Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 9791–9795, October 1995
Immunology

Antibody-targeted superantigens are potent inducers of tumor-infiltrating T lymphocytes *in vivo*

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Communicated by Sune Bergström, Karolinska Institute, Stockholm, Sweden, July 7, 1995

ABSTRACT **Recruitment of antigen-specific tumor-infiltrating lymphocytes (TILs) is a major goal for immunotherapy of malignant tumors. We now describe that T-cell-activating superantigens targeted to a tumor by monoclonal antibodies induced large numbers of pseudospecific TILs and eradication of micrometastases. As a model for tumor micrometastases, syngeneic B16 melanoma cells transfected with the human colon carcinoma antigen C215 were injected intravenously into C57BL/6 mice and therapy with an anti-C215 Fab fragment–staphylococcal enterotoxin A (C215Fab–SEA) fusion protein reacting with the C215 antigen was initiated when visible lung metastases were established. More than 90% reduction of the number of lung metastases was observed when mice carrying 5-day-old established lung metastases were treated with C215Fab–SEA. The antitumor effect of C215Fab–SEA was shown to be T-cell-dependent since no therapeutic effect was seen in T-cell-deficient nude mice. Depletion of T-cell subsets by injection of monoclonal antibody demonstrated that $CD8^+$ cells were the most prominent effector cells although some contribution from $CD4^+$ cells was also noted. C215Fab–SEA treatment induced massive tumor infiltration of $CD4^+$ and $CD8^+$ T cells, while only scattered T cells were observed in untreated tumors. SEA treatment alone induced a slight general inflammatory response in the lung parenchyme, but no specific accumulation of T cells was seen in the tumor. TILs induced by C215Fab–SEA were mainly $CD8^+$ but a substantial number of $CD4^+$ cells were also present. Immunohistochemical analysis showed strong production of the tumorcidal cytokines tumor necrosis factor α and interferon γ in the tumor. Thus, the C215Fab–SEA fusion protein targets effector T lymphocytes to established tumors *in vivo* and provokes a strong local antitumor immune response.**

The existence of tumor-specific T cells has been demonstrated in a number of experimental animal tumor models and in certain human malignancies, such as melanoma and renal carcinoma. However, the frequency of tumor-specific T cells is generally low and insufficient to interfere with progressive tumor growth. T lymphocytes possess various potential anti-tumor effector functions, such as perforin-mediated or fas–fas ligand-induced apoptosis and secretion of growth-suppressive cytokines. Therapy with *in vitro*-expanded tumor-infiltrating lymphocytes (TILs) enriched for T cells with tumor specificity has clearly demonstrated the potency of T cells in tumor-cell eradication (1, 2). However, the clinical use of TILs is hampered by the cumbersome technology and the limited and heterogenous expression of tumor-specific peptide–major histocompatibility complex (MHC) complexes. In contrast, numerous tumor-associated antigens have been demonstrated by the use of monoclonal antibodies (mAbs). We have described (3) the use of antibody–superantigen (SAg) fusion proteins as an approach for T-cell-based tumor therapy. Staphylococcal enterotoxin A (SEA) belongs to a family of bacterial SAgs capable of activating many T cells to cytotoxicity and cytokine secretion (4–6). SAgs bind to MHC class II molecules as unprocessed proteins and subsequently activate T cells expressing particular T-cell-receptor β-chain-variable-region (V_β) families. Since MHC class II products are expressed on normal B cells and monocytes but only to a limited degree on most tumors, we have designed SAgs with tumor reactivity (3). Recombinant fusion proteins of the tumor reactive mAb C215 (Fab fragment) and the SAg SEA (C215Fab–SEA) expressed a hundredfold stronger affinity for the tumor antigen C215 compared to MHC class II molecules (3). By using a tumor model with murine B16 melanoma cells transfected with the C215 antigen, we demonstrated that the C215Fab–SEA fusion protein completely eliminated 1-day-old lung metastases. In the present paper, we have adapted this therapeutic approach for treatment of established B16-C215 lung metastases and characterized the immune response in the tumor. C215Fab–SEA treatment induced a massive infiltration of cytokine-producing T cells into the tumor. The results imply that the antitumor effects of Fab–SEA fusion proteins are dependent on their extremely potent ability to direct activated T cells to the tumor. They might therefore be useful for treatment of nonimmunogenic tumors currently considered beyond the reach for immunotherapy.

MATERIALS AND METHODS

Reagents. Hybridomas secreting anti-CD4 (clone GK1.5) and anti-CD8 (clone 2.43) mAb used for *in vivo* depletion of lymphocyte subsets were obtained from American Type Culture Collection. The hybridomas were cultured *in vitro* and IgG was affinity-purified with a protein G-Sepharose column. Polyclonal rat IgG was purchased from Jackson ImmunoResearch. For immunohistochemical staining the following antibodies were used: anti-CD4, anti-CD8, anti-tumor necrosis factor α (TNF-α) (clone MP6-XT22) and anti-interferon γ (IFN-γ) (XMG1.2). Antibodies were purchased from PharMingen and biotinylated goat anti-rat IgG was from Jackson ImmunoResearch.

Cloning, Expression, and Purification of C215Fab–SEA Fusion Protein. The construction and expression of C215Fab–SEA has been described (3). Briefly, cDNA encoding the heavy-chain V (V_H) and light-chain V regions (V_L) of the C215 mAb was cloned by PCR from the C215 hybridoma cells and ligated to consensus IgG1 heavy-chain-constant-region (C_H) and light-chain-constant-region (C_L) cDNA. The V_H–C_H was fused via a tripeptide spacer to the SEA gene. The V_L–C_L and V_H–C_H–SEA gene constructs were assembled in the vector pKp865. In this vector, transcription of the bicistronic mRNA

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Abbreviations: SEA, staphylococcal enterotoxin A; SAg, superantigens; MHC, major histocompatibility complex; IFN, interferon; TNF, tumor necrosis factor; TIL, tumor-infiltrating T lymphocyte; mAb, monoclonal antibody; V, variable; C, constant.

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is driven by an isopropyl β-D-thiogalactoside-inducible promoter and secretion of the light chain and heavy chain–SEA fusion constructs are directed by an OmpA and a synthetic signal sequence, respectively. The fusion protein is expressed in *Escherichia coli* K-12 UL635 (*ara-14, xyl-7, ΔompT*, $T4^R$). The C215Fab–SEA protein was purified on a protein G-Sepharose column and a Mono S HR 5/5 column (Pharmacia LKB). The fractions containing C215Fab–SEA were pooled and finally passed through a PD-10 column (Pharmacia LKB). The protein was >95% pure as determined by SDS/PAGE.

Transfection with cDNA Encoding the C215 Antigen. The expression vector pKGE839 containing the GA733-2 cDNA (encoding the C215 antigen) and the neomycin-resistance gene (7) was transfected into B16 melanoma cells by using Transfectam (Sepracor, Villeneuve la Garenne, France) and subjected to repeated cell sorting. The C215 high-expressing and metastasizing clone 7.B6 was selected from the B16-C215 cells (3) and the expression of C215 antigen was regularly analyzed by flow cytometry.

Immunohistochemical Staining. To facilitate immunohistochemical analysis, C215Fab–SEA treatment was initiated 18 days after tumor challenge when large lung metastases were present. Animals were sacrificed at various times after C215Fab–SEA treatment and pieces of lung tissue containing B16-C215 tumors were snap-frozen in isopentane cooled in liquid nitrogen. Frozen sections (10 μm) were prepared, and after drying and fixation in ice-cold acetone, the sections were sequentially stained with the indicated mAbs, followed by biotinylated goat anti-rat IgG and avidin-biotinylated alkaline phosphatase complex (8). The slides were developed in the alkaline phosphatase substrate S-5100 (Vector Laboratories) and mounted in DPX medium (Kebo, Stockholm). For intracytoplasmatic staining of TNF- and IFN-secreting cells, the sections were fixed in formalin and stained in the presence of saponin. Sections were analyzed and photographed by standard light microscopy or by UV epifluorescence. The relative T-cell infiltration was analyzed by using the Quantimet 600 image analysis system (Leica, Cambridge, U.K.). The percentage of T cells compared to whole tumor area was measured, and the relative infiltration was graded as no infiltrating cells (0%), low infiltration (<1%), moderate infiltration (1–10%), high infiltration (10–40%), and massive infiltration (40–70%).

Tumor Models. C57BL/6 ($H\text{-}2^b$), C57BL/6 $nu^{+/-}$, and C57BL/6 $nu^{+/+}$ mice were obtained from Bomholtgaard (Ry, Denmark). For induction of lung metastasis, 6- to 10-week-old mice were inoculated intravenously (i.v.) in the tail vein with 100,000 B16-C215 cells in 0.2 ml of PBS containing 1% normal syngeneic mouse serum. Treatment with C215Fab–SEA was initiated various times after tumor inoculation as indicated. The mice were sacrificed 3 weeks after injection of tumor cells and the number of lung metastases were counted.

Depletion of CD4 and CD8 T Cells. Mice were injected intraperitoneally (i.p.) with 200 μg of affinity-purified anti-CD4 (GK1.5) and/or anti-CD8 (2.43) mAb or control rat IgG in PBS containing 1% syngeneic mouse serum 2 and 4 days after tumor inoculation. Depletion of the relevant T-cell subset (>96%) was verified by flow cytometry analysis of peripheral blood lymphocytes from individual mice prior to C215Fab–SEA treatment (data not shown).

RESULTS

Therapeutic Effect of C215Fab–SEA on Established Tumors. As an *in vivo* model for treatment of established metastases, mice were injected i.v. with C215-transfected B16 melanoma cells and the appearance of lung metastases was followed. Five days after injection of tumor cells, visible metastases on the surface of the lung were seen and confirmed to express the C215 antigen by immunohistochemistry (data not shown). Treatment with C215Fab–SEA resulted in >90% reduction in the number of metastases when treatment was started 1, 3, or 5 days after tumor injection (Fig. 1*A*). To optimize the therapeutic regimen, various therapeutic schedules were evaluated. Marginal effects were seen with a single injection, a moderate effect was recorded with two injections, and profound antitumor effects were achieved by three or four injections of C215Fab–SEA (Fig. 1 *B* and *C*). By using various time intervals between the injections, we found that treatment with 1- or 2-day intervals was optimal, while treatment with 4- or 7-day intervals generated only minor therapeutic effects (Fig. 1*C*).

Phenotype of Antitumor Effector Cells. We have shown (3) that the C215Fab–SEA fusion protein inhibits the growth of B16-C215 metastases, whereas neither native SEA nor the nonrelevant C242Fab–SEA generates any therapeutic effects.

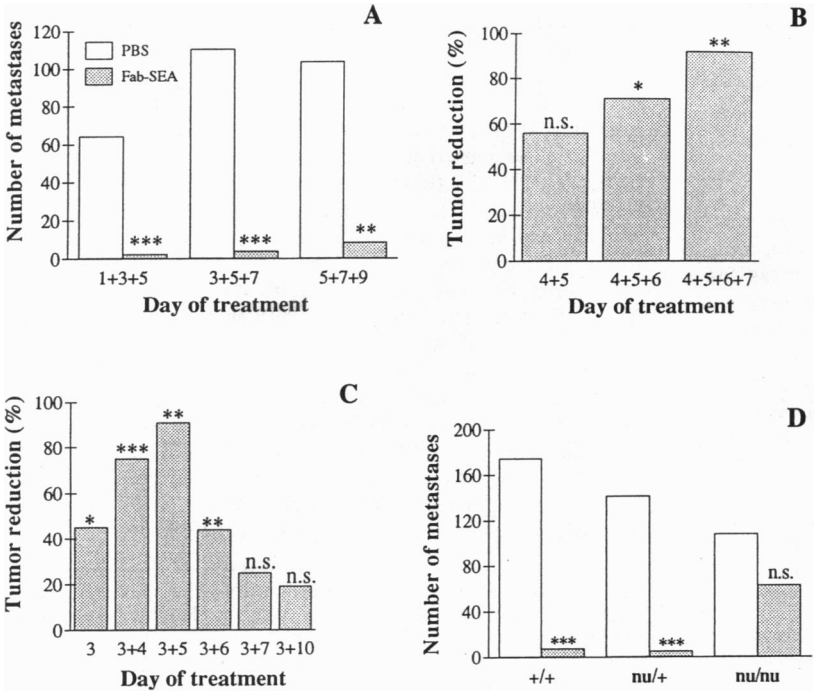

FIG. 1. C215Fab–SEA therapy of B16-C215 lung metastases. (*A*) Therapeutic effect of three injections of C215Fab–SEA at 50 μg per animal or PBS given at various times after tumor inoculation. (*B*) Therapeutic effect of increasing number of injections with 10 μg of C215Fab–SEA starting 4 days after tumor inoculation. Tumor reduction was calculated as the percentage of 90 lung metastases in PBS-treated mice. (*C*) Therapeutic effect of two injections with C215Fab–SEA at 10 μg per animal at various times. Tumor reduction was calculated as in *B* (200 lung metastases in PBS control). (*D*) C215Fab–SEA therapy with 50 μg per animal given on days 1, 3, and 5 after injection of B16-C215 melanoma in normal +/+ C57BL/6 mice, heterozygotic nu/+, and T-cell-deficient homozygotic nu/nu C57BL/6 mice. (*A–D*) Each experimental group contained six to eight mice and the difference from vehicle-treated mice was analyzed with the Mann–Whitney *U* test. *, $0.05 \leq P > 0.01$; **, $0.01 \leq P > 0.001$; ***, $P < 0.001$; n.s., not significantly different.

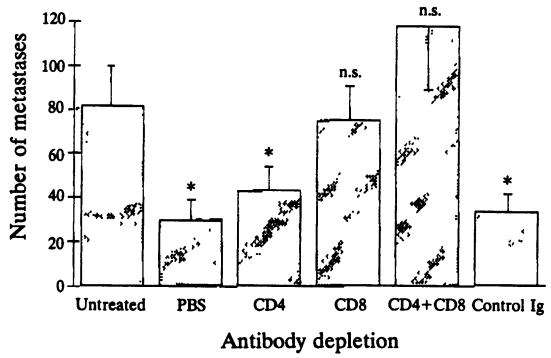

FIG. 2. Role of T-lymphocyte subsets in antitumor effects of C215Fab–SEA. Animals were injected i.p. with 200 μg of affinity-purified anti-CD4, anti-CD8, anti-CD4 + anti-CD8, or control rat IgG 2 and 4 days after tumor inoculation and treated with three consecutive injections of 50 μg of C215Fab–SEA starting 5 days after tumor inoculation. Each group contained eight mice. Error bars indicate SEM. The difference from vehicle-treated mice was analyzed with the Mann–Whitney *U* test. *, $0.05 \leq P > 0.01$; n.s., not significantly different.

This strongly indicated that targeting of T cells to the tumor is dependent on the antigen specificity of the fusion protein. To characterize the phenotype of lymphocytes mediating the antitumor effect, we compared the effect of C215Fab–SEA in normal C57BL/6 mice, heterozygotic nu/+ C57BL/6 mice and in T-cell-deficient C57BL/6 nu/nu nude mice. Highly significant antitumor effects were seen in heterozygotic nu/+, while minimal effects were observed in T-cell-deficient homozygous nu/nu nude mice (Fig. 1*D*). The presence of $CD4^+$ and $CD8^+$ T cells in C215Fab–SEA-treated tumors suggested that both subsets may be involved in the antitumor response. Depletion of $CD4^+$ and $CD8^+$ T cells by injection of mAbs prior to treatment with C215Fab–SEA demonstrated a major role for $CD8^+$ T cells in the antitumor response, whereas $CD4^+$ T cells appeared to be less important (Fig. 2). Depletion of both CD4 and CD8 T-cell subsets completely abrogated the therapeutic effect of C215Fab–SEA.

T-Cell Infiltration and Cytokine Production in the Tumor. To examine whether the apparent T-cell requirement for C215Fab–SEA mediated therapeutic effects correlated with the presence of TILs in the tumor, we performed immunohistochemical analysis of established B16-C215 melanoma metastases. Tumors from vehicle-treated mice contained only scattered infiltrating $CD4^+$ T cells and no $CD8^+$ T cells (Fig. 3 *A* and *B*). Treatment with C215Fab–SEA resulted in massive infiltration of $CD4^+$ T cells and a substantial infiltration of $CD8^+$ T cells (Fig. 3 *C* and *D*, respectively).

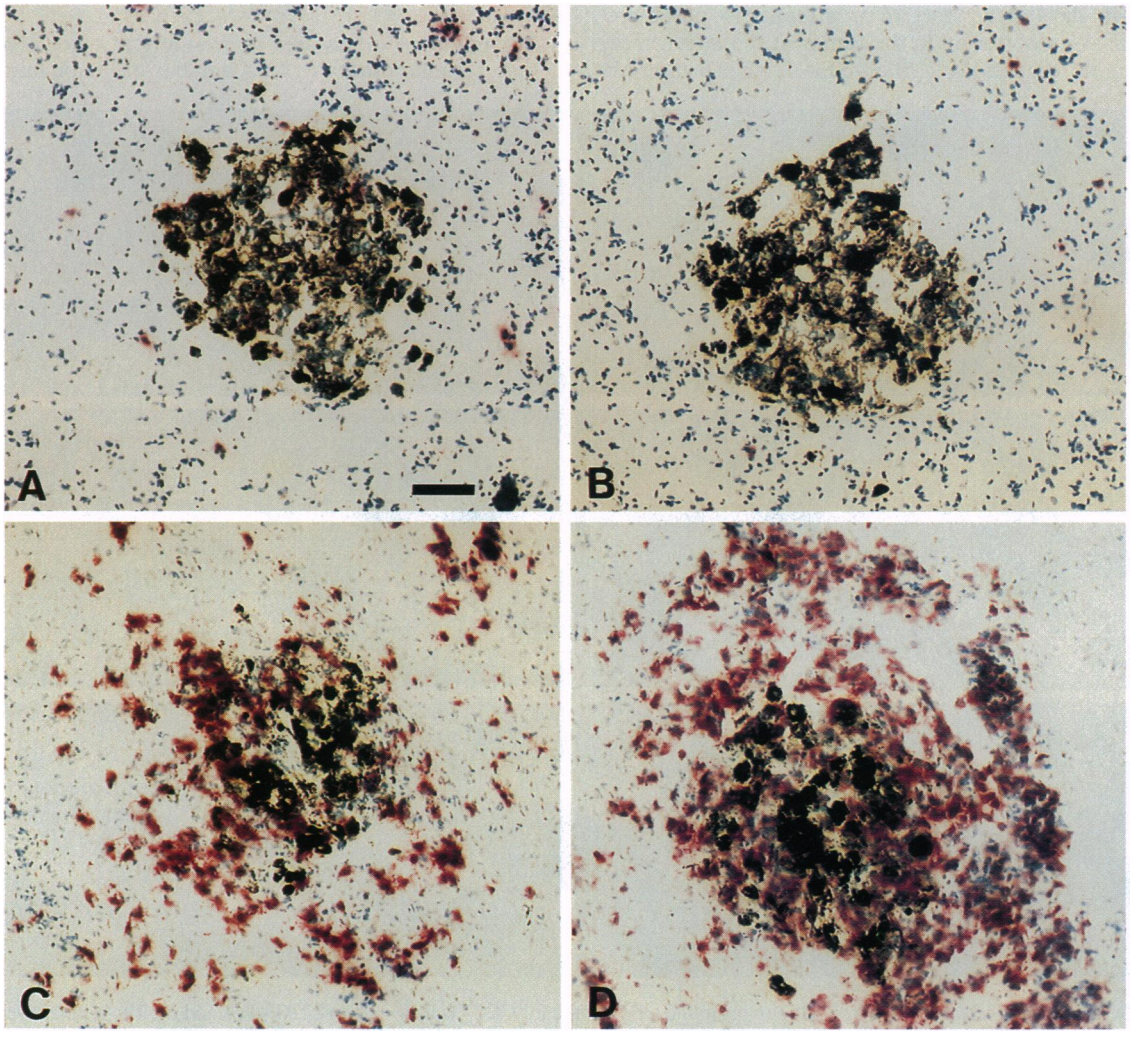

FIG. 3. Micrographs of melanin-pigmented B16-C215 tumors in the lung 18 days after tumor inoculation. (*A* and *B*) Tumor nodule from a PBS-treated mouse showing scattered infiltrating $CD4^+$ cells (*A*) and no infiltrating $CD8^+$ cells (*B*). (*C* and *D*) Tumor taken from a mouse 48 h after a single injection of 50 μg of recombinant C215Fab–SEA, illustrating infiltration of $CD4^+$ (*C*) and $CD8^+$ (*D*) cells in the tumor. [Bar (in *A*) = 50 μm and is valid for *A–D*.]

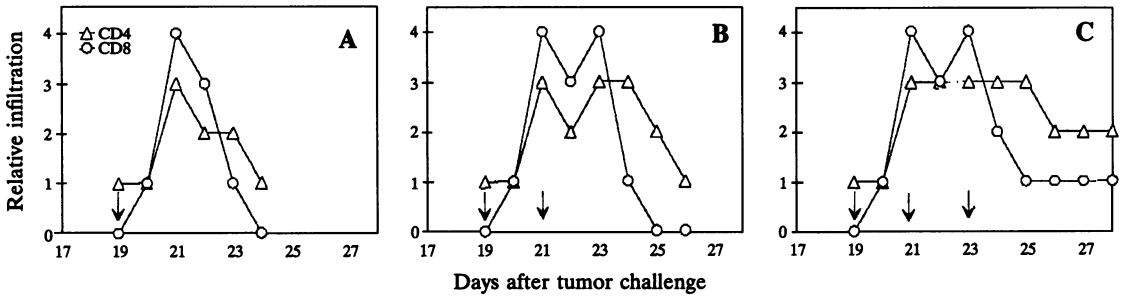

FIG. 4. Immunohistochemical analysis of CD4$^+$ and CD8$^+$ TILs. The times for injection of C215Fab–SEA are indicated by arrows and the relative tumor infiltration of lymphocytes was graded as no (0), low (1), moderate (2), high (3), or massive (4). (*A*) Injection on day 19. (*B*) Injections on days 19 and 21. (*C*) Injections on days 19, 21, and 23.

Kinetic analysis of the appearance of TILs demonstrated significant infiltration 1 day after C215Fab–SEA injection, massive infiltration after 2 days, and a gradual decline in the number of CD8$^+$ TILs after 4 or 5 days (Fig. 4*A*). Treatment with SEA (Fig. 5*B*) or the nonrelevant C242Fab–SEA fusion protein (data not shown) resulted in a slight inflammatory response in the lung parenchyme, but no specific targeting of T cells to the tumor was seen. Repeated injections of C215Fab–SEA resulted in increased lymphocyte infiltration compared to a single injection (Fig. 4*B*). Moreover, the T cells persisted in the tumor and substantial infiltration was recorded 2–6 days after start of treatment. In particular CD4$^+$ T cells were observed in the tumor at the later times. Injection of a third dose of C215Fab–SEA further prolonged the presence of infiltrating T cells (Fig. 4*C*). Certain sections of the lung clearly illustrated extravasation of T cells and apparent trafficking to nearby metastases (Fig. 5*D*). Effector functions of the TILs were assessed by immunohistochemical staining for selected cytokines known to affect B16-C215 cells. Although a single injection of C215Fab–SEA was effective in recruiting TILs, only scattered cells produced TNF-α or IFN-γ. In contrast, a second or third injection of C215Fab–SEA resulted in strong TNF-α (Fig. 5*F*) or IFN-γ production (data not shown) in a large number of TILs.

DISCUSSION

Treatment of metastases is presently a major challenge for tumor immunotherapy. In a number of major human cancer

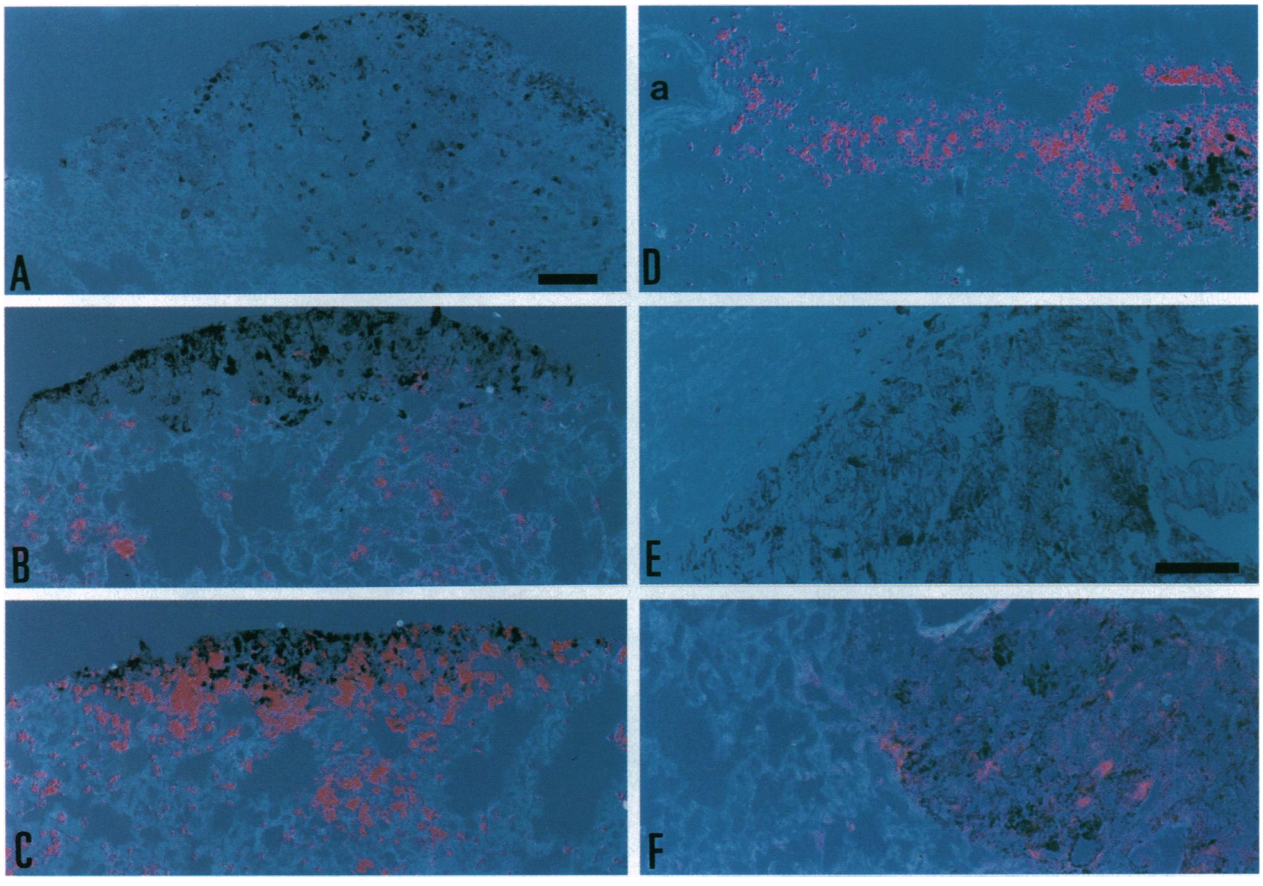

FIG. 5. Micrographs of melanin-pigmented B16 tumors in the lung stained for CD8$^+$ cells (*A–D*) and TNF-α (*E* and *F*). The sections were visualized by UV epifluorescence, which gives a red color of the substrate at positive sites. [Bars: *A* (valid for *A–D*) and *E* (valid for *E* and *F*) = 100 μm.] (*A*) PBS-treated animal showing no CD8$^+$ T cells in the tumor. (*B*) Forty-eight hours after recombinant SEA (18 μg) treatment, moderate numbers of CD8$^+$ cells could be found in the lung but no specific accumulation in the tumor. (*C*) Forty-eight hours after recombinant C215Fab–SEA (50 μg) treatment, a high density of CD8$^+$ cells could be seen within and in close proximity to the tumor. (*D*) CD8$^+$ cells in the lung parenchyma between tumor and blood vessel. (*E*) TNF-α staining of tumor from PBS-treated animal. (*F*) TNF-α staining in tumor 48 h after C215Fab–SEA (50 μg) treatment.

types, the primary tumor may be removed either by surgery, such as in colon cancer Duke stage C and melanoma stage 2–3, or reduced to nondetectable disease by chemotherapy, such as in small cell lung carcinoma. However, due to the presence of micrometastases these conditions often result in clinical relapse and a fatal outcome in a large number of patients. To evaluate the role of antibody-targeted SAgs in animal models mimicking these human malignant conditions, we utilized disseminated metastases of the highly aggressive murine B16-C215 melanoma. The B16 melanoma expresses only minute amounts of MHC class I molecules and is considered to be poorly immunogenic (9). The C215-transfected B16 clones were apparently not immunogenic as indicated by the lack of T-cell infiltration in the tumor and a similar growth pattern in normal and nude mice (data not shown). Consequently, the metastasizing B16-C215 tumor provides a relevant model for poorly immunogenic human tumors. In the present study, we demonstrate that established lung metastases are strongly inhibited by antibody-targeted SAgs. The therapeutic effect most likely involves antibody/antigen-dependent targeting of SEA-reactive pseudo-specific T cells to the tumor since C215Fab–SEA, but not SEA or an irrelevant Fab-SEA fusion protein, inhibited tumor growth. B16 melanoma cells are sensitive to IFN-γ and TNF-α (10) and the massive accumulation of cytokine-producing TILs is likely to be involved in tumor eradication. Local intratumoral release of the tumoricidal cytokines TNF-α and IFN-γ should enable potent antitumor effects with limited systemic toxicity. Moreover, the released cytokines are expected to be particularly important in elimination of antigen-negative tumor cells likely to be present in human tumors irrespective of the antibody used for targeting. Indeed, in preliminary experiments, injections of B16-C215 cells mixed with a significant fraction of mock-transfected B16 cells did not result in loss of therapeutic effect (unpublished observation). Therapy of B16-C215 tumors with C215Fab–SEA in nude mice showed that the therapeutic effect was entirely T-cell-dependent. Immunohistochemical analysis of the tumor demonstrated a massive influx of both $CD4^+$ and $CD8^+$ T cells into the tumor. This is compatible with earlier studies showing that SEA stimulate both $CD4^+$ and $CD8^+$ T cells *in vivo* (11–13). In certain tumor models, it has been shown that $CD4^+$ T cells may actively suppress tumor growth by secreting cytokines while in other models $CD8^+$ T cells mediated the antitumor effect (14, 15). Depletion of these subsets *in vivo* demonstrated that $CD8^+$ T cells are the major effector cells in C215Fab–SEA-based therapy of the B16-C215 tumor, although both subsets were required to obtain optimal effect. We have shown (11–13) that SEA treatment *in vivo* induced cytokine production in both $CD4^+$ and $CD8^+$ T cells while induction of cell-mediated cytotoxicity was confined to $CD8^+$ cells. Thus, the antitumor effect of $CD8^+$ T cells may be related to the release of cytokines and direct cell-mediated cytotoxicity. Moreover, TNF-α and IFN-γ might upregulate cell surface molecules needed for cytotoxic T-cell function. The contribution of cytokines and cytotoxicity in the final outcome of Fab–SEA-based tumor therapy is currently addressed by using cytokine-neutralizing mAbs and perforin knockout mice.

Semiquantitative immunohistochemical analysis of TILs demonstrated a peak response 2 days after injection. The number of TILs gradually declined and completely disappeared after 4 days. Optimal therapeutic effects were obtained with repeated injections given with <2-day interval, while longer intervals were clearly less effective. One interpretation of these findings is that the first injection of C215Fab–SEA induces a rapid but transient expansion of TILs, while the following injections induce local production of cytokines, chemokines, and cytotoxic T cells. The highly organized cellular trafficking from blood vessels to the tumor site frequently observed (Fig. 5*D*) indicates the release of T-cell-specific chemokines produced by C215Fab–SEA-activated cells in the tumor. Prolonged time intervals between C215Fab–SEA injection is inadequate since most T cells then have disappeared from the tumor. Furthermore, pharmacokinetic studies using radiolabeled C215Fab–SEA have shown that the fusion protein is rapidly accumulated in the tumor and maximal uptake is reached 1 day after injection and then declines (data not shown).

The B16 melanoma expresses low amounts of MHC molecules and is a poorly immunogenic tumor. Vaccination with B7 and MHC class II-transfected B16 cells have failed to elicit protective immunity (9), while a number of other tumors have responded to such therapies (14–16). In contrast, C215Fab–SEA bound to the tumor cell surface recruits pseudospecific $CD4^+$ and $CD8^+$ T cells dependent on their T-cell receptor Vβ expression and irrespective of the presence of MHC molecules on the tumor. This suggests that targeting of superantigenicity is highly effective also against tumors considered to be nonimmunogenic and indicates that they also might be useful for treatment of human cancer.

We thank Dr. L. Abrahmsén for contributions in the molecular biology work with the fusion protein and Ms. C. Modéus, E. Folkesson, I. Andersson, and K. Behm for skillful technical assistance.

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