

The eosinophil-mediated antitumor activity of interleukin-4

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Within the last several years *in vivo* assays have been developed to assess the biologic activities of individual cytokine gene products as antitumor agents. One such assay, termed the tumor-cytokine transplantation assay,^{1, 2} was developed to determine whether individual cytokines, when expressed locally, could incite immune and/or inflammatory cells capable of cytotoxic action against transplanted tumor cells. The assay involves the engineering of cytokine genes into expression vectors that are introduced into tumor cell lines; these modified cells are then transplanted into recipient hosts. The inhibition of growth of the cytokine-secreting tumor cells compared with mock-transfected tumor cells constitute the basis for identifying the cytokine as a potential antitumor effector. This assay has several advantages for studying the biology of cytokine-induced antitumor activity. First, it assesses the localized action of a cytokine *in vivo*, and therefore it recapitulates to a somewhat greater extent the normal delivery of cytokines compared with their systemic administration. Further, cytokines that can be shown to have no effect on the growth of tumor cells *in vitro* but that do inhibit tumor growth *in vivo* likely require the involvement of host effector cells in the tumoricidal effect observed. This is the case, for example, with the antitumor effect of interleukin (IL)-4.¹ Histologic analysis involving routine staining methods and immunohistochemical methods can thereby provide clues to the host effector cells important in the observed antitumor response. The ability to mix tumor cells secreting cytokine with nonproducing tumor cells in a "mixed tumor transplantation assay" also allows one to assess whether the secreted cytokine is acting in a non-cell-autonomous fashion, as has been shown for a number of

Abbreviations used

ELAM-1:	Endothelial leukocyte adhesion molecule-1
ICAM-1:	Intercellular adhesion molecule-1
IL:	Interleukin
NK:	Natural killer
TNF- α :	Tumor necrosis factor- α
VCAM-1:	Vascular cell adhesion molecule-1
VLA-4:	Very late antigen-4

cytokines by this assay method. As such, the ability to test a wide spectrum of tumors of diverse histologic types for their susceptibility to the antitumor action of a given cytokine can be rapidly achieved simply by mixing individual tumor cell types with a standardized cytokine-producing cell.

The tumor-cytokine transplantation assay thereby provides a means for screening in a convenient fashion, cytokines or any other gene product for *in vivo* antitumor activity. With this type of assay a number of cytokines have been demonstrated to possess antitumor properties *in vivo*. These include IL-4,^{1, 3, 4} IL-2,⁵⁻⁷ interferon- γ ,^{8, 9} IL-7,¹⁰⁻¹² tumor necrosis factor- α (TNF- α),^{13, 14} granulocyte colony-stimulating factor¹⁵ and granulocyte-macrophage colony-stimulating factor.^{16, 17} Moreover, the assay has demonstrated the lack of localized antitumor activity of other cytokines such as IL-3, IL-5, and IL-10.¹⁸

IN VIVO ANTITUMOR ACTIVITY OF IL-4

The expression of an activated murine IL-4 gene locally at the injection site of a wide variety of transplantable tumor cell types, with the previously described assay, results in a potent antitumor effect with complete killing of most tumor cell types.¹ The histologic tumor types inhibited by IL-4 action *in vivo* include murine plasmacytomas, lymphomas, various adenocarcinomas, melanoma, and sarcomas. Moreover, human tumors, including the HT 1080 fibrosarcoma and a human glioblastoma (U87)¹⁹ are susceptible to IL-4-induced killing, as demonstrated in *nu/nu* mice. The action of IL-4 is clearly dose dependent, with complete

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eradication of tumor requiring high localized concentrations of the cytokine. For example, the degree of in vivo growth inhibition of IL-4-transfected clones of the mammary adenocarcinoma line K485¹ increases with the concentration of IL-4 produced, with an eightfold reduction in tumor size in clones producing 100 U/10⁶ cells per 24 hours compared with clones not expressing IL-4, to almost complete growth inhibition of clones expressing 3000 U of IL-4 activity. The reversal of IL-4-induced killing of tumor cells by the in vivo delivery of the neutralizing anti-IL-4 antibody 11B11 provides further evidence for the requirement of sufficient levels of IL-4 generated locally at the site of tumor cell death.¹ Evidence that the action of IL-4 is not directed against the tumor cell itself is provided by the absence of an inhibitory effect of IL-4 on the growth of tumor cells in vitro and the observation that murine IL-4 can inhibit the growth of *human* tumor cells in nu/nu mice despite the inability of murine IL-4 to bind to the high-affinity human IL-4 receptor.²⁰ These findings therefore suggest a host-dependent mechanism for the antitumor effect of IL-4.

The observation that the antitumor action of IL-4 is operative in T lymphocyte-deficient nu/nu mice suggests that the principal mechanism of IL-4-induced tumor cell killing is not dependent on the action of effector T cells. This finding is further supported by histologic examination of IL-4-producing tumor cells at various time points after their transplantation into syngeneic hosts. Within 48 hours IL-4-producing tumors become extensively infiltrated with host-derived granulocytes. As demonstrated by tissue Giemsa stains, more than 99% of these cells are eosinophils.^{1, 4} Already by this time the great majority of tumor cells are killed, as evidenced by extensive tumor cell dropout, nuclear pyknosis, and absence of mitoses. Few mononuclear cells, either lymphocytes or monocytes, are observed at this point. By 96 hours eosinophil infiltration has typically declined and extensive invasion with macrophages becomes evident, but lymphocytes remain sparse. Taken together, these findings suggest that the predominant antitumor effect incited by IL-4 is a result of the activation of nonlymphoid effector cells. To define further the host cellular effector mechanism responsible for the antitumor effect of IL-4, two series of investigations were undertaken. The first involved the study of IL-4-producing tumor cells in a panel of genetically immunodeficient mouse strains. In addition to nu/nu mice, as discussed previously, bg/bg mice (natural

killer [NK] cell-deficient), bg/nu/xid mice (B cell-, T cell-, and NK cell-deficient), scid mice (B cell- and T cell-deficient) and w/w^v mice (mast cell-deficient) were analyzed. The antitumor activity of IL-4 could be demonstrated in each of these immunodeficient mouse strains with two different tumor types engineered to produce IL-4 (J558L plasmacytoma and B16 melanoma), providing evidence that various T-cell populations, as well as B cells, NK cells, and mast cells, are not required for IL-4-mediated tumor killing.⁴

Given the histologic finding of eosinophil infiltration into tumors with localized IL-4 expression at the time of the bulk of tumor cell death, we sought to determine whether eosinophil depletion would inhibit the action of IL-4. The rat IgG2b monoclonal antibody RB6-8C5, which recognizes an epitope on mature myeloid cells (but not lymphocytes or monocytes) was used to achieve the in vivo depletion of eosinophils.²¹ This antibody has been shown to be cytotoxic for eosinophils in vivo, resulting in the depletion of eosinophils from mice rendered hypereosinophilic by infection with the helminthic parasite *Nippostrongylus brasiliensis*.⁴ Neutrophils, a cell type not observed in our IL-4-induced inflammatory infiltrates, are also depleted by this antibody. With an IL-4-producing plasmacytoma cell line that fails to form tumors in vivo as a result of IL-4 expression,¹ tumorigenicity could be restored in 90% of animals pretreated with RB6-8C5. Tumor formation remained inhibited in control animals receiving an irrelevant IgG antibody. Histologic analysis confirmed the depletion of eosinophils at the tumor site of animals receiving the antieosinophil antibody; viable tumor with numerous mitoses was also restored. With two other independent IL-4 tumor transfectants, RB6-8C5 antibody could be shown to synergize with anti-IL-4 antibody in the restoration of tumorigenicity. These findings suggest that an eosinophil-mediated mechanism appears to be important for the antitumor action of IL-4. The precise mechanism of IL-4-mediated tumor killing by eosinophils, however, remains to be defined. Like other granulocytes, eosinophils are known to possess phagocytic properties and can mediate killing of cellular targets by the generation of superoxide and peroxide oxygen species, and through the release, on activation, of cytotoxic granule proteins (eosinophil cationic protein, major basic protein).²² Cytokines, including IL-1, TNF- α , IL-3, granulocyte-macrophage colony-stimulating factor, and tumor growth factor- β , have also been recently shown to

be secreted by activated eosinophils²³⁻²⁵; their action may possibly contribute to tumor cytotoxicity either by direct action, by autocrine activation of eosinophils, or by the stimulation of additional effector populations.

The mechanism by which IL-4 promotes the localized eosinophil infiltration at the site of IL-4-producing tumor cells also remains to be established. The appearance of localized eosinophil infiltrates in association with IL-4 production appears to relate to a generalized type of inflammatory pattern seen with deregulated IL-4 expression in a number of naturally occurring pathophysiologic states. In patients with vernal conjunctivitis, an ocular allergic disorder, the great majority of CD4⁺ T-cell clones isolated from conjunctival infiltrates express IL-4 (and IL-5, but not interferon- γ) and contain large numbers of infiltrating eosinophils and mast cells.²⁶ Similarly, infection with helminthic parasites such as *Schistosoma* or *Nippostrongylus* is characterized by increased T-cell populations producing IL-4 (and IL-5) and blood, and tissue eosinophilia.²⁷ Human allergic asthma is characterized by eosinophilic infiltrates in the lung, airway hyperreactivity, and lysophospholipase crystal deposition (as a result of eosinophil membrane degradation).²⁸ These pulmonary findings and ocular lesions resembling vernal conjunctivitis are all features of transgenic mice that we have engineered to overexpress constitutively IL-4 in T lymphocytes.²⁹ Thus the proinflammatory action of IL-4 that is necessary for its antitumor activity also appears to be operative in pathophysiologic states. In both cases the action of IL-4 appears to be dependent on its localized expression at the site of inflammation.

Relatively little is known about the mechanisms by which eosinophils accumulate and are activated at local sites of tissue inflammation. Previous studies have established that the adherence of human eosinophils (and basophils) to cultured human endothelial cells share some of the same functional and molecular characteristics, as does neutrophil-endothelial cell adhesion.^{30, 31} Neutrophil-endothelial cell adhesion is thought to be a multistep sequential process initiated by the binding of leukocyte adhesion receptors to counterreceptors on endothelium.³² In many leukocyte types, including neutrophils, monocytes, and lymphocytes, this primary reversible adhesive event ("rolling"^{33, 34}) can be mediated by the interaction of the constitutively expressed L-selectin (leukocyte-endothelial cell adhesion molecule,

LAM-1, Leu-8, gp90-110 homing receptor, MEL-14 antigen) on the leukocyte surface, with the vascular E-selectin (endothelial leukocyte adhesion molecule-1 [ELAM-1]) or P-selectin (CD62) molecules on the endothelium.^{33, 35} These endothelial glycoproteins are upregulated by inflammatory cytokines (such as IL-1 and TNF- α) and specifically bind to the sialylated Lewis-X lectin domain³⁶ of neutrophil L-selectin. Eosinophils, like other leukocytes, have also been shown to constitutively express L-selectin.³⁷ After primary adhesion both neutrophils and eosinophils can be activated to induce or to upregulate the expression of various integrins by the process of E-selectin or P-selectin binding itself, by chemoattractant factors such as formyl-methionine-containing peptides and platelet-activating factor, and, for neutrophils, through the action of intercrine family molecules (e.g., IL-8, hMGSA/gGRO α , platelet factor-4).³² Leukocyte integrin interactions with endothelial counterreceptors have been shown to promote the *secondary*, definitive adherence of leukocytes to the vessel wall, allowing for subsequent extravasation of inflammatory cells into the surrounding tissues.³²⁻³⁴ Integrin molecules identified to participate in definitive granulocyte-endothelial adhesion include (1) the β_2 (i.e., CD18-containing) integrins, such as leukocyte function associated antigen [CD11a/CD18], Mac-1, CR3 [CD11b/CD18], which bind intercellular adhesion molecule-1 (ICAM-1)³⁴ on the endothelial surface; and (2) the β_1 integrin, very late antigen-4 (VLA-4), which binds vascular cell adhesion molecule-1 (VCAM-1).³⁸

Activation of endothelial cells by cytokines such as IL-1 and TNF- α induces adherence of all granulocyte types³⁹⁻⁴²; this cytokine-induced adherence to activated endothelial cells results from increased expression of at least three adhesion molecules on the endothelial cell surface, ELAM-1,^{43, 44} ICAM-1,⁴⁵ and VCAM-1,⁴⁶ all of which are capable of binding eosinophils.⁴⁷ The transcriptional induction of VCAM-1 messenger RNA in cultured endothelial cells by IL-1 and TNF- α has been demonstrated.⁴⁶ IL-4 has been shown to stimulate, alone or synergistically with IL-1 or TNF- α , the upregulation of VCAM-1 on human endothelial cells, whereas ICAM-1 and ELAM-1 are unaffected by IL-4 action.⁴⁸⁻⁵⁰ Moreover, IL-4 can partially inhibit the induction of ICAM-1 and ELAM-1 by IL-1 or TNF- α in vitro,⁵¹ suggesting that IL-4 may impose a selective upregulation of VCAM-1 after cytokine induction of endothelium. Monoclonal antibodies against the β_2 inte-

grins or ELAM-1 partially block the adherence of neutrophils and eosinophils in vitro⁵²; in contrast, anti-VLA-4 or anti-VCAM-1 antibodies block eosinophil adherence in vitro but have no effect on neutrophil adherence.^{48, 50, 52} This selectivity is likely explained by the fact that eosinophils but not neutrophils express the β_1 integrin VLA-4.⁵⁰ These findings may also explain the marked impairment of neutrophil but not eosinophil or lymphocyte extravasation in patients with congenital deficiencies in CD11/CD18 expression (leukocyte adhesion deficiency syndrome).^{53, 54}

As noted, eosinophilic granulocytes predominate the inflammatory lesions observed at the site of IL-4-producing tumors, and the inflammatory lesions in IL-4 transgenic mice, with few neutrophils observed.^{1, 4, 29} It has been recently shown that IL-4 induces the adherence of human eosinophils, but not neutrophils, to endothelium in vitro. On the basis of antibody-blocking studies, binding was shown to be predominantly mediated by VCAM-1 and to a lesser degree by ICAM-1.⁴⁸ In vitro evidence for the requirement of β_2 integrins and VLA-4 for the transmigration of eosinophils across human umbilical vein endothelial cell monolayers has also been provided.⁵⁵ Taken together, these findings suggest that the characteristic eosinophil infiltrate induced by IL-4 may relate to the specific induction of VCAM-1 on the endothelial surface. The IL-4-producing tumor model allows us to study the kinetics of an IL-4-associated inflammatory response with respect to alteration in adhesion molecule expression and the appearance of inflammatory cells within localized vessels and within the surrounding extravascular tissue. With RNA analyses and neutralizing monoclonal antibodies, this model can also provide information as to the importance of VCAM-1 induction in vivo by IL-4 in the generation of the characteristic inflammatory reaction observed. Experiments thus far with neutralizing monoclonal antibodies in vivo against VLA-4 and VCAM-1 have not confirmed the hypothesis that VCAM-mediated adhesion is a critical step in IL-4-induced eosinophil tissue infiltration. However, the inability of these antibodies to inhibit the eosinophil inflammatory response resulting from localized IL-4 expression does not rule out a role for VCAM-1, given potential problems involved with passively administered antibody in vivo. Eosinophil-specific chemotactic factors, for example, the newly described protein eotaxin,⁵⁶ may play a role in the specific eosinophil inflammatory response induced by IL-4. This hypothesis is currently being investigated.

REGRESSION OF ESTABLISHED TUMORS BY THE LOCALIZED PRODUCTION OF IL-4

We established that IL-4 can exert a potent antitumor effect against a wide array of tumor types in vivo, provided a sufficiently high localized concentration of IL-4 can be attained. A key feature in IL-4-mediated tumor killing is that expression of the cytokine by tumor cells themselves is not necessary for IL-4-induced killing. As long as a sufficient concentration of IL-4 is produced locally at the site of tumor growth, growth inhibition will occur.^{1, 4} This is in contrast to strategies that use toxic proteins, for example, diphtheria or ricin toxins, which require intracellular uptake of the toxin for killing. The non-cell-autonomous action of IL-4 is predictable given that IL-4-induced killing acts indirectly through the action of host eosinophils.⁴ We demonstrated this directly by mixing experiments between IL-4-producing and non-IL-4-producing tumor cells; provided that an overall IL-4 concentration of approximately 3000 to 5000 U (30 to 50 ng) per 10⁶ cells per 24 hours (as measured in vitro) is attained locally, complete tumor killing is observed, essentially independent of histologic tumor type.¹ This feature of IL-4 action makes strategies to target cytokine delivery to tumor sites theoretically more tenable. Our initial studies, such as the ones described previously, focused on the failure of tumors to *develop* in the presence of IL-4. Of greater clinical significance is the ability to effect the *regression* of established tumors. We demonstrated the activity of IL-4 in large doses in inhibiting the growth of established tumors, both by the direct administration of purified recombinant cytokine into the tumor mass or by the direct intratumor injection of IL-4-producing cells.⁴ The ability of IL-4 to promote regression of established, sizable tumor masses suggests that IL-4 may have potential clinical utility in the treatment of malignant disorders. One obvious problem that remains is the need to direct IL-4 to the sites of tumor deposits. In relatively confined but surgically inaccessible malignancies, such as some brain tumors, cytokine therapy such as with IL-4 may provide a novel approach. We recently demonstrated the activity of IL-4 against a human glioblastoma stereotactically implanted into the brains of nu/nu mice.¹⁹

With regard to metastatic tumor, the ability to target IL-4 to multiple sites of tumor growth appears to be a prerequisite for effective therapeutic tumor killing. This is because the threshold level of IL-4 production, required for the localized antitumor inflammatory response to be elicited, is not achievable by systemic delivery of IL-4.

Given the specificity of retroviruses for infecting dividing cells, it is possible that relatively selective targeting of metastatic tumor, for example, in the liver and the lungs, may be achievable by the in vivo delivery of either replication-defective retroviruses themselves or producer cell lines capable of retroviral production. A similar approach for the retroviral targeting of in situ brain tumors has been demonstrated by Culver et al.⁵⁷

GENE THERAPY FOR METASTATIC TUMORS

Several features of the antitumor action of IL-4 suggest that studies aimed at targeting the production of IL-4 to sites of metastatic tumor growth in experimental animals may be worthy of investigation as a novel therapeutic approach to disseminated cancer. First, regression of established tumors can occur as a result of IL-4 expression, assuming a threshold level of activity is achieved locally at the site of tumor growth. Even at levels of IL-4 production far less than the threshold for complete tumor killing, significant inhibition of tumor growth can be achieved, provided that IL-4 production is sustained. Second, the broad spectrum of activity of IL-4 against multiple tumor types is noteworthy, as is the ability of IL-4 to elicit a tumoricidal inflammatory response at multiple tissue sites. Finally, the localized expression of cytokine (which declines as IL-4-producing tumor cells, together with neighboring non-IL-4-producing tumor cells, are destroyed) results in no observable systemic toxicity.⁴

Given these features, we performed experiments to determine whether retroviral targeting of experimentally induced liver metastases can be achieved in murine tumor models. The relative selectivity of tumor versus normal tissue at these organ sites is based on the requirement for cell division to achieve retroviral integration and expression.⁵⁸ After the establishment of metastases the hematogenous in vivo delivery of replication-defective retroviruses or, alternatively, retroviral producer cell lines were tested for their tumor-targeting potential.

We used a high efficiency retroviral vector, MFG, expressing an *Escherichia coli* β -galactosidase gene (MFG-*lac Z*, kindly provided by Dr. Richard Mulligan, Whitehead Institute, Cambridge, Mass.) transfected into the ecotropic packaging cell line ψ -CRE.⁵⁹ High titers of the replication-defective recombinant retrovirus CRE-LACZ are achieved from this clonal producer line. The MFG vector preserves the normal

5' and 3' splice signals of the Moloney murine leukemia virus (Mo-MuLV), utilizes the endogenous long terminal repeat for expression of the inserted complementary DNA, and contains no selectable marker; this design has been associated with higher levels of expression when compared with similar vectors that do not possess these features.⁶⁰ In an initial series of experiments we used neomycin-resistance (*neo*) gene transfectants (4JK-*neo*) of the fibrosarcoma line 4JK to establish liver metastases in syngeneic C57B/6 mice. These cells demonstrated a high rate of infectivity with the CRE-LAC Z virus in vitro (69% of cells with viral supernatant with a titer of 0.6×10^6 colony-forming units/ml), as determined by X-gal staining for the β -gal gene product.⁶¹ To establish liver metastases, 1×10^6 4JK-*neo* cells were injected into the head of the spleen in sufficient volume to allow flow of the inoculum into the draining portal circulation. Seventy-two hours after tumor cell inoculation, animals were given a single intrasplenic injection of CRE-LAC Z viral supernatant (0.4 ml) or 1×10^6 CRE-LAC Z producer cells. Ten days after tumor cell inoculation, animals received a perfusion of 2% paraformaldehyde and splenic and hepatic tissues were harvested, frozen in OCT compound, and sectioned (10 μ m), as described by Cepko.⁶¹ Adjacent sections were stained histochemically with X-gal or with hematoxylin and eosin. Although no *lac Z*-positive tumor cells could be demonstrated in liver metastases from animals treated with viral supernatant, *lac Z*-positive tumor cells could be identified in liver metastases from animals receiving retroviral producer cells injections. Several experiments of this nature have established that 80% to 90% of widespread hepatic metastases can be targeted by a single injection of retroviral producer cells. In experiments with producer cells engineered to express IL-4, the characteristic eosinophil inflammatory response can be targeted to these metastatic lesions and inhibition of tumor growth can be demonstrated.

These data suggest that in vivo targeting of experimental metastases by the delivery of retroviral producer cells may be a feasible approach for the selective expression in tumor tissue of cytokines (or other gene products) with antitumor activity.

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Development of markers for human basophils and mast cells

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Mast cells and basophils are the major effector cells in allergic disease, yet the relative role of mast cells and basophils in various clinical condi-

tions is not well understood. Mast cells are positioned in connective tissue or mucosa at potential portals of entry of noxious substances. Although basophils normally reside in the circulation, they can enter tissues at sites of inflammation, particularly during the late phase of allergic reactions and during the early phase of delayed-type hypersensitivity reactions. Traditionally, mast cells and basophils have been understood primarily for their role in the pathogenesis of immediate hypersensitivity, but new evidence suggests that mast

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