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Retroviral transduction of interferon-γ cDNA into a nonimmunogenic murinefibrosarcoma: generation of T cells in draining lymph nodes capable of treating established parental metastatic tumor

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Abstract. Gene modification of tumor cells with the cDNA for interferon γ (IFNγ) has been shown to increase the immunogenicity of some tumor cells. In order to explore further the possible therapeutic relevance of these previous findings, two clones of the nonimmunogenic MCA-102 fibrosarcoma of C57BL/6 origin were retrovirally transduced with the cDNA encoding murine IFNγ: 102.4JK (4JK), a clone with relatively high major histocompatibility complex (MHC) class I expression, and 102.24JK (24JK), a clone with low expression of surface MHC class I molecules. Retroviral transduction of tumor cells with the cDNA encoding for IFNγ resulted in a substantial up-regulation of MHC class I surface expression in the 24JK clone but little change of class I in the 4JK clone. In an attempt to generate antitumor lymphocytes, these gene-modified cells were inoculated into mouse footpads and draining lymph nodes (DLN) were removed, dispersed, and cultured in vitro for 10 days with irradiated tumor cells and interleukin-2. DLN from mice bearing either unmodified tumor or tumor transduced with cDNA encoding neomycin resistance (*Neo*^{R}) or IFNγ, were used to treat recipients harboring 3-day pulmonary metastases induced by the parental, unmodified tumor. Treatment with DLN cells obtained following the injection of 24JK tumor cells modified with the gene for IFNγ significantly reduced the number of pulmonary metastases in four separate experiments, compared to groups treated by DLN cells generated from inoculation of either the unmodified, parental 24JK clone or the same clone transduced with the *Neo*^{R} gene only. In contrast, DLN cells induced either by IFNγ-transduced 4JK (high expression of MHC class I) or an unmodified 4JK tumor (moderate expression of MHC class I) had significant but equal therapeutic efficacy. Although the in vitro growth rate of tumor cell lines was unaffected by the insertion of the mouse IFNγ cDNA, their in vivo (s.c.) growth rates were significantly slower than those of the nontransduced tumors. Thus, after retroviral transduction of the murine IFNγ cDNA into a nonimmunogenic tumor with a very low level of surface expression of MHC class I, modified tumor cells could elicit therapeutic T cells from DLN capable of successfully treating established pulmonary metastases upon adoptive transfer. This strategy significantly confirms previous observations on the potential therapeutic effects of gene modification of tumor cells with IFNγ and extends the realm of therapeutic possibilities to include the use of DLN cells for the development of T-cell based immunotherapies against nonimmunogenic human tumors.

Key words: Draining lymph nodes – T cells – Gene modification – Immunotherapy – Interferon γ

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Introduction

Numerous examples of the recognition of tumor cells by T cells, and of the successful use of these T cells immunotherapeutically, have been documented in both mouse and man [3, 14, 27, 28, 34]. Many tumors, however, have proven to be resistant to T-cell-based immunotherapy. While the reasons for this are only partly understood, it has been observed that some nonimmunogenic tumors express low levels of class I molecules in vivo and in vitro [9]. Attempts have been made to enhance α-chain transcription by transfection or transduction of class I α chains into tumors, or by use of DNA-hypomethylating or -alkylating agents [4, 31, 32, 35, 38, 39]. These studies, directed at increasing the immunogenicity of the tumors involved, have met with mixed success. It is now clear that class I expression alone is not sufficient for the presentation of tumor antigens. Tumor antigens, like other antigens, must be processed from intracellular proteins into appropriately sized antigenic peptides and transported to the appropriate intracellular compartment, the endoplasmic

reticulum. Antigenic peptides associated with class I α chains and β_2-microglobulin can then be transported to the plasma membrane [23, 40].

Interferon γ (IFNγ) is potentially a very useful cytokine in this regard. It up-regulates class I transcription, but it also increases the expression of the molecules thought to be important in antigen processing. These include TAP-1 and TAP-2, with genes encoded in the region of the major histocompatibility complex (MHC), which are members of the ABC transporter family [1, 6, 19, 22, 30, 33]. TAP-1 (previously known as RING 4, Y3, and PSF-1) and TAP-2 (previously known as RING 11, Y1, and PSF-2) are very likely to be involved in the transport of peptides from the cytosol into the endoplasmic reticulum. Two other products of genes of the MHC region, called LMP-7 and LMP-2 (formerly known as Ring 10 and Ring 12 respectively), are also up-regulated by IFNγ. The gene products are physically associated with a large nuclear and cytosolic proteolytic structure, termed the proteasome, and may alter the function or location of the proteasome to favor either the production of antigenic peptides or their delivery to class I molecules [2, 12, 13].

IFNγ transduction of tumor cells has been shown to increase the immunogenicity of tumors in several systems [11, 36]. It has also been demonstrated that a nonimmunogenic tumor, incapable of generating a $CD8^+$ T cell immune response, could be gene-modified to generate a therapeutically useful immune response against the wild-type tumor [25]. These cells could then be adoptively transferred to a mouse bearing established, wild-type tumor. One difficulty in the successful application of these ideas to adoptive cellular immunotherapy for cancer patients is likely to be the lack of sufficient tumor to generate tumor-infiltrating lymphocytes (TIL). This situation might especially be the case when one is dealing with gene-modified tumor, since many gene-modified tumors fail to grow. To overcome this significant problem, we have explored the possibility of generating potent antitumor $CD8^+$ T cells by in vivo priming of the draining lymph node (DLN) with cells of a nonimmunogenic murine sarcoma retrovirally transduced with the cDNA coding for mouse IFNγ. In our experiments we selected a clone termed 24JK, derived from the poorly immunogenic tumor MCA-102, which was virtually devoid of MHC antigens (N. P. Restifo, unpublished results) and was completely nonimmunogenic.

In this report we demonstrate that insertion of the cDNA for murine IFNγ induced up-regulation of MHC class I on the 24JK-transduced tumor clone. $CD8^+$ T cells generated from lymph nodes draining the IFNγ-gene-modified 24JK tumor growing s.c. were effective upon adoptive transfer against established lung metastases from the parental unmodified tumor, while those obtained from lymph nodes draining either unmodified tumor or tumor transduced with the neomycin resistant (*Neo*^{R}) gene alone were ineffective.

Materials and methods

Animals. Female C57BL/6 (denoted B6) mice, 10–12 weeks old, were obtained from the Small Animal Section, Veterinary Resources Branch, National Institutes of Health, Bethesda, Md.

Tumors. MCA-102 is a nonimmunogenic fibrosarcoma of B6 origin that was induced in our laboratory by the intramuscular injection of 0.1 ml of 0.1% methylcholanthrene in sesame seed oil [20, 21]. The 4JK and 24JK cell clones of MCA-102 were produced by limiting dilution. Cells of both clones were analyzed for MHC class I expression by staining with anti-(MHC class I) anti-K^b/D^b mAb (28.8.86s; obtained from Dr. D. Sachs, Boston, Mass.) and analyzed by flow cytometry on a FACS 440 cell sorter (Becton Dickinson, Mountain View, Calif.). These tumors were selected for their difference in MHC class I expression; 4JK expresses relatively high levels of class I compared to the very low level of expression by the 24JK cell line. Tumor lines were maintained in monolayer culture in complete medium (CM) consisting of RPMI-1640 medium, 10% heat-inactivated fetal calf serum, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate (all from Biofluids, Rockville, Md.), 50 μM 2-mercaptoethanol (Aldrich Chemical Co., Milwaukee, Wis.), 0.03% (100 mM) fresh glutamine, 100 U/ml penicillin (both from the National Institutes of Health media unit), 50 mg/ml gentamicin sulfate (Gibco, Grand Island, N.Y.), 0.5 mg/ml amphotericin B (Flow Laboratories, Mclean Va.).

Gene transfer into cell lines. The MCA-102.4JK and MCA-102.24JK cell lines were transduced by the retroviral vectors LXSN or PBC/IFN with polybrene (4 μg/ml) for 18 h at 37°C. The LXSN is a Moloney murine leukemia viral backbone with the *Neo*^{R} gene alone promoted by the simian virus 40 early promoter and enhancer [18] (obtained from Dr. D. Miller, Fred Hutchinson Cancer Research Center, Seattle, Wash.). The murine IFNγ cDNA was introduced into the 4JK and 24JK cell lines with the Moloney-murine-leukemia-based retroviral vector PBC/IFN in which the IFNγ gene is driven by the cytomegalovirus promoter [11]. This vector also contains the neomycin-resistance gene and was supplied by E. Gilboa (Memorial Sloan-Kettering Cancer Center, New York, N.Y.). The gene-modified cells were selected in 400 μg/ml neomycin analog, G418 (Gibco), 48 h after retroviral transfer. After 14 days in selection medium the bulk-transduced lines were subcloned by limiting dilution at 0.3 cell/well in 96-well flat-bottom plates (Costar, Cambridge, Mass.), and maintained thereafter in G418. Secretion of IFNγ by the transduced cell lines was assayed by enzyme-linked immunosorbent assay (ELISA; Gibco BRL mouse IFNγ test kit). In repeated assays the transduced tumors consistently produced more than 10 U/ml IFNγ/10^6 cells in 24 h.

Preparation of DLN cells. Cultures of gene-modified and unmodified tumor cells were harvested and washed. Samples containing 10^6 viable cells were injected into the hind footpad in 0.05 ml Hanks balanced salt solution (HBSS; Biofluids). The draining popliteal lymph nodes were removed aseptically 9 or 10 days after tumor inoculation. Single-cell suspensions were prepared by pressing the nodes with the blunt end of a 2-ml plastic syringe plunger in HBSS under sterile conditions. The DLN cells were then filtered through no. 100 nylon mesh (Nytex; Tetco Inc., Elmsford, N.Y.), washed three times and resuspended in CM for in vitro sensitization.

In vitro sensitization of DLN cells. Fresh DLN cells were stimulated in 24-well plates (Costar) by fresh parental, unmodified tumor cells that were harvested from mice, and digested with the three enzymes 0.1% collagenase, 0.002% DNase, and 0.01% hyaluronidase (all from Sigma, St Louis, Mo.) as described previously [37]. Samples containing 5×10^5 lymphocytes were cultured with 2.5×10^5 irradiated (400 Gy) tumor cells and 10 units (U)/ml human recombinant interleukin-2 (IL-2) in CM (supplied by Cetus Corporation, Emeryville, Calif.). Each well was replenished with fresh CM and IL-2 every 3 days. DLN cells were harvested after 10 days in culture, washed twice and suspended in HBSS for adoptive immunotherapy.

FACS analysis. Cell-surface expression of MHC class I molecules was determined by flow cytometry with a FACScan 440 fluorescence-activated cell sorter (Becton Dickinson). Cultured tumor cell lines were harvested with 0.02% EDTA, washed with HBSS and then 1×10^6 cells were stained for 30 min at 4°C with an anti-(MHC class I) mouse mAb 28.8.6s (anti-K^b and -D^b) or with an isotype-matched control mAb (Bec-

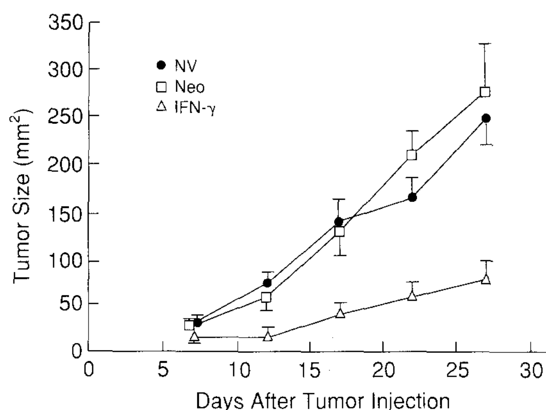

Fig. 1. In vivo growth of interferon-γ (IFNγ)-transduced tumor clones of MCA-102.24JK.Mice were injected subcutaneously with 1×10^6 syngeneic tumor cells. Tumor size was measured every 5 days. Each group represents at least six mice. Mean ± SE values are shown. (●) *NV*, non-transduced tumor; (□) *Neo*, tumor expressing the *Neo*^{R} gene only; Δ *IFNγ*, IFNγ-gene-modified clone

ton Dickinson) followed by a fluorescein-isothiocyanate (FITC)-conjugated goat anti-(mouse IG) mAb (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Lymphocyte subset analysis of DLN was also performed with murine FITc-labelled anti-Thy1.2 and anti-CD8 (LYT2), and phycoerythrin-labelled anti-CD4 PE (L3T4) mAb (Becton Dickinson). Non-viable cells were gated with propidium iodide.

Adoptive Immunotherapy. Normal B6 mice received 5-Gy total-body irradiation and were then injected intravenously with (8–10) x 10^5 fresh parental unmodified tumor cells in 1 ml HBSS to induce pulmonary metastases as described previously [21]. On day 3 after tumor injection, cultured DLN cells were given i.v. in 1 ml HBSS via the lateral tail vein. Mice were also treated with 10 000 U IL-2 in 0.5 HBSS i.p. three times a day on days 3–7. On day 14, mice were ear-tagged, randomized and killed, and pulmonary metastases were enumerated in a blinded, coded fashion as described [21].

Evaluation of in vivo s.c. tumor growth. Samples containing 1×10^6 viable tumor cells suspended in a volume of 0.1 ml HBSS were injected in the shaved skin of the right flank of B6 mice. After tumor inoculation, the mice were ear-tagged, randomized and were monitored in a blinded fashion for tumor growth. Tumor measurements were done every 5 days and tumor size was calculated by multiplying the largest biperpendicular tumor diameters. The values represent a mean of at least six mice per group.

Statistics. The significance of differences in numbers of pulmonary metastases between groups of mice was analyzed by the nonparametric Kruskal-Wallis test, which is an extension of the Wilcoxon rank-sum test for three or more groups [16]. The same test was performed for comparing subcutaneous tumor size. The values represent a mean of at least six mice per group. Two-sided *P* values are presented in all experiments.

Results

Murine IFNγ production by the 4JK And 24JK gene-modified tumors

IFNγ production by the gene-modified 4JK and 24JK tumor clones was followed over time. The 4JK clone secreted between 37.2 U and 53 U IFNγ/10^6 cells in 24 h over a 6-month period. The IFNγ production by the gene-modified 24JK clone was also consistent over time and ranged between 11.4 U and 15.0 U/10^6 cells in 24 h. Neither the unmodified nor the *Neo*^{R} tumors produced measurable IFNγ in repeated assays.

Inhibited growth rate of IFNγ-producing tumors in vivo

Figure 1 shows the results of a representative experiment in which the in vivo growth of parental and gene-modified tumor cells was monitored. While all tumors grew progressively, mice injected with either unmodified or *Neo*^{R}-transfected 24JK cells developed rapidly growing tumors at the injection site. In contrast, the growth of IFNγ-secreting tumors was significantly suppressed and in some experiments tumors were barely palpable 4 weeks after inoculation. This difference in tumor growth rates in vivo contrasted with similar in vitro cell growth kinetics of parental and vector-modified tumors (data not shown). In one experiment, after 4 weeks of growth, tumors were excised, enzyme-digested and cultured in selection medium (G418, 400 μg/ml), and assayed for INFγ production. Cells prepared from the unmodified tumor died in culture within 72 h and no IFNγ activity was detected in the cells cultured from the *Neo*^{R}-transfected tumor. In contrast, 10^6 neomycin-resistant cells from the IFNγ-transduced tumor, secreted 8.9 U /24 h (compared to 15.0 U /10^6 cells in 24 h produced by cultured IFNγ-transduced cells tested in parallel at the same time). This might be caused by differences in the microenvironment between cells grown in vivo and in vitro.

MHC class I expression of 4JK and 24JK tumor cell lines

The presence of MHC class I antigens on the surface of the parental and gene-modified cell lines was analyzed by flow cytometry. Figure 2 shows the profiles of four tumors stained with the mAb 28.8.6s (anti-K^b and -D^b). Both the unmodified 24JK and the 24JK *Neo*^{R} clones expressed negligible quantities (mean channel number < 10) of surface MHC class I molecules. Up-regulation of MHC class I molecules by the 24JK clone occurred following transduction with the cDNA encoding for IFNγ and was comparable to that of the parental 4JK cell line (mean channel number = 123). Retroviral transduction of the IFNγ DNA induced only a modest increase in MHC class I expression on the surface of 4JK cells (data not shown). IFNγ does not up-regulate MHC class II expression in these tumors (data not shown).

Phenotypic profile of in vivo primed DLN cells before and after sensitization in vitro

As shown in Table 1, determination by FACS analysis of the cell-surface phenotype of tumor-primed, freshly isolated DLN cells revealed the following: 93%–96% of the harvested cells were Thy1$^+$, with a nearly equal distribution of CD4$^+$ (39%–43%) and CD8$^+$ (43%–48%) cells. After 10 days in culture in the presence of irradiated parental tumor cells and IL-2, 91% of the cells expressed the

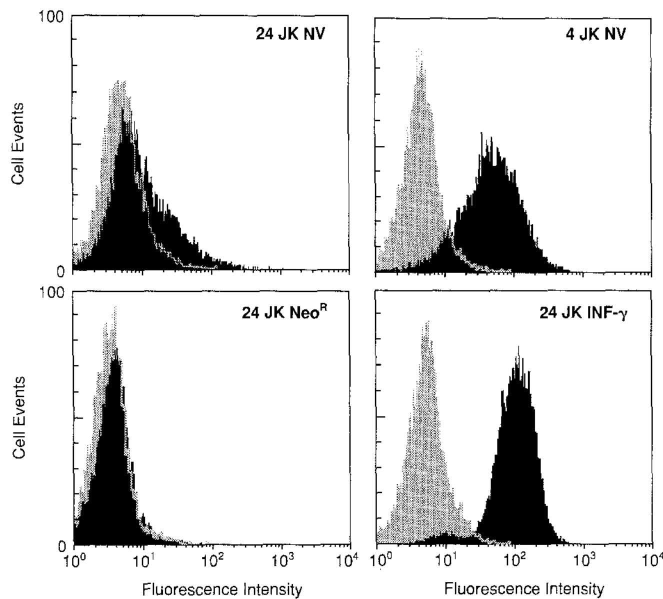

Fig. 2. FACS profiles of 4JK and 24JK sarcoma cells. Gray histograms show tumors stained by control antibody [fluorescein-isothiocyanate-conjugated goat anti-(mouse Ig)]. The anti-(MHC class I) mAb was 28.8.6s (*black histograms*). Profiles are shown for parental 4JK and 24JK cells (*upper panels*) and 24JK clones modified by either the *Neo*^{R} gene only or by the IFNγ gene (*lower panels*). Results are plotted as log (fluorescence intensity) versus cell number units

Table 1. FACS analysis of draining lymph node cells after footpad inoculation of MCA-102. 4JK tumor cells[a]

Marker	Cells positive (%)		
	NV	*Neo*^{R}	IFNγ
Day 0[b]			
Thy1.2	94	93	96
CD8	43	39	40
CD4	43	48	48
Day 10[c]			
Thy1.2	99	99	99
CD8	91	91	90
CD4	1	1	1

[a] Popliteal lymph nodes were harvested 10 days after footpad inoculation of MCA 102.4JK tumor cells. NV (no vector) refers to the non-transduced tumor cells, *Neo*^{R} to cells expressing only the *Neo*^{R} cDNA and IFNγ refers to a clone transduced with the cDNA of murine interferon γ
[b] Lymphocytes phenotyped on the harvesting day before in vitro culture
[c] Cells phenotyped after 10 days culture with interleukin-2 (10 U/ml) and irradiated tumor cells prior to injection

CD8 marker with virtually no CD4+ cells present. The phenotypic profile, before or after in vitro sensitization, was the same whether parental or genemodified cells were used for footpad inoculation.

In vivo antitumor efficacy of DLN cells generated by IFNγ-transduced 4JK and 24JK tumor clones

In an effort to assess the therapeutic activity of DLN cells induced by footpad tumor, we used the adoptive im-

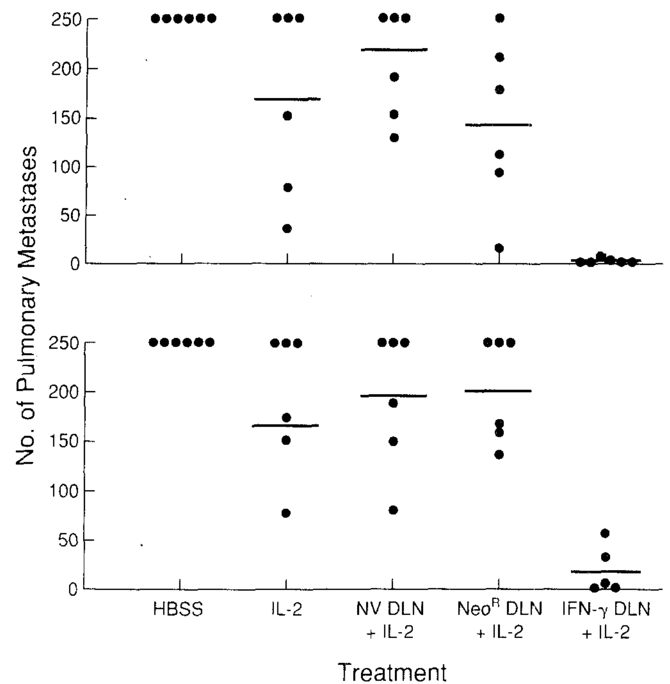

Fig. 3. B6 mice were injected i.v. with 8×10^5 cells of the unmodified, 24JK sarcoma (*NV*) having low MHC class I expression. Mice were then treated, beginning on day 3, with 5×10^6 (*top*) or 1×10^6 (*bottom*) draining lymph node (*DLN*) cells. Interleukin-2 (*IL-2*, 10 000 U/0.5 ml) or saline (HBSS) was given i.p. three times daily for 5 consecutive days. Pulmonary metastases were enumerated in a coded blinded fashion 14 days after tumor injection. Each *dot* represents an individual animal value. *NV DLN*, cells generated by footpad inoculation of non-transduced tumors; *NEO*^{R} *DLN*, neomycin-resistant tumor inoculation; *IFNγ DLN*, cells induced by the IFNγ-transduced tumor. The *horizontal lines* represent the mean of the numbers of pulmonary metastases enumerated in each group of mice shown

Table 2. Adoptive immunotherapy of 3-day pulmonary metastases of MCA-102.24JK with draining lymph node (DLN) cells primed by footpad inoculation of MCA 12.24JK tumor cells

DLN cells induced by inoculation of[a]	In vivo[b] IL-2	Mean no. of pulmonary metastases of MCA-102.24JK (SEM)[c] after transfer of the following no. of cells:			
		None	0.4×10^6	0.8×10^6	3×10^6
–	–	102 (27)			
–	+	55 (16)			
NV	+		41 (8)	28 (6)	6 (6)[d]
IFNγ	+		56 (8)	24 (11)	11 (7)[d]

[a] C57BL/6 mice were inoculated by 1×10^6 tumor cells in the footpad; 9–10 days after inoculation, popliteal lymph nodes were harvested and cultured with IL-2 (100 U/ml) and irradiated tumor cells. Samples containing 5×10^6 DLN cells were transferred i. v. to treat 3-day lung metastases after 10 days in culture. NV, non-transduced tumor cells; IFNγ, tumor cells transduced with the cDNA of murine IFNγ
[b] IL-2 (10 000 U) was given i. p. three times a day for 5 days starting immediately after injection of DLN cells.
[c] 8×10^5 MCA 102.24JK tumor cells were injected i. v. on day 0 to induce lung metastases. Mice were irradiated with 5 Gy total-body irradiation just before tumor injection. On day 14, pulmonary metastases were enumerated.
[d] Wilcoxon rank-sum test of treated groups compared with group receiving IL-2 alone or no therapy (HBSS) $P2 < 0.05$. All other groups were not significant

Table 3. Adoptive immunotherapy of 3-day pulmonary metastases of MCA-102.24JK with DLN cells from footpad inoculation with the 102.24JK tumor cells expressing the cDNA of murine IFNγ

Transferred[a] DLN cells induced by inoculation of	In vivo[b] IL-2	Mean no. of pulmonary metastases (SEM)[c]			
		Expt. 1	Expt. 2	Expt. 3	Expt. 4
–	–	211 (19)	147 (19)	250 (0)	176 (34)
	+	225 (20)	181 (17)	169 (39)	162 (29)
NV	+	225 (16)	133 (25)	205 (22)	161 (29)
Neo[R]	+	221 (38)	147 (17)	145 (35)	130 (20)
IFNγ	+	144 (25)[d]	68 (17)[d]	1 (0.5)[e]	21 (6)[e]

[a] C57BL/6 mice were inoculated by 1×10^6 tumor cells in the footpad; 9–10 days after inoculation, popliteal lymph nodes were harvested and cultured with IL-2 (20 U/ml) and irradiated tumor cells. Sampls containing 5×10^6 DLN cells were transferred i. v. to treat 3-day lung metastases after 10 days in culture. NV, non-transduced tumor cells; IFNγ, tumor cells transduced with the cDNA of murine IFNγ

[b] IL-2 10000 U was given i. p. three times a day for 5 days starting immediately after injection of DLN cells

[c] 8×10 MCA-102.24JK tumor cells were injected i. v. on day 0 to induce lung metastases. Mice were irradiated with 5 Gy total-body irradiation just before tumor injection. On day 14, pulmonary metastases were enumerated

[d] Significantly different from groups treated with IL-2 alone or without treatment $P2 < 0.05$, Wilcoxon rank-sum test)

[e] Significantly different from groups treated with IL-2 alone or without treatment ($P < 0.005$, Wilcoxon rank-sum test)

munotherapy model of 3-day pulmonary metastases [21] (Table 2). DLN cells stimulated by the 24JK IFNγ-gene-modified tumor were effective against pulmonary metastases induced by the i.v. injection of unmodified parental tumor (Fig. 3). DLN cells generated by the unmodified and *Neo*[R]-transduced 24JK tumor lines, however, had no demonstrable impact on the number of pulmonary metastases. As shown in Fig. 3, the adoptive transfer of a relatively small number of effectors (1 x 10^6 cells) was effective in reducing the lung metastases by 93% compared to control groups ($P < 0.01$). Similar findings were demonstrated in three additional consecutive experiments (Table 3). While there was experiment-to-experiment variation, all of the experiments statistically demonstrated that more effective DLN cells were generated after inoculation of IFNγ-transduced tumor. In contrast, the tumor clone with a relatively high MHC class I expression, 4JK, induced DLN cells that significantly reduced the number of lung metastases when 3×10^6 effector cells were injected i.v. ($P < 0.05$). As shown in Table 2, DLN cells generated by the IFNγ-gene-modified 4JK clone had no increased potency in diminishing the number of pulmonary nodules. Similar results were obtained in three successive experiments using the parental and gene-modified 4JK clones for in vivo priming of the DLN cells (data not shown).

Discussion

Decreased expression of MHC class I antigens on tumors may lead to the failure of cytotoxic T lymphocytes (CTL) to protect the host effectively against tumor growth since CD8+ T cells recognize antigens only in the context of self-MHC-class-I molecules. Indeed, in a variety of murine tumor models, a correlation was shown between reduced MHC class I expression and the failure of adoptive immunotherapies [4, 9, 15, 21, 24]. In addition, many naturally occurring human tumors, like breast carcinoma and small-cell cancer of the lung, express minimal or no MHC class I molecules on their surface, and consequently could conceivably escape recognition by CTL [9, 26].

IFNγ, a cytokine with multiple effects on the immune response, has been shown in vivo and in vitro to increase MHC class I expression on several methylcholanthrene-induced sarcomas used in our experiments [10, 37]. This cytokine has also been shown to increase surface expression of tumor-associated antigens as well as reverse certain processing and presentation deficiencies of MHC class I antigen [25]. It was recently reported that the expression of retrovirally transduced cDNA for IFNγ in weakly immunogenic murine tumors inhibited the in vivo growth rate of those tumors [11, 36]. We have now extended this approach to insert the cDNA for IFNγ into a nonimmunogenic tumor to attempt to provoke an immune response in lymph nodes draining a site of tumor inoculation. This strategy is largely based upon the approach of Shu et al., who successfully used DLN to generate effective antitumor T cells [29].

Our study shows that DLN cells induced by the non-transduced or the *Neo*[R] 24JK clones of the MCA 102 sarcoma had no therapeutic effect in vivo against established pulmonary metastases whereas DLN cells induced by the 24JK IFNγ-gene-modified clone could reduce the number of lung metastases from the parental unmodified tumor (Fig. 3, Table 3). In contrast, the inoculation of both the unmodified and the IFNγ-transduced 4JK clones induced DLN cells that significantly reduced the number of lung metastases (Table 2). Both 4JK and 24JK IFNγ-gene-modified tumors produced large amounts of IFNγ in vitro over a period of several months. We have recently retrovirally transduced another nonimmunogenic mouse sarcoma, MCA 101, with the cDNA for IFNγ. This IFNγ-gene-modified tumor, 101.22H, expressed a high level of MHC class I molecules and could be used to generate TIL that were therapeutic in vivo against pulmonary metastatic nodules generated from the wild-type tumor [25]. This 101.22H tumor clone, however, consistently secreted less than 5 U IFNγ/10^6 cells in 24 h. This finding supports the notion that the generation of CD8+ T cells with antitumor activity is induced by modulation of cell-surface molecules, notably MHC class I, or other accessory molecules like ICAM-1 [7, 8] and is not dependent upon extracellular secretion of IFNγ.

The in vitro phenotypic analysis shown in Table 1 indicates that the population of DLN cells at the initiation of the in vitro sensitization was equally divided between CD4+ and CD8+ lymphocytes. However, cultured DLN cells that were injected i.v. and mediated tumor regression in vivo were mainly CD8+ T cells with only 1% CD4+ lymphocytes. There was no difference between the phenotypic profile of DLN cells derived from parental or gene-modified tumors.

Up-regulation of MHC class I surface molecules occurred in the two tumor clones retrovirally transduced with

the IFNγ cDNA (Fig. 1). Only DLN generated from the animals bearing 24JK IFNγ–transduced tumor demonstrated enhanced in vivo antitumor activity compared with DLN cells generated by unmodified or *Neo*^{R} tumor cells (Fig. 3, Table 3). In contrast, the DLN cells primed by the inoculation of IFNγ-gene-modified 4JK tumors did not exhibit augmented therapeutic activity compared with DLN cells from animals bearing unmodified or *Neo*^{R}-transduced 4JK cells (Table 2). These data suggest that 4JK has a sufficient number of surface MHC class I molecules to evoke an immune response and generate potent CTL in the tumor-bearing host. Modest up-regulation of MHC class I antigens did not seem to increase this capacity to promote the generation of effective CD8+ T cells. On the other hand, 24JK is a tumor with essentially no detectable MHC class I molecules on its surface. On the basis of our experiments that show increased density of MHC class I antigens induced by the insertion of the IFNγ cDNA to 24JK cells, and on the basis of the in vivo therapeutic activity of DLN cells primed by these gene-modified 24JK tumor cells, we may hypothesize that an adequate number of complexes between antigen peptide and MHC class I molecules were formed on the 24JK IFNγ-gene-modified cells. Thus, when a certain threshold number of MHC class I molecules is achieved, the gene-modified tumor cells are probably capable of eliciting an immune response against a much weaker immunological stimulus, i.e. the parental unmodified 24JK tumor. Indeed, it was recently demonstrated, utilizing a highly immunogenic variant of another murine fibrosarcoma, that recognition of tumor antigens on the surface of the highly immunogenic variant is responsible for the afferent induction and efferent elicitation of antiparental cross-protective immunity [17]. In addition, there is evidence to suggest that as few as 200 MHC-class-I/peptide complexes on a tumor cell surface are sufficient for T cell recognition following elicitation of an immune response [5].

The findings presented here suggest that IFNγ gene modification can increase class I molecule expression, and that increased immunogenicity is observed with this maneuver. We do not suggest, however, that steady-state cell-surface expression of MHC class I molecules is the sole parameter by which the immunogenicity of a tumor is determined. The immunogenicity of a tumor is likely to be determined by a number of different factors. These factors include but are not limited to the immunogenicity of the expressed tumor-associated antigens, and the levels of expression of important intracellular adhesion molecules are likely to vary from one tumor clone to another.

The strategy described in this report has potential application for the development of T-cell-based immunotherapies for nonimmunogenic human tumors. The tumor histologies used in this approach would have to be chosen carefully. Specifically, this approach might be most useful in histologies expressing low levels of surface class I. Further, such low surface class I levels must be augmentable by IFNγ. Nevertheless, the tumors that express low levels of class I are frequently those that are most nonimmunogenic and resistant to immunotherapeutic responses. Small numbers of viable tumor cells, modified by the insertion of the gene encoding for IFNγ, would be injected into peripheral sites with easily accessible draining lymph nodes. T cells could potentially be recovered from these draining lymph nodes and expanded in culture for the subsequent treatment of patients with advanced cancer.

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