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An antibody–interleukin 2 fusion protein overcomes tumor heterogeneity by induction of a cellular immune response

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ABSTRACT **Antibody-based therapies for cancer rely on the expression of defined antigens on neoplastic cells. However, most tumors display heterogeneity in the expression of such antigens. We demonstrate here that antibody-targeted interleukin 2 delivery overcomes this problem by induction of a host immune response. Immunohistochemical analysis demonstrated that the antibody–interleukin 2 fusion protein-induced eradication of established tumors is mediated by host immune cells, particularly CD8$^+$ T cells. Because of this cellular immune response, antibody-directed interleukin 2 therapy is capable to address established metastases displaying substantial heterogeneity in expression of the targeted antigen. This effector mechanism further enables the induction of partial regressions of large subcutaneous tumors that exceeded more than 5% of the body weight. These observations indicate that antibody-directed cytokine delivery offers an effective new tool for cancer therapy.**

The unique binding capacity of monoclonal antibodies (mAbs) makes these molecules an attractive tool for tumor therapy. However, it became obvious that although mAbs can selectively target malignant cells *in vivo*, most mAbs lack the ability to destroy these cells by immunologic mechanisms (1). Therefore, mAbs have been conjugated to radioisotopes, cytotoxic drugs, or potent toxins to use them more effectively to destroy the targeted tumor cell (1–3). Despite some highly encouraging data obtained with this approach *in vitro*, the general therapeutic efficacy of tumor-reactive mAbs conjugated to such molecules *in vivo* has been rather disappointing. One of the major obstacles thwarting antibody-based cancer therapy is the heterogeneity of target antigen expression within the tumor. Furthermore, mAbs do not sufficiently penetrate large tumor masses because of their pharmacokinetic characteristics (4, 5).

Since becoming available in recombinant form, interleukin 2 (IL-2) has been used as an *in vivo* T cell growth factor either alone or in combination with *in vitro*-activated lymphocytes in the treatment of patients with advanced renal cell carcinoma or melanoma (6, 7). The aim of this partially successful approach is to generate or propagate tumor-reactive lymphocytes. Forni *et al.* (8) demonstrated that injection of a physiological dose of IL-2 directly into tumors caused suppression of their growth. The major advantage of an *in situ* application is that it avoids certain forms of toxicity associated with the systemic use of cytokines. Cancer patients receiving systemic IL-2 often experience potentially life-threatening side effects that limit the total amount that can be administered (6). Recently, *in situ* cytokine therapy has been developed further by transferring cytokine genes into tumor cells (9, 10). The expected goal is that *in vivo* injection of tumor cells transduced with cytokine genes will produce effective local concentrations of the cytokine to generate an antitumor response through the host's immune system, but at systemic concentration levels too low to produce significant side effects. We reasoned that by using the targeting ability of tumor specific monoclonal antibodies, we could develop a technically more simple strategy to achieve effective concentrations of IL-2 in the tumor microenvironment (11).

In this study, we demonstrate the effectiveness of antibody-targeted IL-2 therapy for established pulmonary and subcutaneous melanoma metastases in a syngeneic tumor model. Even metastases displaying heterogenous expression of the targeted antigen could be successfully addressed. Immunohistochemical analysis suggests that eradication of the tumor is mediated by host immune cells. This effector mechanism enables antibody–IL-2 fusion proteins to overcome some of the traditional limitations of antibody-based therapeutics, i.e., antigen heterogeneity and access to large tumor masses.

MATERIAL AND METHODS

Cell Lines, Animals, and Reagents. The murine melanoma cell lines, B16 and B78-D14, have been described (12). B78-D14 was derived from B16 melanoma cells by transfection with genes coding for β-1,4-*N*-acetylgalactosaminyltransferase and α-2,8-sialyltransferase, inducing a constitutive expression of the gangliosides GD2 and GD3. B16 melanoma cells were maintained as monolayers in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM L-glutamine and were passaged as necessary. The culture medium for B78-D14 cells was further supplemented with G418 at 400 μg and Hygromycin B at 50 μg per ml.

Mouse/human chimeric antibodies directed against GD2 (ch14.18) were constructed by joining the cDNA for the variable region of the murine antibodies with the constant regions of the γ1 heavy chain and the κ light chain. From this, the antibody–IL-2 fusion protein ch14.18–IL-2 was constructed by fusion of a synthetic sequence coding for human IL-2 to the carboxyl end of the human Cγ1 gene (13, 14). The fused genes were inserted into the vector pdHL2, which encodes for the dihydrofolate reductase gene. The resulting expression plasmids were introduced into Sp2/0-Ag14 cells and selected in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 nM methotrexate. The fusion proteins were purified over a protein A-Sepharose affinity column.

All other antibodies used are commercially available and have been described in detail by the manufacturer (PharMingen).

C57BL/6 mice were obtained from The Jackson Laboratory at the age of 4–6 weeks. All experiments were performed

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Abbreviation: IL-2, interleukin 2.

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according to National Institutes of Health (Bethesda) guidelines for care and use of laboratory animals.

Experimental Lung Metastases. Single cell suspensions of 5×10^6 tumor cells were injected into the lateral tail vein. To prevent pulmonary embolism caused by injection of tumor cells, mice were anesthetized by halothane inhalation; tumor cells were suspended in 500 μl PBS containing 0.1% BSA and administered i.v. over a period of 60 s. After 7 days, micrometastases were disseminated throughout the lungs and were invading into the pulmonary alveoli. At day 28 after tumor cell injection, grossly visible metastases were present on the surface of the organ.

Subcutaneous Tumors. Tumors were induced by s.c. injection of 5×10^6 murine melanoma cells in RPMI 1640 medium, which resulted in tumors of $\approx$40 μl volume within 14 days.

Immunohistology. Frozen sections were fixed in cold acetone for 10 min followed by removal of endogenous peroxidase with 0.03% H_2O_2 and blocking of collagenous elements with 10% species specific serum in 1% BSA/PBS. The antibodies were then overlayed onto serial sections at predetermined dilutions (usually 20 μg/ml) and the slides were incubated in a humid chamber for 30 min. With PBS washes between every step, a biotinylated link antibody was applied for 10 min followed by an enzyme, i.e., either peroxidase or alkaline phosphatase, linked to streptavidin for 10 min. After another wash, the substrate was added and the slides were incubated in the dark for 20 min. After a wash in PBS, the slides were counter stained, mounted, and viewed using an Olympus (New Hyde Park, NY) BH2 microscope with photographic capabilities.

Statistical Analysis. The statistical significance of differential findings between experimental groups of animals was determined by Student's *t* test. Findings were regarded as significant if two-tailed *P* values were $\leq$0.01.

RESULTS

Characterization of the Antibody–IL-2 Fusion Protein and the Syngeneic Tumor Model. We previously demonstrated that the genetic fusion of IL-2 to the carboxyl-terminal end of an antibody heavy chain changes neither the biological activity of IL-2 nor the binding affinity of the monoclonal antibody (11). Thus, when the concentration of IL-2 in the fusion protein was calculated as two molar equivalents of IL-2 per mole of fusion protein, the same biological effects to equimolar concentrations were observed; 1 μg of fusion protein corresponds to $\approx$3000 units of IL-2 activity.

To test the effect of this antibody–IL-2 fusion protein on melanoma metastases *in vivo*, we used a syngeneic tumor model of B16 melanoma cells that had been transfected with genes coding for β-1,4-*N*-acetylgalactosaminyltransferase and α-2,8-sialyltransferase, resulting in a constitutive expression of the ganglioside GD2, the antigen recognized by ch14.18–IL-2.

Therapeutic Efficacy Against Established Subcutaneous Tumors. One of the major sites for distant metastases of human melanoma, besides lymph node, lung, and liver, is the skin. Therefore, we tested the effect of antibody–IL-2 fusion protein treatment on established subcutaneous tumors. Such tumors induced by inoculation of 5×10^6 of B78-D14 cells grew within 10 days to a volume of $\approx$25 μl (Fig. 1). At this point, animals were treated over a period of 7 days by i.v. administration of ch14.18–IL-2 fusion protein (16 μg per injection). Objective responses could be observed in all treated animals, with 37.5% complete and 62.5% partial remissions (Fig. 1*A*). Even if treatment was delayed as long as 35 days, in which case large subcutaneous tumors of about 1000 mm^3 are present, ch14.18–IL-2 was able to induce a partial regression of these tumors and delay their future growth (Fig. 1*B*). The differences in tumor weight between animals receiving either the fusion

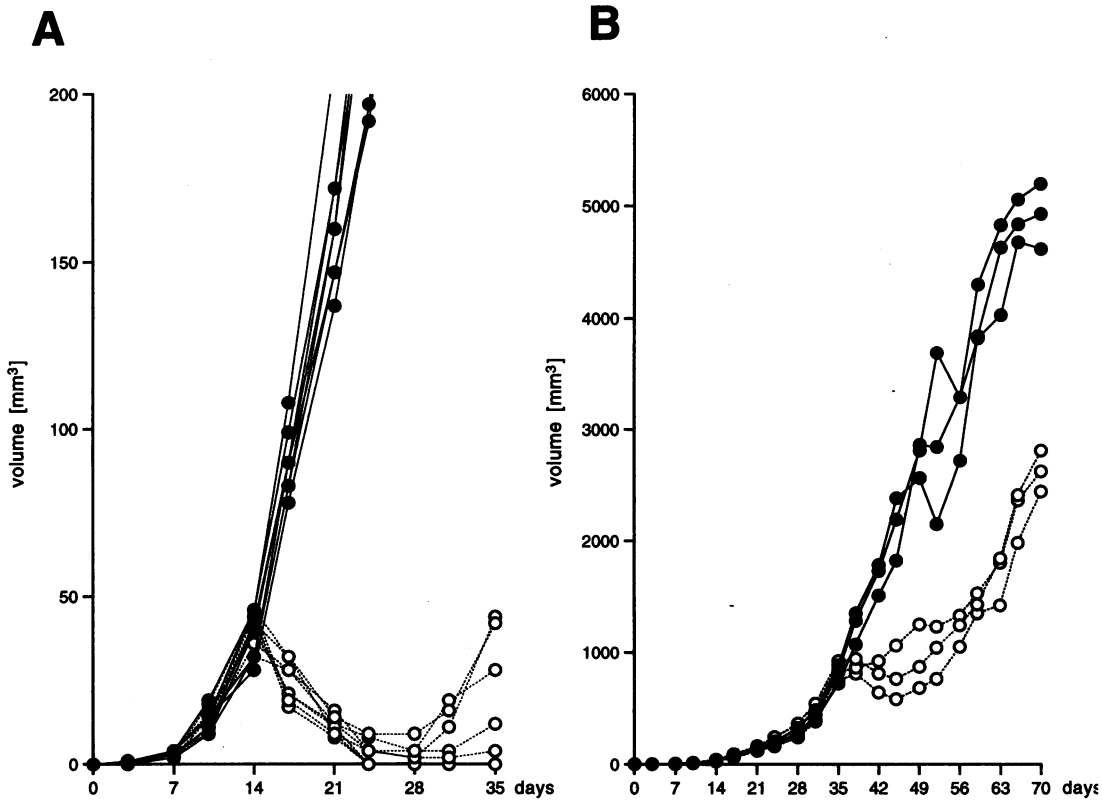

FIG. 1. Effect of ch14.18–IL-2 on the growth of B78-D14 cells. C57BL/6 mice were injected subcutaneously with 5×10^6 B78-D14 melanoma cells. Therapy (○) with i.v. ch14.18–IL-2 fusion protein (16 μg per day for 7 days) was started 10 days (*A*) or 35 days (*B*) after tumor cell inoculation. Control animals (●) received PBS over the same period.

protein or no treatment were statistically significant with two-tailed P values ≤ 0.01.

Histological Evidence of Cellular Infiltrates in Regressing Tumors. The use of a syngeneic tumor model enables the analysis of the effector mechanisms responsible for the observed antitumor effect. Animals bearing 50 μl subcutaneous tumors induced by inoculation of B78-D14 melanoma cells were treated by daily i.v. injections of 8 μg ch14.18–IL-2 fusion protein for 7 d. Tumors and surrounding tissues were excised on 3, 5, and 7 days after initiation of therapy and examined histologically (Fig. 2). Biopsies of tumors from control animals treated with the combination of recombinant IL-2 and ch14.18 at equivalent concentrations showed melanoma cells with essentially no inflammatory response either within or at the periphery of the tumor (Fig. 2 *C* and *D*). In contrast, in tumors of animals treated with the fusion protein marked inflammatory responses, with infiltration of lymphocytes, macrophages, and occasional neutrophils were observed at all time points examined (Fig. 2 *A* and *B*). Staining with an antibody reacting with the CD45 antigen (rat IgG2b, clone 30F11.1) confirmed the presence of leukocytes within the tumor of treated mice (Fig. 3 *E* and *F*). Further characterization of the lymphocytic infiltrate with antibodies reacting with CD4 (rat IgG2a, clone H129.19, Fig. 3 *A* and *B*) or CD8 (rat IgG2a, clone 53–6.7, Fig. 3 *C* and *D*) identified a large portion of these as $CD8^+$ T cells, whereas only a small percentage of the infiltrate could be accounted for by $CD4^+$ cells. CD8 and CD4 were not detectable in tumors obtained from control animals; only a few CD45 cells were detectable. Staining of tumor specimens with NK 1.1 (C3HxBALB/c IgG2a, clone PK136), which identifies natural killer cells in C57BL/6 mice, showed only occasional positive cells in tissues surrounding the tumor and none that infiltrated the tumor; no obvious differences in fusion protein treated or control animals could be detected in this regard (data not shown).

Effect of Antibody–IL-2 Fusion Proteins on Heterogeneous Tumors. One of the major obstacles of antibody-based immunotherapy is the heterogeneity of target antigen expression within the tumor. We previously proposed that successful treatment with antibody–IL-2 fusion proteins may be achieved with only a part of the tumor mass being targeted by fusion proteins because it may serve to elicit a specific cellular immune response. To test whether antibody–IL-2 fusion protein treatment might overcome tumor cell heterogeneity, pulmonary metastases were induced that were heterogeneous with respect to GD2 expression (Fig. 4*A*). This was achieved by i.v. injection of GD2 positive and negative B16 melanoma cells at a ratio of 5:1. This ratio was chosen to compensate for the higher proliferative rate of the GD2 negative wild-type cells, which results in a substantially shorter doubling time than the GD2 expressing variant. Treatment with ch14.18–IL-2 dramatically reduced the number of metastatic foci on the lungs in 62.5% of animals and induced a complete cure in 37.5% of animals (Fig. 4*B* and Table 1). If only GD2 negative cells were used, ch14.18–IL-2 displayed no antitumor effect, proving the specificity of this treatment.

DISCUSSION

The idea of using the targeting ability of certain molecules to direct therapeutics to the desired microenvironment is not new. Ehrlich (15) proposed this concept almost a century ago; more recently, a broad research effort was initiated to prove the feasibility of targeting radioisotopes, cytotoxic drugs, or potent toxins by conjugating them to monoclonal antibodies (1–3, 16). The novelty of our approach lies in its attempt to induce a tumor specific cellular immune response using directed IL-2. Thus, antibody–cytokine fusion proteins combine the unique targeting ability of antibodies with the multifunctional activity of cytokines. In the present report, we demonstrate the therapeutic effectiveness of such constructs in a syngeneic murine melanoma model. Even pulmonary metastases displaying some degree of antigenic heterogeneity or large subcutaneous tumors could be successfully addressed by this form of therapy. Immunohistochemical analysis demonstrated that this antitumor activity is mediated by host immune cells, particularly $CD8^+$ T cells.

A considerable body of knowledge about the effects of IL-2 present in the tumor microenvironment on the induction of an antitumor T cell response has been gained through *in vivo*

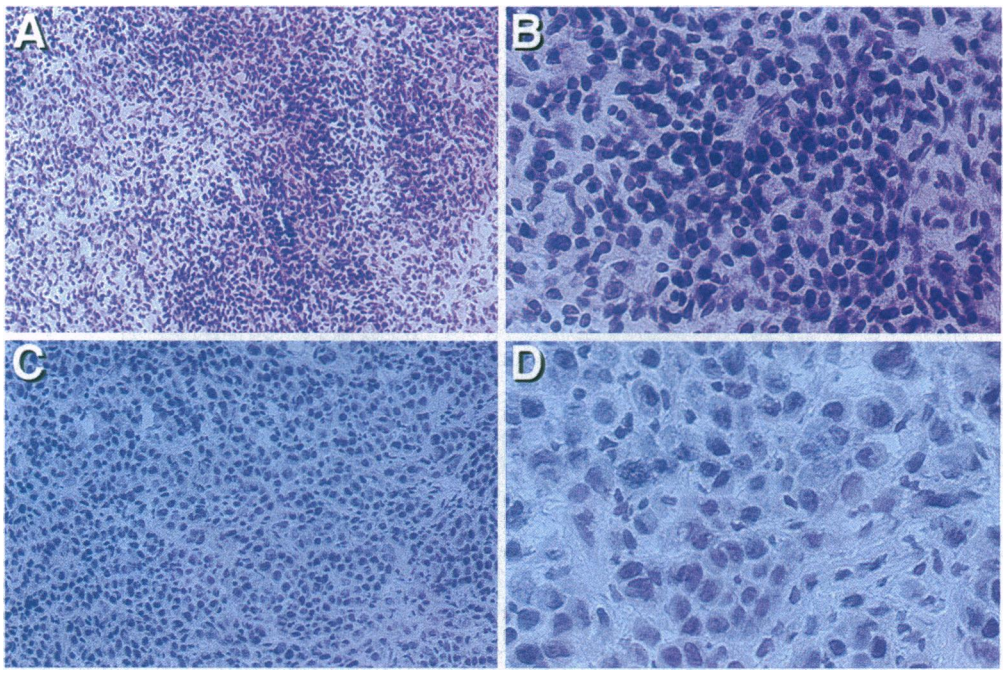

FIG. 2. Presence of massive leukocytic infiltrates in subcutaneous tumors of mice treated with the antibody–IL-2 fusion proteins. C57BL/6 mice were injected s.c. with 5×10^6 B78-D14 melanoma cells. After 14 days, animals received either ch14.18–IL-2 (*A* and *B*) or PBS (*C* and *D*) for 7 days. On day 5, sections (8 μm) of these tumors were prepared and subjected to hematoxylin/eosin staining. (*A* and *C*, ×80; *B* and *D*, ×200.)

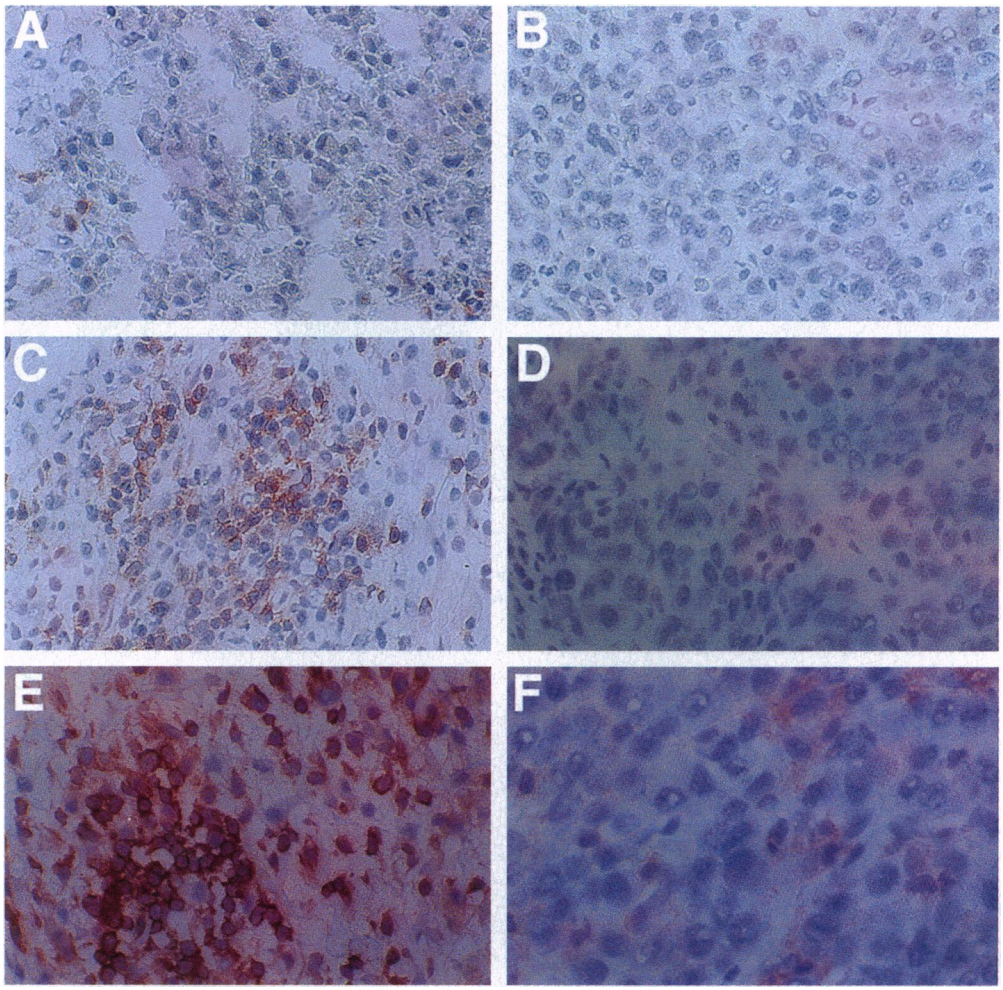

FIG. 3. Immunohistologic characterization of the inflammatory infiltrate of tumors induced by the antibody–IL-2 fusion protein. C57BL/6 mice were injected s.c. with 5×10^6 B78-D14 melanoma cells. After 14 days, animals received either ch14.18–IL-2 (*A*, *C*, and *E*) or PBS (*B*, *D*, and *F*) for 7 days. On day 5, sections (8 μm) of these tumors were prepared and subjected to anti-CD4 (*A* and *B*), -CD8 (*C* and *D*), or -CD45 (*E* and *F*) immunostaining. (×200.)

experiments using tumor cells genetically engineered to produce IL-2 (17–19). Initial reports have shown that IL-2-producing tumor cells induce an antitumor major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocyte response in the absence of CD4 help (17, 20). Although more recent reports suggested that the rejection of IL-2-expressing tumor cells is primarily the result of T cell-independent host defense mechanisms, e.g., macrophages or granulocytes, the published studies to date clearly demonstrate that the elimination of wild-type tumor cells, inoculated either before or after vaccination, is critically dependent on the presence of tumor reactive T cells (17–20). We reported previously that it is possible to achieve effective concentrations of IL-2 at the tumor site *in vivo* by targeting it to the tumor through the binding specificity of monoclonal antibodies (21). Analysis of the biodistribution studies of the ch14.18–IL-2 fusion protein demonstrated that it localizes to subcutaneous tumors, as well as to organs bearing micrometastases, thus providing the means for *in situ* cytokine therapy in a simple and nonpersonalized way.

A successful antitumor T cell response involves induction, recruitment, and effector function of T cells. Antibody-directed IL-2 therapy may influence this process in a number of different ways. First, the tumor cells themselves might interact with naive T cells with IL-2 acting as the second costimulatory signal in the activation of cytotoxic T cells. A recent model proposed by Sprent (22) for the activation of naive T cells provides the rationale for this mechanism. According to this model, high-avidity interactions between peptide–MHC class I complexes and the T cell receptor promote strong crosslinking of T-cell receptor–CD3 complexes, which in turn leads to strong signaling, thereby stimulating the production of cytokines, such as IL-2, and receptors thereof. Costimulation boosts the T cell receptor-mediated signal. If the intensity of signaling is below a certain threshold, e.g., when the density of peptide–MHC complexes or the level of costimulation is low, the responding T cells express only IL-2 receptors, but no IL-2. Hence, these T cells fail to proliferate unless exposed to exogenous IL-2. The second possible scheme for the establishment of T cell activation is based on tumor antigens being processed by antigen-presenting cells. It has been shown that preactivated macrophages, dendritic cells, and granulocytes express receptors for IL-2 and that *in vitro* culture with IL-2 causes functional changes in these cells (23, 24). After arriving at the tumor site, these cells may be activated by the antibody-targeted IL-2 to kill the tumor cells and subsequently present the tumor antigens to T cells. The obvious infiltration of mononuclear cells within the tumor after administration of the antibody–IL-2 fusion protein supports this hypothesis. In addition, antibody–IL-2 fusion proteins are likely to be involved in the recruitment of primed cytotoxic T cells and the activation of their effector function. This is particularly obvious in view of the demonstrated effect of antibody–IL-2 fusion proteins on large subcutaneous tumors.

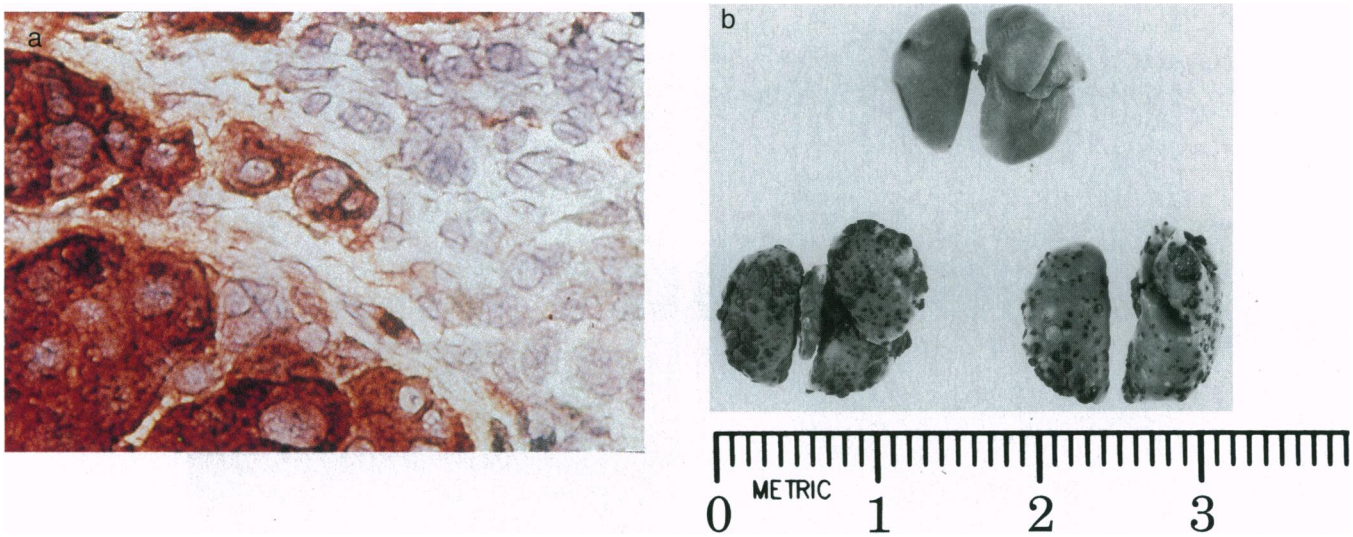

FIG. 4. Effect of ch14.18–IL-2 on heterogeneous metastases. (*a*) Immunohistology of 14-day-old tumors induced by injection of a mixture of B78-D14 and B16 cells (ratio 5:1) stained with mAb 14.18. (*b*) Pulmonary metastases were induced by i.v. injection of either 5×10^6 B16 melanoma cells alone (*Lower Left*) or the mixture of 5×10^6 B78-D14 and 1×10^6 B16 cells (*Upper and Lower Right*). Treatment with 8 μg ch14.18–IL-2 fusion protein was initiated 1 week after tumor cell inoculation (*Upper and Lower Left*). Control animals (*Lower Right*) received PBS over the same period instead. A representative lung specimen for each group is shown.

The therapeutic efficacy of antibody–IL-2 fusion protein against micrometastases expressing antigen heterogeneity can be explained in two ways, either by induction of an immune response directed against both cell types through cells expressing the target antigen or by the presence of IL-2 in close proximity to antigen-negative cells. In support of the latter contention, it is important to note that the carboxyl-terminal lysine residue of the CH3 domain of the antibody heavy chain is accessible to cleavage by plasmin (13). Therefore, it is likely that in the tumor microenvironment a portion of the fusion protein is cleaved into ch14.18 and IL-2. Zatloukal *et al.* (19) have demonstrated that an antitumor immune response can be elicited by coadministration of nonmodified tumor cells and IL-2 transduced fibroblasts. This observation argues against the necessity that the antigenic signal and costimulatory signal have to be delivered by the same cell.

The induction of a cellular antitumor immune response by antibody-targeted IL-2 therapy explains its high therapeutic efficacy against large established subcutaneous tumors as well as heterogeneous pulmonary metastases. In contrast to most other antibody-based therapeutics that rely on the targeteing of all malignant cells (1–3, 16), antibody-mediated cytokine delivery can be effective even if only a small percentage of tumor cells are reached, as these are able to elicit a host immune response. Thus, targeted delivery of cytokines offers a new strategy for tumor immunotherapy that may provide a practicable means to induce eradication of primary and metastatic disease.

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Table 1. Efficiacy of targeted IL-2 therapy on pulmonary metastases displaying antigenic heterogeneity

Treatment*	Tumor	No. of foci†
Experiment 1		
None	B16	>500,>500,>500,>500,>500,>500,>500
rIL2 + ch14.18	B16	129, 145, >500, >500, >500, >500, >500, >500
ch14.18–IL-2	B16	97, 138, >500, >500, >500, >500, >500
None	B78-D14 + B16	>500, >500, >500, >500, >500, >500, >500, >500
rIL2 + ch14.18	B78-D14 + B16	104, 179, 189, >500, >500, >500, >500
ch14.18–IL-2	B78-D14 + B16	0, 0, 2, 7, 9, 12, 21, 43
Experiment 2		
None	B16	173, >500, >500, >500, >500, >500, >500
ch14.18–IL-2	B16	158, >500, >500, >500, >500, >500, >500, >500
None	B78-D14 + B16	>500, >500, >500, >500, >500, >500
ch14.18–IL-2	B78-D14 + B16	0, 0, 2, 5, 9, 12, 18, 29

All experimental groups started with eight mice; animals found dead before the planned date of sacrifice were not included in the evaluation.

*Experimental pulmonary metastases were induced by i.v. injection of 1×10^6 B16 cells or the combination of 1×10^6 B16 and 5×10^6 B78-D14 cells as indicated. Treatment was started 1 week thereafter and consisted of daily i.v. administration either of PBS, 8 μg ch14.18, and 24 000 units recombinant IL-2 or of 8 μg of the tumor-specific fusion protein ch14.18–IL-2 as indicated for 7 consecutive days.

†Differences in numbers of metastatic foci between the group of animals bearing mixed tumors treated with the fusion protein and all control groups were statistically significant ($P \leq 0.002$).

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