Efficacy of Local versus Systemic Application of Antibody-Cytokine Fusion Proteins in Tumor Therapy

Oliver Christ, Simone Seiter, Siegfried Matzku, Christa Burger, and Margot Zöller

Departments of Tumor Progression and Immune Defense, German Cancer Research Center, D-69120 Heidelberg [O. C., M. Z.]; Department of Dermatology, University of Saarland, 66421 Homburg/Saar [S. S.]; Department of Immunology and Oncology, Preclinical Pharma Research, Merck KGaA, 64271 Darmstadt [S. M., C. B.]; and Department of Applied Genetics, University of Karlsruhe, 76128 Karlsruhe [S. M., M. Z.], Germany

ABSTRACT

Application of immunocytokines [fusion proteins (FuPs)] where the cytokine has been coupled to an antibody may not produce the severe side effects frequently observed during systemic application of cytokines in cancer therapy. However, it has not been explored whether FuPs are sufficient for intratumoral activation of leukocytes or whether intratumoral versus systemic application may be of greater efficacy. Interleukin 2 (IL2) or tumor necrosis factor (TNF) coupled to an anti-epidermal growth factor receptor monoclonal antibody (IL2-FuP or TNF-FuP) were tested in SCID mice bearing a human epidermal growth factor receptor-positive melanoma transplant and being reconstituted with human HLA-matched peripheral blood leukocytes. Whole-body autoradiography revealed larger accumulation and prolonged retention of i.v. or intratumorally applied IL2-FuP or TNF-FuP compared with the antibody. Even with low doses of FuP, tumor growth was significantly retarded, with the survival time being further prolonged by the intratumoral application. Furthermore, outgrowth of the tumor was prevented in ~50% of mice as long as they received weekly injections of peripheral blood leukocytes concomitantly with the FuPs, which confirmed that it was the donor leukocytes activated in vivo that retarded tumor growth. An in vitro analysis revealed that the IL2-FuP supported mainly proliferation and lymphokine-activated killer cell activity, whereas TNF-FuP stimulated cytokine production and cytotoxic activity of monocytes and, to a low degree, of T cells. Both TNF-FuP and IL2-FuP significantly accumulated in the tumor, which led to retardation of tumor growth. The therapeutic effect was improved by intratumoral application. Importantly, the efficacy of both IL2-FuP and TNF-FuP depended on the induction of an immune response in vivo.

INTRODUCTION

Considerable effort is being made to add to surgery, radiation, and chemotherapy a fourth biological weapon against cancer (1, 2). Apart from gene therapeutic concepts (3), much hope has been given to immunological approaches, which are based either on vaccination (4) or on the transfer of antigen-presenting cells or effector cells (5, 6). Although these approaches are promising, the question of sufficient recruitment of activated effector cells remains, particularly because it is well known that lymphocytes infiltrating the tumor frequently become anergic (7, 8). Thus, a local stimulus that combines targeting of lymphocytes to the tumor and a concomitant activation stimulus should be favorable, if not essential (9, 10). In this respect, antibodies (11) and cytokines (12) have been taken into consideration, but neither approach has met expectations in preliminary studies. Antibodies by themselves frequently appeared rather ineffective (11). Application of cytokines can be quite effective, but is burdened by the danger of side effects (13–16).

Several concepts are being propagated to efficiently direct effector cells toward tumor target cells, while hopefully avoiding toxic effects on normal tissues. One approach is based on bispecific antibodies, which bind the tumor cell, the T-cell receptor/CD3 complex, and a costimulatory molecule (17–21). T cells covered with chimeric receptors are another means of targeting and focusing effector cells (10, 22). A rather interesting approach is based on fusion proteins, which consist of an antibody that recognizes a surface molecule of the tumor cell and either a superantigen (23–28) or an appropriate cytokine (29). In the latter case, it is expected that the localization of a cytokine at the surface of the tumor cell suffices to replace the second signal in T-cell activation, which otherwise requires ligation of a costimulatory molecule. However, ligands of costimulatory molecules are rarely expressed by the tumor cells (29). Immunocytokines, indeed, have been demonstrated in a variety of murine models, including the “humanized” SCID mouse, to reduce metastasis formation (30–33) as well as growth of the primary tumor (32, 33). Under selective circumstances, curative treatment of established tumors or metastases has been achieved (34).

We used the SCID mouse reconstituted with human PBLs.

1 The abbreviations used are: PBL, peripheral blood leukocyte; EGFR, epidermal growth factor receptor; i.t., intratumoral; TNF, tumor necrosis factor α; IL2, interleukin-2; GvH, graft versus host; FuP, antibody-...
and transplanted with a human EGFR⁺ malignant melanoma to explore the efficacy of two anti-EGFR-cytokine fusion proteins. The anti-EGFR partner molecule was chosen for several reasons: Most epithelial tumors, especially squamous cell carcinomas, overexpress EGFR. A blockade of the EGFR by antibody will be accompanied by growth inhibition (35), which may lead to the eradication of established tumors (36). In particular, we were interested in three questions: (a) whether a therapeutic effect be achieved with low, nontoxic doses of FuP; (b) whether the therapeutic efficacy be improved by i.t. application; and (c) whether an inflammatory cytokine such TNF would exert effects similar to those exerted by the classical IL2 T helper cytokine. Although our experimental setting had a dose and time limit because of the induction of GvH disease, we could demonstrate in the available time window that both FuPs had a significant impact on survival time at nontoxic doses. Furthermore, i.t. application was more efficient than systemic application of a FuP or local application of a mixture of cytokine and antibody. Finally, the TNF-FuP was as efficient as the IL2-FuP, but the underlying mechanisms apparently differed.

MATERIALS AND METHODS

Mice and Tumors. SCID mice (H-2d) were bred at the central animal facilities of the German Cancer Research Center. Animals were kept under specific pathogen-free condition and were fed standard diet and water ad libitum. Animals were used for experiments at the age of 11–12 weeks.

The melanoma lines BLM (37), BLM-gp100 (BLM cells transfected with gp100 cDNA; Ref. 38), A375, and the colon carcinoma line SW480 were cultured in RPMI 1640 supplemented with antibiotics, 1-glutamine, and 10% FCS. Confluent cultures were split after detachment of cells by 0.1% trypsin. The BLM lines and the SW480 line are HLA-A2.1, the A375 line is HLA-A2.1⁺. EGFR is expressed at a medium level on the melanoma lines and at a high level on the SW480 line.

Blood Samples. Heparinized peripheral blood was collected from healthy donors or was obtained from the blood bank. Leukocytes were separated by Ficoll-Hypaque gradient centrifugation. Cells were either used immediately or were frozen in aliquots.

Generation of IL2-FuP and TNF-FuP. For expression of both fusion protein, two vectors were constructed. One vector (pcDNA3.1/huCH3-IL2 and pCNeo/huCH3-TNF) coded for the antibody-heavy chain fused to the IL2 gene and the TNF gene, respectively. The second vector (pCI/dhfr-huL) coded for the humanized light chain of the antibody. The cDNA encoding human IL2 and human TNF was from British Biotechnology LTD. Humanization of mAb 425 has been described (39). To create the vector coding for the heavy chain linked to TNF, the vector pCI-neo was digested with EcoRI and NheI (blunt). An EcoRI-HindIII (blunt) fragment containing the DNA sequence encoding the maB Ab 425CH3-TNF was ligated into the EcoRI-NheI side of pcI-neo. In the linker region between the end of the constant region and TNF, seven amino acids were deleted from the original sequence with a PCR step (SPGK from the heavy chain constant region and MVR from the start of the leaderless TNF sequence). Thus, the TNF sequences in the fusion protein started at amino acid 79 of the TNF precursor protein.

To create the vector coding for the light chain, the neo selection marker in the vector pCI-neo was first exchanged for the DHFR gene. PCI-neo was cut with BsrBI and StvI to remove the neo gene. With a PCR step, a StvI (5) and a BsrBI (3) restriction site were introduced at the ends of the DHFR gene (40). The PCR fragment was ligated into the pCI-neo vector cut with BsrBI and StvI to create the vector pCI-DHFR. The humanized light chain with the human κ constant region gene (39) was cloned into the pCI-DHFR vector cut with Xhol and EcoRI.

The hamster kidney cell line BHK21 (a subclone of CCL-10, obtained from the American Type Culture Collection) was cultivated in DMEM supplemented with 10% FCS and 2 mM glutamine. Cells were transfected by the calcium phosphate precipitation method. Stable transfectants were selected using 1 mg/ml gentamicin and 200 μM methotrexate. Best producers were selected with a sandwich ELISA that detected human IgG1 using a goat antihuman IgG for coating and a peroxidase-conjugated antihuman κ antibody as detector antibody. Fermentation was performed in a semicontinuous manner using serum-free CHO-S-SFMII medium (Life Technologies).

The first purification was performed by affinity chromatography on carrier-bound protein A (Pharmacia) using the extended bed technology. The fusion proteins were eluted with 0.2 M glycine buffer (pH 3.3). The pH was immediately neutralized with 1 M Tris solution and brought up to pH 8–8.5. The second purification step involved cation-exchange chromatography on Fractogel EMD SO3 650(S) (Merck). The fusion proteins were eluted with a NaCl gradient (0–0.6 M). The final purification step involved size-exclusion chromatography on Fractogel BioSec 650(S) in PBS (pH 7.4). Purity was controlled by PAGE analysis and isoelectric focusing.

The functional activity of both FuPs was tested in bioassays, using the IL2-dependent CTL-L2 line for IL2-FuP and the TNF-sensitive breast cancer cell line BT20 for TNF-FuP.

mAbs. The following hybridomas were obtained from the American Type Culture Collection: OKT3 (anti-hCD3, mIGGa), OKT4 (anti-hCD4, mLGGb2), OKT8 (anti-hCD8, mLGGa), and BB7.2 (anti-HLA-A2.1, mLGGb2). The hybridoma 15E8 (anti-human CD28, mLGGa2a) was kindly provided by P. Krammer, German Cancer Research Center (Heidelberg, Germany). Generation and humanization of mAb 425 (anti-human EGFR, mLGGa2a) has been described (39). Culture supernatants were purified by passage over protein G. The eluted mAbs were concentrated, dialyzed, and filter sterilized. Anti
CD40L, biotinylated antihuman IL2, biotinylated antihuman IFNγ, biotinylated antihuman TNF, as well as FITC- or phycoerythrin-labeled secondary antibodies were obtained from PharMingen (Hamburg, Germany). For flow cytometry analysis, 5 × 10⁵ cells were stained according to routine procedures. For the determination of cytokines, cells were fixed and permeabilized in advance. Fluorescence was determined with a FACStar (Becton Dickinson, Heidelberg, Germany).

**Generation of DCs and Priming for gp100-specific T Cells.** A modification (20) of the protocol of Xu et al. (41) was used for the generation of DCs. In brief, freshly harvested PBLs (1.5–2 × 10⁶/ml) were suspended in IMEM containing 10% autologous serum and were seeded in 24-well plates. After an incubation period of 90 min at 37°C, nonadherent cells were removed by washing and the plastic-adherent cells were cultured in IMEM containing 10% autologous serum and 150 units/ml granulocyte macrophage colony-stimulating factor, 50 units/ml IL-4, and 50 units/ml IFNγ (Stratham, Hannover, Germany). The medium was exchanged every second day. After 6–7 days of culture, DCs were loaded with the gp100 peptide YLEPGPVT, which is known to bind to HLA-A2.1 molecules (42), and a peptide covering amino acids 231–243 (KHFLRN-QPLTFAL). This peptide was kindly selected by J. Hammer, Roche Diagnostics (Nutley, NJ), according to the TEPITOPE program (43). Loading was performed for 2 h at a peptide concentration of 10 μg/ml. The peptide-containing medium was aspirated, and DCs were carefully washed with IMEM containing 10% autologous serum. DCs were allowed to mature for an additional 3–4 days in the medium described above. Thereafter, autologous PBLs were added and cultured on the peptide-loaded DCs for 3–7 days.

**Proliferation Assay.** PBLs (2–0.25 × 10⁶/well) were cultured with irradiated (30,000 rads) BLM or BLM-gp100 cells (1 × 10⁶/well) for 3 days, with [³H]thymidine (10 μCi/ml) added during the last 16 h of culture. Where indicated, cultures contained, in addition, 10 μg/ml anti-EGFR, 10 ng/ml IL2, 20 ng/ml TNF, 10 μg/ml anti-EGFR plus 10 ng/ml IL2, 10 μg/ml anti-EGFR plus 20 ng/ml TNF, 2 μg/ml IL2-FuP, or 2 μg/ml TNF-FuP. Cells were harvested, and [³H]thymidine incorporation of triplicate cultures was determined in a beta counter.

**In Vitro Activation of CTLs and Cytotoxicity Assay.** For the in vitro activation of CTLs, freshly harvested PBLs were cultured with irradiated (30,000 rads) BLM or BLM-gp100 cells for 8 days. The ratio of lymphocytes to irradiated tumor cells was in the range 20–10:1. Where indicated, cultures contained 10 μg/ml anti-EGFR, 10 ng/ml IL2, 20 ng/ml TNF, 10 μg/ml anti-EGFR plus 10 ng/ml IL2, 10 μg/ml anti-EGFR plus 20 ng/ml TNF, 2 μg/ml IL2-FuP, or 2 μg/ml TNF-FuP. After 8 days of culture, blasts were separated from dead cells by Ficoll-Hypaque gradient centrifugation and used for the evaluation of cytotoxic activity. LAK activity of PBLs was evaluated after 2 days of culture in the presence of antibody, cytokine, antibody plus cytokine, or FuP at the above-mentioned concentrations. Mφs were enriched by plating in flat-bottomed 96-well plates, with nonadherent cells being washed off after 2 h. For the determination of CTL, LAK, and Mφ cytotoxic activity, melanoma cells were labeled with ⁵¹Cr and washed, and 10⁴ cells were seeded in 96-well round-bottomed plates together with the stimulated PBLs or Mφs at an E:T ratio of 50–3:1:1. Plates were incubated for 6 h (CTLs and LAKs) or 16 h (Mφs) at 37°C. After centrifugation, aliquots were removed, and released radioactivity was determined in a gamma counter. All cytotoxicity assays contained the drugs at the indicated doses. When CTL activity was determined, cultures contained in addition cold target cells (A375, HLA-A2.1⁺, or EGFR⁺; ratio of cold target to target, 10:1). Cytotoxicity was calculated as percentage of cytotoxicity: 100 × [(counts in test well − counts in control well)/(maximum releasable counts − counts in control well)]. Spontaneous release was in the ranges 7–12% (CTLs and LAKs) and 12–20% (Mφs). Standard deviations of triplicates were 1–5%.

**In Vivo Studies.** For in vivo experiments, the melanoma lines BLM and BLM-gp100 and the colon carcinoma line SW480 were used. SCID mice were conditioned by irradiation (300 rads) and anti-asialo-GM1 (WAKO, Osaka, Japan) treatment according to the manufacturer’s suggestion (44). Mice received a single s.c. injection of 5 × 10⁶ BLM or BLM-gp100 or 5 × 10⁶ SW480 cells, which were >100-fold (BLM or BLM-gp100) and 5-fold (SW480) above the dose of cells required to observe tumor growth in 100% of mice. The cell count of the tumor cell inoculum was chosen to allow for full-fledged tumor growth within the period before GVH reactions developed in the SCID mice reconstituted with human PBLs. Unstimulated PBLs or peptide-loaded DCs plus primed T cells (1–5 × 10⁶) were injected i.v. once per week. PBL injections were repeated up to seven times. Where indicated, mice received twice weekly injections of 50 μg of anti-EGFR, 300 ng of IL2, 500 ng of TNF, 50 μg of anti-EGFR plus 300 ng of IL2, 50 μg of anti-EGFR plus 500 ng of TNF, 5 μg of IL2-FuP, or 10 μg of TNF-FuP. The doses 300 ng for IL2 and 500 ng for TNF were chosen as corresponding to the bioactive amounts of the cytokines in 5 μg of IL2-FuP and 10 μg of TNF-FuP, respectively. The antibody, cytokines, and FuPs were injected either i.v. or i.t. as indicated in the individual experiments. Applications of PBLs and the drugs mostly started 5 days after tumor cell application. In some experiments (as indicated), injections started on the day of tumor cell application. Animals were sacrificed when tumor masses with a mean diameter of 25 mm appeared or when the animals became anemic or lost weight. These time points were defined as the survival time.

**Pharmacokinetics.** To evaluate accumulation and degradation/excretion of anti-EGFR and FuP, SCID mice were conditioned as described above and received a s.c. injection of 5 × 10⁴ BLM cells. When the tumor reached a mean diameter of 0.5–1 cm, mice received a single i.v. or i.t. injection of [¹²⁵I]-labeled anti-EGFR, [¹²⁵I]-labeled FuP, or the nonbinding [¹²⁵I]-labeled control antibody 20-5 (45). Where indicated, mice received 5 × 10⁶ PBLs. The purified antibodies and the FuPs were labeled with [¹²⁵I] according to the procedure described by Fraker and Speck (46). Briefly, 100 μg of purified mAb or FuP in 100 μl of sodium phosphate buffer (pH 7.4) were incubated for 5 min with 100 μCi of Na¹²⁵I in iodogen-coated tubes. After the reaction was blocked with 400 μl of 0.05 M sodium phosphate, the labeled mAb/FuP was separated from the free Na¹²⁵I by passage over a NAP-5 column (Pharmacia). The amount of immunoreactive, [¹²⁵I]-labeled antibody and FuP was 75–80%. Mice received a mixture of [¹²⁵I]-labeled and unlabeled antibody (100 μg) or [¹²⁵I]-labeled and unlabeled FuP (10 μg) correspond-
Fig. 1 Intratumoral accumulation and degradation of IL2-FuP and TNF-FuP compared with anti-EGFR. SCID mice received a s.c. injection of $5 \times 10^4$ BLM cells. After 25 days (mean tumor diameter, 1 cm), mice received a single i.v. (A and C) or i.t. (B and D) injection of a $^{125}$I-labeled control antibody, $^{125}$I-labeled anti-EGFR, $^{125}$I-labeled IL2-FuP (A and B), or TNF-FuP (C and D). In A and B, mice received in addition $5 \times 10^6$ human PBLs i.v. Mice were killed after 1–120 h, organs were excised and weighed, and radioactivity was determined in a gamma counter. The percentage of the total radioactivity per gram tissue is shown. In E (IL2-FuP plus PBL) and F (TNF-FuP), the tumor:blood ratios are presented. Values are the means of three mice/group; bars, SD.
ing to 20 × 10^6 cpm/mouse. Mice were killed after 1–120 h. The organs were excised and weighed, and antibody distribution was determined by measuring radioactivity in a gamma counter. Antibody distribution is presented as percentage of total activity ± SD per gram of wet tissue from three mice per group. In parallel settings, mice were killed and frozen. Whole-body sections (5 μm) were mounted on Parafilm and exposed to X-ray films for 48 h.

**Statistics.** Significance of differences was evaluated by the two-tailed Student’s t test (*in vitro* studies) and by the Mann Whitney test (*in vivo* studies).

**RESULTS**

**Distribution of IL2-FuP and TNF-FuP in the SCID Mouse.** In a first experiment we evaluated the distribution of the anti-EGFR and the FuPs in tumor-bearing SCID mice using the doses of antibody and FuPs that were also administered in subsequent therapy experiments (Fig. 1). Compared with the anti-EGFR, both FuPs were eliminated more rapidly, i.e., low levels of radioactivity were recovered from the blood as well as from organs such as lung, liver, and kidney within the first 24 h. The more rapid excretion of the FuPs was independent of whether the drugs had been given i.v. (Fig. 1, A and C) or i.t. (Fig. 1, B and D). The concomitant application of human PBLs (Fig. 1, A and B) did not alter the distribution of FuP except for a high retention in the spleen, which was also quite pronounced after application of the anti-EGFR. The unexpectedly high retention in the spleen most likely was attributable to the fact that in the SCID mouse, Fc-receptors are not naturally occupied by IgG. Comparison of the retention of the FuPs and the anti-EGFR antibody in the tumor revealed a different picture. Within 24 h after i.v. injection, both FuPs had significantly accumulated in the tumor. After 72 h, FuPs reached a tumor-to-blood ratio >4, whereas the tumor-to-blood ratio after application of the anti-EGFR was <2 (Fig. 1, E and F). Accumulation was further increased after i.t. application of the FuPs (Fig. 1, E and F). Additional consequences of i.t. administration deserve being mentioned: nonspecific antibodies (and most likely also nonspecific FuPs, which were not tested) emerged rather rapidly in the circulation as well as in nontarget organs. This is additional evidence for a general retention of the specific antibody in tumor tissue. However, specific antibodies also appeared in the whole organism, although at lower levels. Hence, the nontumor tissue also had some exposure to the FuPs.

Accordingly (Fig. 2), IL2-FuP was detected nearly exclusively in the tumor as early as 48 h after i.v. injection, at which time the slow clearance of the anti-EGFR prevented precise tumor localization. i.t. application of IL2-FuP led to significantly prolonged persistence with heavy staining of the tumor area in autoradiography even 120 h after injection. The whole-body distribution of TNF-FuP in tumor-bearing SCID mice revealed concordant results (data not shown).

**Retardation of Tumor Growth by Systemic Application of FuP.** Mice received s.c. transplants of 5 × 10^4 BLM cells (>100 × the TD_100). Starting at day 5 after tumor cell application, mice received twice per week an i.v. injection of either 50 μg of anti-EGFR, 300 of ng IL2, 500 ng of TNF, 50 μg of anti-EGFR plus 300 ng of IL2, 50 μg of anti-EGFR plus 500 ng of TNF, 5 μg of IL2-FuP, or 10 μg of TNF-FuP, the dose of IL2 and TNF being chosen to equal the amount of bioactive cytokine in the FuP preparations. In addition, mice received once per week an i.v. injection of HLA-A2.1^+ PBLs derived from one donor throughout an experiment. According to the availability, the dose of PBLs varied between 1 and 5 × 10^6 cells per injection. Although the FuPs were not toxic by themselves, i.e., SCID mice not receiving PBLs tolerated high doses of FuPs without any side effects, SCID mice that repeatedly received twice the above-mentioned dose of IL2-FuP or TNF-FuP plus...
PBL developed severe GvH reactions with rough fur, rapid weight loss, cachexia, destruction of the liver, and gastrointestinal bleeding after three to four injections of PBLs. Even with a dose of 5 μg of IL2-FuP or 10 μg of TNF-FuP, we had a loss of 10–20% of mice before any sign of tumor growth. Therefore, in most experiments, application of PBLs was ceased after six to seven injections, i.e., before the majority of mice developed GvH disease. Hence, the FuP doses were determined as mentioned and compared with antibody doses that corresponded to a standard therapeutic dose of 2.5 mg/kg body weight (47) and to cytokine doses that were stoichiometrically equivalent to the cytokine doses of the FuPs.

Animals receiving only BLM cells started to become severely sick after ~3 weeks and rarely survived for longer than 5 weeks (Fig. 3A). When mice received in addition human PBLs, survival time was slightly prolonged. Although the additional application of IL2 had only a minor effect on the survival time, application of anti-EGFR was quite efficient, possibly by initiating an in vivo antibody-dependent cellular cytotoxicity (see below). The simultaneous application of anti-EGFR plus IL2 led to a further prolongation of the survival time. Systemic application of IL2-FuP at the given dose did not significantly alter the survival time compared with the mixture.

A slightly different picture emerged when TNF or TNF-FuP were applied systemically (Fig. 3B). TNF had no effect at all on the survival time. The survival time was prolonged by the mixture of TNF and anti-EGFR, and the same was true for the TNF-FuP, albeit at the lower dose. Because Mφs are supposed to be a major target of TNF (48) and because SCID mice show a significant level of murine Mφs, we also tested tumor growth in nonreconstituted SCID mice. TNF by itself again exerted no effect. TNF-FuP slightly prolonged the survival time, but to a much lesser extent than that observed in PBL-reconstituted mice (data not shown). It should be noted that the survival time was evaluated only for those mice in which the developing tumor necessitated killing the animal, i.e., mice that succumbed with severe GvH disease before developing a tumor were not included.

Effect of i.t. Application of FuP on Tumor Growth. Because the systemic application of FuPs provided no significant advantage over the mixture of anti-EGFR and cytokine with respect to the survival time, we next explored whether a stronger effect would be observed by i.t. application. The expectation was based on the rapid degradation of the FuPs and on the low dose of FuP tolerated by the humanized SCID mouse. Antibody, cytokines, and FuPs were injected s.c. in the surrounding of the tumor cell depot until tumors became palpable (diameters >5 mm) but were injected i.t. thereafter. With this regimen, the survival time was prolonged compared with the local application of the cytokines, the antibody, or the cytokine plus the antibody (Fig. 4, A and B). Notably, TNF, which did not influence the survival time when given systemically, had some therapeutic effect after local application.

To support the higher efficacy of the i.t. application of FuP, the i.t. versus the i.v. application mode were directly compared. When mice received IL2-FuP i.t., tumor growth started with a significant delay, and the growth rate remained low as long as PBL substitution was continued. The survival time, as compared with the systemic application of IL2-FuP, was prolonged accordingly (Fig. 4, C and D). By contrast, the antibody, IL2, and the antibody-cytokine mixtures were similarly effective when applied either locally or systemically.
Effect of Conditioning the Immune System. To this point, we had started with the treatment 5 days after tumor cell application. Thus, it became of interest whether a “conditioning administration” of i.v. or i.t. FuP before the application of tumor cells would further improve the therapeutic efficacy. This experiment was performed with TNF-FuP or the mixture of TNF plus anti-EGFR and two tumor lines, BLM and SW480, with moderate and high EGFR levels, respectively. When mice received PBLs together with either TNF-FuP or TNF plus anti-EGFR (i.v. or i.t.) starting simultaneously with tumor cell inoculation, the survival time was prolonged compared with starting the therapeutic protocol on day 5 (as in the experiments shown in Figs. 3 and 4). Furthermore, in this setting the survival time achieved by the FuP exceeded the survival time observed with the TNF plus anti-EGFR treatment, irrespective of the i.v. versus the i.t. application. Two of 10 mice that received BLM cells and TNF-FuP i.t. remained tumor free (Fig. 5, A and B). With the SW480 tumor line, TNF-FuP was curative in 1 and 3 of 10 mice that were treated systemically or locally with TNF-FuP, whereas none of the animals that received i.v. or i.t. TNF plus anti-EGFR survived (data not shown).

A similar improvement should be observed when applying PBLs that had been primed in vitro by a defined tumor antigen. This hypothesis was endorsed by an experiment where BLM cells transfected with gp100 cDNA were used and PBLs were preincubated with in vitro-generated DCs that had been pulsed with a mixture of MHC class I and MHC class II restricted peptides of gp100 to prime for gp100-specific T cells (Fig. 5, C and D). When SCID mice, 5 days after the tumor cell application, were reconstituted with a mixture of gp100 peptide-loaded DCs plus primed PBLs, the i.t. application of IL2-FuP resulted in a further prolongation of the survival time. None of the animals developed palpable tumors as long as they received PBL plus IL2-FuP, and 50% of animals remained tumor free. In addition, several animals had to be killed because the necrosis resulting from melting down the tumor nodule created an untoward condition. Again, the therapeutic efficacy of the i.t. application of IL2-FuP exceeded the efficacy of the i.v. application.

Statistical Evaluation of the Therapeutic Efficacy of the Drugs. A statistical analysis (Table 1) confirmed that the systemic as well as the local application of 300 ng of IL2 exerted a significant effect on the survival time, whereas 500 ng of TNF was efficient only when administered locally. The efficacy of the antibody plus either IL2 or TNF was higher than that of the individual components. However, differences between the systemic versus the local application of antibody plus cytokine were not significant. Instead, both FuPs were more efficient when applied locally. Accordingly, the local application of the FuPs significantly exceeded the effect of antibody plus cytokine. This accounted for the delayed application of the drugs. When lymphocytes were conditioned, either by the FuPs in vivo or by a nominal antigen in vitro, FuPs were more efficient than the mixture, irrespective of the route of application. Finally, it should be noted that the differences in the survival time between systemically and locally applied FuPs were statistically significant, although not at a high level.

Because both FuPs achieved a significant retardation of tumor growth, questions arose concerning the underlying mech-
anism. First, the efficient induction of a GvH disease in animals receiving either IL2-FuP or TNF-FuP and the suppression of tumor growth as long as mice received the FuPs plus PBLs pointed toward an in vivo activation of T cells. Second, TNF-FuP exerted some therapeutic effect by itself, although it was more efficient when applied concomitantly with PBLs. The former effect could have been attributable to activation of murine Møs or to a cytotoxic/cytostatic effect of TNF-FuP by itself.

**In Vitro Analysis of Immune Response Elements as Induced by FuPs.** To see which elements of the immune system may be triggered by targeting of lymphocytes to tumor cells via FuPs, unstimulated PBLs of the matching (HLA-A2.1) haplotype were incubated with irradiated BLM cells in the presence of IL2-FuP or TNF-FuP and the respective controls. When PBLs alone were cultured with irradiated tumor cells, survival of CD4+ cells was low; they did not express activation markers and produced hardly any IL2 (Table 2). Addition of anti-EGFR did not induce a significant change except for a marked increase in IL2 expression. Addition of IL2 improved blast transformation of CD4+ and CD8+ cells and significantly increased the percentage of cells expressing activation markers, e.g., CD28 and CD40L. Furthermore, expression of intrinsic IL2 was strongly augmented. TNF had a slight effect on expansion of CD4 cells and a strong effect on IFNγ expression. The mixture of either IL2 or TNF and the antibody produced results similar to cytokines alone, except for an up-regulation of CD40L expression by both drug combinations and a significant increase in IL2 expression by the mixture of anti-EGFR and TNF. A higher percentage of cells expressed CD28 and CD40L in the presence of IL2-FuP compared with the mixture of IL2 and antibody. Otherwise, no major differences between FuPs and the mixtures of anti-EGFR and cytokines were observed.

Major differences became apparent at the functional level (Fig. 6). To test for functional changes, freshly harvested PBLs were cultured in the presence of the drugs, which were also added to the assays. Proliferative activity was evaluated after 3 days of culture, LAK activity after 2 days, and CTL activity after 8 days. To differentiate in the latter case from LAK activity, an HLA-A2.1, EGFR cold target cell was added. Møs were enriched by plastic adherence. Their lytic activity was evaluated in a 16-h 51 Cr-release assay.

PBLs cultured with irradiated tumor cells neither proliferated nor displayed a sizable cytotoxic potential. Low proliferative activity was seen in cultures containing anti-EGFR. IL2 mainly influenced proliferation and LAK activity, but also supported the activation of CTLs. TNF by itself had no influence on cytotoxic or on proliferative activity, nor did it exert a cytostatic effect. Cultures containing IL2 plus anti-EGFR showed good proliferative as well as LAK and CTL activity. In the presence of anti-EGFR plus TNF, proliferative and cytotoxic activities

![Fig. 4 Influence of locally applied FuP on the survival time of tumor-bearing SCID mice. A and B, mice (5/group) received a s.c. injection of 5 × 10^4 BLM cells. Starting 5 days thereafter, they received once per week an i.v. injection of 1–5 × 10^6 HLA-matched human PBLs (terminated after seven injections to avoid GvH reactions) and twice per week a peritumoral/i.t. injection of the drugs as described in the legend for Fig. 3. The survival time is recorded. C and D, SCID mice (10/group) received a s.c. injection of 5 × 10^4 BLM cells. Starting 5 days thereafter, they received twice per week an i.v. or a peritumoral/i.t. injection of 5 μg of IL2-FuP or of 50 μg of anti-EGFR plus 300 ng of IL2. Mice received six weekly i.v. injections of 1–5 × 10^6 human PBLs (HLA-A2.1+). The mean tumor diameters (C) and survival times (D) are shown.

![Diagram](image_url)
resembled the activities observed in the presence of anti-EGFR only. IL2-FuP produced a significant increase in the proliferative potential and the lytic activity of LAKs and CTLs. In the presence of TNF-FuP, the proliferation and lytic activity of CTL were augmented. Finally, significant lysis by Mφs was seen with TNF plus anti-EGFR, which was further increased in the presence of TNF-FuP (Fig. 6D). IL2 and IL2-FuP had no effect on Mφ activity.

Thus, under in vitro conditions, IL2-FuP efficiently initiated T-cell activation and stimulated LAKs as well as CTLs. Its potency was superior but not qualitatively different from that of a mixture of anti-EGFR and IL2. TNF-FuP provided a weak stimulus for lymphocyte proliferation, but it had no effect on LAK cells. However, it supported activation of Mφs and, to a minor extent, of CTLs.

**DISCUSSION**

There is ample evidence that cytokines provide an efficient means of initiating an antitumor immune response. However, cytokines are very short lived, and high doses are required, which leads to severe side effects (13–16). Alternatively, vaccination with cytokine cDNA-transfected tumor cells has been proposed (49, 50), as has the application of fusion proteins composed of an antibody entity that recognize a surface molecule on the tumor cell and a cytokine (29). The basic idea in both cases is to activate immune cells in close proximity to the tumor cell and its plethora of tumor “antigens.”

To date, mostly fusion proteins with an IL2 component have been studied (30–34, 51–55), and to our knowledge, FuPs have been applied systemically in all cases. The half-life of most FuPs is much longer than that of cytokines but shorter than that of antibodies. Once in the blood stream, FuPs will stimulate primarily circulating lymphoid cells, which may not be the optimal condition for the initiation of an immune response in the tumor tissue. Hence, we wondered whether local, i.e., i.t., application may be superior to the systemic one. We were also curious to see whether TNF, whose therapeutic use is thought to be based on its cytotoxic/cytostatic activity (56, 57), may exert its natural function as a proinflammatory cytokine (58, 59) when applied as a FuP. We could demonstrate that both FuPs strongly

![Fig. 5](https://example.com/fig5.png)
Antibody-Cytokine Fusion Protein in Cancer Therapy

Table 1  Prolongation of survival time by antibody, cytokines, and FuPs: Statistical evaluation of differences

<table>
<thead>
<tr>
<th>PBLs + stimulus</th>
<th>Mean survival time, days</th>
<th>P (PBL)</th>
<th>P (cytokine + anti-EGFR)</th>
<th>Mean survival time, days</th>
<th>P (PBL)</th>
<th>P (cytokine + anti-EGFR)</th>
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<tr>
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<td></td>
<td>35.2</td>
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<td>Anti-EGFR</td>
<td>44.8</td>
<td>0.002</td>
<td></td>
<td>50.4</td>
<td>0.003</td>
<td>NS</td>
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<td>IL2</td>
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<td></td>
<td>42.4</td>
<td>0.003</td>
<td>NS</td>
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<tr>
<td>IL2 + anti-EGFR</td>
<td>61.5</td>
<td>&lt;0.001</td>
<td></td>
<td>60.6</td>
<td>&lt;0.001</td>
<td>NS</td>
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<td>IL2-FuP</td>
<td>61.4</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>&gt;78.8</td>
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<td>TNF</td>
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<td>TNF + anti-EGFR</td>
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<th>P (cytokine + anti-EGFR)</th>
<th>Mean survival time, days</th>
<th>P (PBL)</th>
<th>P (cytokine + anti-EGFR)</th>
<th>P (i.v.)</th>
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<tr>
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<td></td>
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<td>IL2 + anti-EGFR</td>
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<td>51.5</td>
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<tr>
<td>TNF + anti-EGFR</td>
<td>58.0</td>
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<td>0.049</td>
<td>61.1</td>
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<td>TNF-FuP</td>
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<td>0.003</td>
<td>0.049</td>
<td>&gt;100.0</td>
<td>&lt;0.001</td>
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<th>PBLs + stimulus</th>
<th>Mean survival time, days</th>
<th>P (PBL)</th>
<th>P (i.v.)</th>
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<td>IL2-FuP, i.v.</td>
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<td>&gt;95.8</td>
<td>&lt;0.001</td>
<td>0.031</td>
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*SCID mice were conditioned by irradiation (300 rads) and anti-asialo-GM1 treatment. Mice (10/group) received 5 × 10^4 BLM cells or 5 × 10^7 BLM-gp100 cells or 3 × 10^6 SW480 cells s.c., and starting 5 days thereafter or on the same day (as indicated) weekly i.v. injections of 1–5 × 10^6 PBLs. Mice received in addition, twice weekly, i.v. (systemic) or i.t. (local) applications of 50 μg of anti-EGFR, 300 ng of IL2, 500 ng of TNF, 50 μg of anti-EGFR plus 300 ng of IL2, 50 μg of anti-EGFR plus 500 ng of TNF, 5 μg of IL2-FuP, or 10 μg of TNF-FuP.

*The mean survival times and the significance of prolonged survival times are shown.

NS, not significant.

accumulate in the tumor after systemic application and are retained for a prolonged period of time after i.t. application. In the therapeutic setting, both FuPs lead to an increase in the survival time, the effect being stronger after i.t. application.

Before discussing the efficacy of both FuPs as therapeutic targets, we briefly want to comment on our choice of the humanized SCID mouse model. If one is going for an in vivo system, wherein human lymphocytes can be confronted with a human tumor graft, the SCID mouse model after reconstitution with human lymphoid cells (PBLs in our case) is the only choice. This model is particularly suited to characterize drugs with human lymphoid cells (PBLs in our case) is the only choice. This model is particularly suited to characterize drugs that preferentially attach and retain the cytokine at the tumor cell surface. In the therapeutic efficacy of antibody plus cytokine was not improved by the combination. Additionally, the tumor-to-blood ratio of both FuPs within the tumor was in the same range as the decline of the antibody. Accordingly, the tumor-to-blood ratio of both FuPs within the tumor was in the same range as the decline of the antibody. Additionally, the tumor-to-blood ratio of both FuPs within the tumor was in the same range as the decline of the antibody. Additionally, the tumor-to-blood ratio of both FuPs within the tumor was in the same range as the decline of the antibody. Additionally, the tumor-to-blood ratio of both FuPs within the tumor was in the same range as the decline of the antibody. Additionally, the tumor-to-blood ratio of both FuPs within the tumor was in the same range as the decline of the antibody. Additionally, the tumor-to-blood ratio of both FuPs within the tumor was in the same range as the decline of the antibody. Additionally, the tumor-to-blood ratio of both FuPs within the tumor was in the same range as the decline of the antibody. Additionally, the tumor-to-blood ratio of both FuPs within the tumor was in the same.
if administered i.t. With respect to TNF-FuP, the danger of a Schwartzmann reaction may be avoided, whereas a local collapse of tumor vasculature (60) could further augment the therapeutic efficacy.

It is important to discuss the aspect of GvH reactions in the FuP-treated, humanized SCID mice. When mice repeatedly received higher doses of FuP plus human PBLs, they succumbed after 3–4 weeks with GvH disease. At this time, we never observed even mild signs of a GvH reaction in mice receiving PBLs only, or in mice receiving PBLs together with either cytokine or the antibody or the mixture. With the low dose of FuP that was used in subsequent experiments and with cessation of the application of PBL after 6–7 weeks, we still lost ~20% of mice by GvH disease, whereas we had no loss in any of the control groups. This finding is a strong indication of the high efficacy of the FuPs to support an immune response in vivo, but it also points to the fact that a major portion of FuP function is deployed outside the tumor. The interpretation has been supported by the observation that a high amount of anti-EGFR and a relatively high amount of FuP were transiently retained in the spleen. This was most likely attributable to trapping of the antibody and the FuP by Mφs via FcR binding, because in the SCID mouse, which lacks B cells and consequently immunoglobulin, FcRs are not occupied. Although GvH disease in SCID mice reconstituted with human PBLs is a general phenomenon, not all protocols of treating human tumors in the SCID mouse are essentially burdened with the appearance of GvH disease. This may be partly attributable to the duration of the experiment, i.e., GvH reactions are rarely seen in the first weeks after PBL injection. In addition, GvH disease rarely is observed after a single bolus injection of PBLs, but this is paid for by a rapid loss of the human lymphocytes (61). Furthermore, the problem is avoided a priori if T cell clones are applied. Finally, GvH reactions are mitigated when fully allogeneic tumors are used, where roughly half of the lymphocytes will recognize the allogeneic MHC, i.e., are deviated from the xeno-nogenic host.

The most convincing evidence for the in vivo induction of an immune response in the tumor-bearing SCID mice derived from the observation that, particularly after the i.t. application of FuP, exponential tumor growth started only after cessation of the application of PBLs. We have described before that human PBLs vanish rather quickly in the SCID mouse (61), for which reason we chose the repeated application of PBLs. Blockage of tumor growth was achieved during the period of PBL application as long as IL2-FuP or TNF-FuP was also applied. Hence, when TNF was applied as a fusion protein, it apparently exerted its natural function as a proinflammatory cytokine. However, the therapeutic effect of TNF in the absence of reconstitution, where there is only endogenous Mφs and a regrowth of NK cells, was essentially nil, and TNF-FuP induced only a slight retardation of tumor growth. Finally, the efficacy of the FuPs could be increased when pulsed DCs were applied together with primed T cells compared with unstimulated PBLs. The therapeutic effect also was strengthened when we started the therapeutic regimen at the time of tumor cell application rather than with delay. All of these features strongly support our conclusion that it was not direct toxicity of the FuPs on tumor cells that conferred a therapeutic benefit, but rather the targeted activation of an antitumor response by the transferred PBLs.

| B. Cytokine expression | % stained cells  
|------------------------|--------------------------
| PBLs + stimulus        | IL2 | IFNγ | TNF |  
| None                   | 8.4 ± 2.5  | 22.3 ± 3.7  | 7.9 ± 2.2  |  
| Anti-EGFR              | 19.1 ± 2.5  | 21.3 ± 3.3  | 10.8 ± 1.3  |  
| IL2                    | 42.5 ± 4.6  | 28.3 ± 3.2  | 7.8 ± 1.4  |  
| TNF                    | 28.5 ± 4.2  | 49.4 ± 4.8  | 9.7 ± 2.9  |  
| IL2 + anti-EGFR        | 43.1 ± 3.8  | 29.7 ± 2.8  | 7.1 ± 1.7  |  
| TNF + anti-EGFR        | 37.8 ± 3.9  | 50.1 ± 4.8  | 10.8 ± 2.5  |  
| IL2-FuP                | 46.1 ± 3.8  | 31.0 ± 3.3  | 8.4 ± 2.8  |  
| TNF-FuP                | 40.7 ± 4.5  | 50.4 ± 5.1  | 11.2 ± 3.1  |  

* PBLs were cultured in the presence of irradiated (30,000 rads) BLM cells plus the indicated stimuli. Cultures contained (per ml) 10 μg of anti-EGFR, 10 ng of IL2, 20 ng of TNF, 10 μg of anti-EGFR plus 10 ng of IL2, 10 μg of anti-EGFR plus 20 ng of TNF, 2 μg of IL2-FuP, or 2 μg of TNF-FuP.

* After 3 days of culture, lymphocytes were harvested and stained for flow cytometry. For the evaluation of cytokine expression, cells were fixed and permeabilized before staining. Values represent the mean ± SD of 3–5 experiments.
Antibody-Cytokine Fusion Protein in Cancer Therapy

In the presence of IL2-FuP, we noted expansion of CD4+ tumor cell cultures. Although the gross influence of FuP on the in vitro activation of proliferating and cytotoxic leukocytes. A, human PBLs (1 × 10^6/well) were cultured in the presence of irradiated (30,000 rads) BLM cells (1 × 10^5/well). Cultures contained (per ml) 10 μg of anti-EGFR, 10 ng of IL2, 20 ng of TNF, 10 μg of anti-EGFR plus 10 ng of IL2, 10 μg of anti-EGFR plus 20 ng of TNF, 2 μg of IL2-FuP, or 2 μg of TNF-FuP. Cells were cultured for 3 days, with [3H]thymidine added during the last 16 h. Plates were harvested and counted in a beta counter. The mean cpm of triplicate cultures are shown; rad.

Influence of FuP on the in vitro activation of proliferating and cytotoxic leukocytes. A, human PBLs (1 × 10^6/ml) were cultured for 2 days in the presence of irradiated (30,000 rads) BLM cells (1 × 10^5/ml). Cultures contained the drugs as described in the legend for panel A. Surviving lymphocytes were harvested, washed, counted, and added together with the drugs to 51Cr-labeled BLM cells at an E:T ratio of 50:1. C, human PBLs (1 × 10^6/ml) were cultured for 8 days in the presence of irradiated (30,000 rads) BLM cells (1 × 10^5/ml). Cultures contained the drugs as described in the legend for panel A. Surviving lymphocytes were harvested, washed, counted, and added together with the drugs and HLA-A2.1 cold target cells (cold target:target cell ratio of 10:1) to 51Cr-labeled BLM cells at an E:T ratio of 25:1. D, Møs were enriched by plating human PBLs in flat-bottomed microtiter plates. After 2 h of incubation at 37°C and 5% CO2, nonadherent cells were removed by washing. 51Cr-labeled BLM cells at estimated M6-T ratios of 25:1 and the drugs as described in the legend for panel A were added. Supernatants were harvested after 6 h (B and C) or 16 h (D) and counted in a gamma counter. Values represent the means of triplicate cultures. Significance of differences are indicated by *, * in open columns (anti-EGFR, IL2, and TNF) refer to cultures without any drug; * in gray columns (anti-EGFR plus cytokine) refer to cultures containing only anti-EGFR; * in filled columns (FuPs) refer to cultures containing the mixture of anti-EGFR plus cytokine.

To date, IL2-FuP has been described to activate CD8+ CTLs as well as NK/K cells mediating ADCC (30, 31, 33, 53, 55, 62). One report, from a study using an antibody-lymphotoxin-FuP, explicitly stated that T cells may not be involved but that asialo-GM1+ cells, including NK cells and B cells, appear to be important (63). After vaccination with IL2 cDNA-transfected tumor cells and concomitant application of IL2-FuP, a CD8 memory response was observed (51). No comparable data are available for TNF-FuP. However, TNF by itself has been described to activate the cytotoxic potential of γδ T cells (64), to recruit CD8+ cells into the tumor (65), to synergize with IFNα and IFNγ (66), to initiate NO synthesis by neutrophils and Møs (67), and to restore and/or increase apoptotic programs with bystander killing (68). To provide some hints as to the mechanistic repertoire of IL2-FuP and TNF-FuP in the SCID system, we performed an in vitro analysis in mixed lymphocyte tumor cell cultures. Although the gross in vivo effects obtained with TNF-FuP and IL2-FuP were rather indiscriminate, in vitro analysis clearly pointed toward distinct underlying mechanisms. In the presence of IL2-FuP, we noted expansion of CD4+ cells, up-regulation of activation markers, high-level expression of IL2 and IFNγ, a significantly enhanced proliferative response, very efficient LAK activity, and an unambiguous activation of CTLs. TNF-FuP at a dose equivalent to that in the in vivo studies was not cytostatic. It supported, although less efficiently than IL2-FuP, T-cell activation, which was accompanied by expression of IFNγ. It had no effect on proliferative response or on LAK activity. However, we observed a low level of lytic activity by CTLs and by Møs. We interpret these findings in the sense that both IL2-FuP and TNF-FuP support the activation of T cells, IL2-FuP preferentially of CD4+ cells, and TNF-FuP preferentially of CD8+ cells. Both FuPs also appear to stimulate nonadaptive defense mechanisms, but in a nonoverlapping manner. IL2-FuP supports the activation of LAK cells, whereas TNF-FuP supports the activation of Møs. Clearly, the final proof of an in vivo activation of T cells requires the analysis of lymphocytes regained from FuP-treated, tumor-bearing SCID mice. This will be described in an accompanying article, in which we report on confirmation of the in vitro features of differing activities of IL2-FuP versus TNF-FuP.
IL2-FuP and TNF-FuP are eliminated from nontargeted tissues with kinetics between those of the cytokine and the unmodified antibody. However, they are retained and stabilized in the tumor, which allows for therapeutic efficacy even at low dose. Both FuPs function in concert with the immune system, including adaptive and nonadaptive mechanisms, but they support different leukocyte subsets and different pathways of activation. The superior therapeutic efficacy of i.t. compared with i.v. application can be explained by the highly efficient targeting that results from selective retention within the tumor. Taking into account that in the SCID mouse, the FuPs accelerated GvH reactions and therefore could be given only at a low dose, both FuPs can be expected to be highly efficient in humans, in whom this problem will not exist.

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Efficacy of Local versus Systemic Application of Antibody-Cytokine Fusion Proteins in Tumor Therapy

Oliver Christ, Simone Seiter, Siegfried Matzku, et al.

*Clin Cancer Res* 2001;7:985-998.