowed it in even eight out of eight animals. This is in contrast to the response obtained with GM-CSF-releasing M-3 cells: immunity was already high at moderate secretion and leveled off at maximally obtainable GM-CSF production. This seems to indicate that GM-CSF is a less critical molecule for the generation of cancer vaccines than IL-2. Once the dosage is beyond a certain threshold, generation of optimal protection could be expected.

As shown above, mice inoculated with irradiated tumor cells secreting 6000 units of IL-2 are not protected against tumor challenge. We asked the question of whether high antitumor immunity would be obtained if the inoculum secreting 6000 units of IL-2 were distributed over three rather than one site. When vaccines expressing a protective level of 2000 units of IL-2 were injected at three sites of an animal with a total expression level of 6000 units per mouse, again no protection against tumor challenges was obtained, as if the vaccine had been applied at a single site (Table 1). This indicates that the expression level of IL-2 per mouse is the critical parameter for obtaining maximum possible protection against tumor challenge.

High-Level IL-2-Producing Tumor Cells Prevent Generation of Tumor-Specific CTLs. To understand the negative effect of the high-level IL-2-releasing M-3 cells, we compared the lytic activity of splenocytes from animals that received the protective vaccines producing a medium level of IL-2 with those from animals injected with high-level IL-2 secretors (Fig. 2). Splenocytes derived from both groups were incubated with M-3 cells for determination of specific lysis. Syngeneic but otherwise unrelated KLN 205 cells were used as a control for the unspecific lytic capacity of the spleen cell isolates. The unspecific lysis mediated by splenocytes isolated from recipients of vaccines producing medium- and high-levels of IL-2 was found to be very similar and quite low. In the specific lysis, however, there was a significant difference between both groups. The protective vaccine producing a medium level of IL-2 clearly evoked a representative specific lysis activity, whereas the nonprotective M-3 cells producing a high level of IL-2 do not. This result indicates that M3 cells secreting a high level of IL-2 interfere with the generation of M-3-specific CTLs. It also suggests an explanation for the observed phenomenon, since CTLs have been identified as the major effector cells for antitumor immunity and their clonal selection is essential for the destruction of the challenge tumor burden (ref. 1 and references therein).

Table 1. Comparison of local and systemic IL-2 effects

Total IL-2, units/mouse	Injection sites, no.	IL-2, units/site	Tumor-free/total animals
—	1	—	0/10
2000	1	2000	7/8
6000	1	6000	0/8
6000	3	2000	2/8
2000	3	670	7/8

Comparison of IL-2-secreting vaccines applied at one or three sites. The results show that the level of IL-2 secreted per animal is the important parameter for successful vaccination or for the IL-2 overshoot phenomenon, respectively.

Abrogated Anti-Tumor Immunity Correlates with Systemic Blood Levels of IL-2. We further investigated the consequences from the injection of M-3 cells secreting a high level of IL-2 to identify the reason for the "overshoot" phenomenon. We found that the failure of high-IL-2-secreting M-3 cells to elicit an antitumor effect correlates with the appearance of low, short-term, systemic blood levels of IL-2 (Fig. 3). When M-3 cells secreting 5000, 6000, or 10,000 units of IL-2 per 10^5 cells per day *in vitro* were inoculated into mice, transient systemic blood levels of IL-2 were detected 4 hr after injection. No systemic levels of IL-2 can be detected 24 hr (or later) after application of a 10^5 M-3 cell inoculum (data not shown).

The immunization site, a location of possible interference with the generation of long-lasting antitumor immunity, is apparently not affected by vaccines producing a high dosage of IL-2. Cellular infiltrates, mainly consisting of macrophages accompanied by granulocytes and natural killer cells, and secondary cytokine expression patterns at the injection site (IL-1, IL-6, and IL-10) are identical after vaccination with medium- or high-level-IL-2 producers (data not shown). These data suggest that the failure to generate tumor-specific CTLs at high values of IL-2 secretion may be a late event in the process leading to the appearance of such CTLs. According to the three-step model for the generation of antitumor T lymphocytes (G.M., unpublished data), the first step, which consists of influx of macrophages, natural killer cells, and gran-

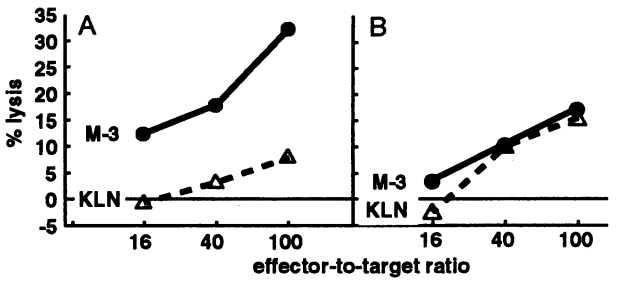

FIG. 2. Generation of M-3-specific CTLs. (*A*) When M-3 melanoma cells express IL-2 at a medium level, the percent lysis of M-3 cells (●) increases with the effector-to-target ratio and exceeds lysis of the syngeneic but otherwise unrelated KLN 205 carcinoma cells, which give the control values for the nonspecific lysis (△) by a large margin. (*B*) In contrast, the lysis efficiency mediated by high levels of IL-2-producing vaccines on M-3 target cells nearly coincides with that on KLN 205 cells and reflects only the nonspecific lytic activity of isolated spleen cells, demonstrating the absence of tumor-specific CTLs.

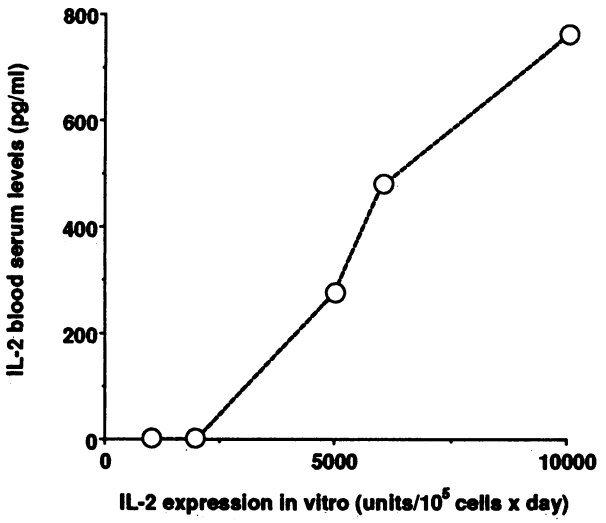

FIG. 3. Detection of systemic serum levels of IL-2. When M-3 cells secreting 5000, 6000, or 10,000 units of IL-2 per 10^5 cells per day *in vitro* are inoculated into mice, transient systemic blood levels of IL-2 are 280, 480, or 760 pg/ml 4 hr after injection. No systemic levels of IL-2 can be detected 24 hr (or later) after application of a 10^5 M-3 cell inoculum.

ulocytes into the vaccination site, remained constant and does not appear to be affected. Therefore, we hypothesize that one or both of the consecutive steps—migration of antigen-loaded antigen-presenting cells into the draining lymph node or the T-cell priming in the lymph node—is affected.

The nature of the interference of tumor cells producing a high level of IL-2 seems to be very specific, since no generalized toxic side effects could be detected, nor were they expected from the transient low levels of systemic IL-2 concentration. Lymphocytes are fully responsive on allogeneic and mitogenic stimuli (unpublished results), hence there is no general paralysis of the immune system. We do not see signs of vascular leakage syndrome, which is an important problem of high-dose recombinant IL-2 therapy (18, 19). Thus, serum transaminase levels, indicators of liver damage, are not altered, and histological analysis revealed the absence of any liver tissue abnormality. We also do not find tissue edema in any of the organs (not shown).

Our findings show that irradiated tumor cells secreting low levels of IL-2 as applied in many reported instances (reviewed in ref. 1) can offer some protection over and above vaccination with nonsecreting irradiated cells. However, this is only a fraction of the maximal possible tumor protection obtainable with appropriate IL-2 levels. Since the negative effects of very high levels of IL-2 expression are apparently connected with an organismal failure to generate CTLs (at least in the M-3 murine melanoma investigated in this study), other tumors may show similar IL-2 efficiency profiles, which might be, however, individually different in strains and species.

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