Tumor Suppression after Tumor Cell-targeted Tumor Necrosis Factor α Gene Transfer

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Summary

The tumor necrosis factor α (TNF-α) gene was introduced by retroviral gene transfer into the TNF-α-insensitive tumor cell line J558L. Production of 40 pg/ml TNF-α by clone J2T12 consistently did not change the growth rate in vitro, but drastically suppressed tumor growth when injected into syngeneic BALB/c mice. Within 2 wk, 90% of the mice inoculated with J558L cells developed a tumor, but none of the mice injected with J2T12 did so. Within the observation period (>3 mo), 60% of the mice inoculated with J2T12 did not develop a tumor. In the other 40% of the mice, tumor manifestation was significantly delayed. Mice injected simultaneously with J2T12 cells and an anti-TNF-α monoclonal antibody developed tumors similar to parental J558L cells. Similarly, the tumor-suppressive effects of TNF-α were abolished, e.g., by injection of an anti–type 3 complement receptor (CR3) monoclonal antibody that is known to prevent migration of inflammatory cells. These results and the observation of tumor-infiltrating macrophages suggest that lack of tumorigenicity of J2T12 cells is due to the TNF-α secretion by the tumor cells and that TNF-α acts indirectly by a mechanism that involves chemotactic recruitment and activation of cells, predominantly of macrophages. In contrast, the tumor growth was not affected when, instead of TNF-α, interleukin 6 was expressed by J558L cells. Together, our results support the concept of tumor cell–targeted cytokine gene transfer as a tool for cancer treatment, and particularly demonstrate that extremely low doses of TNF-α produced by tumor cells are sufficient to inhibit tumor growth without detectable side effects.

Application of cytokines has already been promising in cancer treatment (1). However, it reveals a severe problem resulting from the requirement to apply systematically high doses of the respective cytokines. Application of therapeutically effective concentrations is often accompanied by toxic side effects. This problem may be circumvented by "tumor cell–targeted cytokine gene therapy" (2): the tumor cells are genetically engineered to produce a given cytokine in vitro and upon injection into mice provide a locally enhanced cytokine concentration. This makes use of a more physiological mode of action of the cytokine to eventually chemotactically attract effector cells and/or induce a tumor-specific immune response. Additionally, upon destruction of the tumor cells, the source for the cytokine production is eliminated. Tumor cell–targeted cytokine gene therapy has been successfully used with IL-2, IL-4, and IFN-γ genes (3-7). These experiments demonstrate that in certain cases the failure of the immune system to recognize the tumor cells is due to

insufficient activation rather than to the lack of immunogenic determinants on the tumor cells.

The antitumor activity of TNF-α is well documented (for review, see 8). Originally, it was attributed to a direct cytolytic effect on tumors. Accordingly, in most studies the effect of systemical application of TNF-α on in vitro sensitive tumor cell lines has been investigated. Recently, it has become clear that some effects of TNF-α are mediated by immunological effector mechanisms (9). Tumor-suppressive effects were obtained only with nearly lethal concentrations of systemically applied TNF-α. Furthermore, the systemic application of TNF-α does not make use: (a) of the property of TNF-α to induce chemotactic attraction of macrophages (10) and, consequently, (b) of the potential of macrophages to display a tumoricidal activity (11-13).

We have investigated the potential of TNF-α to suppress the growth of a TNF-α-insensitive tumor cell line. Therefore, the TNF-α gene was expressed in a tumor cell line in

order to locally activate the immune system and thereby to destroy the tumor cells.

Materials and Methods

Cell Lines. J558L is a heavy chain loss variant of BALB/c-derived plasmacytoma J558 (14) and was maintained as described (4). Cell cultivation conditions of virus-packaging cell lines PA317 (15) and psi2 (16) were described (4).

mAbs. The rat anti–mouse TNF-α mAb (IgM/K) was purchased from Innogenetics, Antwerpen, Belgium (no. IKM-90) and contained 1.6 × 10⁶ U/ml. 1 U was defined as the amount of antibody required for 50% neutralization of 1 U TNF-α in the L929 cytotoxic assay. Control antibody was the isotype-matched mAb 23-233 (unpublished results). 5C6 is a mAb (IgG/K) directed against the type 3 complement receptor (CR3) (17). All mAbs were used as ascites fluid.

Retroviral TNF-α Gene Transfer and Expression in J558L Cells. A 1-kb SalI fragment of plasmid pSVd2-3mTNF (18), which contains the mouse TNF-α cDNA minus most of the 3' untranslated region, was inserted into the XhoI restriction site of plasmid pXT1 (19) resulting in pXT-TNF. In pXT-TNF, the TNF-α gene is expressed by the thymidine kinase promotor and the neomycin gene as a selectable marker by the retroviral long terminal repeat (LTR)[1]. PA317 cells were transfected by calcium-phosphate coprecipitation as described (4), selected in G418 (1 mg/ml)-containing medium, and clones were established. Subsequently, psi2 cells were infected with culture supernatant of clone PA317-XT-TNF, selected in G418 (1 mg/ml)-containing medium, and finally clone psi-XT-TNF was established, which produces a viral titer equivalent to 5 × 10⁵ G418ᴿ colonies/ml. Retroviral infection and determination of the viral titer was done as described (4). The virus-containing supernatant of psi-XT-TNF was used to infect 10⁶ J558L cells. After 14 d, selection in G418 (1 mg/ml) clones were established. TNF-α activity was measured by the L929 cytotoxic assay as described (20). 1 U was defined as the amount of TNF-α required for 50% cytolysis. In our assay system, 1 U TNF-α activity corresponded to 30 pg/ml.

IL-6 Gene Transfer and Expression in J558L Cells. A 670-bp fragment of plasmid pHP1B5 (21) containing the mouse IL-6 cDNA coding region up to the translational stop signal was inserted into the BglII cloning site of plasmid pEB.B splice-neo by the use of appropriate oligonucleotides resulting in pEP.B-IL6. pEB.B splice-neo is an expression vector that allows expression of genes by the Ig heavy chain (IgH) enhancer/promoter (W. Müller and T. Blankenstein, unpublished results). 5 × 10⁶ J558L cells were transfected with pEP.B-IL6 plasmid DNA by electroporation as described (22). Transfected cells were selected in 1 mg/ml G418-containing medium and clones were established. The biological activity of IL-6 was measured by proliferation of the mouse plasmacytoma cell line TEPC 1033 (23) as described (24). 1 U was defined as the amount of IL-6 required for half-maximal proliferation.

Analysis of Tumor Cell Growth. 5 × 10⁶ cells were injected subcutaneously into 6-wk-old female BALB/c mice (Bomholtgard, Denmark). Mice bearing a tumor of >0.5 cm in diameter were considered as positive. To study the tumor growth in the presence of mAbs, 0.1 ml mAb was coinjected with the cells and a further 0.1 ml was injected on days 2, 4, 6, and 8 either intraperitoneally (5C6) or intravenously (TNF-α and control mAb). In some experiments, 100 mg/kg body weight Zymosan (Serva, Heidelberg,

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[1] *Abbreviation used in this paper:* LTR, long terminal repeat.

FRG) was injected intraperitoneally the day before the tumor cells were injected subcutaneously.

Immunohistochemistry. Indirect immunoenzymatic staining of 6-μm frozen sections was performed by using the anti-CR3 monoclonal primary antibody MAC-1 (25) and alkaline phosphatase–conjugated secondary and tertiary antibodies. Alkaline phosphatase activity was visualized by New Fuchsin as described (26), and sections were counterstained by hemalaun.

Results

TNF-α and IL-6 Gene Transfer and Expression in Myeloma J558L. The experimental approach for the study of the tumor cell-targeted TNF-α gene therapy is outlined in Fig. 1. A TNF-α-specific retroviral vector (pXT-TNF) was constructed, expressing the neomycin gene as a selectable marker by the retroviral LTR, and the TNF-α gene by the internal thymidine kinase promoter. Virus-producing cells were generated and, subsequently, J558L cells were infected, selected for G418ᴿ, and cloned. Two clones, J2T12 and J2T4, were assayed for TNF-α activity in the culture supernatant three times in intervals of 6 wk. The cells produced 40 pg/ml (J2T12) and 14 pg/ml (J2T4) on the average. The activity did not decrease during culturing. The biological activity in the culture supernatant of J2T12 cells could be neutralized by an anti-TNF-α mAb. Determination of the doubling time showed no difference between J2T12 and parental J558L cells. Additionally, in a proliferation assay with J558L cells in the presence of increasing amounts of TNF-α up to 100 ng/ml, we did not observe cytostatic or cytolytic effects on J558L cells (data not shown). Therefore, the TNF-α production by J558L cells does not change the growth rate of the cells, and J558L cells are resistant to TNF-α in vitro even in a 2,500-fold higher concentration than produced by J2T12 cells. In parallel experiments, the IL-6 gene was expressed in J558L cells. Therefore, a plasmid was constructed that contains the mouse IL-6 cDNA region under the transcriptional control of the IgH enhancer/promoter (pEP-IL-6). J558L cells were transfected by electroporation with pEP-IL-6, and finally a clone was established (J558-IL-6) that produces 500 U/ml IL-6 as determined by the TEPC 1033 indicator line (data not shown).

The Growth of TNF-α- but not IL-6-producing J558L Cells Is Suppressed In Vivo. The tumor progression of J2T12 cells in comparison to J558-IL-6 and parental J558L cells was monitored (Fig. 2). 5 × 10⁶ of either cells were injected subcutaneously into syngeneic BALB/c mice. After 2 wk, 90% (45/50) of the mice injected with J558L cells had developed a tumor. 100% (30/30) of the mice injected with J558L-IL-6 had developed a tumor within 10 d. In contrast, 60% (18/30) of the mice inoculated with J2T12 cells did not develop a tumor during the observation of >3 mo. In the other 40% (12/30), the tumor growth was delayed, the first mice developing a tumor after 4 wk. Similar results were obtained with the second clone, J2T4, but J558L cells infected with a control virus (pXT1) grew as a tumor, like the parental cells (data not shown).

To analyze possible toxic side effects of the TNF-α secreted by J2T12 cells, mice injected with J2T12 cells were weighed
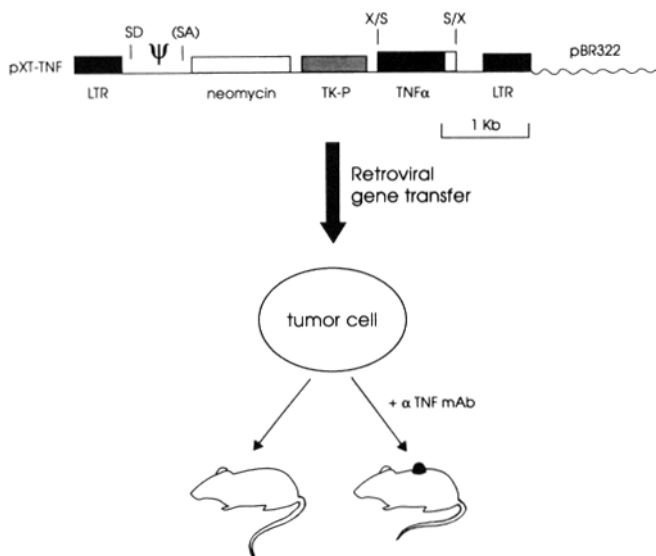
Figure 1. Experimental approach for the study of TNF-α-mediated tumor suppression. LTR, long terminal repeat; TK-P, herpes simplex virus–derived thymidine kinase promotor; SD, splice donor site; (SA), cryptic splice acceptor site; X, XhoI; S, SalI.

and compared with naive mice or mice injected with J558L cells, since high amounts of TNF-α are known to cause drastic weight loss (27). We could neither observe a significant weight loss in J2T12-injected mice, nor did they show any obvious symptoms of cachexia.

mAbs against both, TNF-α and CR3 Revert the Phenotype of J2T12 Cells. BALB/c mice were injected with J2T12 cells (5 × 10⁶ cells/mouse) simultaneously with an anti-TNF-α mAb and, as control, with an isotype-matched mAb. 80% (4/5) of the mice treated with the anti-TNF-α mAb developed a tumor within 20 d, but only 20% (1/5) of the mice that had been treated with the control mAb did (Fig. 3). Subsequently, the tumor-suppressing mechanism induced by TNF-α was analyzed. J2T12 cells were injected subcutaneously into BALB/c mice simultaneously with 5C6, an anti-
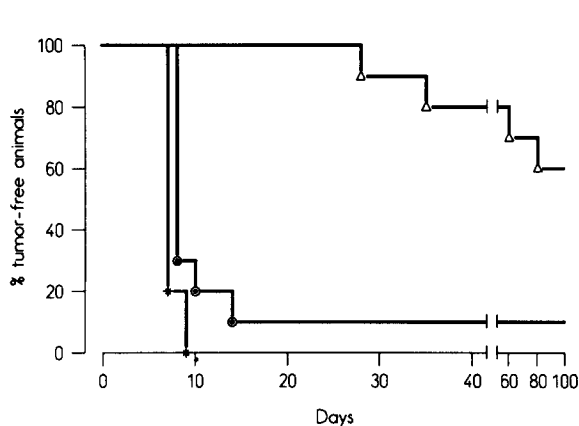
CR3 mAb that is known to block migration of inflammatory cells such as macrophages, neutrophils, or NK cells (17). As can be seen from Fig. 3, mAb 5C6 completely abolished tumor suppression. Consistently, injection of Zymosan, which is known to induce an inflammatory process, reversed the nontumorigenic phenotype of J2T12 cells (Fig. 3) when it was injected intraperitoneally the day before mice received J2T12 cells subcutaneously.

Macrophage Infiltration at the Tumor Site Induced by TNF-α. The observation that the anti-CR3 mAb neutralized the antitumor activity of TNF-α suggests that the tumor suppression acts indirectly via CR3⁺ cells of the host. To demonstrate cellular infiltration at the tumor site and to identify the respective cells, immunohistochemical analysis was performed. BALB/c mice were injected subcutaneously with 5 × 10⁶ J2T12 or J558L cells. Tissue from the injection site was excised at different time points and stained with a mAb against CR3. CR3⁺ cells, morphologically identified as macrophages, were visible in mice injected with J2T12 cells on day 2 (Fig. 4) and increased to a massive infiltrate on day 5. In contrast, in tissue sections derived from mice injected with J558L cells, moderate infiltration consisting predominantly of granulocytes could be observed.

Discussion

The severe toxicity of high concentrations of systemically applied cytokines and the well-established fact that cytokines, under physiological conditions, act locally and in a narrow range necessitates the need to find new experimental approaches for immunotherapy in cancer. These criteria are met by tumor cell–targeted cytokine gene therapy, which is achieved by transfer and expression of appropriate genes into tumor cells in vitro followed by the analysis of their growth characteristics in vivo. This approach provides a sensitive assay: (*a*) to detect the antitumor activity of cytokines; (*b*) to titrate cytokine concentrations required to exert antitumor activity without toxic side effects; (*c*) to elucidate the type of tumor cells that are susceptible or resistant for cytokine gene therapy;
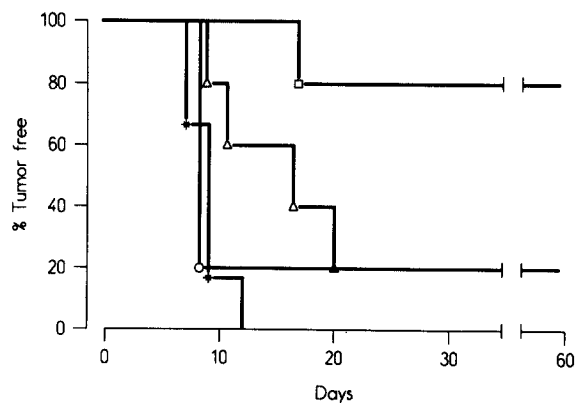

Figure 2. Kinetic of tumor progression in BALB/c mice injected subcutaneously with 5 × 10⁶ J558L (⊙), J558-IL6 (∗), or J2T12 (△) cells. 50 J558L, 30 J558-IL6, and 30 J2T12 cell–injected mice were analyzed.

Figure 3. Kinetic of tumor progression in BALB/c mice injected subcutaneously with 5 × 10⁶ J2T12 cells and simultaneously with an anti-CR31 receptor mAb (O), an anti-TNF-α mAb (△), a control mAb (□), or zymosan (∗). There were five mice per group.
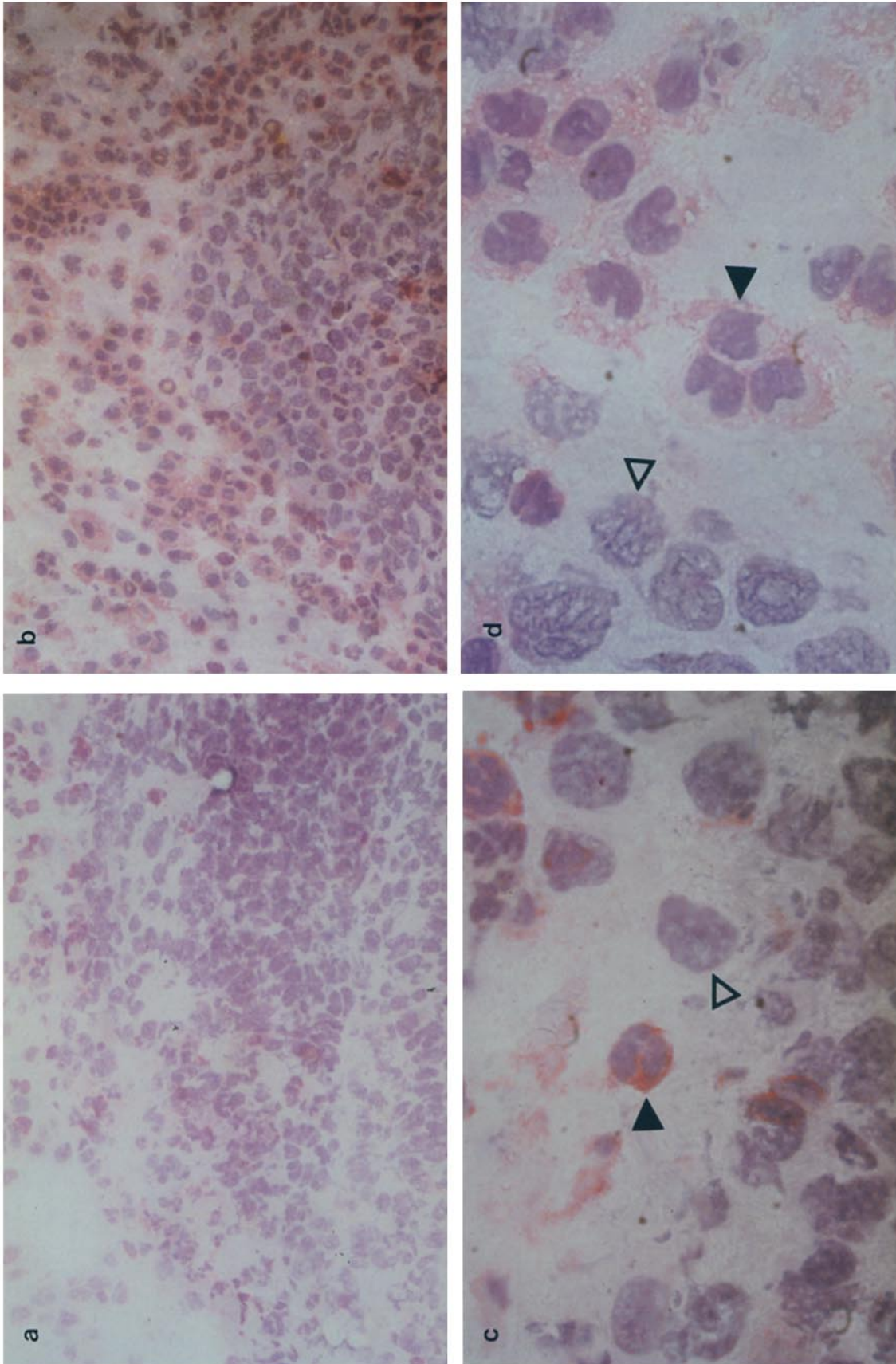
Figure 4. Immunohistochemical analysis of the site of tumor injection at day 2 using an anti-CR3 mAb. (*a*) Site of J558L cell injection. (*b*) Site of J2T12 cell injection demonstrating CR3-positive cells surrounding tumor cells. At higher magnification, the CR3-positive cells at the site of J558L cell injection display a polymorphonuclear morphology (*c*, *filled arrow*). In contrast, at the site of J2T12 cell injection, the CR3-positive cells were morphologically identified as macrophages (*d*, *filled arrow*). Open arrows in *c* and *d* indicate tumor cells.

(*d*) to elucidate the mechanisms by which the antitumor activity of cytokines operate and, finally; (*e*) to analyze whether different cytokines cooperate or synergize in their properties to induce a tumor-specific immune response.

Several arguments underline the importance of thoroughly studying cytokine-mediated tumor suppression. The deleterious consequences of aberrant cytokine production acting in an autocrine or paracrine manner have been shown in a number of studies (28). The tumor cell–targeted cytokine gene therapy studied so far indicated that different cytokines induce different tumor-specific immune responses. For instance, IL-2 produced by tumor cells seems to act primarily by bypassing T helper cell function and inducing a cytotoxic T lymphocyte response (6), whereas IL-4, when secreted by tumor cells, induces (at least partially) a T cell–independent immune response (3, 4). The main anti-tumor effector cells induced by IL-4 are thought to be eosinophils and macrophages. Moreover, some tumor cell types seem to be more susceptible to IL-4 treatment than others. In another study, IFN-γ produced by tumor cells induced a tumor-specific immune response involving mainly CTL but not activated macrophages (7). We have investigated tumor cell–targeted cytokine gene therapy with TNF-α and IL-6 genes in the mouse plasmacytoma J558L. The IL-6 expression by J558L cells did not show any sign of tumor suppression. In contrast, tumor growth seemed slightly accelerated, confirming the growth-promoting activity of IL-6 for plasmacytomas (23). These results are not necessarily in contrast to the recently described antitumor activity of IL-6 on a variety of tumor cells (29), but demonstrate the requirement to test different tumor cells types for their susceptibility to different cytokines.

The expression of TNF-α instead of IL-6 by J558L cells strongly suppressed the tumor growth, allowing the following conclusions: (*a*) the antitumor activity of TNF-α is not restricted to cells that are sensitive to TNF-α in vitro and, therefore, it acts indirectly. (*b*) The antitumor activity that was observed is induced by TNF-α, since it could be neutralized by an anti-TNF-α mAb. (*c*) TNF-α induces an immune response in the tumor-bearing animals that seems to operate at least partially by the chemotactic recruitment of inflammatory cells such as neutrophiles, macrophages, or NK cells, which bear CR3. Therefore, an increased local concentration of TNF-α seems to be important. Since TNF-α has been reported to act chemotactically on macrophages (10), and also to augment tumoricidal activity on macrophages (11–13), it may play an important role in the host's tumor defense. This is supported by the massive infiltration of macrophages in the tumor tissue (Fig. 4), by the observation that activated macrophages accumulate in the peritoneal cavity of mice that had been injected with J2T12 cells, and by the cytolytic activity of cells that have been isolated from such mice (data not shown). Further effector cells may exist since the antitumor effect was much weaker in BALB/c *nu/nu* mice (data not shown). (*d*) Extremely low (nontoxic) doses of TNF-α are sufficient for tumor rejection. Therefore, the retroviral gene transfer seems to be an appropriate choice because it generally results in a single virus integration and often in moderate gene expression. Low TNF-α concentrations are not only sufficient, but also important, because high TNF-α production by a tumor cell (~1,000 times higher than the one reported here) leads, after injection into mice, to cachexia and the death of the animals (27). (*e*) The antitumor activity of TNF-α seems to induce an immune response that differs, at least partially, from the ones observed with IL-2, IL-4 or IFN-γ. In conclusion, our results are reminiscent of the name originally proposed for TNF-α.

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