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Interferon γ (IFNγ) gene transfer of an EMT6 tumor that is poorly responsive to IFNγ stimulation: increase in tumor immunogenicity is accompanied by induction of a mouse class II transactivator and class II MHC

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Abstract Interferon γ (IFNγ) is an important cytokine with immunomodulatory properties that include activation of immune cells and induction of class I and class II major histocompatibility complex antigens. In this study a retroviral vector was used to introduce the IFNγ gene into EMT6 tumor cells to assess the effect of IFNγ gene expression on tumor immunogenicity. Transfectants were selected in G418-containing tissue-culture medium and were determined to express the inserted IFNγ gene by reverse transcriptase/polymerase chain reaction. Flow-cytometric analysis revealed that parental unmodified EMT6 cells constitutively expressed only class I MHC and were poorly responsive to exogenous IFNγ stimulation, whereas class II MHC was induced in IFNγ-transfected cells. The induction of class II MHC in IFNγ-transfected cells correlated with the expression of a mouse class II transactivator gene that was dormant in unmodified or mock-transfected cells. In addition, IFNγ-gene-transfected tumor cells were found to secrete up to 17 ng IFN (equivalent to 75 units/10^6 cells) by enzyme-linked immunosorbent assay (ELISA). Whereas parental EMT6 cells grew unchecked, the growth of genetically modified tumor cells was significantly inhibited in immunocompetent mice. Rechallenge of animals that rejected an IFNγ-transfected EMT6 clone (EMT6-B17) with parental EMT6 cells resulted in tumor rejection, suggesting that IFNγ-transfected EMT6 cells were able to induce long-term immunity. Mixing experiments using gene-transfected and unmodified tumor cells demonstrated that 10% of IFNγ-transfected cells in the population was sufficient to protect mice against subsequent challenge with tumorigenic EMT6 cells. These studies demonstrate that the immunogenicity of tumor cells that are poorly responsive to exogenous IFNγ can be enhanced by inserting and expressing the IFNγ transgene. These findings also suggest a role for class II MHC in reducing tumorigenicity of the EMT6 tumor and inducing long-term tumor immunity.

Key words Interferon γ · MHC · EMT6 · Class II transactivator (CIITA) · Gene therapy

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Introduction

The role of class I and class II major histocompatibility complex (MHC) molecules in immune recognition has been widely documented. $CD8^+$ cytotoxic T lymphocytes (CTL) recognize antigens in association with class I whereas $CD4^+$ T-helper lymphocytes are activated by antigens presented by antigen-presenting cells on class II MHC [10]. One mechanism by which tumors may evade immune recognition is down-regulation of MHC molecules [11, 12, 16]. Reduced levels of MHC molecules on tumor cells may lead to poor presentation of tumor-associated antigens in association with class I and class II MHC. By increasing the expression of MHC antigens on tumor cells, it is possible to augment the ability of the host to recognize tumor-associated antigens and eliminate the tumor. MHC molecules can be induced on normal and neoplastic cells by exogenous IFNγ stimulation or by transfer of interferon γ (IFNγ) [3, 7, 8, 14, 17, 21, 26] or MHC [2, 15] genes.

IFNγ treatment or gene transfer preferentially increases expression of class I MHC in various tumor types [7, 8, 14, 17, 21, 26]. These studies have also demonstrated that enhancement of class I expression reduces tumorigenicity [7, 14, 17, 21, 26]. Unlike class I MHC, only a few studies have examined the role of class II MHC in tumor immunogenicity [3, 15].

The aim of this study was to determine the effect of expression of a murine IFNγ transgene on the tumorigenicity of the EMT6 mammary carcinoma. The EMT6 tumor is a moderately immunogenic carcinoma that forms progressing tumors in syngeneic animals. These studies demonstrate that the EMT6 tumor is poorly responsive to exogenous IFNγ stimulation whereas IFNγ gene transfer induces class II MHC antigens, increases immunogenicity and causes the acquisition of long-term immunity in the host. We also show a correlation between class II MHC expression in IFNγ-gene-transfected EMT6 cells and induction of a murine class II transactivator (CTIIA) gene.

By expressing class II MHC antigens, IFNγ-transfected EMT6 cells may function as antigen-presenting cells and play an important role in the generation of tumor-specific immunity.

Materials and methods

Mice

Female BALB/c mice, 6–8 weeks old (The Jackson Laboratory, Bar Harbor, Me.) were used in these studies. Mice were housed in the University of Arizona Animal Facility and fed food and water ad libitum.

Tumors

The EMT6 mammary tumor and the B16-F10 melanoma cell lines were kindly provided by Dr. Sara Rockwell (Yale University, New Haven, Conn.) and Dr. Mary Hendrix (St Louis University, St Louis, Mo.) respectively. These tumor cell lines were routinely passaged in vivo by subcutaneous (s.c.) injection in BALB/c and C57BL/6 mice respectively and were subsequently disaggregated and cryopreserved at −70 °C. Prior to each experiment, an aliquot of frozen cells was thawed and passaged minimally in vitro. The cells were maintained in α minimal essential medium (α-MEM) (Gibco, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (FBS; Intergen, Purchase, N.Y.). The EMT6 clone B17 cell line was derived by transfection of the parental EMT6 cell line with a retroviral vector containing the murine IFNγ gene and the neomycin-resistance (Neo^r) gene as a selectable marker. Control cells (EMT6-neo) were transfected with vector containing only the Neo^r gene. Tumor cells were maintained in α-MEM supplemented with 10% FBS and 400 μg/ml geneticin (G-418; Gibco, Gaithersburg, Md.).

In vitro stimulation with interferon γ

The effect of IFNγ stimulation on class I and class II MHC expression by EMT6 and B16-F10 tumor cells was determined by incubating 5×10^5 tumor cells for 48 h in the presence of IFNγ (Genzyme Co., Boston, Mass.) at various doses ranging from 10 U/ml to 1000 U/ml in culture medium. After incubation, the cells were trypsinized, collected and stained for MHC class I and II expression by flow cytometry as described below.

Transfection of EMT6 tumor cells

A replication-defective retroviral vector containing the mouse IFNγ transgene (Viagene Inc. San Diego, Calif.) was used to introduce the IFNγ gene into EMT6 cells. The expression of the IFNγ gene was under the control of a Moloney leukemia virus (MuMoLV) long terminal repeat 5′ promoter. The presence in the vector of the Neo^r gene under the control of the simian virus (SV40) early promoter allowed for selection of transfectants in G418-containing tissue-culture medium. To create the bulk IFNγ-transfected EMT6 cell line, 2×10^5 EMT6 cells were incubated for 24 h in α-MEM supplemented with 10% FBS (α10-MEM). The culture medium was then replaced with fresh α10-MEM supplemented with 4 μg/ml polybrene (1,5-dimethyl-1,5-diazaundecamethylene-polymethobromide; Sigma, St. Louis, Mo.). The virions containing the retroviral construct were added at a multiplicity of infection of 5:1. Infection of EMT6 cells was carried out overnight. Tumor cells were then selected at a concentration of 400 μg/ml in α10-MEM for 2 weeks. Transfected cells expressing MHC class II antigens were isolated by fluorescence-activated cell sorting (FACS) analysis and cloned by limiting dilution.

Flow-cytometric analysis

Cell surface expression of class I and class II MHC molecules was determined by flow cytometry with a FACScan fluorescence-activated cell sorter (Becton Dickinson, Mountain View, Calif.). Parental EMT6 and transfected (EMT6-neo, EMT6 clone B17) tumor cells as well as B16-F10 melanoma cells were harvested with trypsin/0.02% EDTA, washed and resuspended in phosphate-buffered saline (PBS) at 5×10^6 cells/ml. All antibody-labeling steps were carried out on ice in the dark. Antibodies were used at a concentration of 1 μg/10^6 cells. Approximately 5×10^5 EMT6 tumor cells were stained with fluorescein-isothiocyanate (FITC)-conjugated anti-class II MHC monoclonal antibody (mAb) (FITC-I-A^d; Becton Dickinson, Mountain View, Calif.) and R-phycoerythrin(PE)-conjugated anti-class I MHC mAb (PE-H-$2D^d$; Pharmingen, San Diego, Calif.) for 30 min. B16-F10 melanoma cells were similarly stained with anti-I-A^b and anti-H-2 K^bD^b monoclonal antibodies (kindly provided by Dr. S. Ostrand-Rosenberg, University of Maryland, Baltimore, Md.) followed by FITC-labeled goat anti-mouse Ig antibody. Expression of IFNγ receptors on EMT6 cells was accomplished using anti-IFNγ-receptor-specific antibodies kindly provided by Dr. Robert Schreiber (Washington University, St. Louis, Mo.). The cells were washed twice with PBS containing 0.1% sodium azide and 0.5% bovine serum albumin. Labeled cells were then resuspended in this buffer and analyzed by flow cytometry. When sorting of IFNγ-transfected cells expressing class II MHC antigens was performed by FACS analysis, 1×10^7 transfected cells were stained according to the aforementioned procedure and bright double-positive cells (for class I and II MHC) were collected. Sorted cells were cultured for 48 h before cloning by limiting dilution.

Analysis of IFNγ gene expression in transfected cells

Total RNA was extracted by the Trizol method (Gibco BRL, Gathersburg, Md.). The resulting RNA was routinely treated with DNase (Boehringer Mannheim, Indianapolis, Ind.) and quantified by spectrophotometry. Equal amounts of RNA (6 μg) from each source were reverse-transcribed using the Invitrogen cDNA cycle kit (Invitrogen Corp., San Diego, Calif.). The cDNA (4 μl) was then amplified by the polymerase chain reaction (PCR) using the following IFNγ primers, designed using the OLIGO program (National Biosciences, Playmouth, Minn.):

IFNγ sense: 5′ GACTCCTTTTCCGCTTCCTG 3′;
IFNγ antisense: 5′ CTGGCAAAAGGATGGTGACA 3′.

Histone primers were included as positive controls in the PCR. The PCR reaction mixture consisted of 0.2 mM dNTP, 1 × Taq DNA polymerase buffer (Boehringer Mannheim Corp., Indianapolis, Ind.), 5 μl cDNA, 5 μl 2 μM IFNγ sense primer (0.2 μM final concentration), 5 μl 2 μM IFNγ antisense primer (0.2 μM final concentration), and 1.5 U Taq DNA polymerase (Boehringer Mannheim Corp., Indianapolis, Ind.) in a 50-μl total reaction volume. The reaction mixtures were treated with 35 cycles of 93 °C for 5 s, 56 °C for 30 s, and 72 °C for 30 s, following addition of Taq DNA polymerase at 80 °C, using a thermal cycler (MJ Research Inc., Watertown, Mass.) with an in-sample temperature probe. Samples comprising 15 μl resulting PCR

products were electrophoresed through a 2% agarose gel, stained with ethidium bromide and photographed.

Analysis of CIITA gene expression in transfected cells

Total RNA was extracted by the Trizol method (Gibco BRL, Gathersburg, Md.) and cDNA was prepared as described above. The cDNA was amplified by touchdown reverse transcriptase/PCR (RT-PCR) [19] using human CIITA primers [4]. The reactions comprised 45 cycles of denaturation at 94 °C for 8 s, annealing at 55 °C for 30 s and extension at 75 °C for 30 s. The annealing temperature was reduced to 42 °C in decrements of 0.3 °C per cycle. This was followed by an additional 10 cycles consisting of 94 °C for 8 s, 42 °C for 25 s, 75 °C for 25 s (following addition of Taq DNA polymerase at 80 °C); 10-µl samples of the resulting PCR products were analyzed by electrophoresis through a 1.5% agarose gel. For sequencing, a 470-base-pair product was ligated to the pGEM-T vector (Promega, Madison, Wis.). Sequencing of the ligated product was performed on both DNA strands using Sequenase (U.S. Biochemical) as described by the manufacturer. The identification of mouse CIITA mRNA was based on the alignment of the nucleic acid and amino acid sequences with the human MHC class II transactivator (CIITA) sequence from GenBank.

IFNγ ELISA

Parental (EMT6) and transfected (EMT6-neo and EMT6 clone B17) tumor cells were harvested, washed and adjusted to 1×10^6 cells/ml in α10-MEM. Aliquots of 10^6 cells were seeded in 24-well plates (Falcon 3047; Becton Dickinson, Mountain View, Calif.) in triplicate and incubated for 48 h. The plates were centrifuged and the supernatants were harvested, pooled and frozen at −77 °C prior to analysis. Thawed supernatants were assessed for IFNγ production by ELISA (Genzyme Co., Boston, Mass.). EMT6 clone B17 tumor cells were also assessed for long-term production of IFNγ after treatment with 50, 100, or 200 Gy irradiation. Supernatants were harvested from irradiated cultures on days 2, 4 and 6.

Determination of biological activity in EMT6 clone B17 supernatant

Supernatants were collected from 48-h cultures of IFNγ-producing EMT6 clone B17 and concentrated ten times on a Centriprep concentrator (Amicon Inc., Beverly, Mass.). Prior to use, the supernatants were diluted to the original concentration in α10-MEM medium. Biological activity was assessed by measuring membrane expression of class I and class II MHC antigens by IFNγ-responsive B16-F10 tumor cells incubated 48 h earlier with cell supernatant.

In vivo tumor growth studies

Prior to these studies, the in vitro growth kinetics of EMT6 clone B17, EMT6-neo and parental EMT6 cells were compared. Their growth rates were not significantly different (data not shown). BALB/c mice were injected s.c. in the right flank with 10^5 viable IFNγ-gene-transfected tumor cells (EMT6 clone B17) in 0.1 ml PBS. Control animals received 10^5 EMT6 cells transfected with vector containing the neomycin-resistance gene (EMT6-neo). Tumor growth was monitored weekly for 21–28 days. Animals that were tumor-free after 4 months received a s.c. challenge of 10^5 viable EMT6 cells. Tumor burden was expressed in terms of tumor volume and calculated using the formula (length $\times$ width^2)/2.

Immunization and tumor challenge studies

Naive BALB/c mice were immunized twice s.c. in the right flank on day 0 (first immunization) and on day 10 (second immunization) with 5×10^6 irradiated (100 Gy) EMT6-neo, EMT6 clone B17 cells or a mixture of the two at various ratios. Five different tumor inocula were prepared for immunizations, one for each group of mice: 100% EMT6-neo, 100% EMT6 clone B17, 90% EMT6-neo + 10% EMT6 clone B17, 80% EMT6-neo + 20% EMT6 clone B17, 50% EMT6-neo + 50% EMT6 clone B17. Fourteen days after the second immunization all groups received a challenge injection consisting of 1×10^5 viable EMT6 in the left flank. Tumor growth was then monitored for 26 days.

Statistical analysis

Statistical significance of immunization and tumor challenge studies was determined by logistic regression analysis [13]. This method relates the logarithm of the odds of tumor rejection to some function of the mixture concentration. The logarithms of the odds of tumor rejection were proportional to the logarithm of the mixture concentration, so that the corresponding logistic regression model used was: log(odds of rejection) = $-2.5658 + 0.6998 \times$ log(mixture concentration) where the coefficient 0.6998 was statistically significant with a P value of 0.004.

Results

Response of EMT6 cells to exogenous IFNγ stimulation

Our first objective was to determine whether EMT6 cells were responsive to IFNγ stimulation. For this purpose parental and mock-transfected (EMT6-neo) tumor cells were incubated for 48 h with recombinant murine IFNγ ranging from 10 U/ml to 1000 U/ml and assessed for class I and class II MHC expression. The data (Fig. 1A) show that the level of constitutively expressed class I MHC antigens (panels a, b, dashed line) was not increased after IFNγ stimulation (panels a, b, solid line). Class II MHC expression was not induced by IFNγ treatment (panels c, d, solid line) compared to unstimulated cells (panel c, d, dashed line). In contrast, B16-F10 melanoma cells that were included as controls (Fig. 1B) demonstrated a significant increase in class I MHC expression (panel a, solid line). Furthermore class II MHC was induced in these cells (panel b, solid line). These results demonstrate that whereas B16-F10 melanoma cells are responsive to IFNγ stimulation after 48 h, EMT6 cells are not. However, continuous exposure of EMT6 cells to IFNγ (100 units/ml) for 7 days induced a bimodal expression of class II MHC with a mean fluorescence channel number of 384, which was still lower than that of IFNγ-transfected (EMT6 clone B17) cells (mean channel number, 520). This high dose (4000 units) of IFNγ over 7 days resulted in only 14% of class II MHC protein expression (data not shown).

Expression of MHC antigens on IFNγ-gene-transfected EMT6 cells

Following the failure to demonstrate responsiveness by EMT6 cells to exogenously administered IFNγ after 48 h, we decided to ascertain the effect of IFNγ transgene on MHC expression. The level of class I MHC expression was not affected by IFNγ gene transfection since the fluorescence intensity of control gene-modified cells (EMT6-neo) and EMT6 clone B17 cells were similar (Fig. 2a, b). The

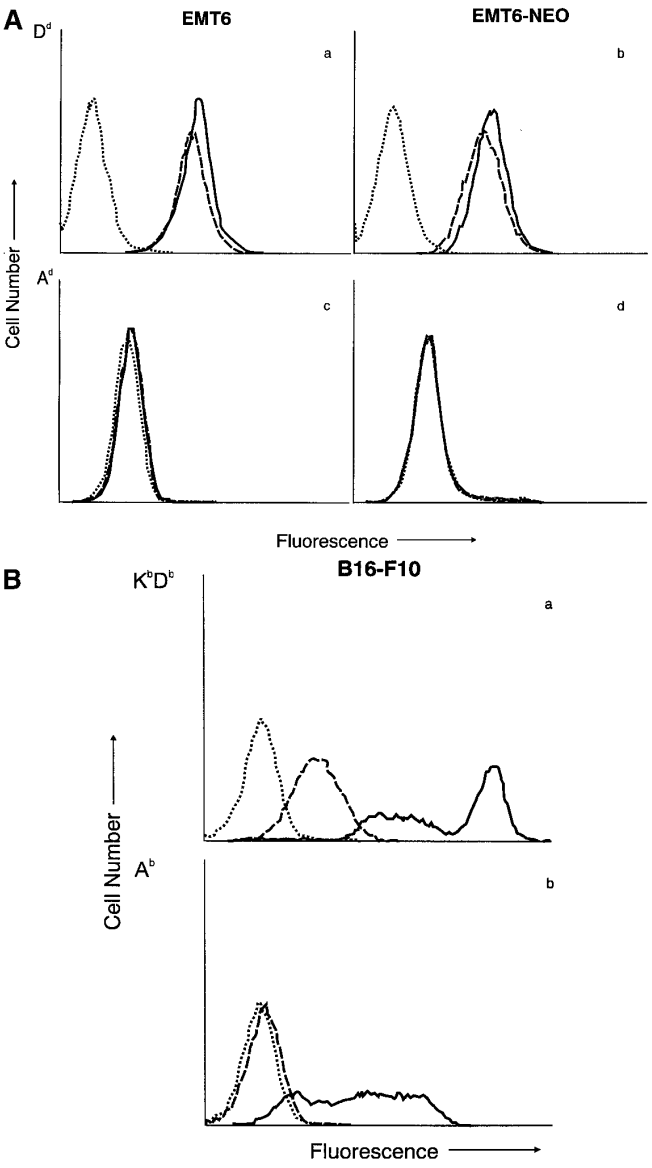

Fig. 1A, B Response of EMT6 tumor cells to exogenous interferon γ (IFNγ) stimulation. Parental EMT6 and EMT6-neo tumor cells were incubated for 48 h with recombinant murine IFNγ ranging from 10 U/ml to 1000 U/ml (100 U/ml IFNγ treatment only is shown as a representative experiment) and assessed for class I and class II MHC expression. EMT6 tumor cells were stained with fluorescein-isothiocyanate(FITC)-conjugated anti-class II MHC mAb (FITC-I-A^d, Becton Dickinson) (**A**: **c**, **d**) and R-phycoerythrin-conjugated anti-class I MHC mAb (PE-H-$2D^d$, Pharmingen) (**A**: **a**, **b**) as described in Materials and methods. B16-F10 melanoma cells were included as controls and similarly stained with anti-I-A^b (**B**: **a**) and anti-H-2 K^b D^b (**B**: **b**) monoclonal antibodies followed by FITC-labeled goat anti-(mouse Ig) antibody. ··· Isotype controls for class I and class II MHC staining respectively, - - - unstimulated cells, —— IFNγ-stimulated cells. *Abscissa* four log cycles of fluorescence intensity (10^1–10^4)

transfer of the IFNγ gene into EMT6 cells resulted in induction of class II MHC (I-A^d; EMT6 clone B17, panel d). In contrast, cells transfected with vector containing the selectable marker (Neo^r) gene (EMT6-neo) did not express class II (panel c), and the constitutive expression of class I was unchanged (panel a).

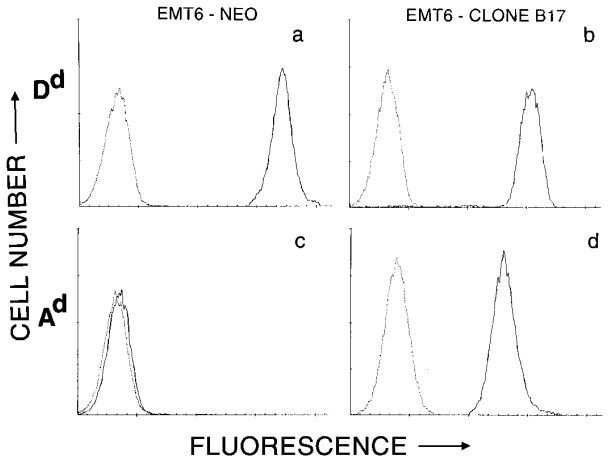

Fig. 2a–d Analysis of MHC class I and II expression on EMT6 IFNγ-transfected cells. Parental EMT6, EMT6-neo and EMT6 clone B17 tumor cells were stained with FITC-conjugated anti-class II MHC mAb (FITC-I-A^d, Becton Dickinson) (**c**, **d**) and R-phycoerythrin(PE)-conjugated anti-class I MHC mAb (PE-H-$2D^d$, Pharmingen) (**a**, **b**) as described in Materials and methods. ··· Isotype controls IgG2a-PE and IgG2b-FITC for class I and class II MHC staining respectively. *Abscissa* four log cycles of fluorescence intensity (10^1–10^4)

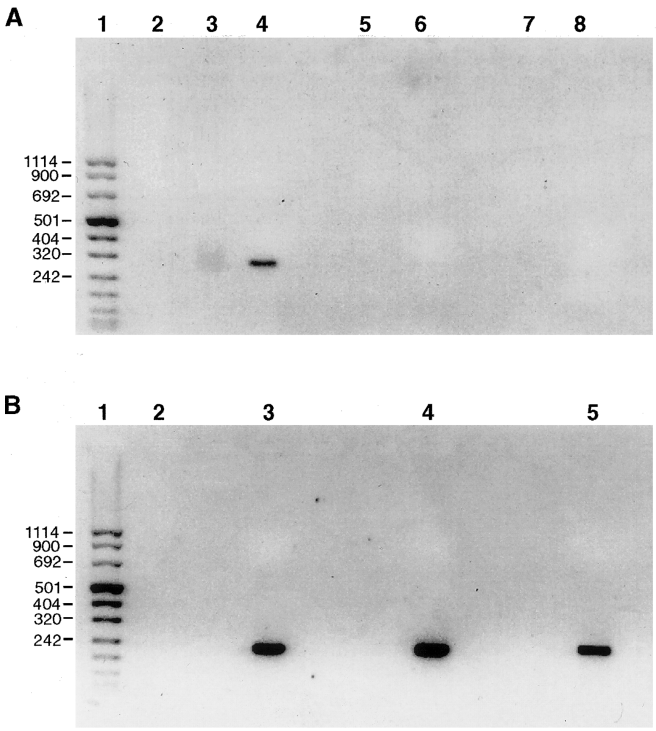

Fig. 3A, B Gene expression by IFNγ-transfected EMT6 cells. IFNγ-transfected EMT6 tumor cells (EMT6 clone B17) were analyzed for gene expression by reverse transcriptase/polymerase chain reaction (RT-PCR). The PCR product of IFNγ is 293 bp. *Lane 1* 1-kb ladder; *lane 2* PCR control (no cDNA template); *lanes 3, 5, 7* RT controls (mRNA, without reverse transcriptase, obtained from EMT6 clone B17, and unmodified EMT6 and EMT6-neo tumor cells respectively); *lane 4* cDNA obtained from EMT6 clone B17; *lane 6* cDNA obtained from unmodified EMT6 tumor cells; *lane 8* cDNA obtained from EMT6-neo tumor cells. As a control, PCR was performed using histone primers (**b**) on cDNA from EMT6 clone B17 (*lane 2*), EMT6 (*lane 3*), and EMT6-neo (*lane 4*) cells

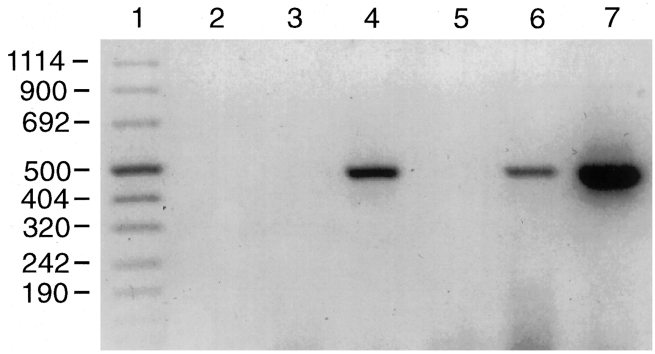

Fig. 4 Expression of CIITA gene in IFNγ-transfected and IFNγ-stimulated EMT6 cells. cDNA obtained from IFNγ-transfected and IFNγ-stimulated EMT6 cells was analyzed by touchdown RT-PCR using human CIITA primers (*lanes 3–7*). The PCR product of CIITA is 471 bp. *Lane 1* molecular weight marker VIII; *lane 2* PCR control (no cDNA template); *lane 3* cDNA obtained from EMT6 cells; *lane 4* cDNA obtained from EMT6 cells stimulated with 100 U/ml IFNγ; *lane 5* cDNA obtained from EMT6-neo cells; *lane 6* cDNA obtained from EMT6-neo cells stimulated with 100 U/ml IFNγ; *lane 7* cDNA obtained from IFNγ-transfected EMT6 cells (EMT6 clone B17) tumor cells

Gene expression by IFNγ-transfected EMT6 cells

In order to determine whether the transgene was expressed in transfected tumor cells, total RNA was isolated, reverse-transcribed to cDNA and analyzed by RT-PCR. The results (Fig. 3A) demonstrated that IFNγ-transfected EMT6 clone B17 cells (lane 4) expressed the IFNγ transgene. In contrast, unmodified parental EMT6 (lane 6) and EMT6-neo tumor cells (lane 8) did not express the IFNγ gene. As a control, PCR was performed using histone primers (Fig. 3B) on cDNA from EMT6 clone B17 (lane 2), EMT6 (lane 3) and EMT6-neo (lane 4).

Expression of CIITA gene in transfected EMT6 cells

Since it has been shown that IFNγ induction of class II MHC requires expression of the class II transactivator (CIITA) in humans [4, 23], we hypothesized that class II MHC expression in IFNγ-transfected mouse cells (EMT6

Fig. 5A,B Sequence alignment and protein homology of mouse CIITA gene. **A** Alignment of mouse CIITA (*top strand*) and human CIITA (*bottom strand*, 2902–3330 bp) nucleotide sequence. *Dashes* indicate identity. **B** Polypeptide sequence similarity between mouse and human CIITA. Identical amino acids are indicated by *dashes* and similar amino acids are indicated by *dots*. The human CIITA primers are not represented in the figure

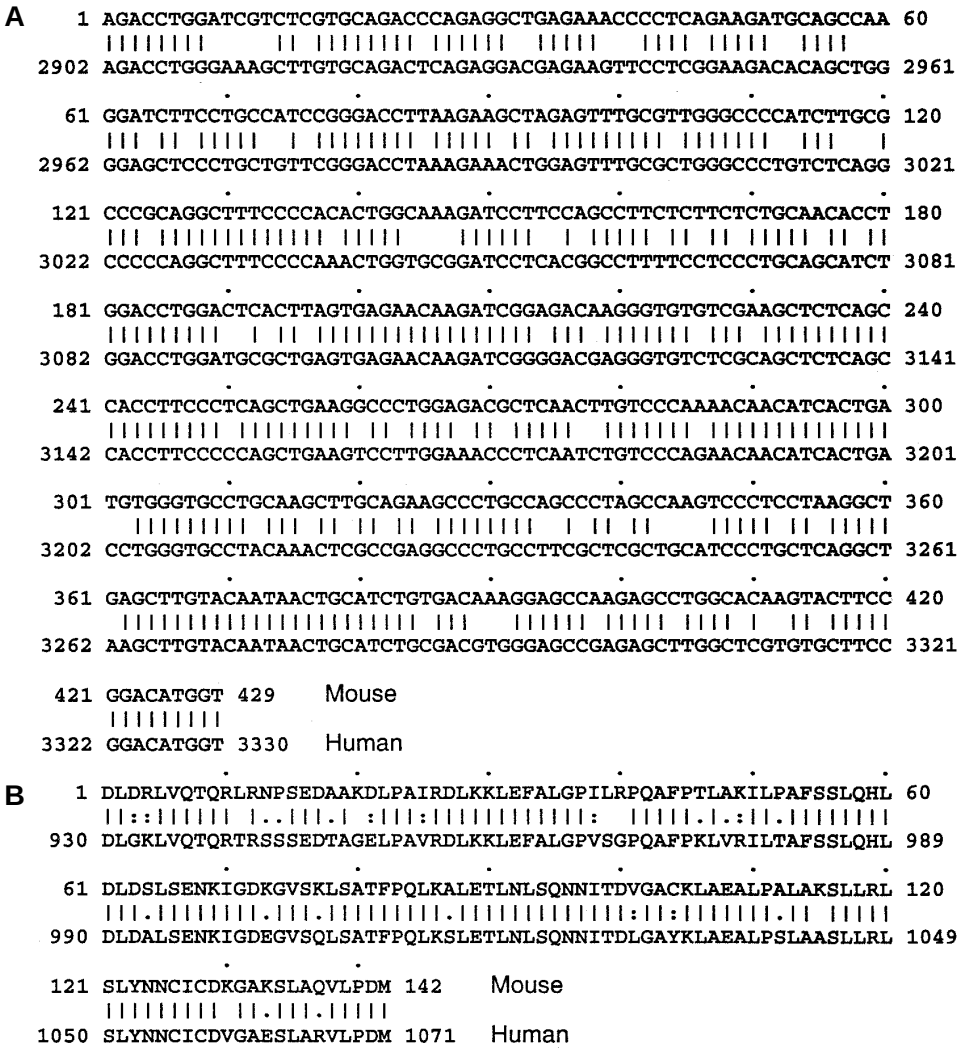

Table 1 Interferon γ (IFNγ) production by IFNγ-transfected EMT6 cells. IFNγ production was determined by ELISA (Genzyme) in 48-h cell-culture supernatants obtained from EMT6, EMT6-neo and IFNγ-transfected EMT6 clone B17 tumor cells. *ND* not detectable (< 125 pg/ml). Values represent means ± SD of triplicate supernatants

Cell line	IFNγ production	
	(pg ml^{-1} (10^6 cells)$^{-1}$	U ml^{-1} (10^6 cells)$^{-1}$
EMT6	ND	ND
EMT6-neo	ND	ND
EMT6 clone B17	16,700 ± 1.33	74.8 ± 0.00

Table 2 Effect of irradiation on IFNγ production by EMT6 clone B17. EMT6 clone B17 tumor cells were examined by ELISA (Genzyme) for long-term production of IFNγ after treatment with increasing doses of gamma irradiation. Values represent means ± SD of triplicate supernatants

Dose (Gy)	IFNγ production		
	Day 2 U ml^{-1} (10^6 cells)$^{-1}$	Day 4 U ml^{-1} (10^6 cells)$^{-1}$	Day 6 U ml^{-1} (10^6 cells)$^{-1}$
50	11.7 ± 1.78	11.6 ± 0.61	12.5 ± 0.40
100	6.63 ± 0.74	8.34 ± 1.78	8.83 ± 0.12
200	8.52 ± 1.61	13.2 ± 1.08	15.5 ± 0.12

clone B17) would correlate with expression of the CIITA gene. Since the mouse CIITA homolog has not been characterized, we used human CTIIA primers to assess gene expression by touchdown PCR. The data show that EMT6-B17 cells and also IFNγ-stimulated EMT6 and EMT6-neo cells expressed a 471-base-pair product (Fig. 4, lanes 7, 4, 6) that was not detectable in unstimulated EMT6 and EMT6-neo cells. (Fig. 4, lanes 3, 5). Similar expression of CIITA was observed in all class-II-expressing clones analyzed (data not shown). Search of GenBank revealed that the sequenced RT-PCR product showed highest similarity to human CIITA cDNA. Sequence alignment revealed 79% identity with the human CTIIA gene spanning base pairs 2902 and 3330. (Fig. 5a). The identity at the protein level was 81% (Fig. 5b). These findings demonstrate that IFNγ-transfected EMT6 cells in which class II MHC antigens are induced also express a mouse gene that is similar to the human CIITA gene.

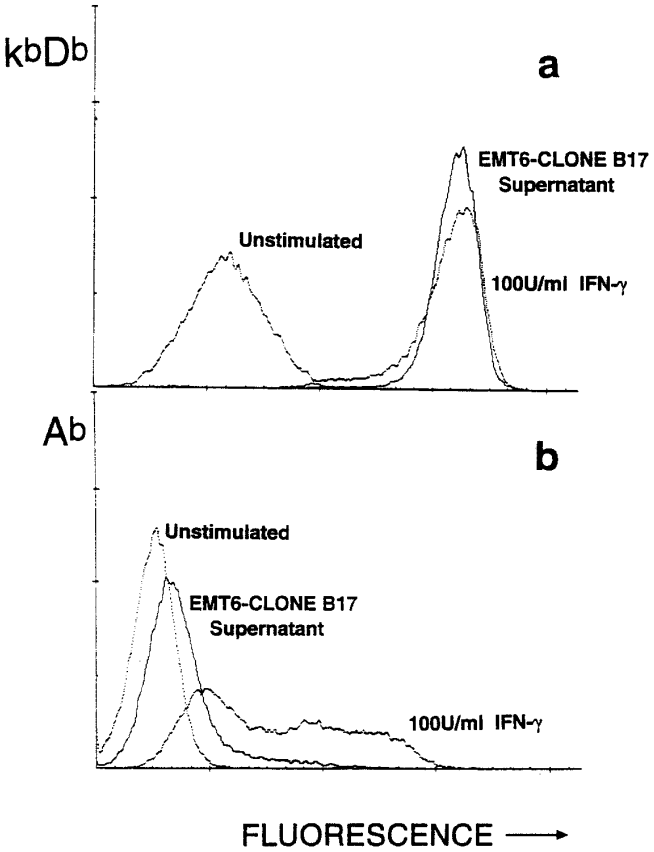

Fig. 6a, b Determination of biological activity in EMT6 clone B17 culture supernatants. Supernatants were collected from 48-h cultures of IFNγ-producing EMT6 clone B17 and concentrated ten times. Prior to use, the supernatants were diluted to the original concentration in α10-MEM medium. Biological activity was assessed by measuring the membrane expression of class I and class II MHC antigens by IFNγ-responsive B16-F10 tumor cells incubated 48 h earlier with cell supernatant. B16-F-10 melanoma cells treated with recombinant murine IFNγ (100 U/ml) were included as controls. Tumor cells were stained anti-H-2 K^b D^b (**a**) and with anti-I-A^b (**b**) monoclonal antibodies followed by FITC-labeled goat anti-(mouse Ig) antibody. *Abscissa* four log cycles of fluorescence intensity

Secretion of IFNγ by gene-modified EMT6 cells

The amount of IFNγ secreted by transfected EMT6 cells was determined by ELISA (Genzyme) on supernatants of 48-h cell cultures obtained from parental EMT6 and transfected (EMT6-neo and EMT6 clone B17) tumor cells (Table 1). The results showed that 10^6 EMT6 clone B17 tumor cells secreted 16 700 pg/ml IFNγ (equivalent to 75 U/ml) whereas EMT6-neo and parental EMT6 cells did not produce any detectable IFNγ (less than 125 pg/ml). The presence of biological activity in IFNγ secreted by transfected cells was confirmed by the ability of supernatants collected from 24-h EMT6 clone B17 cultures to increase membrane class I MHC expression (Fig. 6a) and induce class II MHC expression (Fig. 6b) in B16-F10 tumor cells.

Effect of irradiation on IFNγ secretion by transfected EMT6 cells

We assessed the effect of gamma irradiation on the ability of EMT6 clone B17 cells to secrete IFNγ since non-replicating cytokine-producing tumor cells would constitute the most desirable vaccine in the clinical setting. For this purpose IFNγ-producing EMT6 clone B17 cells were gamma-irradiated and analyzed for IFNγ production. The data (Table 2) show that, even at high doses of irradiation (200 Gy), transfected cells still retained the ability to secrete IFNγ albeit at much lower levels. Irradiation doses above 50 Gy did not further decrease IFNγ production. Furthermore, IFNγ was detectable in culture supernatants

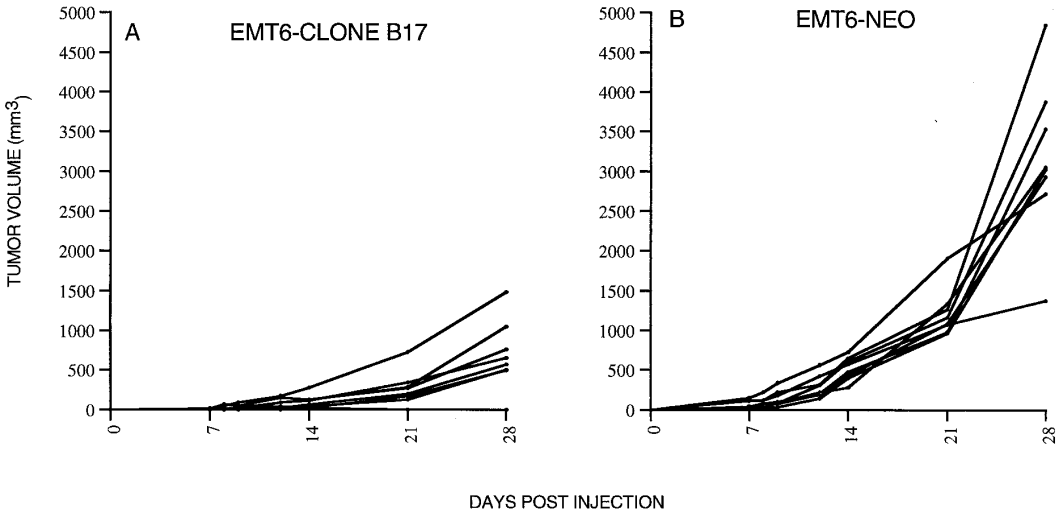

Fig. 7A, B In vivo growth of EMT6 clone B17 and EMT6-neo tumor cells. Sixteen BALB/c mice (8 animals/group) were injected with either 10^5 viable EMT6 clone B17 (**A**) or EMT6-neo (**B**) in 0.1 ml PBS in the right flank. Tumor growth was monitored for 28 days. Each line represents tumor growth in individual animals

for up to 6 days after high-dose irradiation (200 Gy). Significantly, irradiation did not affect the level of expression of class II MHC (data not shown).

In vivo tumor growth of IFNγ-transfected EMT6 cells

In vivo growth of IFNγ-transfected EMT6 cells was determined in euthymic BALB/c mice. Mice were injected either with 10^5 viable EMT6 clone B17 (Fig. 7a) or EMT6-neo (Fig. 7b). Tumor growth was monitored for 28 days. The data demonstrated that tumor growth was inhibited in 7 out of 8 mice injected with EMT6 clone B17 (100% positive for MHC class II expression) with rejection occurring in 1 mouse. In contrast tumors grew progressively in all 8 animals injected with EMT6-neo cells. By day 28, EMT6-neo tumors had reached a maximum of 4800 mm^3 whereas the majority of tumors in animals injected with the IFNγ-transfected EMT6 clone B17 were below 1000 mm^3.

Development of tumor resistance in vaccinated animals

The ability of IFNγ-transfected tumor cells to induce immunity to wild-type EMT6 cells was studied using a vaccine-type approach (Fig. 8). Naive BALB/c mice were immunized twice s.c. on day 0 (first immunization) and at day 10 (second immunization) with 5×10^6 irradiated (100 Gy) EMT6-neo, EMT6 clone B17 tumor cells or a mixture of both at various ratios. Animals were challenged 14 days later with an inoculum of 1×10^5 parental EMT6 cells and tumor growth was monitored for 26 days. When mice were immunized with 100% EMT6-neo, 11 out of 12 animals developed tumors. As the percentage of EMT6 clone B17 cells in the immunization mixture increased from 10% to 100%, the number of animals bearing tumors decreased in a dose response-fashion (Fig. 8). Immunizations with 100% EMT6 clone B17 cells followed by challenge with parental EMT6 cells resulted in tumor rejection in 67% of animals (8 out of 12) and in the lowest mean tumor volumes (709 mm^3) (Fig. 8). Animals that rejected the tumor challenge remained tumor-free until they were terminated on day 56. These results demonstrate that EMT6 clone B17 cells are capable of inducing protection against subsequent challenge with parental, unmodified tumor cells, and that 10% EMT6 clone B17 tumor cells

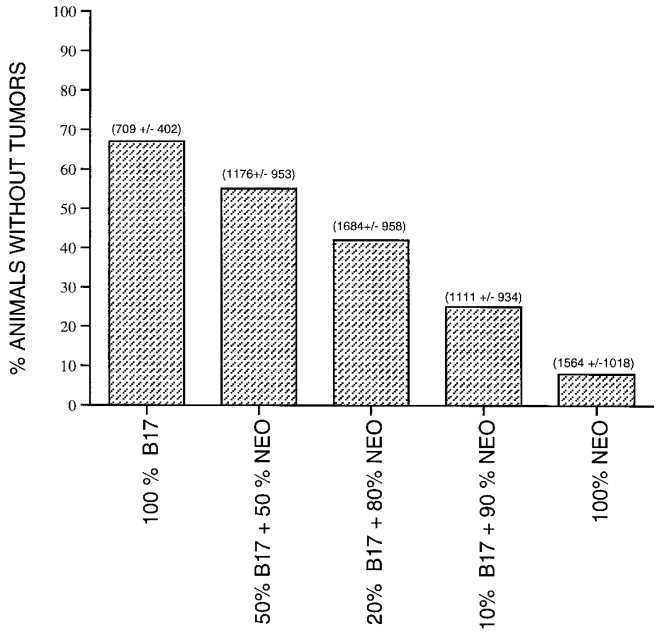

Fig. 8 Inhibition of tumor growth in immunized mice. BALB/c mice (12 per group) were immunized twice s.c. on day 0 and day 10 with 5×10^6 irradiated (100 Gy) EMT6-neo or EMT6-B17 tumor cells or a mixture of the two at various ratios. Fourteen days after the second immunization all groups received a s.c. challenge consisting of 10^5 viable parental EMT6 cells. Tumor growth was monitored for 26 days after tumor challenge. Numbers in parentheses represent mean tumor volume (mm^3) in those animals that did not reject tumors in each treatment group

in the immunization mixture is sufficient to induce immunological resistance to wild-type tumor cells.

Discussion

In this study we sought to increase the immunogenicity of a tumorigenic cell line that is poorly responsive to exogenous IFNγ stimulation by transfer of the IFNγ gene. The results indicate that expression of the transgene induces class II MHC antigens and considerably increases tumor immunogenicity. The failure of either the class-I-MHC-expressing EMT6 cells or mock transfectants (EMT6-neo) to induce tumor immunity strongly suggests that IFNγ-induced class II expression plays a significant role in the measured immune response.

The demonstration in the current study that irradiated IFNγ-transfected cells continue to secrete IFNγ supports the findings of Gastl et al. using human renal cell carcinoma [9]. What is more important, we provide direct evidence that immunization with these cells results in the development of immunity capable of rejecting wild-type tumorigenic cells. The ability to induce antitumor responses with irradiated gene-transfected cells is necessary for this approach of immunopotentiation to be used in the clinic for the treatment of cancer. Vaccinating patients with viable replicating tumor cells is not a feasible option. In experiments in which various percentages of parental EMT6 cells were mixed with IFNγ-secreting cells, we showed that a vaccine preparation consisting of only 10% irradiated gene-modified cells was able to confer partial or complete protection against a rechallenge with parental cells. As it can be technically difficult and time-consuming to obtain 100% gene-modified tumor cells, our experiments indicate that significant clinical benefit may be achieved using a small fraction of transfected cells.

Our studies demonstrating induction of class II MHC following IFNγ gene transfer are different from other studies in which either class I was induced or the levels of constitutively expressed class I were increased [7, 14, 17, 21, 26]. In all instances, increased class I MHC expression resulted in enhancement of tumor immunogenicity [7, 14, 17, 21, 26]. The induction of class II MHC and the attendant immunogenicity of transfected cells offer the intriguing possibility that IFNγ-producing, class-II-positive tumor cells may be acting as antigen-presenting cells. In this role, the tumor cell could either present exogenous peptides (which may arise from degradation products of dead tumor cells) or endogenous peptides to $CD4^+$ T cells [5, 10, 15]. This property may serve to complement the well-described immunostimulatory effects of secreted IFNγ on effector cells mediating tumor rejection [1, 7, 25].

The interesting finding that IFNγ gene transfer induced class II MHC antigen in EMT6 cells whereas exogenously supplied IFNγ was unable to do so after 48 h suggests that the continued presence of IFNγ is necessary to elicit a biological response. This is supported by the observation that continuous exposure of EMT6 cells to IFNγ for 7 days induces class II MHC.

The transcription of a mouse class II transactivator (CIITA) gene in IFNγ gene-transfected tumor cells and not in unstimulated parental or mock-transfected cells suggests a primary role for IFNγ in class II MHC expression that involves CIITA as previously reported by others [4, 23]. The finding that CIITA is expressed in parental or mock-transfectants stimulated with exogenous IFNγ for 48 h without concomitant detection of membrane class II MHC antigens is consistent with the hypothesis that CIITA is required for, and is transcribed prior to the expression of class II MHC [4, 23]. The transcription of CIITA in these cells suggests that the pathway of signal transduction involving JAK and STAT proteins [4, 6, 23] upstream of CIITA is intact. However, the absence of detectable class II antigens after 48 h on these cells may be because the levels of CIITA are insufficient to induce class II gene expression [4].

The induction of class II MHC antigens following CIITA gene expression in transfected cells could be a consequence of continued intracellular synthesis of IFNγ. The mechanism of action may be via binding of the secreted protein to its membrane receptors or via binding of cytoplasmic IFNγ to components of an intracellular pathway that bypasses these receptors. Previous reports have demonstrated that human IFNγ introduced intracellularly by microinjection or gene transfection was able to induce class II MHC in murine macrophages and L cells presumably by interacting with transcription factors [20, 22]. In addition, expression of the IFNγ transgene may induce transcription of as yet unidentified class II MHC promoter-DNA-binding proteins. Since CIITA does not by itself bind the MHC class II promoter DNA [24], it has been proposed that these DNA-binding proteins form an obligate complex with CTIIA to activate class II genes [18, 27]. These studies indicate that the poor responsiveness of EMT6 cells to IFNγ stimulation (demonstrated by its failure to express class II MHC after 48 h) can be rectified by inserting the IFNγ transgene, which induces class II MHC gene expression presumably via a CIITA-mediated pathway [4, 23]. Experiments are in progress to clone the mouse CIITA gene and to assess its role in tumor immunogenicity.

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