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Induction of tumor immunity by autologous B lymphoma cells expressing a genetically engineered idiotype

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A fusion protein containing a B cell lymphoma idiotype (Id) and granulocyte–macrophage colony-stimulating factor (GM-CSF) is a potent stimulator of tumor immunity. In three different tumor models we show that immunization with autologous lymphoma cells that have been engineered to express the Id in the context of GM-CSF is much more effective than immunization with an equivalent dose of the purified protein. The lymphoma Id could be modified by introducing the GM-CSF gene into the immunoglobulin (Ig) heavy chain locus via gene targeting. This approach circumvents the isolation of the rearranged immunoglobulin variable genes from the tumor and the preparation of tumor-specific vector constructs. The low production of Id/GM-CSF fusion proteins by transfected cells, which is a major obstacle in the use of purified fusion proteins for immunotherapy, is due to the presence of the cytokine gene in the immunoglobulin locus. Low production, however, is not limiting in the cell-based setting, because upon in vivo administration of the modified autologous cells, even minute expression levels are sufficient to induce tumor immunity.

Keywords: *gene therapy; GM-CSF; homologous recombination; B cell lymphoma*

Introduction

The growing understanding of antigen presentation and T cell activation mechanisms has led to a renewed interest in immunotherapy of cancer. Despite their expression of a variety of tumor-associated antigens (TAAs),[1,2] malignant cells may escape immune surveillance, because their presentation of TAAs is insufficient,[3] or because they are unable to provide the requisite costimulatory signals for T cell activation.[4,5] Numerous approaches have been evaluated to improve the presentation of tumor-derived peptides by professional antigen-presenting cells (APCs) or to activate directly tumor-specific T cells by the use of cytokines.[6,7] To minimize systemic side-effects, whole tumor cells genetically engineered to express cytokines have been used as cancer vaccines.[8–10] Thus, immune effector systems could be recruited specifically to the tumor site by high cytokine concentrations in the local environment of the malignancy. A comparative study showed that among a wide variety of cytokine genes tested, the GM-CSF gene when introduced into B16 melanoma cells was most effective in protecting mice from a subsequent challenge with wild-type tumor.[11] GM-CSF is thought to exert its immunostimulatory effect through the recruitment of professional APCs, such as macrophages and dendritic cells, that activate resting T cells by virtue of their capability of providing efficient costimulation (Ref. 11 and reviewed in Ref. 12).

Also in a B cell lymphoma model, vaccination with GM-CSF gene-transduced tumor cells could confer efficient tumor protection.[13] Lymphomas of the B cell lineage are characterized by a tumor-specific antigen, namely the immunoglobulin Id that results from unique rearrangements of Ig heavy (H) and light (L) chain variable (V) gene segments. Whereas cytokine gene transduction results in immunization against undefined TAAs,[13] Id-specific responses could be induced by a variety of active immunization protocols.[14–18] The low immunogenicity of the Id, however, necessitated injection of Id protein together with adjuvant. Alternatively, an anti-Id response was elicited by immunization with a protein containing the lymphoma Id covalently coupled to GM-CSF.[19] As with other idiotypic vaccination protocols, this approach suffers from the drawback that the IgV genes have to be isolated from the tumor and that the expression of the fusion protein in sufficient amounts is a tedious procedure.

To circumvent these obstacles, we have developed a strategy that allows the expression of an Id/GM-CSF fusion protein in an autologous lymphoma cell. Hereby, a construct containing the lymphoma-derived V_H and the GM-CSF gene is transferred into the autologous tumor

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cell, or the GM-CSF gene alone is introduced into the IgH chain locus of the tumor by gene targeting.[20,21] Using these engineered tumor cells rather than the purified fusion protein as vaccines permits the redirection of cytokine-induced effector systems to the local environment of the tumor cell and obviates the need for cumbersome *in vitro* production and purification steps.

Results

Poor expression from Ig/GM-CSF fusion constructs

Studying the expression of Ig/GM-CSF fusion proteins in transfected cells, we first evaluated the influence of the heterologous gene on the Ig expression level using a well characterized model vector containing a V gene with anti-Thy-1.2 specificity and the human IgG1 constant (C) region. Transcription was driven by a V_H promoter and the Igμ and IgH 3′ enhancers. This vector was chosen just as a model construct, because it yields efficient expression upon transfection into the Sp2/0 nonproducer cell line together with the corresponding κ chain vector.[22] The vector was compared with an analogous construct whose C_H3 exon was ligated in-frame to GM-CSF. Whereas Sp2/0 cells transfected with the chimeric construct without GM-CSF secreted up to 3 μg/ml antibody into the culture supernatant, the highest expression obtained with the Ig/GM-CSF fusion construct was reduced by a factor of 100. This direct comparison between the two plasmids, which differed only in the presence of the GM-CSF gene, indicates that down-regulation of the expression level is exclusively due to the presence of the heterologous gene within the IgH locus. Thus, the poor expression may be attributable to transcriptional suppression related to intragenic control elements or to inefficient folding of the fusion protein.

Expression of Id/GM-CSF fusion proteins in lymphoma cells

To combine the potent effect of the GM-CSF fusion protein with the advantage of a local delivery and to obviate the tedious preparation of the immunogen in cell culture we generated an autologous tumor cell vaccine expressing its Id in the context of GM-CSF. We transfected the murine 38C13 lymphoma with the construct p3159 which contained the 38C13 V_H gene and the human IgG1 C region fused to mouse GM-CSF.[19] This construct was originally created to express an Id/GM-CSF fusion protein in a nonproducer cell line for vaccination studies of mice with soluble protein. After stable transfection of 38C13 cells with this vector the endogenous κ chain was assembled with the endogenous μ chain, as well as with the transferred GM-CSF-containing IgH chain. The best producer (1H7) was selected and further analyzed. The expression level (250 ng/ml fusion protein in culture supernatant) and the integrity of the product were demonstrated by binding to the anti-idiotypic antibody E4 and to anti-human IgGFc and anti-GM-CSF antibodies, by Western blotting and by an assay testing for the functional activity of the GM-CSF moiety (not shown). After separation of the Id/GM-CSF construct from the parental mouse IgM molecule by protein A chromatography, syngeneic C3H mice were vaccinated with the soluble fusion protein. Upon challenge with a lethal dose of wild-type 38C13 cells, 33% of the mice developed tumors (Figure 1a). This result is in good agreement with earlier studies.[19] In the next step, mice were immunized with irradiated 1H7 cells rather than the purified protein. In contrast to immunization with the soluble protein, 100% of the mice successfully rejected the 38C13 tumor and all but one of the protected mice survived a tumor rechallenge given after 100 days.

These results could be reproduced in a second lymphoma model. A20 is derived from the BALB/c strain and expresses IgG2a. The V_H gene from A20 was cloned by PCR and ligated to the human IgG1/mouse GM-CSF cassette. The construct was transfected into A20 cells, and the resulting transfectant 5C12 was characterized as outlined above, using the anti-idiotypic antibody 6C10 (see Materials and methods). Although the expression rate of 5C12 was 10-fold lower than that of 1H7, all mice vaccinated with irradiated 5C12 cells survived a subsequent A20 tumor cell inoculation (Figure 1b). Again, a significantly lower survival benefit was observed after immunization with soluble 5C12 protein. Thus, minute expression levels were sufficient for inducing tumor protection as long as the vaccine was given in the form of the gene-modified cells. In addition, the tumorigenicity of live 5C12 cells was significantly reduced with only one of six mice succumbing after 29 days.

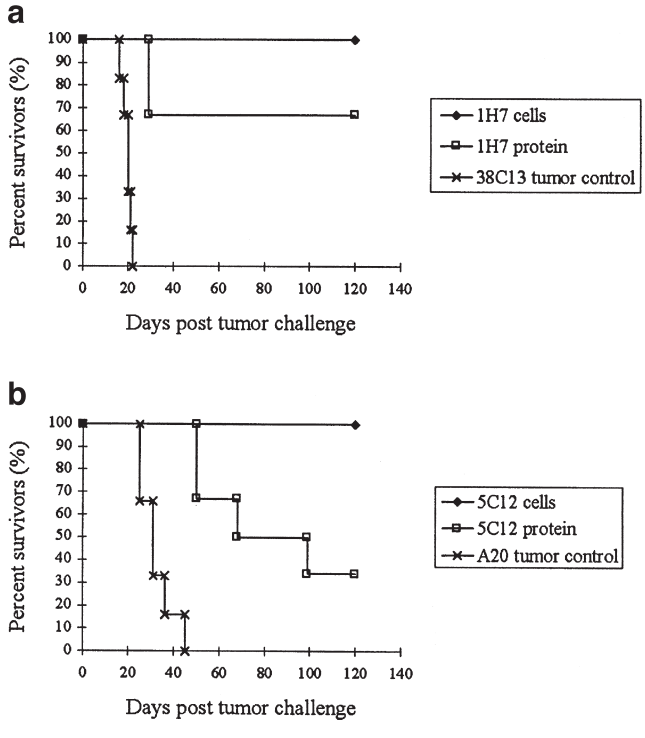

Figure 1 *Protection from wild-type 38C13 (a) or A20 (b) lymphoma cells in mice immunized with 5×10^6 irradiated autologous cells expressing an Id/GM-CSF fusion protein or with 50 μg of the purified protein. C3H (a) or BALB/c (b) mice were vaccinated twice at 3-week intervals and challenged after another 7 days with 200 38C13 or 10^5 A20 cells, respectively. No difference was observed between immunization with 50 or 10 μg of the soluble fusion protein. The difference between the cell and the protein immunization was significant in each experiment ($P = 0.04$). Irradiated wild-type 38C13 or A20 cells alone were not able to impart tumor immunity. In each experiment a tumor control group without vaccination is included. Representative results from at least two independent experiments are shown.*

Modification of lymphoma cells by gene targeting

To circumvent the isolation of V genes from the lymphoma cells and the generation of tumor-specific gene constructs, we next modified lymphoma cells to express their Id in the context of GM-CSF by introducing the cytokine gene into the IgH locus via gene targeting. This system is based on the exchange of the murine IgH C exons with other gene segments and has earlier been applied for generating mouse/human chimeric antibodies in hybridoma cell lines.[20,21] Integration vectors were constructed that contained a murine homology flank derived from the Igμ intron, a human IgG1 C region fused to mouse GM-CSF and a eukaryotic selection marker (Figure 2). Site-specific vector integration via the homology flank will give rise to the modified IgH locus shown in the lower part of Figure 2. These vectors will be universally applicable to all B cell lymphomas so that the generation of individual-specific vector constructs should become dispensable.

We showed earlier that an isotype exchange in B cell hybridomas by homologous recombination can dramatically impair Ig expression.[20] Furthermore, in view of the data presented above, it must be kept in mind that expression may be further reduced by introducing the GM-CSF gene into the IgH locus. Indeed, the integration of the GM-CSF gene into the 38C13 IgH locus seemed to suppress Ig expression to a level below detection. After transfection of the recombination vector into 38C13 cells that secrete very low IgM amounts *per se* and screening of the stable transfectants by ELISA, no targeted recombinants could be found. On the other hand, homologous recombination was readily detected in 38C13 after transfer of the vector pSVgpt-huγ1-A5 which only introduced the human IgG1 C exons without GM-CSF.[21]

We, therefore, selected a lymphoma (MPC11) whose Ig secretion is 1000-fold higher than that of 38C13 (our unpublished observation). In this case, targeted recombinants were detected with a frequency of 3.0%; the 4A1 line was selected for further study. The predicted size (about 72 kDa) and structure of the purified protein were shown by Western blotting and ELISAs using anti-human IgGFc and anti-GM-CSF. Quantification in the biological GM-CSF assay demonstrated functional cytokine gene expression (exemplified in Figure 3). The parental IgG2b chain of MPC11 was no longer produced and the endogenous κ chain was correctly paired with the chimeric IgH chain. The production rate was decreased to only 5 ng/ml culture supernatant. Nevertheless, the tumorigenicity of 4A1 was significantly reduced in syngeneic mice with 33% of the animals surviving an i.p. injection of 10^5 cells (not shown) for more than 100 days, whereas all mice succumbed to the same dose of MPC11 wild-type cells by day 35. A tumor challenge after two preimmunizations with 2×10^7 irradiated 4A1 cells showed that the mice developed systemic immune responses with 100% long-term survivors. By using 5×10^6 4A1 cells, 50% of the animals developed tumor resistance (Figure 4). Furthermore, a tumor rechallenge given after 100 days was successfully rejected in 83% of the immune mice. Essentially the same results were obtained when mice received only one preimmunization (not shown).

Assuming that *in vivo*, the irradiated 4A1 cells will produce the protein for 3 to 4 days at a rate similar to that measured *in vitro*, injection of 5×10^6 irradiated 4A1 cells per mouse will give rise to an accumulated protein dose of as few as 10 ng. Such low amounts delivered as purified protein did not confer any tumor protection (Figure

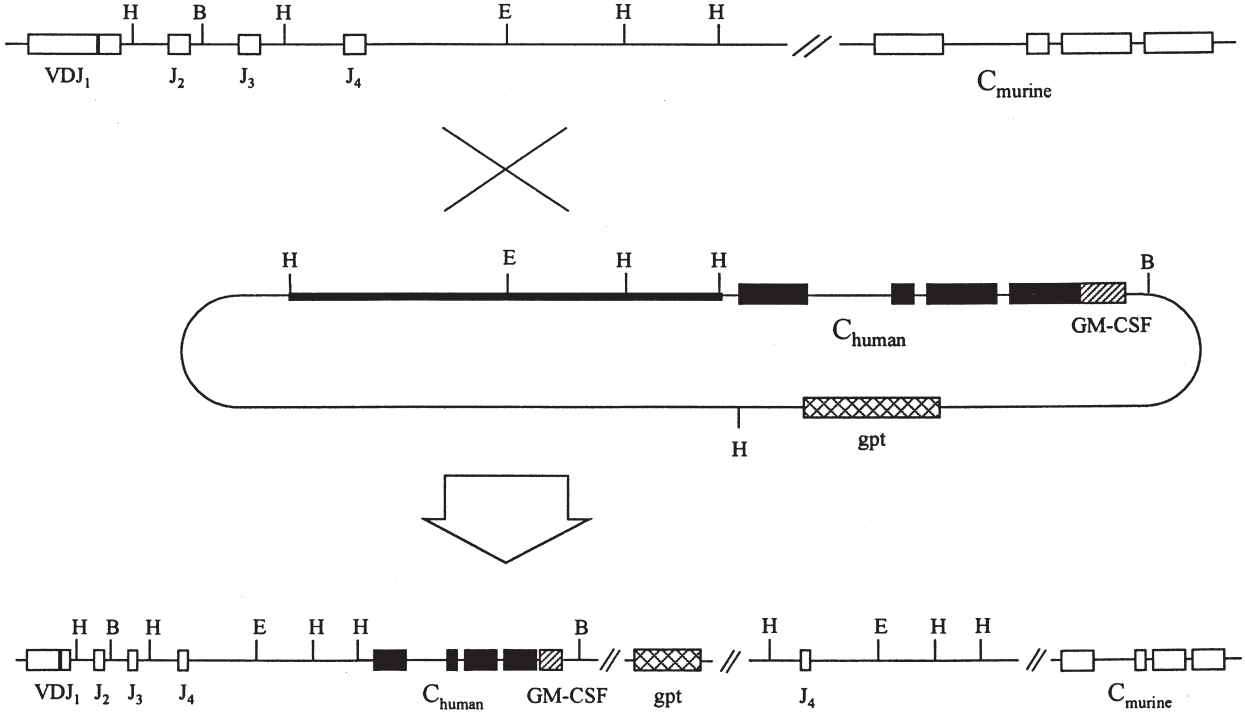

Figure 2 *Introduction of the GM-CSF gene into the IgH locus by gene targeting. The integration vector pSVgpt-huγ1(CH3)-GMCSF(ΔL)-A5 (middle part) recombines via the indicated break point into the mouse IgH locus (upper part). The bolt line in the vector denotes the homology flank. Open boxes, mouse exons; black boxes, human exons. Restriction sites: E, EcoRI; B, BamHI; H, HindIII.*

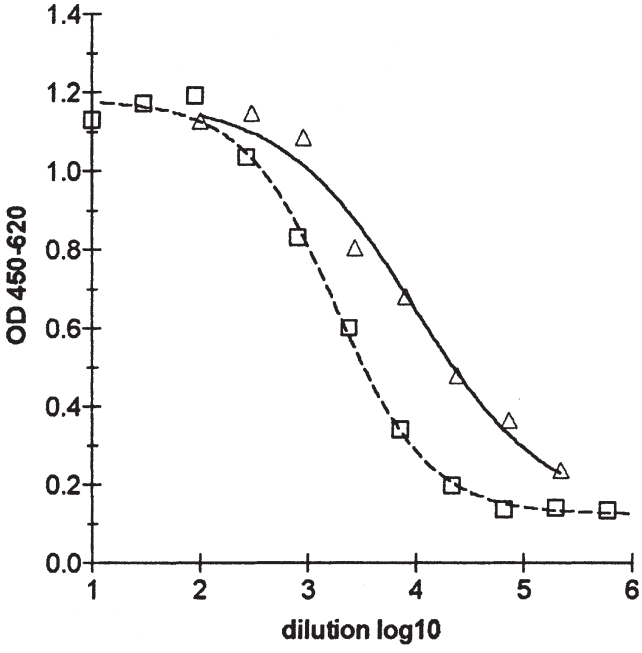

Figure 3 *Cell proliferation assay demonstrating the functional activity of GM-CSF in the Ig/GM-CSF fusion molecule. Purified fusion protein (triangles) and recombinant murine GM-CSF (squares) were serially diluted with a starting concentration of the quantification standard of 10 ng/ml. Cell proliferation is indicated as the OD at 450 nm corrected for the background extinction measured at 620 nm.*

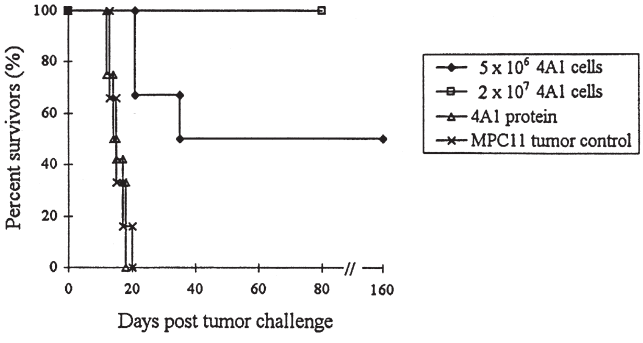

Figure 4 *Tumor protection in mice vaccinated with 5×10^6 or 2×10^7 4A1 cells or 10 ng of 4A1 protein. After two immunizations, mice received 10^5 wild-type MPC11 cells. A tumor control group is included. The outcome of a second experiment was identical. Unmodified irradiated MPC11 cells had no effect.*

4). This again underscores our hypothesis that the low productivity of the cells does not affect the immunizing potential of the Id/GM-CSF protein, provided the vaccine is delivered in the form of gene-modified cells.

Vaccination with gene-targeted cells was also successful in eradicating a simultaneously injected tumor inoculum. Mice received 2×10^7 irradiated 4A1 cells mixed with a lethal MPC11 dose. Also in this setting, 50% long-term survivors were observed. The specificity of the immune response was demonstrated by challenging 4A1-treated animals with the syngeneic A20 tumor. All mice succumbed to this unrelated B cell lymphoma (not shown).

Specificity of the immune response

It was reported that lymphoma cells that were engineered to secrete GM-CSF alone, were capable of conferring tumor immunity.[13] For comparison, we also generated cellular vaccines by transferring a vector encoding only GM-CSF into A20 and MPC11 cells. Modified cells were selected for vaccination that secreted cytokine at levels comparable to 5C12 or 4A1 cells. The survival benefit observed following wild-type tumor challenge was similar to that obtained after immunization with 5C12 and 4A1 cells, respectively (not shown). We reasoned, however, that tumor immunity induced by Id/GM-CSF fusion protein-expressing cells should be more specific in terms of mounting an anti-Id response. Therefore, we tested for the presence of a humoral anti-Id response in mice having received two immunizations with modified cells. There were, indeed, significantly higher anti-Id titers when mice received fusion protein-secreting cells rather than GM-CSF-expressing cells (Table 1). In the MPC11 system, the titers were generally lower than in the A20 model, which might be explained by the decreased expression rates of the modified MPC11 cells. In individual mice, the antibody response was independent of whether or not the animals were protected against the tumor and did not reflect the differential survival times observed after immunization with 5C12 protein *versus* 5C12 cells. The advantage of the cell-based vaccine, however, is clearly demonstrated by the survival benefit and may be related to a cellular response (see below). Because of the low or (in some animals) undetectable anti-Id titers, the role of antibodies in the induction of tumor protection cannot be unequivocally assessed. Also the antibody response against whole cells was very weak. A direct comparison of anti-Id and anti-cell reactivities, however, is difficult because different determination systems were utilized. In summary, anti-Id titers are not necessarily predictive of tumor rejection, but indicate that a more specific immunity was induced by the fusion protein approach.

To elucidate the role of T lymphocytes, mice were depleted of T cells before or following immunization with 4A1 cells. In both settings, depletion of $CD8^+$ T cells totally abrogated the protective effect, while treatment with anti-CD4 antibodies resulted in a 50% survival. This may be explained by the fact that anti-CD4 antibodies can spare cells that have been activated.[23] In contrast, following pan T cell depletion, tumor protection was completely abolished. These data indicate that T lymphocytes

Table 1 Humoral anti-Id response in mice immunized with A20 or MPC11 cells that were engineered to express an Id/GM-CSF fusion protein or GM-CSF alone

	A20 model	*MPC11 model*
Id/GM-CSF fusion protein	235.0 (34.1)	8.3 (0.8)
GM-CSF alone	9.9 (7.2)	0.0 (0.0)

Titers were determined by ELISA and expressed as reciprocal serum dilutions giving two-fold background signals. Average titers from five to nine mice from two to three independent experiments each are shown. Standard deviations are indicated in brackets.

are crucial for the induction of immunity, as well as for the effector phase of tumor cell elimination.

Discussion

The use of autologous tumor cells modified to secrete an Id/GM-CSF fusion protein may open the way for more widespread application of anti-Id immunization for lymphoma therapy. This approach has been hampered to date by the tedious *in vitro* production and purification procedures required to generate fusion proteins for vaccine use. We show here that the low productivity of transfected cells may be an inherent problem related to the presence of the heterologous gene segment in the Ig locus. If gene-modified cells are used as a cell-based vaccine, however, the low expression levels are not limiting, because minute secretion rates by the transfected cells are sufficient *in vivo*. In three different tumor models, the comparison between the soluble and the cellular immunogen demonstrated that the cellular vaccine was more effective (Figures 1 and 4), although the protein amount delivered by the irradiated transfected cells *in vivo* was assessed to be 50-fold lower (Figure 1) than that injected in the experiments using soluble protein. Even 4A1 cells, whose expression has been even more down-regulated due to homologous recombination, could exert an immunostimulatory effect, whereas an equivalent quantity of purified protein was ineffective (Figure 4).

Several factors may contribute to the superior effect of GM-CSF-modified cells. GM-CSF has been identified as the most potent inducer of tumor immunity due to its stimulatory effect on the differentiation of monocytic and dendritic progenitor cells that subsequently function as highly efficient APCs.[11] Apart from the specific uptake of the covalently fused Id via GM-CSF receptors,[12] these cells may also ingest and process other TAAs when they are locally recruited to the tumor cell environment. Thus, immunization against undefined TAAs may act synergistically with the anti-Id response. As we could demonstrate, immunity is strictly T cell-dependent. Whether the humoral response, whose Id-specific component was markedly stimulated with fusion protein-expressing vaccines, is also instrumental for tumor resistance or merely reflects immunization, cannot be decided at the moment. If the humoral response plays a role in the immune mechanism, low titers of anti-Id antibodies together with unspecified anti-cell antibodies would be sufficient to contribute to protection.

Antitumor immunity in the cell-based setting may be further stimulated by the inclusion of the xenogeneic Fc portion of Ig into the fusion molecule. When administered as a soluble protein, a xenogenized Id had no immunostimulatory effect.[19] When expressed in an autologous lymphoma cell, however, it is conceivable that T cells can be activated more effectively, because peptides are presented not only by class II molecules, but also by class I molecules on cells that endogenously synthesize and process the immunogen. Consistent with this view is the finding that DNA vaccination with Id plasmids is more effective than immunization with the corresponding protein, provided the mouse C region has been substituted with the xenogeneic human Fc portion.[24]

In a previous study, we reported that injection of autologous lymphoma cells that were engineered to express their Id in the form of a bispecific Ig is much more effective in inducing tumor protection than administration of the purified modified Id.[25] We hypothesize that this might be a common principle contributing to generation of effective responses.

Because transferred IgH/GM-CSF fusion constructs assemble with the endogenous IgL chains, the V_L gene has not to be isolated from the tumor. Moreover, in the case of gene targeting, neither the V_L nor the V_H chain has to be isolated. There is no need to produce custom-made vectors, because one recombination vector is suitable for all lymphomas. Homologous recombination at the IgH locus is more rapid than cloning of the V genes into expression vectors. A requirement of the gene targeting approach, however, is that the parental lymphoma cell must exhibit a minimum level of Ig expression. If the expression is too low, such as in the case of 38C13, the sensitivity of the ELISA for identifying targeted recombinants will be insufficient. In this study we have targeted lymphoma cell lines. For homologous recombination in primary tumor cells, suitable long-term culturing systems will be required. This should not be an obstacle, since in our hands, human lymphoma cells can be grown for several weeks on a layer of CD40L-transfected fibroblasts in the presence of cytokines. Moreover, it will be necessary to use, for example, retroviral gene transfer systems, that can ensure high transduction efficiencies. Whether an enrichment for targeted Id/GM-CSF-secreting recombinants will then still remain a necessity must be evaluated experimentally.

Materials and methods

Vector construction

The construct pSVgpt-huγ1-A5 has been described previously.[21] A *Sal*I site was engineered at the 3′ terminus of the C_H3 domain of this vector by PCR thereby giving rise to the construct pSVgpt-huγ1(CH3)-A5. The murine GM-CSF gene[26] was cloned into pSP72 (Promega, Madison, WI, USA) via *Pst*I ends. To eliminate a putative leader sequence[26] and to introduce a *Sal*I restriction site at the 5′ boundary of the gene, the 0.4 kb *Eco*RV/*Xma*I fragment was excised from pSP72-GMCSF and substituted with an *Eco*RV/*Xma*I-digested PCR fragment. The GM-CSF gene was isolated from the resulting construct pSP72(ΔEV)-GMCSF(ΔL) with *Sal*I and ligated into the *Sal*I-digested vector pSVgpt-huγ1(CH3)-A5. This vector was designated pSVgpt-huγ1(CH3)-GMCSF(ΔL)-A5. A V_H gene with specificity for Thy-1.2 was cloned into this construct by transferring a 2.1 kb *Pvu*I/*Sac*I fragment from pSVgpt-Eμ2-V_HMmT1-huγ1[22] into the *Pvu*I/*Sac*I-digested vector pSVgpt-huγ1(CH3)-GMCSF(ΔL)-A5 thereby creating pSVgpt-V_HMmT1-huγ1(CH3)-GMCSF(ΔL)-A5. Finally, a 1.0 kb fragment comprising the IgH 3′ enhancer[27] was ligated into this plasmid via *Bam*HI ends.

The vector p3159 has been described elsewhere.[19] For generating the recombination vector, pSVgpt-DNShuG1-GMCSF(St), the 5′ part of the mouse Igμ intron was excised from pSVgpt-huγ1(CH3)-A5 as a *Pvu*I/*Eco*RI fragment and ligated to the *Pvu*I/*Eco*RI fragment encompassing the human IgG1/murine GM-CSF fusion cassette that has been prepared from p3159 (kindly provided by R Levy, Stanford University School of Medicine, Division of Oncology, Stanford, CA, USA).

To express the Id of the A20 lymphoma in the context

of GM-CSF, the V_H gene of A20 was amplified by RT-PCR, sequenced and cloned into pBluescript II KS+ (Stratagene, Heidelberg, Germany) via *Sal*I/*Spe*I ends. A 3.3 kb *Xba*I/*Not*I fragment comprising the IgG1/GM-CSF fusion cassette was excised from p3159 and ligated into the modified Bluescript vector. Finally, the V_HA20 gene together with the fusion cassette was transferred to BCMGSNeo[28] as a *Not*I/*Xho*I fragment thus giving rise to BCMGSNeo-V_HA20-huγ1-GMCSF. To express GM-CSF alone the vector BCMGSNeo-GMCSF was constructed by inserting a PCR fragment containing the GM-CSF gene with an *Xho*I and a *Not*I site into BCMGSNeo.

Cell culture

38C13 is a mouse lymphoma derived from the C3H strain expressing IgM, A20 and MPC11 are BALB/c lymphomas expressing IgG2a and IgG2b, respectively.[29–31] Sp2/0 is a nonproducing mouse myeloma cell line.[32] The rat IgG2a monoclonal antibody, 6C10, with specificity for the A20 Id was generated by immunizing Lou/C rats with A20 cells and fusing splenocytes to X63-Ag8.653 cells. The E4 antibody has been earlier described.[33] All cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mm glutamine and 50 μm mercaptoethanol in a 37°C CO_2 incubator. For transfection, $3–10 \times 10^6$ cells were suspended in 700 μl RPMI 1640, mixed with 3–20 μg of DNA that was linearized with *Pvu*I (V_H expression vectors with exception of the BCMGSNeo-based construct) or *Bam*HI (recombination vectors) and exposed to a single pulse of 200 V (Sp2/0), 220 V (38C13, MPC11) or 250 V (A20) at a capacity of 960 μF or 500 μF (for MPC11 cells) in a BioRad gene pulser apparatus (BioRad, München, Germany). After incubation for 10 min on ice, the cells were distributed in 96-well plates at a density of 10^4 cells per well. After 48 h, selection with xanthine, hypoxanthine and escalating concentrations of mycophenolic acid or with 0.5 mg/ml G418 (for BCMGSNeo) was initiated.

Antibody purification

Antibodies and fusion proteins were purified from culture supernatants by chromatography on protein A sepharose. Bound proteins were eluted with 0.1 m citric acid pH 3.5 and dialyzed extensively against PBS.

Enzyme-linked immunosorbent assay (ELISA) and Western blotting

ELISA plates were coated with the antiidiotypic antibodies E4 (for 38C13) or 6C10 (for A20) or with polyclonal goat anti-human IgGFc (Dianova, Hamburg, Germany) or with the mouse anti-GM-CSF antibody 22E9 (Pharmingen, San Diego, CA, USA). After incubation with culture supernatant or purified protein fractions, the proteins were detected with peroxidase-labeled anti-human IgGFc or goat anti-mouse IgM (Dianova) or the biotinylated anti-GM-CSF 31G6 antibody (Pharmingen). For quantification recombinant murine GM-CSF (Genzyme, Cambridge, MA, USA) was used as a standard. The color reaction was initiated with *o*-phenylenediamine (Sigma, München, Germany) and H_2O_2.

Sera of immune mice were assayed for anti-Id responses using as capture antibodies the purified 38C13, A20 or MPC11 immunoglobulins and as detecting antibodies peroxidase-labeled polyclonal anti-mouse IgGFc that was absorbed against IgG2a (for A20) or IgG2b (for MPC11). Reactivity of the sera with constant domains was excluded by ELISAs using irrelevant IgM, IgG2a or IgG2b, respectively, as the capturing antibodies. Cellular ELISAs were performed to measure the humoral response against unknown TAAs. MPC11 or A20 cells were incubated with immune sera and then with peroxidase-coupled goat anti-mouse IgGFc absorbed against IgG2b or IgG2a.

For Western blotting, approximately 1 μg of purified proteins were reduced, separated on 10% SDS-polyacrylamide gels and blotted on to nitrocellulose membranes that were subsequently developed with either peroxidase-labeled anti-human IgGFc or rat monoclonal anti-GM-CSF antibody (No. 1723-01, Genzyme, Cambridge, MA, USA).

GM-CSF bioassay

Biological activity was tested in serially diluted samples using the GM-CSF/IL-3 responsive FDCP1–1 cell line and recombinant murine GM-CSF (Genzyme) as a quantification standard.[34] Cell proliferation was measured in a soluble tetrazolium/formazan-based colorimetric assay according to the manufacturer's protocol (EZ4U assay; Biomedica, Wien, Austria). The GM-CSF specificity of the bioassay was controlled with the neutralizing antibody 22E9.

Animal studies

BALB/c and C3H mice were purchased from Bommice (Ry, Denmark). Groups of six female animals were injected i.p. with 10–50 μg of the purified fusion protein or with $5 \times 10^6–2 \times 10^7$ gene-modified cells that were irradiated at a dose of 20 Gy from a Cs137 source. A boost was given after 3 weeks and following another 7-day interval, the mice were challenged i.p. with a lethal dose of wild-type tumor cells (200 38C13 cells, 10^5 A20 or MPC11 cells). In some experiments, tumor cells were injected simultaneously with 2×10^7 gene-modified cells. In T cell depletion experiments, mice were injected i.p. with 0.2–0.5 mg of the anti-CD4 mAb RmCD4.2,[35] the anti-CD8 mAb RmCD8.2[35] or the pan T cell mAb MmT1.[36] Depletion before immunization was done four times beginning 5 days before the first immunization and discontinuing 7 days before the boost. As shown by FACS analysis, depletion was at least 99% and T cells had recovered at the time of tumor challenge. Depletion after immunization was done by injecting mAb four times every 7–10 days starting 3 days before tumor inoculation. All experiments were done at least in duplicate. Statistical survival analysis was made using the log-rank test.

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References

1 Coulie PG. Human tumour antigens recognized by T cells: new perspectives for anti-cancer vaccines? *Mol Med Today* 1997; **3**: 261–268.

2 Boon T, van der Bruggen P. Human tumor antigens recognized by T lymphocytes. *J Exp Med* 1996; **183**: 725–729.
3 Tsioulias GJ *et al.* Expression of HLA class I antigens in sporadic adenomas and histologically normal mucosa of the colon. *Cancer Res* 1993; **53**: 2374–2378.
4 Chen L *et al.* Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell* 1992; **71**: 1093–1102.
5 Schwartz RH. A cell culture model for T lymphocyte clonal anergy. *Science* 1990; **248**: 1349–1356.
6 Borden EC, Sondel PM. Lymphokines and cytokines as cancer treatment. Immunotherapy realized. *Cancer* 1990; **65**: 800–814.
7 Schiller JH *et al.* A direct comparison of immunological and clinical effects of interleukin-2 with and without interferon-α in humans. *Cancer Res* 1993; **53**: 1286–1292.
8 Schmidt-Wolf G, Schmidt-Wolf IGH. Cytokines and clinical gene therapy. *Eur J Immunol* 1995; **25**: 1137–1140.
9 Colombo MP, Forni G. Cytokine gene transfer in tumor inhibition and tumor therapy: where are we now? *Immunol Today* 1994; **15**: 48–51.
10 Tepper RI, Mule JJ. Experimental and clinical studies of cytokine gene-modified tumor cells. *Hum Gene Ther* 1994; **5**: 153–164.
11 Dranoff G *et al.* Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific and long-lasting antitumor immunity. *Proc Natl Acad Sci USA* 1993; **90**: 3539–3543.
12 Steinmann RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 1991; **9**: 271–296.
13 Levitsky HI *et al.* Immunization with granulocyte–macrophage colony-stimulating factor-transduced, but not B7–1-transduced, lymphoma cells primes idiotype-specific T cells and generates potent systemic antitumor immunity. *J Immunol* 1996; **156**: 3858–3865.
14 Campbell MJ *et al.* Idiotype vaccination against murine B cell lymphoma. Humoral and cellular requirements for the full expression of antitumor immunity. *J Immunol* 1990; **145**: 1029–1036.
15 George AJT, Folkard SG, Hamblin TJ, Stevenson FK. Idiotypic vaccination as a treatment for a B cell lymphoma. *J Immunol* 1988; **141**: 2168–2174.
16 Campbell MJ *et al.* Idiotype vaccination against murine B cell lymphoma. Humoral and cellular responses elicited by tumor-derived immunoglobulin M and its molecular subunits. *J Immunol* 1987; **139**: 2825–2833.
17 Brown SL, Miller RA, Levy R. Antiidiotype antibody therapy of B-cell lymphoma. *Semin Oncol* 1989; **16**: 199–210.
18 Kwak LW *et al.* Induction of immune responses in patients with B-cell lymphoma against the surface-immunoglobulin idiotype expressed by their tumors. *New Engl J Med* 1992; **327**: 1209–1215.
19 Tao M-H, Levy R. Idiotype/granulocyte–macrophage colony-stimulating factor fusion protein as a vaccine for B-cell lymphoma. *Nature* 1993; **362**: 755–758.
20 Mocikat R *et al.* Unaltered immunoglobulin expression in hybridoma cells modified by targeting of the heavy chain locus with an integration vector. *Immunology* 1995; **84**: 159–163.
21 Kardnal C *et al.* Integration vectors for antibody chimerization by homologous recombination in hybridoma cells. *Eur J Immunol* 1995; **25**: 792–797.
22 Mocikat R, Kütemeier G, Hoffmann-Fezer G, Thierfelder S. A mouse model for the preclinical evaluation of immunosuppressive effector functions of human isotypes. The human IgG1 isotype is superior to IgG3. *Transplantation* 1994; **57**: 405–411.
23 Chace JH, Cowdery JS, Field EH. Effect of anti-CD4 on CD4 subsets. I. Anti-CD4 preferentially deletes resting, naive CD4 cells and spares activated CD4 cells. *J Immunol* 1994; **152**: 405–412.
24 Syrengelas AD, Chen TT, Levy R. DNA immunization induces protective immunity against B-cell lymphoma. *Nature Med* 1996; **2**: 1038–1041.
25 Mocikat R, Selmayr M, Thierfelder S, Lindhofer H. Trioma-based vaccination against B-cell lymphoma confers long-lasting tumor immunity. *Cancer Res* 1997; **57**: 2346–2349.
26 Stanley E *et al.* The structure and expression of the murine gene encoding granulocyte–macrophage colony-stimulating factor: evidence for utilisation of alternative promoters. *EMBO J* 1985; **4**: 2569–2573.
27 Mocikat R, Kardinal C, Klobeck, HG. Differential interactions between the immunoglobulin heavy chain μ intron and 3′ enhancer. *Eur J Immunol* 1995; **25**: 3195–3198.
28 Karasuyama H, Kudo A, Melchers F. The proteins encoded by the V_{preB} and λ_5 pre-B cell-specific genes can associate with each other and with μ heavy chain. *J Exp Med* 1990; **172**: 969–972.
29 Bergman Y, Haimovich J. Characterization of a carcinogen-induced murine B lymphocyte cell line of C3H/eB origin. *Eur J Immunol* 1977; **7**: 413–417.
30 Kim KJ *et al.* Establishment and characterization of BALB/c lymphoma lines with B cell properties. *J Immunol* 1979; **122**: 549–554.
31 Laskov R, Scharff MD. Synthesis, assembly and secretion of gamma globulin by mouse myeloma cells. I. Adaptation of the Merwin plasma cell tumor-11 to culture, cloning and characterization of gamma globulin subunits. *J Exp Med* 1970; **131**: 515–541.
32 Shulman M, Wilde CD, Köhler G. A better cell line for making hybridomas secreting specific antibodies. *Nature* 1978; **276**: 269–270.
33 Demanet C *et al.* Treatment of murine B cell lymphoma with bispecific monoclonal antibodies (anti-idiotype × anti-CD3). *J Immunol* 1991; **147**: 1091–1097.
34 Naparstek E *et al.* Induction of growth alterations in factor-dependent hematopoietic progenitor cell lines by cocultivation with irradiated bone marrow stromal cell lines. *Blood* 1986; **67**: 1395–1403.
35 Reinecke K, Mysliwietz J, Thierfelder S. Single as well as pairs of synergistic anti-CD4 + CD8 antibodies prevent graft-versus-host disease in fully mismatched mice. *Transplantation* 1994; **57**: 458–461.
36 Kremmer E *et al.* Neutralization of immunosuppression by antibodies against variable as well as constant regions of monoclonal anti-Thy-1 xenoantibodies and their ability to be suppressed by initial T cell depletion. *Transplantation* 1989; **47**: 641–646.