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Review article

Antibody–cytokine fusion proteins for the therapy of cancer

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Abstract

Advances in genetic engineering and expression systems have led to rapid progress in the development of antibodies fused to other proteins. These 'antibody fusion proteins' have novel properties and include antibodies with specificity for tumor associated antigens fused to cytokines such as interleukin-2 (IL2), granulocyte/macrophage colony-stimulating factor (GM-CSF), and interleukin-12 (IL12). The goal of this approach to cancer therapy is to concentrate the cytokine in the tumor microenvironment and in so doing directly enhance the tumoricidal effect of the antibody and/or enhance the host immune response (T-cell, B-cell or NK) against the tumor. In the past decade, multiple antibody–cytokine fusion proteins have been developed with different specificities targeting a broad variety of tumors. These novel molecules retain both antibody and cytokine associated functions. In addition, in animals bearing tumors, antibody–cytokine fusion proteins are able to target the tumor and to elicit a significant anti-tumor response that in some cases results in a complete elimination of the tumor. These results suggest that antibody–cytokine fusion proteins have potential for use in the treatment of human cancer. In the present review, we describe strategies for construction of antibody–cytokine fusion proteins and discuss the properties of several antibody–cytokine fusion proteins with IgG genetically fused to the cytokines IL2, GM-CSF or IL12.
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1. Introduction

The management of residual disease is a central problem in the treatment of cancer. Despite improvements in treatment protocols, relapse remains a critical and generally fatal problem in high risk cancer patients. Chemotherapeutic strategies are necessarily limited by various toxicities and of limited efficacy. Therefore, additional modalities are needed to achieve disease containment or elimination. Systemic treatment with cytokines such as IL2, IL12 and GM-CSF can render some non-immunogenic tumors immunogenic, activating a protective immune response (Rosenberg et al., 1998; Ruef and Coleman, 1990; Tsung et al., 1997). However, when

Abbreviations: Ab, antibody; Ag, antigen; TAA, tumor-associated antigen; IL2, interleukin-2; rhIL2, recombinant human IL2; IL12, interleukin-12; rmIL12, recombinant murine IL12; GM-CSF, granulocyte/macrophage colony-stimulating factor; rmGM-CSF, recombinant murine GM-CSF; Ig, immunoglobulin; NK, natural killer; DNS, *N*,*N*-dimethyl-1-aminonaphthalene-5-sulfonyl chloride (dansyl); LAK, lymphokine activated killer; E/T, effector target; Id, idiotype; i.p., intraperitoneal; s.c., subcutaneous; i.v., intravenous; PBS, phosphate-buffered saline; ADCC, antibody dependent cell-mediated cytotoxicity; MAb, monoclonal antibody; CTL, cytotoxic T lymphocyte; scFv, single chain Fv; KSA, KS antigen; Ep-CAM, epithelial cell adhesion molecule; CHO, chinese hamster ovary; MHC, major histocompatability complex; NHL, non-Hodgkin's lymphoma; PBMC, peripheral blood mononuclear cells

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cytokines are given systemically there are frequently problems with severe toxic side effects that make it impossible to achieve an effective dose at the site of the tumor (Cohen, 1995; Maas et al., 1993; Ruef and Coleman, 1990; Siegel and Puri, 1991).

More effective treatment with cytokines could be achieved if methods were developed to provide effective concentrations in the tumor while limiting generalized toxicity. Direct injection into the site of the tumor has been one approach to this problem (Cortesina et al., 1988; Forni et al., 1987; Maas et al., 1989, 1991; Pizza et al., 1984; Rutten et al., 1989). However, this requires that the tumor be localized and accessible and direct injection into micrometastases is not possible. Another approach has been cytokine gene therapy whereby tumor cells are removed from the patients, transduced or transfected with the cytokine of interest, and reintroduced into the patient with the expectation that a systemic immune response will be elicited against the tumors (Dranoff and Mulligan, 1995; Hurford et al., 1995; Maass et al., 1995; Schmidt et al., 1995; Soiffer et al., 1998; Su et al., 1994; Zatloukal et al., 1995). Although the results suggest that this immunization strategy has potential application in the treatment of minimal residual disease, the ex vivo genetic modification and reintroduction of cells into patients is limited by its patient specific nature. Additionally, it is technically difficult, time consuming and expensive to expand primary autologous human tumor cells to the numbers required for vaccination (Hrouda et al., 1999; Simons et al., 1997, 1999; Soiffer et al., 1998). While in vivo gene delivery using viral vectors has been considered, the in vivo low transfer efficiency of viral vectors and their immunogenicity and safety limit their use (Hrouda et al., 1999). In addition, surface glycoproteins of many viral vectors bind to receptors prevalent on a variety of cells, such non-specific interaction directly decreases the transfection efficiency in vivo (Smith and Wu, 1999). Moreover, a significant fraction of the human population carries preexisting antibodies to viral vectors and such unfavorable immune responses decrease the half-life of vectors (Piedra et al., 1998). Thus the challenge is to develop an alternative approach for achieving effective local concentrations of cytokines.

Tumor specific Abs genetically fused to cytokines provide an alternative approach for concentrating in the region of the tumors quantities of cytokine sufficient to elicit a significant anti-tumor activity without accompanying systemic toxicity. In fact, in the past decade, we and others have developed several Ab-cytokine fusion proteins specific for different TAAs. In preclinical trials using murine model such Ab-cytokine fusion proteins have been shown to be very effective anti-cancer agents suggesting that they may be useful in the treatment of human cancer. As the number and diversity of Ab-cytokine fusion proteins has dramatically increased in the last years the present review cannot include all of them. Instead we will focus this review on a subset of Ab-cytokine fusion proteins consisting of IgG genetically fused to the cytokines IL2, GM-CSF or IL12.

2. Ab-(IL2) fusion proteins

Originally known as ‘T cell growth factor’, IL2 is a cytokine produced by T helper cells which stimulates T cells to proliferate and become cytotoxic (Grimm et al., 1982; Hank et al., 1990; Lotze et al., 1981; Yron et al., 1980) and NK cells to respond with increased cytotoxicity toward tumor cells (Grimm et al., 1982). These properties suggest that Ab-IL2 fusion proteins targeting cancer cells may be effective for cancer treatment. Indeed, among the Ab-cytokine fusion proteins, Ab-IL2 fusion proteins have been the best characterized and most broadly used in successful anti-tumor experiments using animal models.

Although our long term goal was to provide tumor specific therapeutics, the first IgG3-IL2 fusion protein that we produced in the laboratory was specific for the hapten DNS (Harvill and Morrison, 1995) and had human IL2 was fused after the end of the C_H3 domain of human IgG3. Human IgG3 as used since its extended hinge region is expected to provide spacing and flexibility (Dangl et al., 1988). In addition, IgG3 is the most effective of the human isotypes in complement activation (Tao and Morrison, 1989) and like IgG1 binds all three FcγRs (Roitt et al., 1989). The anti-DNS IgG3-IL2 expressed in myeloma cells was properly assembled and secreted. Anti-DNS IgG3-IL2 retains the ability

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to bind Ag, activate complement and bind FcγRI (Harvill and Morrison, 1995). It resembles rhIL2 in its ability to stimulate proliferation of the IL2 dependent cell line CTLL-2 (Harvill and Morrison, 1995). Surprisingly, anti-DNS IgG3-IL2 was more effective than rhIL2 in generating LAK cell activity, eliciting more efficient killing than a 10 fold higher concentration of rhIL2 at all E/T ratios (Harvill and Morrison, 1995). This increased activity may reflect the fact that anti-DNS IgG3-IL2 exhibits approximately a 4-fold higher affinity than native IL2 for the α chain of the high affinity IL2 receptor (Table 1) (Harvill and Morrison, 1996).

Anti-DNS IgG3-IL2 has a half-life in mice of approximately 7 h (Harvill et al., 1996). Although this half-life is shorter than the half-life of IgG3, it is 17-fold longer than the half-life reported for IL2 (Donohue and Rosenberg, 1983; Talmadge et al., 1987) and is similar to that observed for polyethylene glycol-modified IL2 (PEG-IL2) which in some cases exhibits superior anti-tumor efficacy compared with IL2 (Katre et al., 1987; Mattijssen et al., 1992). Indeed this increased half-life may contribute to a better therapeutic effect (Lotze et al., 1985). We also found that, anti-DNS-IL2 was effective in potentiating the immune response to dansylated antigens (Harvill et al., 1996).

To test the effectiveness of IgG3-IL2 fusion proteins in treating the highly malignant murine B cell lymphoma 38C13 (Penichet et al., 1998), the variable regions of the anti-DNS IgG3-IL2 fusion protein were replaced with the variable regions of a murine MAb specific for the Id of the Ig expressed on the surface of 38C13. The resulting anti-Id IgG3-IL2 exhibited in vitro properties and in vivo half-life similar to anti-DNS IgG3-IL2.

Gamma camera imaging studies showed that ^{131}I-anti-Id IgG3-IL2 has the ability to target the tumor thereby concentrating the IL2 at this site (Fig. 1) (Penichet et al., 1998). A small amount of ^{131}I-anti-DNS IgG3-IL2 was localized to the upper abdomen of the animal in the region of the spleen, an organ rich in IL2 receptors 24 h after injection (Fig. 1A). In contrast, the majority of ^{131}I-anti-Id IgG3-IL2 fusion protein present at 24 h was localized to the right flank, the site of the tumor (Fig. 1B) with a little localized in the region of the spleen. At 4 days

Table 1
Rate constants[a]

Ligand	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (M)
rhIL-2	7.0×10^4	0.013	1.9×10^{-7}
IgG3-IL2	7.5×10^4	0.004	5.3×10^{-8}

[a] A soluble form of the α subunit of the IL2 receptor was immobilized on the surface of an IAsys optical biosensor. Association of both rhIL-2 and IgG3-IL2 at various concentrations to the a subunit was monitored over time. Following association the cuvette was washed with PBS/Tween, and dissociation followed. k_{on} values calculated using the FastFit program were plotted against the concentration with the slope of the best fit line yielding k_a. k_d was determined both from the y intercept of the graph and from direct analysis of the dissociation curves using the FastFit program. K_D was calculated as k_d/k_a.

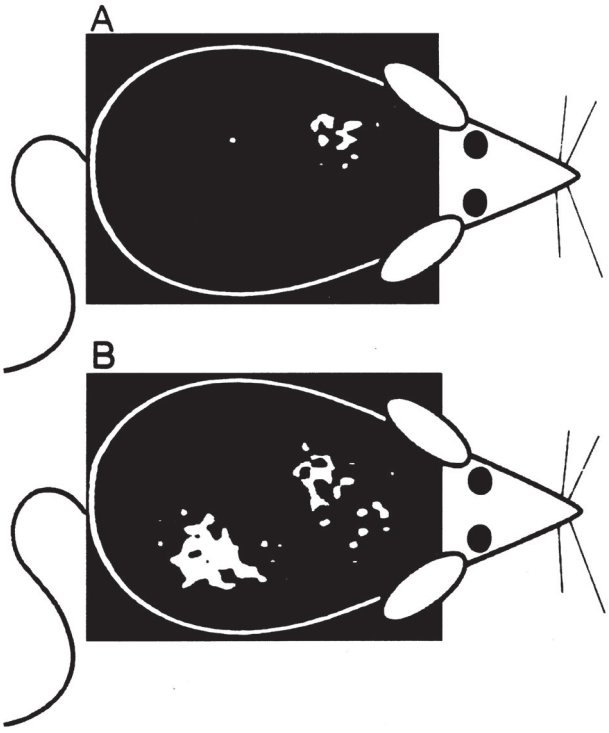


Fig. 1. Tumor localization of Ab fusion proteins. C3H/HeN mice bearing s.c. 38C13 tumors of 0.5 cm diameter on the right flank were given a single i.p. injection of 10 μCi (2 μg) ^{131}I-labeled anti-DNS IgG3-IL2 (A) or anti-Id IgG3-IL2 (B). Then, 24 h later mice were visualized with a Raytheon Spectrum 91 gamma camera. The mice were sedated and immobilized in a prone position with the pinhole collimeter positioned over the torso. A total of 50 000 count images were accumulated and analyzed using Siemens/Microdelta and Adobe software. Reprinted from Penichet et al., 1998. An IgG3-IL2 fusion protein recognizing a murine B cell lymphoma exhibits effective tumor imaging and anti-tumor activity. J. Interferon Cytokine Res., 18: 597–607.

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^{131}I-anti-Id IgG3-IL2 could still be seen at the site of the tumor (data not shown). Less definitive imaging was obtained with the ^{131}I-anti-Id IgG3 which remained distributed throughout the abdomen at 24 h and exhibited only modest accumulation at the site of the tumor at 4 days (data not shown). Images taken immediately after injection showed similar uniform distribution throughout the abdomen for all three proteins (data not shown). These results suggest that tumor specific Ab-IL2 fusion proteins may also be effective imaging agents.

The superior tumor imaging of ^{131}I-anti-Id IgG3-IL2 compared to ^{131}I-anti-Id IgG3 may reflect several parameters. The more rapid clearance of the Ab-IL2 fusion protein as compared with the Ab may result in the elimination of unbound protein, thereby decreasing the background. In addition, IL2 has been shown to increase vascular permeability causing the efflux of intravascular fluids to the extravascular compartments (Damle and Doyle, 1989; Ohkubo et al., 1991). Therefore, the IL2 concentrated in the tumor area might increase the tumor uptake of the radiolabeled molecule. In fact, it has been reported that pre-treatment of nude mice bearing the Raji lymphoma with a IgG1–IL2 fusion protein (chLym-1/IL2) containing the variable region of the anti-human B-cell lymphoma MAb Lym-1 enhances the uptake of the radiolabeled mouse albumin and mouse–human chimeric IgG1 Lym-1 (chLym-1) at the tumor site. The chLym-1/IL2 is thought to alter the permeability of tumor vessels (Hu et al., 1996). Similarly, pretreatment with an Ab-IL2 fusion protein directed against DNA enhances the delivery of therapeutic molecules to solid tumors (Hornick et al., 1999). These results suggest that Ab-IL2 fusion proteins can be used to enhance the targeting of secondary anti-tumor molecules for the treatment of human malignancies.

Anti-Id IgG3-IL2 shows enhanced anti-tumor activity compared to the combination of Ab and IL2 administered together (Penichet et al., 1998). Treatment of mice bearing i.p. tumors with a single i.p. injection of 10 μg of fusion protein prevented tumor growth in 50% of the mice (Table 2, experiment 1). Rechallenge of the survivors indicated immunologic memory with varying degrees of protection consistent with the possibility of a specific T cell response. Consistent with this conclusion immunohistochemical analysis of the tumor from one long term survivor showed extensive infiltration of $CD8^+$ cells. In addition similar treatment of nude mice defective in the ability to mount a T cell immune response did not yield improved survival. In an attempt to improve the efficacy of the therapy, mice with s.c. tumors were treated five times on consecutive days with i.p. injection of 10 μg anti-Id IgG3-IL2. Indeed this approach was more effective resulting in 87% disease-free survivors (Table 2, experiment 2). Although the tumor specific fusion protein was dramatically more effective than non-tumor specific anti-DNS IgG3-IL2 showing the importance of tumor targeting, anti-DNS IgG3-IL2 was more effective than IL2 alone with two of eight treated mice disease-free survivors (25%) (Table 2, experiment 2). This result suggests that IL2 with a much longer half-life might elicit an effective anti-tumor response without direct tumor targeting.

In contrast to what was observed following a single dose of 10 μg of fusion protein, rechallenge of the mice surviving after five doses provided little evidence for immunologic memory (Penichet et al., 1998) suggesting that specific cytotoxic T cells did not play a significant role in providing protection.

Table 2
Results of in vivo therapy experiments

Group	Treatment	Disease free survivors[a]	
		Experiment 1[b]	Experiment 2[c]
1	PBS	0/6 (0%)	0/8 (0%)
2	Anti-Id IgG3	1/6 (16.7%)	2/8 (25%)
3	IL-2	0/6 (0%)	0/8 (0%)
4	Anti-Id IgG3 + IL-2	0/6 (0%)	4/8 (50%)
5	Anti-Id IgG3-IL2	3/6 (50%)	7/8 (87.5%)
6	Anti-DNS IgG3-IL2	Not done	2/8 (25%)

[a] Animals surviving 60 days without evidence of tumor were considered to be tumor free.

[b] Groups of six C3H/HeN mice were injected i.p. with 1000 38C13 cells. The following day each group received single i.p. injections of: PBS, 10 μg of anti-Id IgG3, 30 000 IU of rhIL-2, both 10 μg anti-Id IgG3 and 30 000 IU of rhIL-2, or 10 μg of anti-Id IgG3-IL2.

[c] Groups of eight C3H/HeN mice were injected s.c. with 1000 38C13 cells. The following day each group received the first of five daily i.p. injections. Treatments included PBS, 10 μg of anti-Id IgG3, 30 000 IU of rhIL-2, both 10 μg anti-Id IgG3 and 30 000 IU of rhIL-2, 10 μg of anti-Id IgG3-IL2 or 10 μg of an irrelevant human IgG3-IL2 fusion protein without binding specificity for 38C13 (anti-DNS IgG3-IL2).

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These results are consistent with the previous reports that low doses of IL2 activate T cells while higher doses lead to NK activation (Maas et al., 1993; Talmadge et al., 1987). The dose dependence of the type of response elicited by IL2 may explain the different results obtained upon rechallenge of the survivors from the first and second trial. In fact using IL2 expressing transfected cells as cancer vaccines, it has been observed that high levels of IL2 production are associated with a failure to generate tumor specific CTLs (Schmidt et al., 1995).

Studies from other laboratories have shown that a mouse–human chimeric anti-Id IgG1–mouse IL2 fusion protein (chS5A8-IL2) was more effective in the in vivo eradication of the 38C13 tumor than the combination of the anti-Id Ab and IL2 or irrelevant Ab-IL2 fusion protein (Liu et al., 1998). Interestingly an anti-Id scFv–IL2 fusion protein (scFvS5A8-IL2) containing the variable regions of the chS5A8-IL2 failed to confer protection suggesting the relevance of the Fc effector functions such as ADCC in anti-tumor activity against 38C13 (Liu et al., 1998).

Remarkable success in preclinical trials using syngeneic murine models bearing melanoma, neuroblastoma, and colorectal carcinoma and treated with specific mouse–human chimeric or humanized Ab-human IL2 fusion proteins has been reported by both the Reisfeld and Gillies groups (Becker et al., 1996a,b,c; Lode et al., 1998; Xiang et al., 1997, 1999). To evaluate the in vivo anti-tumor activity of the mouse–human chimeric anti-GD_2 IgG1-IL2 fusion protein ch14.18-IL2, B16 melanoma cells were transduced with human genes encoding the two enzymes in the last stages of diasialoganglioside GD_2 biosynthesis (Becker et al., 1996a). The resulting cell line expressed GD_2, a ganglioside present on the surface of human melanoma but not murine melanoma. Treatment of mice bearing pulmonary and hepatic metastases as well as subcutaneous GD_2 expressing B16 melanoma with ch14.18-IL2 resulted in specific and strong anti-tumor activity. This anti-tumor activity was highly significant compared to the controls such as Ab (ch14.18) and IL2 or irrelevant Ab-IL2 fusion proteins and resulted in the complete eradication of the tumor in a vast number of animals (Becker et al., 1996b,c). It was also found that the immune response induced in tumor-bearing animals receiving treatment with ch14.18-IL2 was $CD8^+$ T-cell dependent and was followed by long-lived transferable protective immunity (Becker et al., 1996a,b,c). The encouraging preclinical results obtained with ch14.18-IL2, have led to Phase I clinical trial using a humanized version of ch14.18-IL2 (Hu14.18-IL2) in patients with GD_2 melanomas (Ostendorf et al., 2000).

GD_2 is also extensively expressed on human but not murine neuroblastoma. A murine neuroblastoma expressing GD_2, NXS2, was established by hybridization of dorsal root ganglional cells of C57BL/6J mice with C1300 mouse neuroblastoma cells (Lode et al., 1998). Using NXS2, ch14.18-IL2 was found to induce a cell-mediated anti-tumor response that effectively eradicated established bone marrow and liver metastases in a syngeneic model. However, the mechanism involved was exclusively NK dependent (Lode et al., 1998) illustrating the fact that different mechanisms of anti-tumor activity may result when different murine tumors expressing the same Ag are treated with the same Ab–cytokine fusion protein.

To evaluate the treatment of colorectal carcinoma using a human TAA as target and immunocompetent mice, the murine colon carcinoma cell line CT26 was transduced with the gene encoding human KSA, an Ep-CAM expressed in a human colon carcinoma and other human malignancies derived from epithelial tissue (Xiang et al., 1997). Like the results obtained with ch14.18-IL2 against melanoma, treatment of mice bearing CT26-KSA hepatic and pulmonary metastases with the humanized anti-KSA Ab-IL2 fusion protein huKS1/4-IL2 directs IL2 to the tumor microenvironment in concentrations that are sufficient to elicit a specific $CD8^+$ T-cell-mediated eradication of established metastases and long-lived T cell memory (Xiang et al., 1997, 1999).

3. Ab-(GM-CSF) fusion proteins

GM-CSF is a cytokine associated with the growth and differentiation of hematopoietic cells. It is also a potent immunostimulator with pleiotropic effects, including the augmentation of Ag presentation in a variety of cells (Blanchard and Djeu, 1991; Fischer et al., 1988; Heufler et al., 1988; Morrissey et al., 1987; Smith et al., 1990; Steis et al., 1990), increased expression of MHC class II on monocytes

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and adhesion molecules on granulocytes and monocytes (Arnaout et al., 1986; Grabstein et al., 1986; Young et al., 1990), and amplification of T-cell proliferation (Santoli et al., 1988). All of these features suggest that localization of GM-CSF at the site of tumor by Ab-(GM-CSF) fusion proteins that recognize TAA may lead to an enhanced tumor specific immune response.

To expand the clinical potential of a promising mouse–human chimeric IgG1 (chCLL-1) directed against a human MHC class II expressed in a high percentage of human B-cell NHL, chronic lymphocytic leukemia, and multiple myeloma cell lines it was joined to human GM-CSF (chCLL-1/GM-CSF) (Hornick et al., 1997). chCLL-1/GM-CSF not only retained the Ab and GM-CSF bioactivity, but also showed enhanced ADCC activity compared to Ab (chCLL-1) using human mononuclear cells (Hornick et al., 1997). In addition, biodistribution and imaging studies in nude mice bearing the human myeloma cell line ARH-77 indicated that the fusion proteins specifically targets the tumors (Hornick et al., 1997) suggesting that this Ab fusion protein may be useful for the treatment of B-cell malignancies and other related cancers. Unfortunately, an animal tumor expressing the Ag recognized by chCLL-1 is not available (Hornick et al., 1997) and human GM-CSF is not active in mice (Ruef and Coleman, 1990) making it difficult to evaluate the anti-tumor activity of chCLL-1/GM-CSF in vivo.

To be able to investigate the in vivo efficacy in a variety of syngeneic tumor models, murine GM-CSF was genetically fused to the carboxyl terminus of a chimeric rat anti-mouse transferrin receptor Ab, ch17217 (Dreier et al., 1998). The ch17217-(GM-CSF) fusion protein retained both Ab and GM-CSF related activities and most importantly demonstrated effective anti-tumor activity by suppressing the growth of hepatic metastases of mouse neuroblastoma NXS2 cells and pulmonary metastases of mouse colon carcinoma CT26 cells in syngeneic A/J and BALB/c mice, respectively. However, it should be noted that the controls of Ab alone (ch17217) or rmGM-CSF were not included in these studies making it impossible to distinguish the in vivo role of the Ab-(GM-CSF) fusion protein from that of the Ab and/or GM-CSF (Dreier et al., 1998). Due to the universal expression of the transferrin receptor on mouse tumor cell lines, constructs like ch17217-(GM-CSF) should prove effective against a wide variety of murine tumors. However, the transferrin receptor is also expressed on all normal cells except mature red blood cells (Dowlati et al., 1997) and antigens whose expression is restricted to tumors may be a more appropriate target for Ab–cytokine fusion proteins. Further studies are required to evaluate the effectiveness and mechanism of action of Ab–(GM-CSF) fusion proteins in this and other tumor models.

4. Ab–(IL12) fusion proteins

IL12, a cytokine normally released by professional Ag-presenting cells, promotes cell-mediated immunity (Trinchieri, 1995) by inducing naive $CD4^+$ T cells to differentiate into Th1 cells (Gracie and Bradley, 1996; Hsieh et al., 1993). In addition, IL12 has the ability to enhance the cytotoxicity of NK and $CD8^+$ T cells (Farrar and Schreiber, 1993; Gately et al., 1994). Moreover, the IFN-γ produced by IL12 stimulated T and NK cells can retard tumor growth by inhibiting tumor angiogenesis (Voest et al., 1995) and enhancing immune recognition of tumor cells through up-regulated MHC expression (Wong et al., 1984). However, in contrast to cytokines like IL2 and GM-CSF, IL12 is a disulfide-linked heterodimer of two subunits p35 and p40. Bioactive IL12 requires the expression of two separate genes, p40 and p35, and correct heterodimer assembly making the construction of an Ab–IL12 fusion protein more complicated (Gubler et al., 1991).

To address this issue, Gillies et al. (1998) constructed an Ab–IL12 fusion protein in which the p35 subunit was fused to the carboxy terminus of an Ab; the p40 subunit was expressed as a separate polypeptide which must then assemble with the p35 subunit. Although this Ab–IL12 fusion protein was functional, the IL12 bioactivity was significantly lower than rmIL12 (Gillies et al., 1998).

As an alternative approach, we fused single-chain murine IL12 to anti-HER2/*neu* IgG3 (mscIL12.her2.IgG3) (Peng et al., 1999). The use of single-chain IL12 may confer greater stability to the fusion protein and ensures equimolar concentrations of the two IL12 subunits facilitating large scale

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production of the Ab–IL12 fusion protein. The cDNA coding for the murine IL12 fusion protein IL12.p40.L.Dp35 with the two subunits joined with a $(Gly_4,Ser)_3$ linker was used (Lieschke et al., 1997). This protein showed a specific activity comparable to that of rmIL12 while a related construct with a different subunit order (IL12.p35.L.Dp40) showed very low activity (Lieschke et al., 1997). In an ovalbumin–IL12 fusion protein in which the p40 subunit was fused to ovalbumin, a 50-fold lower IL12 activity was observed (Kim et al., 1997) suggesting that constraint of the p40 subunit in a fusion protein may disrupt the interaction between IL12 and the IL12 receptor. Although the p40 subunit was not fused to the antibody in the Gillies et al. (1998) Ab–IL12 fusion protein, the direct fusion of the p35 subunit to the carboxy terminus of the Ab without any type of flexible linker may make the p40 subunit somewhat less accessible for receptor binding possibly explaining the 2-fold lower IL12 activity they observed. To further insure accessibility, the murine single chain IL12 was fused to the amino terminus of heavy chain of anti-HER2/*neu*-IgG3 (Peng et al., 1999). At this position proteins which require N-terminal processing or folding for activity such as NGF (McGrath et al., 1997) and human B7-1 (Challita-Eid et al., 1998) remain active. We found that mscIL12.her2.IgG3 was assembled and correctly secreted and that the presence of IL12 at the amino terminus did not interfere with the Ab′s ability to bind HER2/*neu*. In addition, mscIL12.her2.IgG3 binds the IL12 receptor and resembles recombinant IL12 in its ability to stimulate proliferation of PBMC (Peng et al., 1999).

Although our long-term goal was the production of Ab–IL12 fusion proteins for therapeutic use in humans, murine IL12 was used for these initial studies because it has activity on both human and murine cells, while human IL12 has activity only on human cells. The use of murine IL12 makes it possible not only to carry out assays using human PBMC to test biologic activity, but also to perform in vivo studies to examine the effects against human HER2/*neu*-expressing murine tumors using immune competent mice. The in vivo anti-tumor activity of mscIL12.her2.IgG3 was evaluated using murine colon carcinoma cell line CT26 transduced with the cDNA encoding human HER2/*neu* (Penichet et al., 1999) which grows in immunocompetent mice while maintaining the expression of human HER2/*neu* (Penichet et al., 1999). Fig. 2 shows that mscIL12.her2.IgG3 has significant anti-tumor activity in immune competent BALB/c mice under conditions in which the anti-HER2/*neu* Ab (her2.IgG3) failed to confer protection. We observed better anti-tumor activity when treatment was started after the tumors were established (mean diameter of 8–9 mm) than when treatment was started the day after inoculation with tumor cells. This lends support to previous studies by others (Tsung et al., 1997; Zitvogel et al., 1995; Zou et al., 1995) in which better anti-tumor activity of IL12 was observed when tumors were established. They proposed that this may be because effector cells are first recruited to the tumor site, and are then activated by IL12. Further work is being carried out using CT26-HER2/*neu* and other murine tumors expressing human HER2/*neu* to determine the mechanism of action of mscIL12.her2.IgG3.

5. Conclusions

In the last decade many studies have shown that the genetic fusion of anti-cancer Ab with cytokines results in novel proteins which retain both Ab and cytokine functions and show superior anti-cancer activity compared with equivalent amount of free Ab and cytokines or non tumor specific Ab cytokine fusion proteins. These findings support the hypothesis that Ab–cytokine fusion proteins can specifically target the cytokine to the tumor microenvironment and in so doing stimulate the immune effector cells. In some cases the stimulus is sufficient to achieve complete eradication of the tumor suggesting that these unique molecules will be effective in the treatment of human cancer. However, many Ab–cytokine fusion proteins such as Ab–(GM-CSF) and Ab–IL12 fusion protein are still in a very early phase of development and additional preclinical studies are required for a better understanding of how these novel molecules work. Of special interest will be the potential synergistic effects between different Ab–cytokine fusion proteins or between Ab–cytokine fusion proteins and other anti-cancer strategies such as chemotherapy, gene therapy and tumor vaccines.

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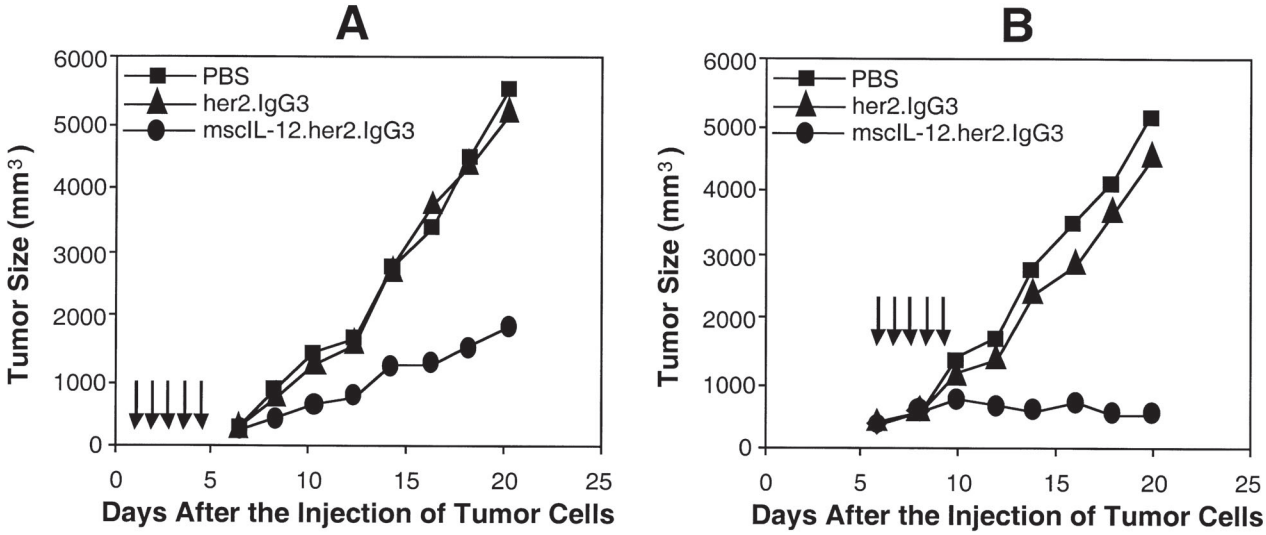


Fig. 2. In vivo anti-tumor activity. BALB/c mice were injected with 1×10^6 CT26-HER2/*neu* cells s.c. on day 0. Beginning on day 1 (A) or day 6 (B), groups of ten mice were treated i.v. with either mscIL-12.her2.IgG3 (at a concentration equivalent to 1 μg IL-12/day), her2.IgG3 (at a concentration equivalent to the Ab concentration of mscIL-12.her2.IgG3 administered/day), or PBS for 5 days. Tumor growth was measured with a caliper beginning on day 6 and tumor volume calculated. The average tumor volumes of the ten mice used in each treatment group were plotted against time. Graphs adapted from Peng et al., 1999. A single chain IL-12 IgG3 antibody fusion protein retains antibody specificity and IL-2 bioactivity and demonstrates anti-tumor activity. J. Immunol., 163: 250–258. Copyright 1999, The American Association of Immunologists.

Obviously, the ultimate use of Ab–cytokine fusion proteins for the treatment of human cancer will depend on the results obtained when these proteins are evaluated in Phase I and II clinical trials.

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