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Gene transfer of human interferon gamma complementary DNA into a renal cell carcinoma line enhances MHC-restricted cytotoxic T lymphocyte recognition but suppresses non-MHC-restricted effector cell activity

DJ Schendel[1], CS Falk[1], E Nößner[1], B Maget[1], S Kressenstein[1], S Urlinger[2,3], R Tampé[2] and B Gansbacher[4]

[1]*Institute of Molecular Immunology, GSF National Research Center for the Environment and Health;* [2]*Institute of Physiological Chemistry, Philipps-University Marburg; and* [4]*Institute for Experimental Oncology and Therapy Research, Technical University, Munich, Germany*

Even though renal cell carcinomas (RCC) are thought to be immunogenic, many tumors express variations in surface molecules and intracellular proteins that hinder induction of optimal antitumor responses. Interferon gamma (IFNγ) stimulation can correct some of these deficiencies. Therefore, we introduced the complementary DNA (cDNA) encoding human IFNγ into a well-characterized RCC line that has been selected for development of an allogeneic tumor cell vaccine for treatment of patients with metastatic disease. Studies were performed to determine how endogenous IFNγ expression influences tumor cell immunogenicity. IFNγ transductants showed minimal increases in surface expression of MHC class I and adhesion molecules but expression of class II molecules was induced. Proteins of the transporter associated with antigen processing (TAP) and low molecular weight polypeptide (LMP) were constitutively expressed at high levels. The transductants stimulated allospecific cytotoxic T lymphocytes (CTL); however, they were not better than unmodified tumor cells in this capacity. Endogenous IFNγ expression enhanced tumor cell recognition by MHC-restricted, tumor antigen-specific CTL but suppressed recognition by non-MHC-restricted cytotoxic cells. Thus, the functional consequences of IFNγ expression varied with respect to the type of effector cell and were not always beneficial for tumor cell recognition. Gene Therapy (2000) **7,** 950–959.

Keywords: *interferon-gamma; allogeneic vaccine; renal cell carcinoma*

Introduction

Currently, there is much interest in using genetically engineered tumor cells to enhance antitumor immune responses. Tumor cells can be altered genetically either *in vivo* or *ex vivo* to express new MHC molecules, adhesion molecules or costimulatory molecules.[1,2] They can also be modified to express cytokines that influence immune responses.[3–5] Following vaccination with genetically engineered tumor cells or introduction of such genes into tumors *in vivo*, activated lymphocytes should develop that are able to recognize and destroy unmodified tumor cells. Animal tumor models have proved this approach to be valid.[3,5] There is good reason to hope that such strategies may be of clinical benefit in renal cell carcinomas (RCC) since there is evidence that some patients can respond immunologically to their tumors; spontaneous tumor regression has been documented in a number of cases and tumor regression was induced in some patients treated systemically with cytokines or interferons (reviewed in Ref. 6).

The nature of immune responses that lead to RCC regression are poorly understood. Various cytotoxic effector cell types have been identified in patients with RCC that are capable of recognizing and destroying autologous tumor cells (reviewed in Ref. 7). One type that is prevalent among tumor-infiltrating lymphocyte (TIL) populations of many RCC patients shows a non-MHC-restricted pattern of specificity. Such T cells lyse not only autologous tumor cells but also a variety of allogeneic tumor cells, seemingly irrespective of MHC sharing. In fact, a prominent characteristic of these T cells is their ability to recognize the Daudi cell line which does not express MHC class I molecules.[8–11] A similar cytotoxic population can be generated by culturing peripheral blood lymphocytes (PBL) with high levels of recombinant interleukin-2 (IL-2) to generate lymphokine-activated killer (LAK) cells. Both $CD3^-$ and $CD3^+$ LAK cells show non-MHC-restricted specificity.[12,13] While the molecular

Correspondence: DJ Schendel, Institut für Molekulare Immunologie, GSF Forschungszentrum für Umwelt und Gesundheit, Marchioninistraße 25, 81377 Munich, Germany
[3]*Present address: Institute for Microbiology, Friedrich-Alexander-University Erlangen-Nürnberg, Germany*
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basis of tumor cell recognition by these effector cell types remains to be defined it has been found that the adhesion molecules ICAM-1 (CD54) and leukocyte-associated function molecule 3 (LFA-3, CD58) are important for optimal effector–target cell interactions.[14–16] More rarely, classical MHC-restricted CTL have been identified in RCC patients.[17–24] CTL lysis of autologous tumor cells is dependent upon triggering of their T cell receptors (TCR) with a ligand composed of a tumor-associated peptide complexed with an MHC molecule.[25,26]

Interferon gamma (IFNγ) has several important immune modulating capacities that might contribute to the optimal development of both types of antitumor response in RCC patients. It can induce expression of MHC class II molecules in epithelial cells that do not constitutively express such molecules.[27–29] This in turn enables their interaction with $CD4^+$ cells that supply cytokines, like IL-2, that support the development of both types of cytotoxic effector cell. IFNγ can up-regulate the expression of MHC class I molecules and adhesion molecules, including ICAM-1 and LFA-3, that are important in effector cell–target cell interactions mediated by both MHC-restricted and non-MHC-restricted effector cells.[14,16,30] It also increases transcription of the TAP and LMP genes.[31,32] These genes encode proteins that play critical roles in the processing and presentation of endogenous peptides by MHC class I molecules, thereby improving ligand presentation for MHC-restricted CTL.[33,34] These properties make IFNγ an interesting candidate for inclusion in genetically engineered tumor cell vaccines. Genetic modification of murine fibrosarcomas and human melanomas to express IFNγ was shown to enhance immunogenicity for T cells but to be detrimental for NK cell recognition.[35–38] Introduction of IFNγ into other tumor lines did not improve responses and could even be detrimental to the tumor host.[39,40] Therefore, it is important to evaluate directly how IFNγ modification impacts on various immune cells directed against a given tumor type, particularly if clinical application is foreseen.

Results

Generation of IFNγ transductants using the RCC-26 tumor cell line

In order to assess the feasibility of using an allogeneic tumor cell vaccine for patients with metastatic RCC we carried out extensive analyses of effector–tumor cell interactions using various RCC lines. The RCC-26 cell line was selected for further development because it was found to be recognized by MHC-restricted CTL derived from different RCC patients, demonstrating that it expressed several distinct immunogenic epitopes[7] (and unpublished data). T cells recognizing one HLA-A2-restricted ligand were found to utilize highly characteristic T cell receptors (TCR), providing a molecular marker to track the development of CTL responses[41] and earlier genetic engineering of this cell line to express human recombinant IL-2 showed that strong enhancement of tumor cell recognition by both MHC-restricted and non-MHC restricted T cells could be achieved.[42]

To determine how modification of RCC-26 cells to express IFNγ would influence immunogenicity, we introduced the cDNA for human IFNγ into two separate bulk cultures of RCC-26 cells by retroviral transduction using a vector that was shown previously to generate IFNγ-secreting RCC.[43] The two independently derived cultures were designated as RCC-26/IFNγ1 (endoγ1) and RCC-26/IFNγ2 (endoγ2). A control population (RCC-26VC) carrying the identical vector without the IFNγ sequence was used for comparison.

Uncloned populations with stable vector integration were selected in bulk by growth in G418-containing medium for extended periods of time. When IFNγ secretion of the uncloned populations was analyzed by ELISA, no cytokine secretion could be detected (data not shown). Therefore, the bulk cultures were analyzed for the presence of IFNγ transcripts by RT-PCR. Unmodified tumor cells, RCC-26VC cells and PBL stimulated with 1% PHA for 72 h were included as controls. A band of the expected 226 bp size was found in the PHA-activated PBL but not in unmodified RCC-26 and RCC-26VC cells (Figure 1a). Bands of expected size were also present in the endoγ1 and endoγ2 transductants, demonstrating that these tumor cells were transcribing the IFNγ transgene. When template material was generated in the absence of RT, no bands were detected, certifying that the positive PCR signals did not arise due to DNA contamination. IFNγ protein was also found to be present in the IFNγ transductants using intracellular immunofluorescence analysis with an antibody specific for IFNγ. The shift in fluorescence intensity, as compared with control tumor cells, indicated that intracellular cytokine was present in the transductants even though its secretion could not be detected by ELISA (Figure 1b).

These low intracellular cytokine levels were adequate enough to have a regulatory impact on the expression of several molecules in the tumor cells, as assessed by flow

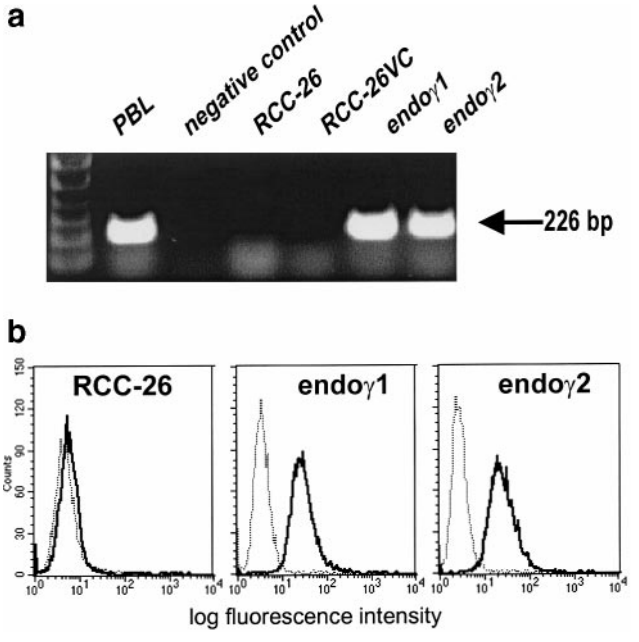

Figure 1 *Expression of IFNγ mRNA and intracellular protein in RCC-26 transductants. (a) RT-PCR analysis using IFNγ-specific oligonucleotide primers and RNA isolated from control PBL stimulated with PHA, RCC-26, RCC-26VC and two IFNγ transductant cell lines of RCC-26, endoγ1 and endoγ2. The negative control reaction did not contain cDNA. (b) Indirect immunofluorescence analysis of permeabilized cells using IFNγ-specific antibody was compared in both transductants with unmodified RCC-26 cells. The x-axis represents the mean fluorescence intensity.*

cytometry and Western blotting. Unmodified RCC-26 (Figure 2a–d) cells constitutively express high levels of MHC class I molecules and substantial amounts of ICAM-1 and LFA-3 (not shown). They do not express MHC class II molecules, as expected for epithelial cells. This same pattern characterized the RCC-26VC cells (Figure 2e–h). Following stimulation with exogenous IFNγ, increased expression of MHC class I and ICAM-1 molecules was seen for both RCC-26 and RCC-26VC cells. Increases in LFA-3 expression were not seen (data not shown). Furthermore, MHC class II expression was induced in both cell lines (Figure 2c, g). These levels of class II expression were obtained only when tumor cells were exposed to at least 50 U/ml of exogenous IFNγ for 96 h, but not through continual exposure of RCC-26 cells to low levels of IFNγ (1 U/ml, added biweekly over 7 weeks of culture; data not shown). While the endoγ1 and endoγ2 cells only showed levels of MHC class I and ICAM-1 expression here that were comparable with those of the RCC-26VC control cells, some increases in these molecules could be measured in other experiments. Good MHC class II expression was consistently seen in the endoγ1 cells and levels varying from low to high were detected when the endoγ2 cells were analyzed in multiple experiments (Figure 2k, o; and data not shown). Thus, even in the absence of secreted IFNγ, the endoγ1 and endoγ2 lines showed alterations in cell surface expression, indicating functional activity of the transgenes.

Since both cell lines were generated independently and represented uncloned populations they are composed of cells with different sites of viral integration. Multiple colonies emerged in each line after initiation of selection in G418-containing medium. The surface expression of MHC and adhesion molecules showed a normal distribution in the transduced lines. Therefore, it is unlikely that the site of viral integration or selection of unique clones accounted for these regulatory effects of IFNγ in the transductants. Furthermore, such alterations in surface marker expression were not seen when the RCC-26 cells were engineered to express IL-2,[42] interleukin-7 or granulocyte–macrophage colony-stimulating factor (GM-CSF), demonstrating that the changes were due to IFNγ expression (data not shown).

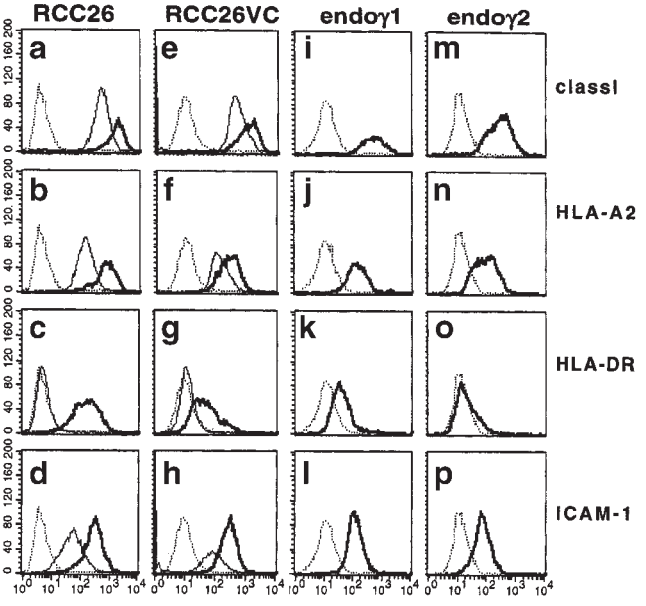

Figure 2 *Cell surface expression of MHC class I, II and adhesion molecules. RCC-26 (a–d), RCC-26VC (e–h) and the endoγ1 (i–l) and endoγ2 (m–p) cells were tested for expression of class I molecules, HLA-A2 molecules, HLA-DR class II molecules and ICAM-1 expression using the reagents listed in Materials and methods. In RCC-26 and RCC-26VC cells isotype control binding is depicted by the stippled curves (. . . .), specific antibody binding on unstimulated cells by the thin-line curves (——) and cells stimulated with exogenous IFNγ and stained with specific antibody by the thick-line curves (——). Endoγ1 and endoγ2 cells stained with isotype control antibodies are depicted with stippled curves (. . . .) and with specific antibodies by the thick-line curves (——).*

IFNγ-transduced tumor cells show enhanced TAP and LMP expression

LMP and TAP molecules contribute to the amount and quality of MHC-peptide ligands through their influence on the proteolytic processing and transport of peptides.[31,32] Reduced expression of TAP proteins has been observed in some tumor cells including RCC, providing one mechanism that may allow cells to escape specific T cell recognition.[44,45] Since IFNγ has been shown to upregulate transcription of TAP and LMP genes[31,32] better expression of these molecules could contribute to improved CTL priming and recognition. Therefore, the IFNγ transductants and exogenously treated tumor cells were compared for expression of TAP1, TAP2, LMP2 and LMP7 proteins in Western blots (Figure 3). Only faint bands for both TAP and LMP proteins were detected in untreated RCC-26 cells but high levels were induced following exogenous IFNγ stimulation. An equally strong induction of these proteins was seen following exogenous IFNγ stimulation of RCC-26VC cells (data not shown). The endoγ1 and endoγ2 transductants were found constitutively to express even higher levels of TAP and LMP proteins than those induced by exogenous IFNγ treatment. Stable, high expression of these proteins was maintained in the transductants over long periods of time

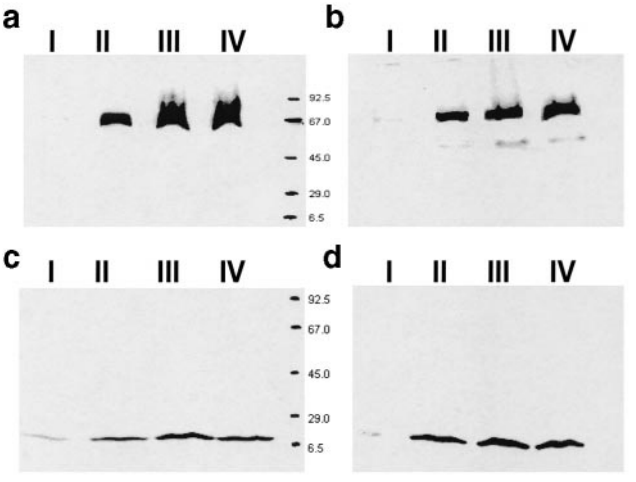

Figure 3 *Influence of IFNγ on the expression of TAP1 (a), TAP2 (b), LMP2 (c) and LMP7 (d) proteins. Total cell lysates were separated by SDS-PAGE and protein expression determined by Western blot analysis. Lane I: untreated RCC-26 cells; lane II: RCC-26 cells treated with 1000 U/ml exogenous IFNγ; lanes III and IV: IFNγ-transduced tumor cells, endoγ1 and endoγ2, respectively. Positions of the marker proteins are indicated in the middle.*

(data not shown). Thus, endogenous expression of IFNγ could dramatically alter deficits in some components of the peptide processing and presentation machinery of the tumor cells.

Unmodified tumor cells and IFNγ-transduced cells induce comparable allospecific CTL responses

The ability of IFNγ-transduced tumor cells to influence immune function was first assessed by analyzing the ability of the tumor cells to induce CTL responses in allogeneic mixed lymphocyte tumor cell cultures (MLTC). PBL of two healthy, control donors were cocultured with either unmodified RCC-26 tumor cells or the endoγ1 cells. The endoγ1 cells were selected for these experiments because they consistently showed better levels of MHC class I and II expression when tested in multiple flow cytometry experiments. Endoγ1 cells were compared as stimulating cells with unmodified tumor cells since this represents the situation that would prevail in a clinical setting. After three rounds of restimulation in MLTC the primed lymphocytes were analyzed for the presence of cytotoxic effector cells using RCC-26, endoγ1, Daudi and K562 as target cells in a 4 h chromium release assay. The results from one donor are shown in Figure 4; comparable results were obtained using PBL of the second donor (data not shown). High levels of cytotolytic activity were detected against both RCC-26 (Figure 4a) and endoγ1 (Figure 4b) cells. The levels of lysis were somewhat higher for unmodified tumor cells and there was no apparent improvement in CTL generation using the endoγ1 tranductant for stimulation of PBL when compared with unmodified RCC-26 cells. Since only weak levels of nonspecific lysis of Daudi and K562 cells were detected we surmised that these effectors cells represented allospecific CTL. This conclusion was supported by assessing the fine specificity of target cell recognition, whereby the CTL were tested for their ability to recognize allogeneic LCL that shared single HLA-A or B allotypes with RCC-26 cells. Three of the four allogeneic LCL were lysed by the CTL, reflecting recognition of mismatched MHC class I molecules of the responding cell donor and RCC-26 cells. This donor carries the HLA-B*5101 allele, accounting for the lack of development of CTL against this self MHC molecule.

This experiment showed that unmodified as well as IFNγ-transduced tumor cells were able to induce strong responses, representing a spectrum of different allospecificities. However, the endogenous expression of IFNγ in the tumor cells did not improve the levels of cytolytic activity or alter the fine specificity of the alloresponse induced in the MLTC.

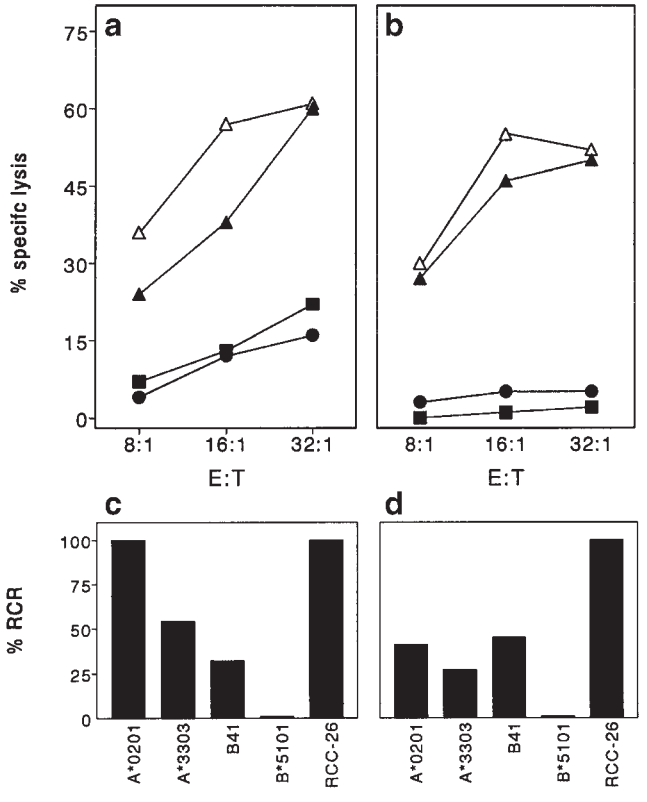

Figure 4 *Induction of allospecific CTL by RCC-26 and endoγ1 cells. Activated PBL isolated from a MLTC using responding lymphocytes of donor JB (HLA-B*5101) were stimulated with (a, c) unmodified RCC-26 cells or (b, d) endoγ1 cells. CTL activity was measured in a standard 4 h chromium release assay using RCC-26 cells (△), endoγ1 cells (▲), Daudi (■) and K562 (●) cells. The fine specificity of allorecognition was determined using LCL matched for single HLA-A (HLA-A*0201 or *3303) or HLA-B (HLA-B41 or *5101) allotypes with RCC-26 cells.*

IFNγ transduction enhances tumor-antigen specific CTL lysis

In a second series of functional experiments the IFNγ transductants and exogenously stimulated tumor cells were compared as target cells for cytotoxic T cells of patient 26 that were obtained from an autologous tumor infiltrating lymphocyte (TIL) population. TIL-26 cells which were isolated from the primary tumor were shown previously to represent a population of HLA-A2 restricted, tumor antigen-specific CTL.[21] Thus, they lysed RCC-26 cells but not Daudi cells (Table 1); they also did not lyse autologous normal kidney cells of patient 26 (NKC-26) and K562 cells.[21] Improved recognition by TIL-26 cells was detected when RCC-26 cells were pretreated with exogenous IFNγ and tested as target cells in a chromium release assay. The RCC-26VC cells were less well recognized by TIL-26 in this experiment; however, pretreatment with exogenous IFNγ substantially improved recognition. The endoγ1 and endoγ2 cells were better recognized than the vector control cells, being lysed at levels comparable with or greater than those attained following pretreatment of RCC-26VC cells with 1000 U/ml exogenous IFNγ. The endoγ1 cells were consistently found to be better target cells when compared with endoγ2 cells. The IFNγ-mediated enhancement of lysis was confirmed in six independent experiments (Figure 5a). The same hierarchy in improved sensitivity to lysis by TIL-26 was observed throughout, showing that exogenously treated tumor cells were most susceptible followed by endoγ1 and endoγ2 cells. The susceptibility to lysis of tumor cells exposed either exogenously to 1000 U/ml IFNγ or modulated intracellularly by low levels of cytokine was significantly greater than that of untreated control cells.

Since the IFNγ-transduced lines did not secrete measurable cytokine the exogenously stimulated cells were washed free of exogenous cytokine to eliminate any direct influence of IFNγ on the effector cells. In order to simulate the effects of IFNγ secretion by tumor cells, we added exogenous IFNγ to the culture medium during the

Table 1 Recognition of RCC-26-derived target cells by MHC-restricted CTL

Exp.	*CTL*	*E:T*	*IFNγ[a] exo U/ml*	*Daudi*	*RCC-26*	*RCC-26[b] exoγ*	*RCC-26VC*	*RCC-26VC[b] exoγ*	*RCC-26 endoγ1*	*RCC-26 endoγ2*
1	TIL-26	4:1	—	2	24	33	8	24	36	24
		2:1	—	3	13	23	6	18	19	15
		1:1	—	2	9	12	3	11	13	8
		4:1	5	0	15	35	11	23	35	20
2	TIL-26	8:1	—	1	20	48	10	37	45	34
		4:1	—	1	18	37	10	28	37	20
		2:1	—	0	10	25	4	22	20	11
		4:1	10	5	18	51	9	32	35	24
		4:1	1	0	14	48	10	29	42	24

[a]Concentration of exogenous IFNγ added to medium during 4 h chromium release assay.
[b]Target cells were pretreated for 72 h with 1000 U/ml IFNγ before use in chromium release assay.

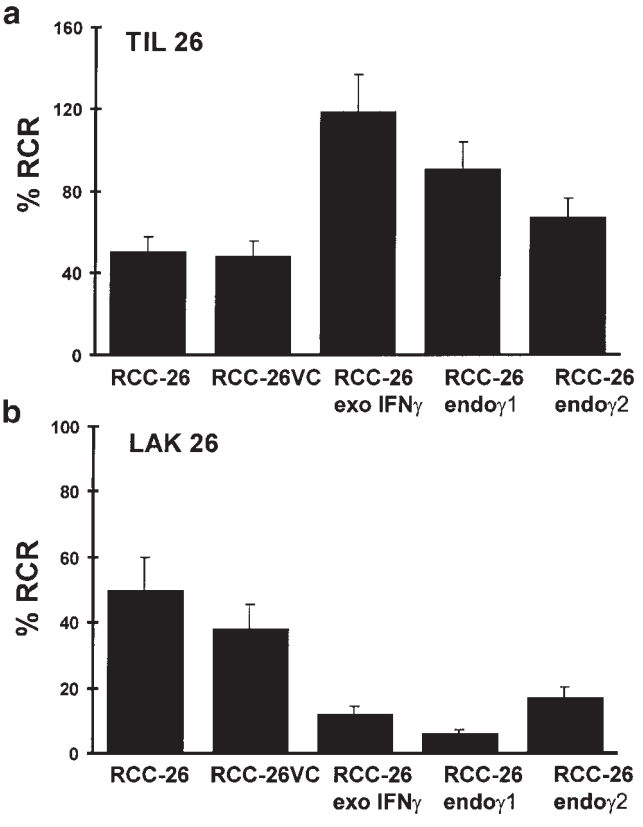

Figure 5 *Cytotoxic activity of TIL-26 and LAK-26 directed against RCC-26 target cells exposed to exogenous (1000 U/ml) or endogenous IFNγ. (a) The mean and standard deviation of the relative cytotoxic response (RCR) of TIL-26 was calculated for six independent experiments using the specific lysis (ranging from 9 to 24% at an E:T of 2:1) of untreated RCC-26 cells as a 50% reference value for each individual experiment, according to published methods.[66] (b) The mean and standard deviation of the RCR of LAK-26 in four independent experiments was determined using the specific lysis (ranging from 10 to 24% at an E:T of 5:1) of untreated RCC-26 as the 50% reference value for each individual experiment.*

chromium release assay. The levels of IFNγ that were added represented a range that would be reached if transduced tumor cells secreted 500–5000 U/ml/10^6 cells. Despite these high relative concentrations of IFNγ we could not observe significant alterations in the lytic capacity of the CTL.

IFNγ transduction suppresses recognition by non-MHC-restricted effector cells

In order to assess how endogenous IFNγ expression in the tumor cells influenced non-MHC-restricted effector cells, LAK cells were prepared using autologous and allogeneic PBL of RCC patients and tested with the same panel of target cells. At the time of use in the cytotoxicity assays, the LAK cells were comprised only of $CD3^+$ T cells (data not shown). Both the autologous LAK-26 and allogeneic LAK-53 cells lysed the class I negative cell line Daudi, RCC-26 and RCC-26VC cells (Table 2). However, when the tumor lines were pretreated with exogenous IFNγ they became highly resistant to LAK-mediated cytotoxicity. A similar resistance was found with the endoγ1 and endoγ2 transductants. Addition of exogenous IFNγ to the culture medium during the 4 h chromium release assay had no influence on the LAK-26 cell activity directed against the untreated tumor cells and it was not able to reverse the IFNγ-induced target cell resistance. The inhibition of tumor cell lysis to LAK-26 cells induced by exogenous and endogenous IFNγ was confirmed in four independent experiments (Figure 5b). The endoγ2 cells retained a low, but consistent degree of susceptibility to the non-MHC-restricted LAK cells. However, if they were pretreated with exogenous IFNγ they became fully resistant to LAK-26 mediated killing (data not shown), suggesting that the level of endogenous IFNγ might influence target cell resistance. Interestingly, the interpretation that the endoγ2 cells express less endogenous cytokine would be consistent with the observations of a lower increase in MHC class I and class II surface expression as compared with endoγ1 cells (Figure 2).

A general alteration in target cell sensitivity to cytolytic mechanisms seemed unlikely to explain these results since the IFNγ transductants were highly susceptible to lysis by allospecific and tumor antigen-specific CTL (see Figure 4 and Table 1). The decrease in susceptibility was also not due to some unexpected alteration caused by their genetic manipulation since both unmodified RCC-26 and RCC-26VC showed similar patterns, ie enhanced recognition by TIL-26 and resistance to LAK-26 mediated lysis following exogenous IFNγ treatment.

Table 2 Recognition of RCC-26-derived target cells by non-MHC-restricted LAK cells

Exp.	*LAK*	*E:T*	*IFNγ[a] exo U/ml*	*Daudi*	*RCC-26*	*RCC-26[b] exoγ*	*RCC-26VC*	*RCC-26VC[b] exoγ*	*RCC-26 endoγ1*	*RCC-26 endoγ2*
1	LAK-26	20:1	—	56	32	3	25	6	0	16
		10:1	—	57	18	1	12	5	0	9
		5:1	—	43	11	0	11	6	0	6
	LAK-53	20:1	—	43	32	4	29	10	1	12
		10:1	—	25	16	2	14	7	0	8
		5:1	—	27	10	0	9	4	0	5
2	LAK-26	20:1	—	64	NT	NT	23	7	2	2
		10:1	—	40	NT	NT	12	5	1	1
		5:1	—	24	NT	NT	7	3	0	0
		10:1	10	43	NT	NT	10	4	2	1
		10:1	5	46	NT	NT	12	5	0	1
		10:1	1	30	NT	NT	10	7	0	0

[a]Concentration of exogenous IFNγ added to medium during 4 h chromium release assay.
[b]Target cells were pretreated for 72 h with 1000 U/ml IFNγ before use in chromium release assay.
NT, not tested.

Discussion

In the absence of effective chemotherapies or radiation therapies for RCC recent efforts have been devoted to the development of immunotherapies for treatment of metastatic disease. Systemic application of rIL-2 alone, or in combination with interferon alpha (IFNα), has been beneficial for some RCC patients. However, clinical benefit is not long lasting and is often accompanied by serious toxicity.[6,46–48] One study reported good clinical responses following systemic IFNγ treatment[49] but similar results have not been seen in other patients.[6,50,51] Because of the toxicities associated with systemic interferon application this form of treatment is not suitable for patients with low performance status or as an adjuvant therapy for patients with minimal residual disease. Recent efforts have been directed to the development of vaccines or *in vivo* transfer of transgenes into tumors to enhance tumor cell immunogenicity. Animal studies revealed that it is particularly important to induce MHC-restricted $CD4^+$ and $CD8^+$ T cells because they retain a memory potential providing long lasting immunity.[52–55] On the other hand, non-MHC-restricted cytotoxic cells have been identified as the only source of effector cells in many RCC patients.[18,56] While their dependence on $CD4^+$ T cell help has not been studied, they are dependent on IL-2. Since it is not known a priori how patients respond to their tumors and how these responses will be altered through vaccination with genetically engineered tumor cells or by introduction of cytokine genes directly into tumor cells *in vivo*, we felt it important to compare the influence of IFNγ tumor cell modification on the functional activity of different effector populations, including both MHC-restricted and non-MHC-restricted cytotoxic lymphocytes.

The use of IFNγ appeared promising because of its multi-faceted potential to influence immune responses through enhanced expression of molecules that contribute to both specific and non-specific effector cell–tumor cell interactions.[57–59] Indeed, the introduction of the IFNγ cDNA into our tumor vaccine line enhanced expression of several cellular proteins that should foster improved effector–target cell interactions.

An enhanced recognition of RCC-26 cells by MHC-restricted, tumor antigen-specific CTL was seen consistently following exogenous IFNγ treatment and improved recognition of the IFNγ transductants was also achieved. IFNγ-induced increases in TAP and LMP could contribute to enhanced target cell recognition through improved peptide ligand generation. The minimal alterations in class I and ICAM-1 expression would not appear to be contributing factors nor does class II expression seem to be revelant here because the CTL are $CD8^+$ and class I restricted. Surprisingly, improved recognition of the transductants was comparable with or greater than that achieved when the vector control cells were prestimulated with 1000 U/ml exogenous IFNγ, even though the transductants did not secrete detectable IFNγ. The alterations in MHC class II expression were not seen in transductants made using the same vector carrying the cDNA encoding IL-2, IL-7 or GM-CSF, demonstrating that modulation was specific for IFNγ (data not shown). IFNγ has been found previously to influence gene regulation in other non-secreting cells following its introduction as a transgene; it may exert such regulatory effects intracellularly through a private autocrine loop.[60,61]

In contrast to the enhancement of MHC-restricted CTL recognition, no improvement in the induction of allogeneic CTL or in tumor cell recognition by non-MHC-restricted effector cells could be detected. In fact, this genetic modification led to tumor cell resistance to lysis by both autologous and allogeneic LAK cells. This was not due to a general alteration in the tumor cell lines caused by retroviral transduction since introduction of the cDNA for IL-2 was shown previously to improve susceptibility of RCC-26 cells to both tumor antigen-specific CTL and non-MHC-restricted LAK-26 cells.[42] IFNγ was shown previously to induce resistance to NK-mediated lysis in other tumor types; in some cases this was ascribed to enhanced MHC class I expression, particularly when unmodified tumor cells expressed low levels of MHC class I molecules.[37,38,62,63] Since RCC-26 cells constitutively express substantial levels of HLA class I molecules, IFNγ induced only minor increases in class I expression on occasion. Whether alterations in the expression of particular allotypes account for the induced resistance to lysis by non-MHC-restricted T cells remains to be determined.

The results of these functional studies reveal that caution must be taken in assuming that a particular genetic modification will necessarily enhance antitumor immunity. This is particularly critical when the molecular basis of tumor cell recognition is poorly understood, as is often the case in gene therapy protocols using autologous tumor cell vaccines. While the vector used to generate the IFNγ transductants was successfully employed to develop other RCC lines secreting interferon, despite multiple attempts we were unable to obtain stable IFNγ secretion from RCC-26 cells. We have noted that exogenous treatment with IFNγ has a cytostatic effect on RCC-26 cells (data not shown); thus continual exposure may preclude the survival *in vitro* of transductants secreting IFNγ. When the non-secreting IFNγ transductants were cultured with exogenous IFNγ during the chromium release assay to mimic the effect of cytokine secretion, we could not substantially enhance CTL activity nor could the resistance to non-MHC-restricted cells be reversed. Thus, the different functional outcomes appear to be a direct result of alterations in the tumor cells. Therefore, it is conceivable that *in vivo* application of IFNγ vectors into RCC could create a situation that allows enhanced MHC-restricted CTL recognition but is detrimental for non-MHC-restricted effector cell activity. We have seen a comparable development of resistance to non-MHC-restricted effector cells in a second RCC line that was genetically engineered to express endogenous IFNγ, revealing that this effect is not unique for RCC-26 cells (data not shown).

In the case of vaccine development utilizing established tumor cell lines that are modified *ex vivo*, such as our approach using RCC-26 cells, detailed functional studies can be performed to evaluate the impact of genetic modification. Here, it seems particularly important to analyze immune reactivity of various effector cells that may contribute to responses in patients *in vivo*. Our results suggest that IFNγ is not a suitable candidate for inclusion in this genetically engineered RCC vaccine. Furthermore, caution is warranted in applying IFNγ vectors in RCC patients *in vivo*. The functional consequences of IFNγ stimulation revealed by our studies may provide some insight into the clinical outcome in RCC patients treated with interferons.[6] While IFNα has shown positive clinical benefit in a number of trials, benefit has rarely been achieved with IFNγ. The capacity of IFNγ to render tumor cells fully resistant to lysis by non-MHC-restricted effector cells, which predominate in many RCC patients, may contribute to this failure. Preliminary studies (data not shown) reveal that no or only minimal inhibition of non-MHC-restricted effector cells (both NK and T cells) is seen when tumor cells are pretreated with IFNα in contrast to a full inhibition seen with IFNγ.

Materials and methods

Establishment of tumor cell lines and cytotoxic effector cells

The RCC-26 tumor line was established from a primary carcinoma of a stage I (T1, G2, N0, M0) patient as described[21] and cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium-pyruvate and 10% heat-inactivated fetal calf serum (FCS). These tumor cells were modified to express IFNγ endogenously by transduction with the human IFNγ cDNA using the retroviral system described previously.[43] As controls, transductants were made using the same vector without IFNγ cDNA or carrying instead the cDNA for human rIL-2.[42]

PBL obtained at the time of surgery from patients undergoing tumor resection were used for the generation of LAK cells by culture in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM pyruvate, 15% heat-inactivated, pooled human serum, 1% phytohemagglutinin (PHA) and 1000 U/ml rIL-2 (Proleukin; Cetus, Emeryville, CA, USA). TIL were obtained from the primary tumor as described previously,[21] cultured in RPMI 1640 with 7% FCS, 7% heat-inactivated, pooled human serum, 5% conditioned medium obtained from PHA-activated lymphocytes[64] and 25–50 U/ml rIL-2. The tumor-specific CTL were restimulated every 7–10 days with irradiated (100 Gy) autologous tumor cells.

Mixed lymphocyte tumor cultures (MLTC) were established using PBL of healthy control donors and irradiated (100 Gy) tumor cells, plating 0.5×10^6 responding cells and 3×10^4 irradiated tumor cells per well of a 24-well culture plate. The presence of allospecific CTL was assessed using a panel of Epstein–Barr virus-derived lymphoblastoid cell lines (LCL) that were individually selected to match the tumor cells by only one MHC class I allele.

Generation of RCC transductants

The retroviral vector N2/CMV/IFNγ has been described previously.[65] It contains the neomycin gene as a selection marker under the control of the Moloney murine leukemia virus long terminal repeat and the cDNA of human IFNγ driven by the internal cytomegalovirus promoter. Retroviral infection and selection in G418-containing medium was done as described. The RCC lines were continuously maintained in G418-containing medium (0.7 mg/ml).

Exogenous cytokine stimulation and endogenous expression of IFNγ

For exogenous treatment, tumor cells were cultured in medium containing 1000 U/ml of recombinant IFNγ for 96 h (Dr Rentschler, Biotechnologie GmbH, Laupheim, Germany). A commercial ELISA (sensitivity of 16 pg/ml) (Laboserve, Giessen, Germany) was used to measure IFNγ in the culture medium of the transduced tumor cells.

Detection of IFNγ by reverse transcriptase polymerase chain reaction (RT-PCR) was performed using total RNA isolated from 1×10^6 cells with RNazol B (Cinna/Biotecx, Houston, TX, USA) and cDNA was synthesized by reverse transcription (Gibco BRL Superscript, Gaithersburg, MD, USA). PCR was performed using oligo-dT primed cDNA and IFNγ-specific oligonucleotides (sense: 5'CAGATGTAGCGGATAATGGA3'; antisense: 5'AGCTT TTCGAAGTCATCTCG3') starting with 3 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 60°C, 2 min at 72°C, and finishing with 5 min at 35°C. The 226 bp amplicon was visualized on a 2% agarose gel stained with ethidium bromide.

Intracellular IFNγ was measured using a cell permeabilization protocol adapted from intracellular cytokine staining of lymphocytes. To enrich the intracellular IFNγ concentration, tumor cells were cultured for 4 h with

100 ng/ml monensin (M5273; Sigma Chemical Co, Munich, Germany). After trypsin harvesting, cells were washed with phosphate-buffered saline (PBS) and incubated in Hepes medium (H-3375; Sigma, Steinheim, Germany) containing 1% saponin (16109; Riedel de Haen, Seelze, Germany) for 30 min, washed twice with PBS and incubated with a 100-fold dilution of an anti-IFNγ antibody (166-5 ascites, kindly provided by D Novick, Weizmann Institute, Rehovot, Israel) in HEPES/saponin solution. Following 90 min incubation at room temperature with continuous shaking, cells were washed twice and incubated with FITC-conjugated goat anti-mouse $F(ab)_2$-immunoglobulin (F313; Dakopatts, Copenhagen, Denmark), fixed with PBS/1% paraformaldehyde and analyzed using flow cytometry (FACSCalibur; Becton Dickinson, Mountain View, CA, USA).

Cell-mediated lysis assay

Cell-mediated lysis was quantified in a standard 4 h chromium-51 release assay.[66] Spontaneous release was determined by incubating target cells alone in complete medium. Total release was determined by directly counting an aliquot of labeled cells. The percent cytotoxicity was calculated according to the formula: % lysis = (experimental c.p.m. – spontaneous c.p.m./total c.p.m. – spontaneous c.p.m.) × 100. Duplicate measurements of three-step titrations of effector cells were used for all experiments.

To compare data from independent cytotoxicity experiments a percent relative cytotoxic response (% RCR) was calculated using the specific lysis of untreated RCC-26 cells in each experiment as a reference value of 50%. The specific lysis of all other target cells in the experiment was determined in relation to the reference value and expressed as a % RCR, as described previously.[66] The mean and standard deviations were calculated and graphically depicted using the Excel 7.0 program.

Indirect immunofluorescence

Tumor cells were tested for surface expression of MHC class I, HLA-A2, HLA-DR, and ICAM-1 by flow cytometry using the following mAb: W6/32 specific for MHC class I molecules (American Type Culture Collection (ATCC), Rockville, MD, USA); L243 specific for HLA-DR (ATCC) and mAb P3.58B specific for ICAM-1 (kindly provided by JP Johnson, Institute of Immunology, University of Munich, Germany). HLA-A2 expression was measured using mAb mA2.1 (ATCC). mAb UPC-10 (Sigma) specific for a mouse myeloma protein was used as a negative control. Cells were incubated with mAb for 90 min on ice, washed twice with PBS and then incubated with fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse $F(ab)_2$-immunoglobulin (F313; Dakopatts) for 30 min. Cells were analyzed using flow cytometery.

Western blot analysis of TAP and LMP using enhanced chemiluminescence (ECL) detection

5×10^6 tumor cells were lysed in 100 μl TE-buffer (10 mM Tris-HCl, 2 mM EDTA, pH 7.6), supplemented with 3 μl of protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 2.5 mM benzamidine) and 100 μl of 3 × SDS-PAGE buffer containing β-mercaptoethanol.[67] To facilitate lysis the suspension was passed several times through an 18-gauge needle and heated at 37°C for 5 min. Ten μl of each sample was separated by 8–18% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and visualized by Ponceau staining. The positions of the molecular weight standard proteins were marked. Immunodetection of LMP and TAP proteins was performed using enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, UK) according to the manufacturer's instruction. For the detection of TAP1 and TAP2 proteins, the membranes were incubated overnight at room temperature with either anti-TAP1 mAb 148.3 (1:10 dilution of hybridoma supernatant)[68] or anti-TAP2 mAb 429.3 (1:20 dilution of hybridoma supernatant),[69] followed by a 1 h incubation with a 1:8000 dilution of horseradish peroxidase-conjugated goat anti-mouse polyclonal antibody (A4416; Sigma) at room temperature. After ECL detection the membrane was stripped of bound antibodies by incubation in a solution containing 60 mM Tris-HCl, 2% SDS, 100 mM β-mercaptoethanol, pH 6.7 at 50°C for 30 min. The membranes were reprobed using polyclonal antibodies against LMP2 (1:200 dilution of rabbit antiserum) and LMP7 (1:200 dilution of rabbit antiserum)[70] followed by a 1 h incubation with a peroxidase-conjugated polyclonal goat anti-rabbit antibody (A6154; Sigma).

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