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Original Paper

Tumour Cell Vaccines That Secrete Interleukin-2 (IL-2) and Interferonγ (IFNγ) are Recognised by T Cells While Resisting Destruction by Natural Killer (NK) Cells

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The inoculation into mice of genetically engineered tumour cells that secrete IL-2 or IFNγ results in rejection, while unmodified parental tumour cells grow progressively. *In vivo* studies demonstrated synergy between IL-2 and IFNγ leading to the rejection of the transduced tumour cells. IL-2 is required for T cell proliferation and differentiation. IFNγ induced the upregulation of MHC class I molecules that present peptides to CD8$^+$ T cells. Furthermore, IFNγ can correct defects in antigen processing. Thus, for T cells, IL-2/IFNγ-secreting double cytokine tumour cell vaccines might serve as class I$^+$ peptide/antigen presenting depots for developing effector cells. In contrast to T cells, NK cells exert spontaneous killing and kill class I$^+$ targets less well than those that are class I$^-$. For this reason, they may actually have a detrimental effect by destroying a class I$^+$ tumour cell vaccine before adequate T cell stimulation occurs. Based upon this rationale, we tested the hypothesis that an unrecognised benefit of increased class I expression by tumour cells in response to IFNγ secretion would be to enable cytokine-secreting vaccine cells to resist destruction by NK cells. Our results demonstrated that T cells recognised tumour cells secreting IFNγ better than those secreting IL-2. NK cells, in contrast, were inhibited by tumour cells that secreted IFNγ, but not by those that secreted IL-2. The findings suggest that, in addition to upregulating adhesion molecules, MHC molecules, and correcting defects in antigen presentation pathways, IFNγ secretion may protect tumour cell vaccines from early NK-mediated destruction, keeping them available for T cell priming.

Key words: gene therapy, T cells, tumour vaccines, IL-2, IFNγ
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INTRODUCTION

THE EXPRESSION of diverse surface proteins on tumour cells, including major histocompatibility complex (MHC) antigens, costimulatory molecules, and adhesion determinants, is required to induce effective immune responses. While, for some cells, expression of these molecules is constitutive, for others they are strongly influenced by the cytokines secreted. We and others have shown in animal models that while parental tumour cells grow progressively, the inoculation of genetically engineered tumour cells that secrete interleukin-2 (IL-2) or interferon γ (IFNγ) results in rejection [1–3]. Both T and natural killer (NK) cells were involved. Recently, we reported more rapid rejection of primary tumours transduced with a retroviral vector containing both IL-2 and IFNγ cDNAs compared to tumour cells secreting similar levels of either cytokine alone [4]. While the underlying mechanism is not fully understood, the known actions of these cytokines provide some hints. Evidence from many systems has shown that IL-2 can augment effector activity of both T [5] and NK cells [6]. IFNγ also indirectly stimulates T cells by upregulating MHC expression [7] and influencing antigen processing, thereby favouring peptide presentation to cytotoxic T lymphocytes (CTL) [8]. NK cells, in contrast, are less able to lyse target cells as MHC expression is increased [9,10]. In humans, the benefits of gene therapy with cytokine-secreting tumour cell vaccines most likely will not bear on the rejection of primary tumours, but rather on the destruction of distant micrometastases by T cells. Thus, vaccines secreting cytokines favouring the sensitisation of T cells, while limiting the destruction of the vaccine itself by NK cells, seem promising.

Cytokines are pleiotropic molecules that exert either positive or negative effects depending upon the particular setting. Cytokines used in vaccination approaches must be carefully selected, since they influence the nature of the subsequent immune response that develops. T cells are thought to be

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especially important in antitumour responses, because of their unique properties of specificity and memory. $CD8^+$ T cells recognise peptides presented by MHC class I molecules and require IL-2 to proliferate [11,12]. Some tumour cells used to stimulate T cells have been shown to have defects in the antigen presentation pathways which can be reversed by the addition of IFNγ [8]. Thus, for T cells, an IL-2/IFNγ-secreting double cytokine tumour cell vaccine might serve to convert a tumour cell incapable of presenting antigen into one able to fulfil this function, at least for memory T cells, enabling effector cell differentiation. In contrast, NK cells exert spontaneous killing and do not need to see a source of antigen in order to mature. As part of the innate immune system, they may be of special benefit early in the antitumour response, before the T cells have had the time to differentiate into CTL. In fact, NK cells actually could be detrimental by destroying a tumour vaccine before optimal T cell stimulation has had a chance to occur. For this reason, an additional advantage of IFNγ expression might be to make tumour cell vaccines less susceptible to NK-mediated lysis by increasing surface class I levels.

Previously, we reported that IL-2-secreting tumour cells were rejected by T cell deficient nude mice [13]. By contrast, double cytokine (IL-2 and IFNγ)-secreting vaccines were rejected by mice that had been depleted of $CD4^+$ cells, but not by those depleted for $CD8^+$ cells, even if they had NK cells [4]. Mice that had been depleted only of NK cells rejected the double cytokine-secreting tumour cells, but in a much delayed manner compared with control undepleted mice. Taken together, the results of these *in vivo* studies suggest that IL-2- and IFNγ-secreting tumour cells could be rejected by $CD8^+$ cells alone, but not by NK cells alone. However, the delayed rejection caused by NK depletion suggests that they might co-operate with $CD8^+$ cells in the antitumour response. This led to the working hypothesis that IL-2/IFNγ-secreting tumour cell vaccines with upregulated MHC class I levels might be more resistant to early destruction by NK cells, further increasing their chance to prime T cells.

To test this, we evaluated the effects of IL-2 and IFNγ secretion, singly or together, on the susceptibility of tumour cells to T cell- versus NK cell-mediated lysis. The results demonstrated that CTL recognised tumour cells secreting IFNγ better than those secreting IL-2. In contrast, NK cells lysed tumour cells secreting IL-2, but were inhibited by tumour cells that secreted IFNγ. These findings suggest that, in addition to upregulating adhesion and MHC molecules and correcting defects in antigen presenting pathways, another benefit of IFNγ secretion might be to protect tumour cell vaccines from premature NK-mediated destruction. *In vivo*, this might contribute to the time span during which T cells could be primed against the tumour vaccine.

MATERIALS AND METHODS

Tumour cell lines and mice

CMS5 is a methylcholanthrene induced fibrosarcoma of BALB/c ($H\text{-}2^d$) origin grown in DMEM medium (Bio Whittaker, Walkersville, Maryland, U.S.A.) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah, U.S.A.). BALB/c mice were obtained from a colony bred at Sloan-Kettering Institute from which the CMS5 tumour originated. C3H/Hen mice ($H\text{-}2^k$) were purchased from Charles River Breeding Facility (Wilmington, Massachusetts, U.S.A.).

Retroviral-mediated transduction of tumour cells

The generation of the IL-2, IFNγ, and IL-2/IFNγ-secreting CMS5 tumour cells using the N2 retroviral vector has been described previously [4,14,15]. Cytokine production was assessed initially using a bioassay [16] and then quantified using ELISA. Two hundred sixty-eight pg/10^6 cells/24 h of IFNγ and 53 pg/10^6 cells/24 h of IL-2 were secreted, respectively, from single cytokine producers. Tumour cells secreting both cytokines produced 159 pg/10^6 cells/24 h of IFNγ and 58 pg/10^6 cells/24 h of IL-2.

Monoclonal antibodies and flow cytometry

To determine the expression of adhesion molecules, 0.5×10^6 spleen cells were stained with purified anti-ICAM-1 antibody (CD54, hamster IgG, Pharmingen), anti-ICAM-2 (CD102, rat IgG2a, Pharmingen, San Diego, California, U.S.A.), or culture supernatant from the mouse homologue of LFA-3 (CD48, a kind gift from Dr Hans Reiser). An anti-B7-1 monoclonal antibody (1G10, rat IgG2a, Pharmingen) was used to screen cells for B7-1 expression. A monomorphic anti-class I monoclonal antibody (42.3.9.8 HLK, rat IgG2a, Pharmingen) and anti-class II antibody (B21-2, IgG2b, Pharmingen) were used as supernatants to detect these MHC products. To detect $H\text{-}2K^d$ and $H\text{-}2D^d$, supernatants of the allele-specific antibodies 34-5-8S (IgG2a) and SF1-1.1 (IgG2a) were used, respectively. Cells were reacted with 100 ng of purified antibody or 100 μl of culture supernatant from the desired antibody for 30 min at 4°C. Samples were washed twice in FACS buffer (PBS with 3% FBS and 0.2% sodium azide) and then appropriate FITC-conjugated Fab'_2 fragments (Biosource International, Camarillo, California, U.S.A.) were added. After another 30 min at 4°C they were washed as for the primary antibody. Samples were analysed for fluorescence using a Coulter Profile II flow cytometer. Results shown are representative of two to three experiments.

Generation of CTL, NK cells and cell-mediated lympholysis (CML)

To raise anti-$H\text{-}2^d$ CTL 15×10^6 C3H ($H\text{-}2^k$) splenocytes depleted of erythrocytes were stimulated with 12×10^6 X-irradiated (2000 rad) BALB/c ($H\text{-}2^d$) splenocytes in RPMI supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 0.1 mM NEAA, and 5×10^{-5} β-mercaptoethanol for 6 days at 37°C with 6% CO_2. Activated NK cells were generated by culturing Nylon wool depleted C3H spleen cells at 2×10^6/ml in the same medium as described above, but supplemented with 1000 U/ml of rIL-2 (Chiron, Emeryville, California, U.S.A.) for 3 days. At this point, the non-adherent cells were discarded and the adherent cells allowed to grow in the same medium for an additional 3 days. Cytotoxicity of CTL and NK cells were assayed in a 4-h chromium release assay carried out in u-bottom 96-well microtiter plates. Target cells were prepared by culturing cells for 1.5 h at 37°C in 6% CO_2 in the presence of 150 μCi of ^{51}Cr sodium chromate (NEN, Boston, Massachusetts, U.S.A.), washed twice and counted. Ten thousand labelled target cells per well were mixed with effector lymphocytes to yield several effector to target (E:T) cell ratios and incubated for 4 h. Per cent specific lysis was calculated as:

$$\frac{\text{cpm experimental wells} - \text{cpm spontaneous release}}{\text{cpm maximum release} - \text{cpm spontaneous release}} \times 100\%$$

RESULTS

Expression of adhesion and costimulatory molecules by IFNγ and IL-2-secreting tumour cells

ICAM-1 and ICAM-2 are ligands for the adhesion molecules LFA-1 [17] and CD48, the murine homologue of CD2 [18]. These monomorphic molecules play an important role in non-specifically increasing the strength of the binding between the T cell receptor and MHC class I or class II and peptide. B7-1 is a costimulatory molecule that provides a second signal to T cells that have received signal 1 via the occupancy of their T cell receptor by specific peptides [19]. Signal 2 is believed necessary to prevent the development of anergy [20]. To determine the effect of IFNγ versus IL-2 on their expression, tumour cells were stained with monoclonal antibodies directed against ICAM-1, ICAM-2 (Figure 1a), CD48, or B7-1 (Figure 1b) followed by a secondary antibody conjugated with FITC. Class I expression served as our positive control and staining with the secondary antibody alone served as our negative control. Fluorescence was analysed via flow cytometry. We observed that levels of ICAM-1 were modestly increased by cells secreting IFNγ alone or together with IL-2, whereas no increase of ICAM-2, B7-1 or CD48 was detectable on any of the populations.

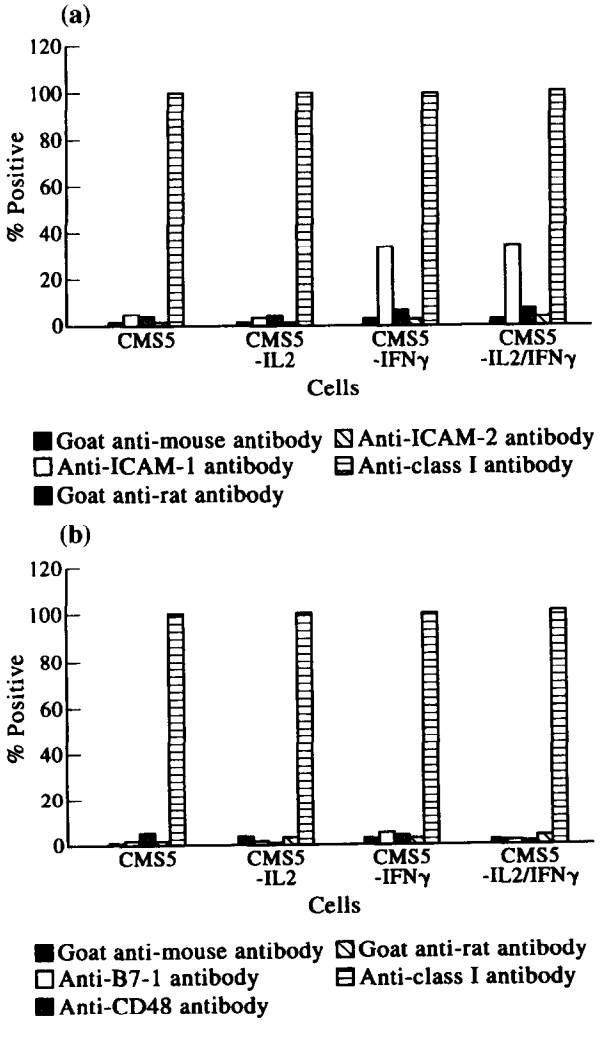

Figure 1. (a) ICAM-1 but not ICAM-2 is upregulated by IFNγ. (b) Neither B7-1 nor CD48 is upregulated by IFNγ or IL-2. The bars represent fluorescence obtained from cells stained with the secondary antibody alone, or with a specific antibody and a secondary antibody.

The density of class I but not class II MHC molecules is increased on IFNγ-secreting cells

The ability of IFNγ to upregulate MHC expression is well known and has direct bearing on functional responses elicited [7]. For example, class II expression is required to present peptides to $CD4^+$ T cells [19] and class I expression is necessary to confer susceptibility to lysis by $CD8^+$ CTL, since the latter recognises peptides only when they are presented in the context of class I [11,12]. In contrast, lysis of target cells by certain NK cells is inhibited by the expression of class I [9,10]. Parental CMS5 cells are class I^+ and are class II^-. When the expression of MHC antigens on IFNγ or IFNγ/IL-2-secreting tumour cells was examined, we observed strong upregulation of class I density, reflected by the increase in the log mean fluorescence intensity (MFI), but only weak or no upregulation of class II (Figure 2). The surface phenotype of tumour cells that secreted IL-2 alone resembled parental tumour cells (results not shown).

Recently, it has been reported that the expression of different class I loci does not influence susceptibility to NK killing equivalently. In particular, the expression H-$2D^d$ but not H-$2K^d$ molecules has been associated with resistance to killing of target cells by NK cells that expressed Ly49, a surface receptor believed to deliver a negative signal following MHC recognition by NK cells [21,22]. To determine whether all H-$2D^d$ and H-$2K^d$ molecules were upregulated equally on CMS5 cells, allele-specific monoclonal antibodies were used for staining. Our results demonstrated that the expression of H-$2D^d$ was increased preferentially compared to that of H-$2K^d$, reflected by the increase in the log MFI (Figure 3). Based upon these findings, we were interested in assessing susceptibility of the tumour cells to lysis by CTL versus NK cells.

Susceptibility of cytokine-secreting tumour cells to CTL versus NK cells

To determine how the altered surface antigen pattern resulting from IL-2 versus IFNγ secretion influenced the susceptibility of CMS5 cells to lysis, anti-H-2^d CTL or activated NK cells were generated. Effector cells were then tested for their ability to lyse parental, IL-2, IFNγ, or IL-2/IFNγ-secreting tumour targets in a conventional ^{51}Cr-release assay.

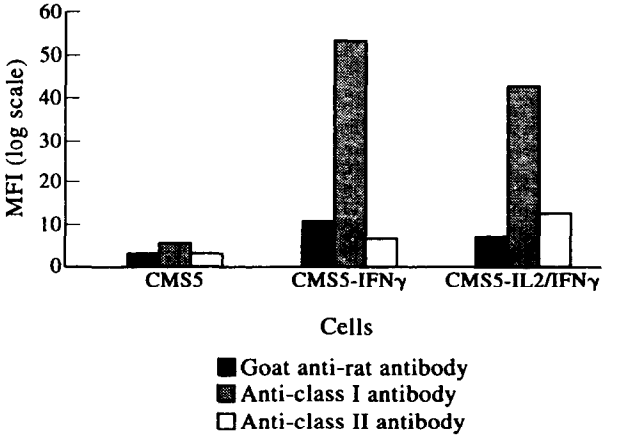

Figure 2. Upregulation of class I but not class II by tumour-derived IFNγ. All cells were greater than 93% positive for class I.

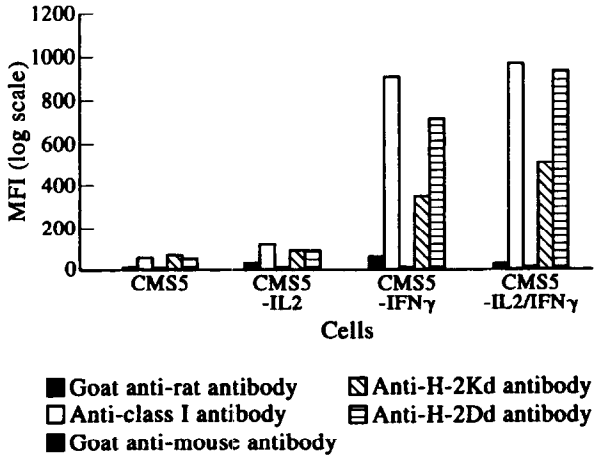

Figure 3. H-2D^d class I is preferentially upregulated on CMS5 by IFNγ.

NK and CTL populations were readily distinguished from one another based upon the generally higher levels of lysis by the NK cells (note the difference in the E:T ratios for each population) and upon the pattern of lysis on P815 (H-2^d) and YAC-1 (H-2^k), the latter a prototypic NK target (Table 1). The anti-H-2^d CTL lysed P815 cells well, but lysed class I$^-$ YAC-1 cells far less efficiently. In contrast, the activated NK cells lysed P815 cells poorly, but the YAC-1 cells strongly. As expected, based upon the increased levels of surface class I, target cells secreting IFNγ or IL-2/IFNγ were somewhat more strongly killed by CTL than were parental cells. The relatively modest increase in killing of IFNγ secretors compared to parental CMS5 cells may have been a result of the fact that the latter were greater than 93% class I$^+$. In contrast, NK killing of IFNγ or IFNγ/IL-2-secreting tumour cells was markedly reduced compared to that of parental or IL-2-secreting cells. The reduced killing of the INFγ/IL-2 secreting tumour cells compared to those secreting IFNγ was associated with increased levels of H-2D^d expressed by the double cytokine secreting cells.

DISCUSSION

Immunisation with cytokine-secreting tumour cells offers a novel approach to the immunotherapy of cancer. Much has been written about the ability of a wide variety of cytokines to cause the rejection of transduced tumour cells following their injection into healthy animals in diverse model systems [1–3]. In selected cases, investigators have succeeded in obtaining regression of established tumours by immunising tumour-bearing mice with cytokine-secreting tumour cell vaccines [23–27]. While these studies contributed to establishing the power of the approach to influence the generation of anti-tumour responses, they may have been less relevant to the application of cytokine gene therapy to human cancer, given the frequent immune suppression of tumour-bearing hosts. In previous studies, we reported diminished levels of p56^{lck}, p59^{fyn}, and/or ζ proteins in mice bearing parental tumours, but not in mice bearing slowly growing tumours that secreted moderate levels of IL-2 [28]. These T cells failed both to transduce activation signals through the T cell receptor and to develop normal function responses [29]. Based upon these results, and those of other groups, our working hypothesis is that immunotherapy with cytokine-secreting tumour vaccines would be of benefit as adjuvant therapy to prevent the outgrowth of micrometastases in patients whose tumours have been resected and in whom immune responsiveness has been restored [30]. Given the number of patients who remain healthy for long periods of time following tumour resection and chemotherapy, only to relapse at a future point, the application of gene therapy to this population would potentially benefit large numbers of patients. In such circumstances it would be desirable for the tumour cell vaccine to persist *in vivo* as long as possible, in order to stimulate most efficiently the activation and differentiation of the T cells that could migrate into the periphery and kill tumour cells found at distant sites.

IL-2 and IFNγ are used in many vaccine approaches because of the demonstrated effect of IL-2 on effector cell proliferation and differentiation [6] and of IFNγ on MHC class I upregulation [7] and the reversal of defects in antigen presentation [8]. Each of these biological effects is important in eliciting effective T cell responses. However, the reported inverse relationship between the expression of class I MHC and susceptibility to NK-mediated lysis [9,10], suggests that an increase in class I expression by IFNγ could have a negative influence on target cell lysis. For this reason, we hypothesised that an additional effect of IFNγ secretion by tumour cell vaccines might be to inhibit their destruction by NK cells.

Previous *in vivo* studies demonstrated that, although NK

Table 1. Lysis of cytokine-secreting tumour cells by CTL and activated NK cells

Effectors		Target Cells CMS5	CMS5-IL2	CMS5-IFNγ	CMS5-ILS2/IFNγ	P815	YAC
C3H × BALB/c CTL (anti-H-2^d)	E:T*						
	100:1	31.1†	38.5	46.1	50.9	83.5	43.6
	50:1	21.1	27.6	30.2	34.2	73.2	26.7
	25:1	10.4	18.6	21.4	18.8	44.6	23.3
	12.5:1	5.1	12.4	16.8	16.9	36.7	21.7
Activated NK	15:1	50.1	51.2	35.2	17.6	13.6	53.5
	7.5:1	31.8	40.6	24.6	9.5	9.5	37.3
	3.75:1	27.7	29.2	15.9	6.2	3.1	50.4
	1.88:1	19.6	18.5	11.4	3.2	1.9	39.9

*E:T, effector : target cell ratio. †Data represent per cent lysis. These results are from one of three similar experiments done.

cells could reject IL-2-secreting tumour cells [13], $CD8^+$ cells were required for rejection of IL-2/IFNγ-secreting cells [4]. NK cells without $CD8^+$ were not sufficient. This suggested that the combination of IL-2 and IFNγ made the tumour cells less susceptible to NK-mediated lysis. The results of this study demonstrated that tumour cells that secreted IFNγ, with or without IL-2, were more readily recognised by T cells but were less sensitive to NK-mediated lysis compared to parental tumour cells. The findings provide a mechanism to explain earlier results that showed that double cytokine-secreting tumour cells persisted longer in mice depleted of NK cells than they did in untreated mice or mice depleted of $CD4^+$ cells [4]. Thus, along with augmenting the ability of tumour cells to function as target cells for T cells, IFNγ secretion may protect tumour cells from destruction by NK cells, making them available to prime T cells longer. In this way, the balance between the amount of source of IL-2 and IFNγ can have a major impact on the eventual antitumour response [31,32].

One could imagine that in cancer patients, cytokine-secreting vaccines might educate CTL that then migrate into the periphery and kill parental tumour cells. Alternatively, cytokine-secreting vaccines might induce the generation of T helper cells able to secrete cytokines and influence the antitumour response in other ways, for example, by activating antigen-presenting cells, or by upregulating surface determinants necessary for CTL-mediated lysis. Taken together, the results shown in this study emphasise the power of gene transfer to exploit the well defined biological effects of each cyotkine. This should help to bring us a step closer to the generation of a more effective-anticancer vaccine.

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