Liposomes Containing Interferon-Gamma as Adjuvant in Tumor Cell Vaccines

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Purpose. Liposomal systems may be useful as a cytokine supplement in tumor cell vaccines by providing a cytokine reservoir at the antigen presentation site. Here, we examined the effect of liposome incorporation of mIFNγ on its potency as adjuvant in an established tumor cell vaccination protocol in the murine B16 melanoma model. Adjuvanticity of the mIFNγ-liposomes was compared to that achieved by mIFNγ-gene transfection of the B16 tumor cells. Furthermore, we studied whether liposomal incorporation of mIFNγ indeed increases the residence time of the cytokine at the vaccination site.

Methods. C57B1/6 mice were immunized with i) irradiated IFNγ-gene transfected B16 melanoma cells or ii) irradiated wild type B16 cells supplemented with (liposomal) mIFNγ, followed by a challenge with viable B16 cells. The residence time of the (liposomal) cytokine at the subcutaneous (s.c.) vaccination site was monitored using radiolabeled mIFNγ and liposomes.

Results. Immunization with irradiated tumor cells admixed with liposomal mIFNγ generated comparable protection against B16 challenge as immunization with mIFNγ-gene modified tumor cells. Irradiated tumor cells admixed with soluble mIFNγ did not generate any protective responses. Radiolabeling studies indicated that free mIFNγ rapidly cleared from the s.c. injection site. Association of [^{125}I]-mIFNγ with liposomes increased the local residence time substantially: liposomal association of mIFNγ resulted in a prolonged local residence time of the cytokine as reflected by a 4-fold increase of the area under the curve. The amount of released cytokine in the optimal dose range corresponds to the amount released by the gene-transfected cells. Moderate but significant CTL-activity against B16 cells was found for mice immunized with irradiated cells supplemented with mIFNγ-liposomes compared to untreated control animals.

Conclusions. Prolonged presence of mIFNγ at the site of antigen presentation is crucial for the generation of systemic immune responses in the B16 melanoma model. These studies show that liposomal encapsulation of cytokines is an attractive strategy for paracrine cytokine delivery in tumor vaccine development.

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ABBREVIATIONS: mIFNγ, murine interferon-gamma; s.c., subcutaneous; MHCII, major histocompatibility complex class II; NK, natural killer; IL-2, interleukin-2; EPC, egg-phosphatidyl choline; EPG, egg-phosphatidyl glycerol; RP-HPLC, reversed-phase high performance chromatography; B16* cells, irradiated (50 Gy) B16F10 cells; CTL, cytotoxic T-lymphocyte; AUC, area under the curve.

KEY WORDS: liposomes; cancer vaccines; cytokines; immunotherapy; interferon gamma; gene transfer.

INTRODUCTION

In the application of autologous tumor cells as tumor vaccines, co-delivery of immunostimulatory cytokines as adjuvant has shown to be critical for induction of an effective vaccine-induced response against the tumor cells (1). Currently, the adjuvant activity of cytokines added as supplement to tumor cell vaccines has not proven to be very successful. This may be due to the rapid elimination and biodegradation of the cytokines at the vaccination site (2,3). Preclinical studies in murine tumor models have demonstrated that autologous tumor cells, genetically engineered to secrete certain cytokines, elicit systemic immune responses capable of eliminating small foci of established tumors (4,5). Long lasting systemic anti-tumor immunity has been observed after vaccination with tumor cells that have been genetically modified to produce interleukin-2 (IL-2) (6,7), interferon-gamma (IFNγ) (8) or granulocyte-macrophage stimulating factor (GM-CSF) (9).

The positive effect of cytokine-gene transfection of tumor cells may be attributed to a prolonged presence of the cytokine at the vaccination site. Liposomal systems may also provide a prolonged cytokine presence at the vaccination site by virtue of their established potential for slow release of entrapped agents (10–12). Therefore, liposomal systems are interesting candidates as adjuvant in tumor cell-based vaccines. A liposome-based cytokine supplement strategy is advantageous over cytokine-gene transfection of tumor cells, as the genetic engineering approach is laborious and technically more complex.

The present study was undertaken to investigate the applicability of mIFNγ-liposomes as adjuvant in tumor-cell based vaccines. Murine IFNγ (mIFNγ) is a cytokine known for its role in a number of immunological processes, such as increasing the expression of MHC class I and II molecules on a wide variety of cells, and generation of CD8+ cytolytic T cells (CTLs). In addition, IFNγ is an important activator of macrophages and natural killer cells (NK cells) leading to non-specific cell-mediated mechanisms of host defense (13).

The effect of liposomal encapsulation of mIFNγ on its potency as adjuvant was investigated in an established tumor cell vaccination protocol in the murine B16 melanoma model. Adjuvanticity of the mIFNγ-liposomes was compared to that achieved by mIFNγ-gene transfection of B16 tumor cells. Furthermore, we studied whether liposomal incorporation of mIFNγ indeed increases the residence time of the cytokine at the vaccination site.

MATERIALS AND METHODS

Materials

Egg-phosphatidylcholine (EPC) and egg-phosphatidylglycerol (EPG) were donated by Lipoid GmbH (Ludwigshafen, Germany). Murine recombinant IFNγ ($1–2 \times 10^7$ U/mg) was expressed in E. coli and purified through six sequential chromatography steps. Purity was assessed by reversed phase chromatography and SDS-PAGE. [^{125}I]-mIFNγ with a specific activity of 7–8 mCi/mg was prepared using the Bolton-Hunter method

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(14). 1α,2α(n)-[^{3}H]-cholesteryloleylether (specific activity 1.71 TBq/mmol) was supplied by Amersham (Buckinghamshire, UK). Hionic-Fluor, Soluene-350 and Plasmasol were purchased from Packard Instruments (Downers Grove, IL). Trypsin was obtained from Sigma (Zwijndrecht, The Netherlands), Dulbecco's modification of Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from Gibco-BRL (Gaithersburg, MD). All other reagents were of analytical grade.

Animals

Female C57B1/6 mice (8 to 12 weeks old) were obtained from Bomholtgård (Denmark). Animals were housed under specific pathogen free conditions.

B16 Melanoma Cells

B16F10 murine melanoma tumor cells were originally obtained from Dr Fidler (M.D. Anderson Hospital, Houston, US). The cells were cultured in DMEM supplemented with 10% fetal calf serum and 2 mM L-glutamine in a humidified incubator in 5% CO_2 at 37°C. Before vaccination cells were trypsinized, washed in phosphate buffered saline and resuspended in serum free Ringers' solution.

The plasmid DNA encoding for the IFNγ-gene was inserted into the tumor cells using an adenovirus enhanced transferrinfection (AVET) system as described previously (4,7,15). The transfected cells were irradiated (50 Gy) to prevent proliferation of the cells, harvested, washed, counted, aliquoted and stored in liquid nitrogen. *In vitro*, the mIFNγ-gene transfected cells produced 120 ng mIFNγ per 24 hours per 10^5 cells as determined by ELISA (Endogen, Cambridge, MA). Expression of the protein was transient, with a peak production of mIFNγ during the first day. Subsequently, the production of mIFNγ decreased to 20% and 10% of the peak amount at day 2 and day 3, respectively. Cytokine production was detectable in cell culture up to 7 days after transfection (Table 1).

Preparation of mIFNγ-Liposomes

Liposomes composed of EPC and EPG in a molar ratio of 9 to 1 were prepared using the classical film hydration method (16). The film was hydrated with a sterile solution of mIFNγ (100 μg/ml) in 5% glucose (w/v) in 10 mM succinate-buffer, pH 5.0 (glucose buffer). Non-liposomal mIFNγ was removed by ultracentrifugation (2 × 60 minutes at 250,000*g*) in a Beckman ultracentrifuge, model L5-65, (Palo Alto, CA) in a 10% sucrose (w/v) solution in 10 mM succinate-buffer at pH 5.0 (sucrose buffer). Finally, liposomes were resuspended in glucose buffer, and were used within 8 days of storage at 4°C. Empty control liposomes were prepared by hydrating the lipid film with sterile glucose buffer only.

Murine IFNγ content in the liposomes was determined by isocratic reversed phase high-performance liquid chromatography (RP-HPLC). To eliminate lipophilic compounds which interfere with the chromatographic analysis of mIFNγ, an extraction of the lipids was performed as described by Bligh and Dyer (17). The HPLC system consisted of a 250 × 4.6 mm Macrosphere reversed-phase C18 column with a pore size of 300 Å and a particle size of 5 μm. The column was protected with a 1 cm reversed-phase (C18) guard column (Alltech, Laarne, Belgium) and was kept at 40°C in a water-bath. The mobile phase was composed of 52% acetonitrile in water (w/w) supplemented with 10 mM sodium perchlorate and 100 mM perchloric acid and the flow was maintained at 1 ml/min. A 783A UV detector at 205 nm (Applied Biosystems, NJ) was used for detection.

Phospholipid concentrations were determined according to the method of Rouser (18).

Table 1. *In Vitro* mIFNγ Production by mIFNγ-Gene Transfected B16 Cells

Time (hours)	mIFNγ production per 2×10^5 B16 cells (ng)[a]
0–24	240
24–48	80
48–72	40
72–168	<10

[a] The production of mIFNγ by gene-transfected B16 cells was measured by ELISA.

Determination of the Amount of mIFNγ Exposed on the Outer Membrane of the Liposomes

Liposomes were incubated in the presence of the proteolytic enzyme trypsin to determine the fraction of liposome-bound mIFNγ exposed on the outer surface of the liposomes. Briefly, to 100 μl (100 μg/ml) of the liposomal mIFNγ-dispersion, 20 μl (1 mg/ml) trypsin in phosphate buffered saline (pH 7.4) was added (100 μg/ml trypsin was sufficient to degrade 250 μg/ml protein). After a 1-hour incubation at 37°C, samples were subjected to the lipid extraction procedure to remove the phospholipids. The amount of encapsulated mIFNγ protected from enzymatic digestion was determined by HPLC.

Immunization Protocol

Female C57B1/6 mice were immunized twice subcutaneous (s.c.) on the right flank with: i) 2×10^5 irradiated mIFNγ-gene transfected B16 (IFNγ/B16*) cells, producing 240 ng mIFNγ per 24 hours or ii), 2×10^5 irradiated B16 cells (B16*) supplemented with free or liposomal mIFNγ. Used doses were 40 ng, 200 ng, 1 μg or 4 μg mIFNγ for liposomal mIFNγ, and 200 ng, 1 μg and 4 μg for free mIFNγ. Animals in control groups received buffer only, irradiated tumor cells only, irradiated tumor cells supplemented with 1 μg free mIFNγ admixed with empty liposomes, or irradiated cells supplemented with empty liposomes. The mice were vaccinated 14 and 7 days before the challenge with a tumorigenic dose of 1×10^5 wild type B16 cells, given s.c. at the contralateral site on day 0. Tumor development was monitored visually until 10 weeks after the tumor challenge.

CTL Assay

Splenocytes were isolated from untreated C57B1/6 mice and mice immunized with: i) irradiated B16 cells supplemented with mIFNγ liposomes (doses of 300 ng or 3.8 μg), or ii) irradiated B16 cells without supplement, 13 days after the last immunization. Splenocytes were restimulated for 5 days with PFA-fixed B16 cells in culture medium (RPMI 1640, 10% FCS,

2 mM L-glutamine and 1 μg/ml gentamycin, supplemented with 0.5% conditioned medium (supernatant of transformed myeloma cells secreting murine IL-2 (19). Cytolytic activity activity was determined using an europium (Eu^{3+}) release assay as described previously (20).

Biodistribution Studies

Two types of labeled mIFNγ-liposomes were prepared: one with [^{3}H]-labeled bilayers (mIFNγ-[^{3}H]-liposomes) and with [^{125}I]-labeled cytokine ([^{125}I]-mIFNγ-liposomes). *mIFNγ-[^{3}H]-liposomes*: [^{3}H]-cholesteryl-oleylether was added as a marker of the lipid phase. This labeled lipid is a reliable marker to monitor the fate of liposomes *in vivo* (21,22). The radioactivity of the [^{3}H]-labeled liposomes was assayed in Hionic-Fluor scintillation mixture after decolorization in H_2O_2 and counted in a Philips PW 4700 liquid scintillation counter. *[^{125}I]-mIFNγ-liposomes*: A lipid film was hydrated in sterile glucose buffer, containing 50 μg/ml mIFNγ and a trace amount of [^{125}I]-mIFNγ. Because of the high association efficiency of the cytokine (approx. 90%), no further washing steps were performed. The radioactivity of [^{125}I]-mIFNγ-liposomes was counted in a Packard MultiPrias-2 γ-counter (Packard Instruments, Groningen, The Netherlands).

C57B1\6 mice were injected s.c. at the right flank with 100 μl of [^{125}I]-mIFNγ-liposomes, mIFNγ-[^{3}H]-labeled-liposomes or free [^{125}I]-labeled mIFNγ (total mIFNγ dose was 5 μg). To visualize the draining lymph nodes, 10 μl of a 1 mg/ml Violet Patent Blue (Sigma, Zwijndrecht, The Netherlands) solution in 5% glucose containing 500 units/ml of heparin (Leo Laboratories Ltd, Princes Risborough, UK) was administered s.c. at the same injection site, 3 minutes prior to the animals were anesthetized with ether and killed by cervical dislocation. Blood was drawn from the thoracic cavity, after opening the intrathoracic inferior caval vein. The s.c. injection site, draining (inguinal) lymph nodes, liver, spleen and kidneys were collected and assayed for radioactivity.

Results are expressed as percentage of injected radioactivity dose (%ID), both for the [^{3}H] and [^{125}I] labels, and represent the mean value of samples from 3–4 mice ± SD respectively.

Statistics

To evaluate the statistical differences in survival of tumor-free animals after immunization with the different vaccine preparations, Kaplan-Meier survival curves were constructed and compared by the log-rank test. The SPSS software package version 7.5.2 (SPSS Inc., Chicago, IL) was used for statistical calculations.

RESULTS

Liposome Characterization

Murine IFNγ liposomes were prepared by hydrating a lipid film containing the lipids in the appropriate molar ratio (EPC:EPG 9:1). The association efficiency of mIFNγ was 88.9 ± 2.1% (mean ± SD of 3 different dispersions). The final mIFNγ-liposome dispersions contained approximately 40 μmol/ml phospholipid and 80 μg/ml mIFNγ. No leakage of the protein was observed during storage at 4°C over a 14-day period.

The relative amount of associated-mIFNγ present on the outer liposome surface was estimated to be 74 ± 9% (mean of 3 dispersions) using a method based on exposure of the mIFNγ liposomes to the enzyme trypsin (23).

Release Characteristics of mIFNγ Liposomes *In Vivo*

To study the *in vivo* release of mIFNγ from liposomes after s.c. injection in mice, both the liposomal carrier and the associated cytokine were radiolabeled in two separate experiments. In the first experiment, the *liposomal carrier* was labeled with a tracer amount of [^{3}H]-cholesteryloleylether. In Fig. 1 it is shown that the non-sized [^{3}H]-labeled mIFNγ-liposomes remain at the injection site throughout the observation period of 7 days. A small fraction of [^{3}H]-labeled liposomes containing mIFNγ is drained from the injection site rapidly after injection. This fraction most likely contained the relatively small vesicles that were able to enter the lymphatic capillaries.

In the second experiment, the *liposomal cytokine* was labeled with ^{125}I, and the release and level of the ^{125}I-label was monitored in time (Fig. 1). In case of s.c. injection of free, soluble [^{125}I]-mIFNγ, the majority (about 95%) of the injected dose rapidly cleared from the injection site within 4 hours after administration. Association of [^{125}I]-mIFNγ with liposomes increased the local residence time substantially: liposomal association of mIFNγ resulted in a prolonged local residence time of the cytokine as reflected by a 4-fold increase of the area under the curve (AUC). Approximately 70% of the injected dose was cleared from the injection site in an initial rapid disappearance phase of 8 hours. A slow disappearance phase of about 160 hours followed, with complete clearance of the remaining amount of the injected dose at 168 hours after administration. At any time point evaluated, low but detectable amounts of ^{125}I-label were found in blood, liver, spleen, kidneys, and inguinal lymph nodes.

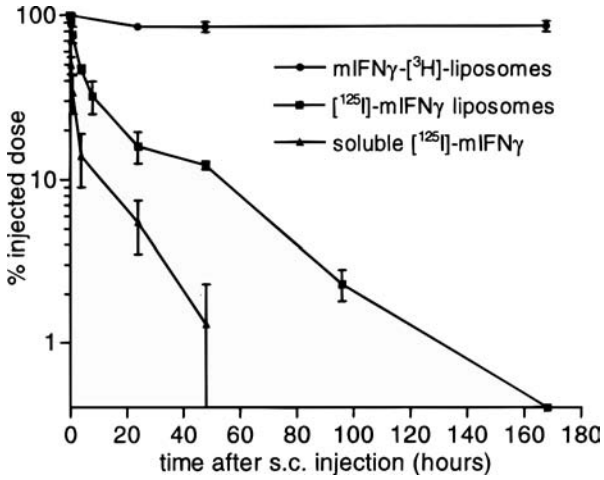

Fig. 1. Cytokine release from s.c. administered IFNγ-liposomes: Percentage of subcutaneously injected dose at injection site of mIFNγ [^{3}H]-liposomes, [^{125}I]-mIFNγ liposomes and soluble [^{125}I]-mIFNγ. A single dose of radiolabeled liposomes (EPC:EPG, 9:1, MLV) or soluble radiolabeled cytokine was injected s.c. into the flank of C57B1/6 mice. At preset time points, animals were killed and the injection site was assayed for radioactivity. Values represent the mean percentage ± sd of 3 animals (^{3}H-label), and 4 animals (^{125}I-label).

Adjuvant Effect: mIFNγ-Liposomes Versus Soluble mIFNγ

The adjuvant potential of mIFNγ-liposomes in tumor-cell based vaccines was evaluated in the B16 melanoma model. Mice were vaccinated twice at a 7-day interval followed by a tumorigenic dose of wild-type B16 cells given at the contralateral flank.

In the control group (no vaccination), all animals developed a lethal tumor (Fig. 2). Vaccination with irradiated tumor cells alone, or admixed with empty liposomes did not confer protection against the tumor challenge. Combination of irradiated cells with mIFNγ-liposomes or free mIFNγ resulted in protection of 7 out of 16 animals or 2 out of 16 animals ($p = 0.0042$), respectively. A bell-shaped dose response curve was observed with a maximal protection at doses ranging between 200 and 1000 ng liposomal mIFNγ (Fig. 3). Lower (40 ng) or higher (4000 ng) doses of mIFNγ were not effective. Remarkably, some animals in the groups immunized with a combination of irradiated B16 cells and mIFNγ liposomes developed a strong vitiligo at both the immunization and the contralateral challenge site. These animals remained tumor free until the end of the observation period of 10 weeks.

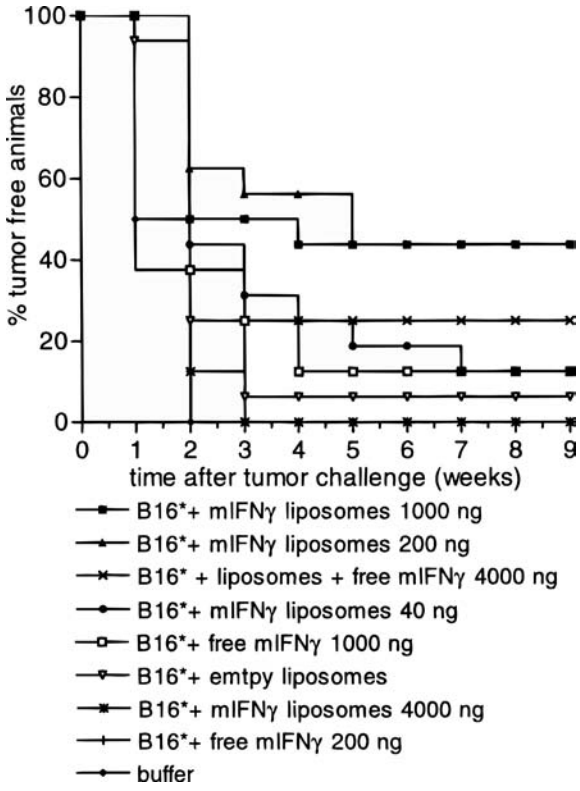

Fig. 2. Protection of C57Bl/6 mice against a lethal challenge with B16 melanoma cells. Mice were vaccinated twice, 14 and 7 days before a challenge with 1×10^5 viable B16 cells. The vaccines were composed of 2×10^5 irradiated B16 melanoma cells supplemented with soluble or liposomal mIFNγ at the indicated doses. $P = 0.0023$ between empty liposomes and liposomal mIFNγ (200 and 1000 ng) admixed with irradiated B16 cells. $P = 0.0042$ between soluble and liposomal mIFNγ (200 ng) admixed with irradiated B16 cells. Difference in tumor free survival between mice immunized with irradiated B16 admixed with liposomal mIFNγ or soluble mIFNγ admixed with empty liposomes is not considered statistically significant ($p > 0.059$). $n = 16$, except ($n = 8$) for three groups which received soluble mIFNγ 4 μg, soluble mIFNγ 200 ng or empty liposomes + 4 μg soluble mIFNγ as adjuvant.

Adjuvant Effect: mIFNγ-Liposomes Versus mIFNγ-Gene Transfection of Cells

The adjuvanticity induced by supplementing irradiated B16 cells with mIFNγ-liposomes or by transfection of B16 cells with the mIFNγ-gene in the same immunization protocol were investigated in the B16 model. The results of the studies are shown in Fig. 4. In gene-transfected cells, cytokine release was 240 ng/2×10^5 cells in the first 24h (Table 1). Seven out of 16 animals immunized with the mIFNγ-gene transfected cells remained tumor free for a period of more than 10 weeks. The protective effect was comparable to the one seen with a vaccine consisting of irradiated B16 cells supplemented with mIFNγ-liposomes at doses of 200 and 1000 ng mIFNγ. The tumor-free survival of mice immunized with gene-modified cells or irradiated B16 cells admixed with liposomal mIFNγ was significantly higher compared to the survival of mice which received non-modified irradiated B16 cells only ($p = 0.0043$). Similarly to the dose response curve described for the adjuvanticity of mIFNγ liposomes, a bell-shaped dose response curve was observed for gene-modified tumor cells (described earlier in (4)) which released between 100–300 ng mIFNγ/2×10^5 cells *in vitro* during the first 24 hours after transfection. Again, vitiligo was observed, albeit less pronounced, in one of the surviving mice in the group of animals immunized with the gene-modified cells (Fig. 3).

CTL-Activity Against B16 Cells

To gain more insight into the immunological mechanisms behind the enhanced antitumor response conferred by supplementing irradiated B16 cell vaccines with mIFNγ-liposomes, immunized mice were analyzed for CTL-activity. Previous experiments had shown that maximal CTL-activity was found when the splenocytes were isolated 10–14 days after vaccination (4). Moderate but significant CTL-activity against B16 cells was found in mice immunized with irradiated cells supplemented with mIFNγ-liposomes (Fig. 5). The CTL-activity observed at a medium dose of 300 ng liposomal mIFNγ (a dose in the dose range which resulted in the highest protection) was superior compared to CTL activity at the higher liposomal cytokine dose (3.8 μg), correlating well with the differences in antitumor protection at the two dose levels (see Fig. 2). Splenocytes of animals immunized with irradiated B16 cells only, did not show any enhanced CTL activity.

DISCUSSION

Many cytokines are paracrine factors physiologically active in close proximity of the target immune cell (e.g., APCs, T cells). Prolonged presence of mIFNγ at the site of antigen presentation may therefore be crucial for enhancement of the immune response. In this study we examined the effect of liposome incorporation of mIFNγ on its potency as adjuvant in an established tumor cell vaccination protocol in the murine B16 melanoma model. Adjuvanticity of the mIFNγ-liposomes was compared to that achieved by mIFNγ-gene transfection of B16 tumor cells, an established effective approach to induce

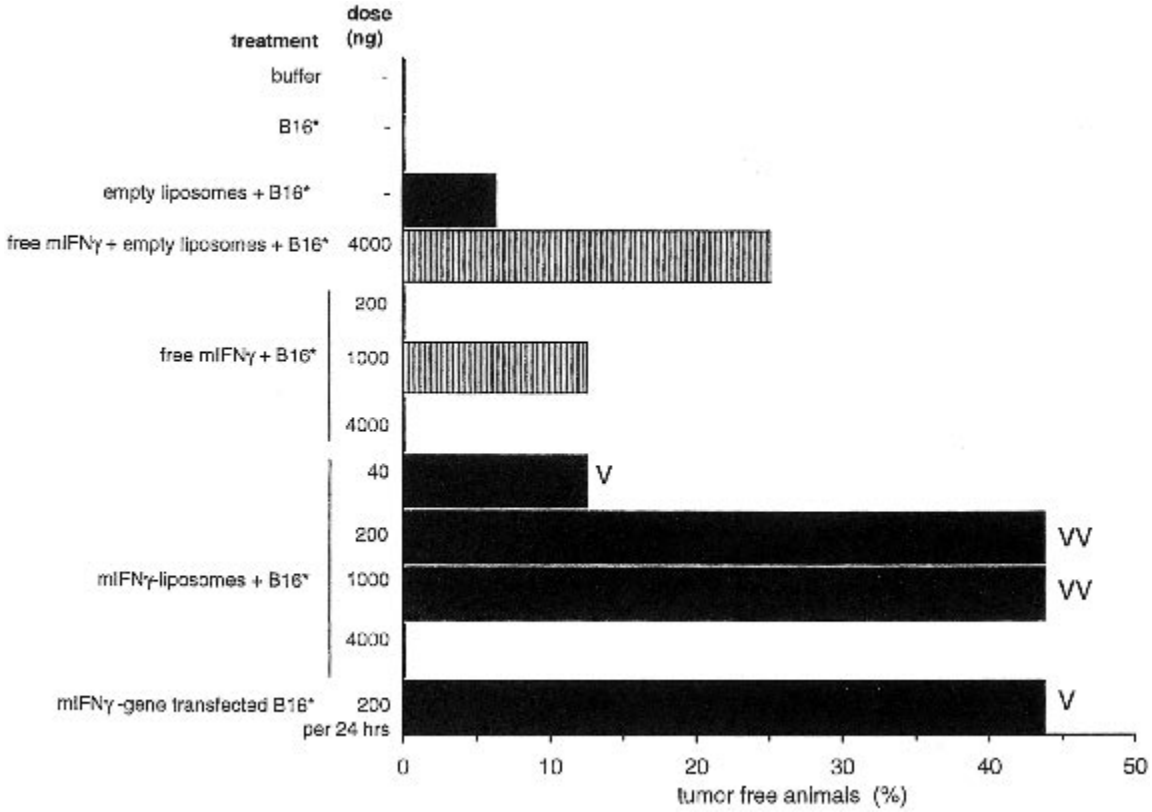

Fig. 3. Protection of C57Bl/6 mice against a lethal challenge with B16 melanoma cells. Bars indicate the percentage of animals without tumor, 10 weeks after the tumor challenge. For detailed information on the dose and formulations used, see legend of Figure 2. Each 'V' indicates the occurrence of vitiligo in an immunized animal.

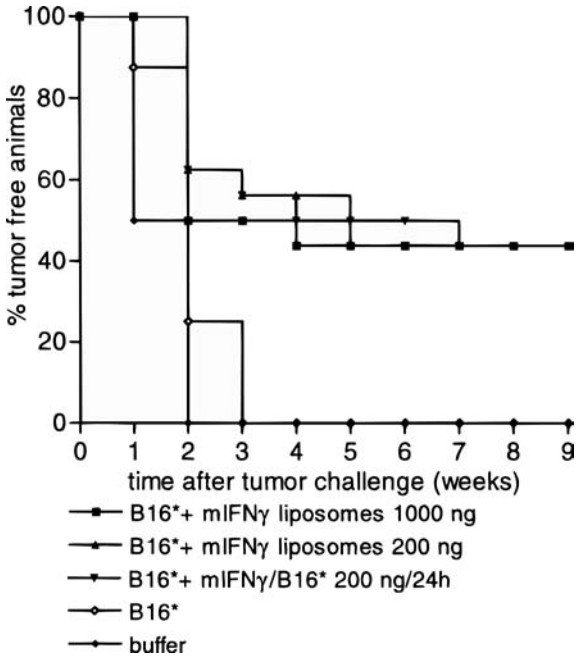

Fig. 4. Protection of C57Bl/6 mice against a lethal challenge with B16 melanoma cells. Mice were vaccinated twice, 14 and 7 days before a challenge with 1×10^5 viable B16 cells. The vaccines were composed of 2×10^5 irradiated B16 melanoma cells supplemented with soluble or liposomal mIFNγ at the indicated doses. $P < 0.001$ between IFNγ-gene modified B16 cells and irradiated non-modified B16 cells. $n = 16$.

protective immunity in this model. Furthermore, we studied whether liposomal association of mIFNγ indeed increases the residence time of the cytokine at the vaccination site.

The murine B16 melanoma model was used to evaluate the adjuvant properties of the mIFNγ formulations in tumor cell-based vaccines. This model represents a poorly immunogenic tumor in which vaccination with irradiated wild type tumor cells produces very limited protective immunity against a challenge with live wild-type tumor cells at a distant site (4). Soluble mIFNγ admixed with irradiated tumor cells did not induce a significant protective effect. Irradiated tumor cells supplemented with liposomal mIFNγ, and mIFNγ-gene transfected tumor cells generated a similar systemic immune response and subsequent tumor rejection.

The *in vitro* production of mIFNγ by the gene transfected B16 cells was about 240 ng per 2×10^5 cells in the first 24 hours after transfection. The *in vivo* release experiments (Fig. 1), indicated that in the first 24 hours after s.c. injection the cytokine-liposomes released about 85% of their content, which corresponds to 170 ng for the injected dose of 200 ng mIFNγ liposomes. Thus, both vaccine formulations may have generated similar sustained cytokine levels at the vaccination site, which may explain their similar efficacy at comparable dose ranges.

The lack of adjuvant effect of soluble mIFNγ alone may be the result of a rapid elimination of the cytokine from the injection site (Fig. 1) (24,25). To our surprise, we observed that after s.c. injection of liposome-bound [^{125}I]-labeled mIFNγ, 70% of the [^{125}I]-labeled mIFNγ did also rapidly disappear from the injection site. After the first burst-like release, the

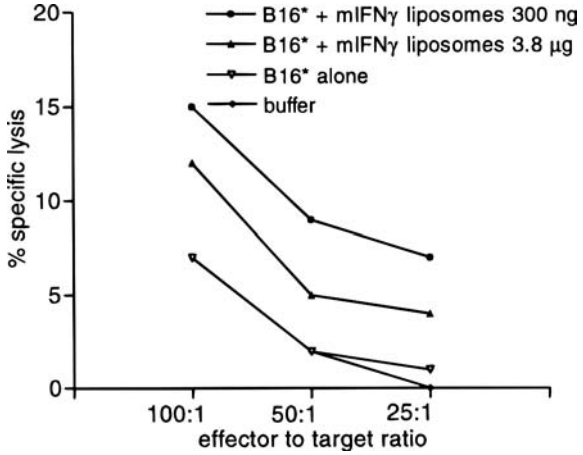

Fig. 5. CTL activity induced against B16F10 melanoma cells by splenocytes of immunized mice (n = 4). Splenocytes were isolated 13 days after the second vaccination, pooled, and re-stimulated *in vitro* for 5 days. Cytolytic activity against the specific target B16F10 melanoma cells was determined by using an Europium (Eu^{3+}) release assay as described above.

release profile became more sustained after 10 hours and the label was still detectable at the injection site after 7 days. The fraction of externally adsorbed cytokine (74 ± 9%) corresponds with the fraction of injected liposomal cytokine disappearing rapidly from the injection site. This suggests that the initial release phase is the result of desorption of cytokine from the outer liposomal surface.

One of the control groups concerned immunization with irradiated tumor cells supplemented with soluble mIFNγ admixed with empty liposomes. Unexpectedly, systemic protection was induced in 2 out of 8 the animals. *In vitro* binding experiments revealed that positively charged mIFNγ shows a strong tendency to adsorb onto the surface of negatively charged preformed empty liposomes when incubated for 5 minutes at room temperature, most likely due to electrostatic interactions. These data suggest that injection of empty liposomes admixed with soluble mIFNγ resulted in release of the protein in a slightly decelerated manner.

In the *in vivo* release experiments, minimal radioactivity was recovered from blood and organs following s.c. injection of liposomal [^{125}I]-mIFNγ. Analysis of urine samples of killed animals, revealed high activity levels in mice which had received liposomal or soluble mIFNγ in the first hours after injection (data not shown). The radioactivity measured in urine collected at later time points after s.c. injection of the mIFNγ formulation was reduced to near-background levels. This points to a very fast renal clearance of the cytokine, as has been described previously in literature (26). The finding by Zatloukal *et al.* (7) that s.c. inoculation of 1×10^5 IL-2-gene transfected M3 melanoma cells does not lead to detectable serum IL-2-levels, whereas mice can be effectively protected against a tumor challenge after immunization with these transfected M3 cells, suggestes that the cytokine induces its immune potentiating effect locally at the vaccination site.

The cytokine dose of mIFNγ-gene transfected tumor cells used in this study (240 ng/2 $\times$ 10^5 cells/24 hr) has shown to generate optimal protection in this murine melanoma model (4). Both higher and lower doses resulted in less systemic protection (data not shown). Similarly, a dose optimum of 200–1000 ng was observed for liposomal mIFNγ. Doses of liposomal mIFNγ below (40 ng) as well as above (4000 ng) the optimal range resulted in less protection. In several tumor models, it has been shown that systemic immunity is associated with the clonal selection of tumor-specific $CD8^+$ or $CD4^+$ T cells. Low concentration of mIFNγ at the site of antigen presentation may not result in a significant enhancement of the cellular immune response, and therefore rejection of the tumor inoculum does not occur (7). In this study, specific CTL responses were observed in the spleens of mice immunized with irradiated tumor cells supplemented with a cytokine dose of mIFNγ-liposomes (300 ng mIFNγ) within the optimal dose range for antitumor protection (Fig. 5).

No protection against a challenge with viable tumor cells was observed in animals immunized with irradiated tumor cells combined with the highest dose of liposomal mIFNγ (4000 ng). The CTL response was lower at the high dose of liposomal mIFNγ (3.8 µg) as compared to a lower dose tested (300 ng), which correlates with the lack of protective effect in the animal model at the highest dose. Schmidt *et al.* (27) observed a similar negative effect of high doses of IL-2-gene transfected M3 murine melanoma cells. Doses outside the optimal dose range failed to generate a significant antitumor immune response.

Remarkably, some surviving mice immunized with an effective dose of liposomal mIFNγ or mIFNγ-gene transfected tumor cells developed vitiligo at the site of vaccination and/or the site of tumor inoculation. This phenomenon has also been observed in mice immunized with an autologous tumor cell based vaccine supplemented with liposomal IL-2 as adjuvant (23). Although still controversial (28), the occurrence of vitiligo has been proposed as a prognostic factor for antitumor responses in melanoma patients (29). It is thought that the $CD8^+$ CTLs generated against the tumor cells in the immunization inoculum recognize antigens like MART-1, gp100, and gp75, antigens which are also expressed on normal melanocytes (30), resulting in lysis of normal as well as malignant cells.

In conclusion, the present results demonstrate that cytokines encapsulated in liposomes may be useful for paracrine cytokine delivery in a tumor vaccination protocol. An attractive point in favor of liposomes over gene-transfection is that the use of liposomes eliminates the need for patient-individualized gene transfer. Another limitation in the translation of the gene-transfer strategy to large-scale human tumor vaccine therapy is the fact that *in vitro* propagation of autologous tumor cells and subsequent cytokine gene-transfection with appropriate vectors can be cumbersome and laborious. Moreover, significant variability exists between tumor cells of different patients with regard to transfection efficiency. In contrast, liposomal cytokines can be simply admixed at any dose with irradiated tumor cells and offer therefore an attractive alternative to cytokine-gene transfection of tumor cells. Currently, studies are in progress to optimize the release characteristics of the liposomal cytokine formulation to improve their adjuvanticity in tumor-cell based vaccines.

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