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Disseminated Human Malignant Melanoma in Congenitally Immune-Deficient (bg/nu/xid) Mice

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Congenitally immune-deficient bg/nu/xid (BNX) mice are severely compromised in their ability to mount T-cell, B-cell, and lymphokine-activated killer (LAK) cell responses. Successful engraftment of BNX mice with human hematopoietic stem cells has been demonstrated recently. We have investigated the potential use of BNX mice for studies relating to the biology and immunotherapy of human malignant melanoma. The intravenous injection of fresh single-cell suspensions of human malignant melanomas into mice resulted in widely disseminated disease. Metastatic spread of human melanoma in BNX mice mimicked that observed in patients: eg, there were numerous tumor nodules identified in the subcutaneous tissues as well as in a variety of visceral organs, including spleen, kidneys, thyroid, adrenals, lungs, heart, and brain. BNX mouse lymph nodes were replaced consistently by human malignant melanoma cells. The presence of human tumor cells in these mice was confirmed by histologic analysis and microcytofluorometry analyses using human melanoma-specific monoclonal antibodies (MAbs). Moreover, human melanoma cells passaged in BNX mice remained lysable in vitro by specifically cytolytic, autologous human tumor-infiltrating lymphocytes (TILs). The capacity of fresh human malignant melanoma to disseminate widely in BNX mice may prove valuable not only for study of the biology of metastatic spread but also for studies of the immunotherapy of human melanoma using melanoma-specific MAbs and chemotherapeutic agents, as well as human TILs and LAK cells with or without retrovirus-mediated gene transfer modification. [J Natl Cancer Inst 83:350–355, 1991]

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Human melanoma has been shown to metastasize to virtually any organ or tissue in patients (*1*). Common sites of metastatic spread by the melanoma include skin, subcutaneous tissues, lungs, liver, bone, and brain. At autopsy, pancreas, thyroid, and adrenals often are also found to have metastatic melanoma involvement. Animal models used for study and possible therapeutic intervention of human malignant melanoma have not been characteristic of the human disease state. For example, although a number of studies have used athymic nude mice for the propagation of human tumors subcutaneously (SC), fresh human melanomas have not disseminated widely to visceral sites upon intravenous (IV) infusion (*2,3*). Athymic nude mouse models of visceral sites of metastases often have employed human melanoma variants that were generated either after prolonged in vitro tissue culture passage and selection or after serial in vivo passage and selection of lung-colonizing cells (*4-7*).

Successful adoptive immunotherapy for advanced melanoma has been achieved in certain patients by the transfer of lymphokine-activated killer (LAK) cells or tumor-infiltrating lymphocytes (TILs) in combination with administration of interleukin-2 (*8,9*). In addition, selective trafficking of TILs to sites of metastasis by the melanoma in patients has been observed following the labeling of TILs with indium 111 (*10*) or with the use of TILs marked by retrovirus-mediated transduction of the bacterial gene coding for neomycin resistance (*11,12*). It would be advantageous to be able to study in mice immunologic agents such as autologous human TILs and LAK cells or monoclonal antibodies (MAbs) for therapeutic efficacy against metastases from fresh human melanoma. In recent studies, severe combined immune-deficient (SCID) mice have been engrafted successfully with a human immune system (*13-15*). Moreover, characteristics of (*16*) systemic lupus erythematosus and (*17*) primary biliary cirrhosis could be transferred to SCID mice with the use of peripheral blood lymphocytes from patients with these diseases. These studies raised the intriguing possibility that fresh human melanoma cells transferred IV into congenitally immune-deficient mice may show organ tropisms similar to those often found in patients.

The triple-immune-deficient beige (bg)/nude (nu)/xid mouse not only is functionally and phenotypically depleted of T cells and B cells but, unlike the SCID mouse, is also lacking in precursors of LAK cells (*18,19*). This added defect allowed bg/nu/xid (BNX) mice to be repopulated successfully with cells from the human myeloid lineage (*20*). In this report, we describe the growth and metastatic spread of fresh human melanomas in these BNX mice and discuss the potential use of these mice for study of the biology and immunotherapy of human cancer.

Materials and Methods

Mice. Triple-immune-deficient bg/nu/xid ($H\text{-}2^q$) male mice (BNX mice) were obtained from the animal production colonies of the NCI-Frederick Cancer Research and Development Center, Frederick, Md, and the Charles River Breeding Laboratories, Wilmington, Mass. Mice were maintained under pathogen-free conditions and were 6 through 8 weeks of age when used in the experiments.

Tumors. Human melanomas received directly from the operating room were minced into fragments of 1 to 5 mm in diameter and either were directly implanted into mice SC under general anesthesia with the use of a trochar or were digested by a filter-sterilized enzyme mixture containing deoxyribonuclease, collagenase, and hyaluronidase for 6 through 24 hours into a single-cell suspension for SC or IV injection (*21,22*). Viable tumor and mononuclear cells were separated from the final cell suspension on a Ficoll-Hypaque gradient (*21,22*) and were washed twice with Hanks' balanced salt solution (Biofluids, Rockville, Md).

Metastatic tumor model. BNX mice received 5×10^6 to 10×10^6 human melanoma cells suspended in 1.0 mL of Hanks' balanced salt solution injected IV via the lateral tail vein.

Phenotypic analyses of tumors. Human melanoma deposits in BNX mice were confirmed by microcytofluorometry (FACS) using MAbs to melanoma-associated antigens. MAb 48.7 [IgG1; anti–high-molecular-weight proteoglycan melnoma-associated antigen (*23*)] was provided by Drs K. E. Hellström and I. Hellström (Oncogen, Seattle, Wash), and MAb R24 [IgG3; anti-GD3 ganglioside melanoma-associated antigen (*24*)] was purchased from Signet Laboratories, Dedham, Mass. Counterstaining of the MAb was performed with fluorescein-conjugated goat anti-mouse immunoglobulin (GAMIg-FITC; Becton Dickinson Immunocytometry Systems, Mountain View, Calif). Phenotype analyses were performed on fresh single-cell suspensions either following enzymatic disaggregation of excised melanoma deposits, as described above, or following expansion of the tumor fragments in vitro by culture in AIM-V medium (GIBCO, Grand Island, NY) supplemented with 10 μg of gentamicin per milliliter (GIBCO), 50 μg of streptomycin per milliliter (GIBCO), 50 U of penicillin per milliliter (GIBCO), 1.25 μg of fungizone per milliliter (Flow Laboratories, McLean, Va), and 2 m*M* L-glutamine (Flow Laboratories).

Histologic assessment. Organs suspected of containing melanoma deposits as well as SC melanoma nodules were removed from BNX mice and fixed in 10% formalin (National Institutes of Health Media Unit, Bethesda, Md). Hematoxylin–eosin-stained slides of sectioned tumor were prepared and evaluated in a coded, blinded fashion by a pathologist (W. D. T.).

Cytotoxicity assays. TILs were grown from human melanoma freshly excised from patients as described previously (*21,22*). Human LAK cells were generated by the incubation of peripheral blood mononuclear cells from normal human donors in culture medium containing 6000 IU of recombinant interleukin-2 per milliliter (Cetus Corp, Emeryville, Calif) for 2 or 6 days as described previously (*25*). The cytotoxic activity of TILs and LAK cells was tested in 4-hour chromium-51 release assays against targets that consisted of autologous and allogeneic human tumor cells derived from patients as well as from nodules growing SC in BNX mice (prepared as described above), or of the natural killer (NK)-resistant Daudi B-cell lymphoma line. Specific cell lysis was determined as described previously (*25*). The spontaneous release of chromium-51 from thawed, BNX mouse-derived human

melanoma cells was similar to that of the respective patient-derived melanoma cells and ranged between 20% and 35%.

Results and Discussion

Growth of Transplanted Fresh Human Malignant Melanoma in BNX Mice

Fresh tumor preparations from 12 consecutive patients with advanced malignant melanoma were inoculated SC into BNX mice as either single-cell suspensions following enzymatic disaggregation or as small tissue fragments. Ten of 12 fresh human melanomas grew progressively following transplantation; 2 of these 10 melanomas were thawed from cryopreserved samples (data not shown). None of the tumors metastasized to distant sites, and all tumors were well encapsulated. Latent periods of progressive tumor growth varied between 2 and 8 weeks. The percentage of tumor cells in the fresh single-cell suspensions varied from 37% to 97%. Two of the human melanomas transplanted SC into the mice failed to grow; these tumor preparations contained 60% and 97% tumor cells, respectively. All growing human melanomas could be successfully passaged SC in BNX mice; latent periods were 1 through 3 weeks following SC injection of 1×10^7 tumor cells into the mice.

In Vitro Lysis by Autologous Human TILs of Human Malignant Melanoma Cells Passaged in BNX Mice

Single-cell suspensions were prepared from three human melanomas (from patients 923, 954, and 965) growing SC in BNX mice for two transplant generations; the cells were then cryopreserved. After thawing, these tumor cells, as well as the cultured NK-resistant Daudi cell line, were used as targets in a 4-hour chromium-51 release assay. As shown in Table 1, all targets were readily lysed by LAK cells generated from peripheral blood mononuclear cells from two normal human donors. More importantly, human melanoma cells (from patients 923 and 965) passaged in BNX mice were lysed specifically by the respective autologous TILs grown from the original tumor harvested at the time of the patient's surgery. In separate assays, the TILs from patients 923 and 965 also specifically lysed their respective fresh autologous melanoma target derived from the original tumor biopsy specimen taken from the respective patient, but they did not lyse one additional fresh allogeneic melanoma or K562 cells (data not shown). When compared to the use of thawed cell suspensions of cryopreserved autologous human melanoma cells, passage of the three fresh melanomas in mice did not select cells with enhanced sensitivity to lysis by TILs or LAK cells (data not shown). Thus, human malignant melanoma cells, following serial passage in BNX mice, retained their capacity to be recognized specifically and lysed by autologous human cytotoxic T cells.

Dissemination of Fresh Human Malignant Melanoma Cells in BNX Mice

Twelve BNX mice received an IV injection of human melanoma cells from patient 954 (six mice received a preparation of cells from the original fresh surgical specimen, and 6 mice received cells derived from first-passage SC tumor in BNX mice). In addition, each of six mice received melanoma cells from patients 923, 954, and 965 that had undergone one SC transplant passage in BNX mice. Widely disseminated metastases of human malignant melanoma were observed in all BNX mice by 8 weeks. IV injection of fresh melanoma cells from patient 954 into NIH athymic nude mice failed to generate widespread disease (data not shown). Gross observation revealed that all mice had numerous SC tumor nodules as well as lymph node (including cervical, popliteal, aortic, axillary and brachial, and inguinal) replacement by human malignant melanoma (Fig 1). The extent of metastatic spread to the viscera varied among the melanomas injected into the mice; generally, the organs

Table 1. Specific lysis by autologous human TILs of human melanoma cells passaged in BNX mice

Experiment No.	Effector*	Tumor targets†, $LU_{30}/10^7$ cells‡			
		923	954	965	Daudi B-cell line
I	923 TILs	417	<1	<1	<1
	LAK cells	20	44	95	100
II	965 TILs	NT	<1	952	<1
	LAK cells	NT	232	526	528

*TILs from patients 923 and 965 were cultured for 35 and 50 days, respectively, at the time of assay. Two- and 6-day LAK cells were used in experiments I and II, respectively.

†Melanoma cells from patients 923, 954, and 965 were derived from second-passage SC tumor nodules in BNX mice. Targets were obtained from thawing cryopreserved single-cell suspensions.

‡One lytic unit (LU) = No. of effector cells that caused 30% specific lysis of 10^4 target cells. NT = not tested.

Table 2. FACS analyses of human melanoma metastases in BNX mice

Patient No.*	Metastasis†	Mean channel No. fluorescence		
		Control‡	R24 MAb§	48.7 MAb§
923	Lymph node	3.34	65.3 (94.7)	90.8 (96.1)
	Brain	3.15	71.4 (86.3)	93.2 (97.0)
	Lungs	3.19	76.4 (94.7)	93.1 (96.2)
954	Lymph nodell	2.80	342.9 (99.8)	83.2 (96.6)
	Brain	3.00	190.7 (99.4)	37.8 (99.5)
	Spleen	2.89	198.5 (99.4)	42.6 (99.4)
965	Lymph node	2.58	ND	127.0 (98.5)
	Kidneys	3.18	ND	96.3 (96.9)
	Lungs	3.08	ND	92.8 (97.0)

*Each patient No. represents a separate experiment analyzed on different days.

†Location in BNX mouse.

‡GAMIg-FITC alone; few, if any, cells were of BNX mouse origin, as confirmed by lack of staining with MAb 28.1.48 (anti-L^q) plus GAMIg-FITC.

§No. in parentheses represents the percentage of positive cells. ND = not determined.

‖Metastasis in the lymph node was analyzed from a different BNX mouse on a different day than were the analyses of metastases in the brain and spleen.

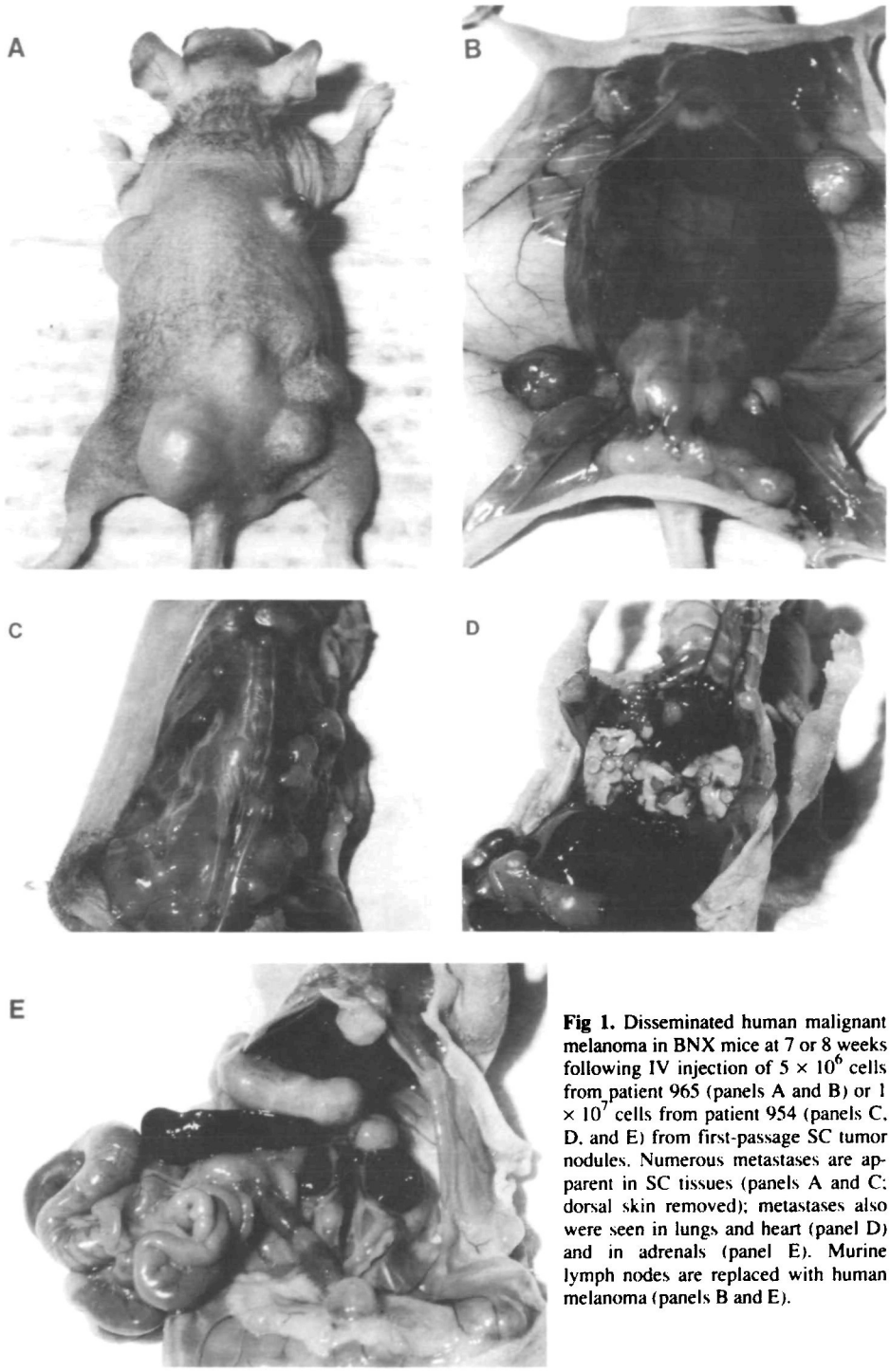

Fig 1. Disseminated human malignant melanoma in BNX mice at 7 or 8 weeks following IV injection of 5×10^6 cells from patient 965 (panels A and B) or 1×10^7 cells from patient 954 (panels C, D, and E) from first-passage SC tumor nodules. Numerous metastases are apparent in SC tissues (panels A and C; dorsal skin removed); metastases also were seen in lungs and heart (panel D) and in adrenals (panel E). Murine lymph nodes are replaced with human melanoma (panels B and E).

involved included heart, spleen, kidneys, thyroid, adrenals, lungs, eye, and brain.

The presence of human malignant melanoma in BNX mice was confirmed by both histologic and FACS analyses. Fig 2 shows the histology of tissues from BNX mice given an IV injection of melanoma cells from patient 954. Widespread organ involvement is evident. MAbs R24 and 48.7 specific for human melanoma-associated antigens were used to phenotype metastases in randomly selected organs of BNX mice that had received melanoma cells IV from patients 923, 954, and 965. The FACS results are shown in Table 2. All samples were stained brightly by R24 and 48.7 MAbs, with greater than 95% of the cells often being positive for either or both of the human melanoma-associated antigens.

Our data in this report show that fresh human melanoma cells injected IV can disseminate widely in the triple-immune-deficient BNX mouse strain. Metastatic spread occurred after the infusion of melanoma cells prepared directly from a patient's surgical specimen or prepared from SC tumor cells passaged in BNX mice. Human melanoma nodules growing in these mice were positive for known melanoma-associated antigens defined by specific MAbs and could be specifically lysed in vitro by autologous TILs. These latter findings are important because it may now be possible to study the therapeutic efficacy and tumor-localization capacity of specific human TILs or MAbs in mice by use of this metastatic solid-tumor model (*8-12,26,27*); the potency of new chemotherapeutic drugs also can be examined. It is conceivable that a potential limitation of this model for study of human effector cells may be the complex issue of trafficking of human LAK and T cells in the mouse. The possibility exists for limited human lymphoid and murine endothelial cell interactions resulting from species differences in adhesion and homing molecules (*28*). Although other data (*13-16,29*) would argue for at least some trafficking of fresh human lymphocytes to organ sites after intraperitoneal (IP) or IV administration into BNX and C.B-17 SCID mice, it is not known whether cultured human lymphoid cells with antitumor activity, particularly TILs and LAK cells, can localize selectively into autologous human tumors growing at visceral and subcutaneous sites and persist long term in these mice. Studies on these issues are currently under way. In a preliminary experiment, we administered 1×10^8 TILs (from patient 923) with in vitro cytolytic specificity (Table 2) IP into BNX mice with disseminated autologous melanoma from patient 923 followed by 5 consecutive days of treatment with IP recombinant interleukin-2 (10 000 U given twice daily). This treatment resulted in a significant enhancement in survival of these mice (median survival in days: treatment with Hanks' balanced salt solution, 74; treatment with recombinant interleukin-2 alone, 70; treatment with TILs plus recombinant interleukin-2, 97; $P<.001$).

The BNX and C.B-17 SCID mouse strains may prove valuable in studies of the cellular and molecular processes involved in the metastatic spread of fresh

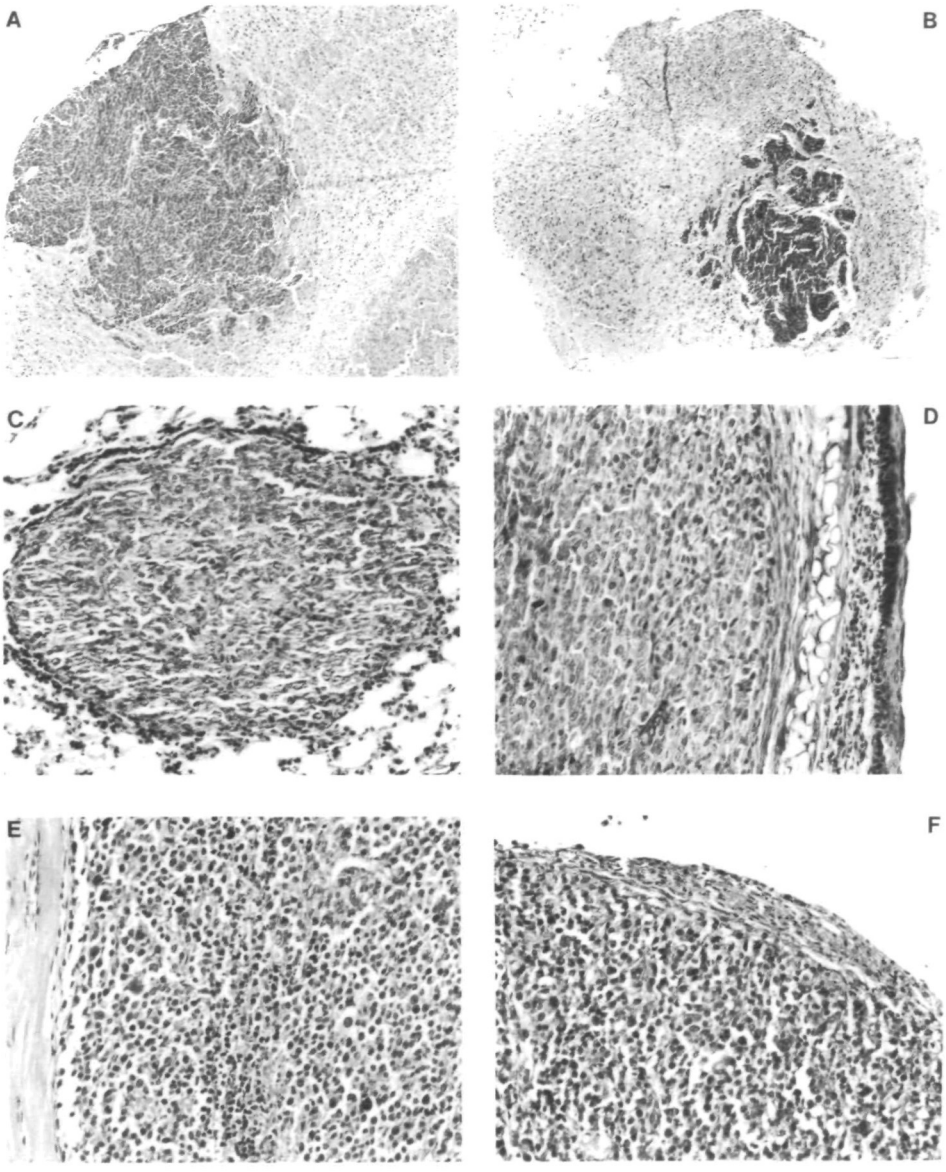

Fig 2. Histologic analyses reveal metastatic tumor in BNX mice consistent with human malignant melanoma (patient 954) in the brain (panels A and B), lung (panel C), dermis of the skin (panel D), soft tissue adjacent to skeletal muscle (panel E), and lymph node (panel F). BNX mice received IV 1×10^7 freshly prepared melanoma cells derived from excised melanoma obtained from the patient (original magnification: panels A and B, ×62; panels C through F, ×250).

human tumors (*29*). We are currently investigating the growth and metastatic properties of human solid tumors of differing histologies (ie, colon and renal adenocarcinomas) in congenitally immune-deficient mice (Jicha DL, Mulé JJ, Yannelli JR, et al: submitted for publication). The growth and distribution of human acute lymphoblastic leukemia in C.B-17 SCID mice have been reported (*30*). We are investigating whether similar metastatic spread of fresh human melanoma occurs in the C.B-17 SCID mouse. These mice have been shown to possess LAK cell precursors (*19*) that may complicate attempts to expand preferentially human lymphocytes in vivo by the administration of recombinant cytokines (eg, interleukin-2, interleukin-4 and interferons). Finally, the fact that most fresh human melanoma cells when injected SC grow progressively in BNX mice means that there is a readily available source of autologous tumor cells that can be used for restimulation of TIL cultures and for targets in cytotoxicity assays. The need for autologous tumor cells has been emphasized recently by our studies demonstrating that the optimum growth of murine TILs with in vitro cytolytic specificity and increased in vivo therapeutic potency requires restimulation with irradiated tumor cells in the presence of low concentrations of interleukin-2 (*31,32*).

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A Hypothesis: Nonsteroidal Anti-Inflammatory Drugs Reduce the Incidence of Large-Bowel Cancer

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Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit prostaglandin synthesis and tumor growth in the rodent colon. We assessed NSAID use in relation to risk of human large-bowel cancer in a hospital-based, case-control study of 1326 patients with colorectal cancer and 4891 control patients. For regular NSAID use that continued into the year before interview, the multivariate relative risk estimate was 0.5 (95% confidence interval, 0.4 to 0.8); the estimate decreased as the duration of use increased, but the trend was not statistically significant. Similar results were obtained whether cancer or noncancer controls were used, and the inverse association was apparent for both colon cancer and rectal cancer in men and women and in subjects younger and older than 60 years. Regular NSAID use that had been discontinued at least 1 year previously and nonregular use were not associated with risk. Almost all regular NSAID use was of aspirin-containing drugs. The present data suggest that the sustained use of NSAIDs reduces the incidence of human large-bowel cancer. [J Natl Cancer Inst 83:355–358, 1991]

Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the synthesis of prostaglandins, which play a role in cell proliferation, neoplasia, and immune response (*1-4*). These drugs, including aspirin, indomethacin, and piroxicam, reduce levels of prostaglandins and inhibit tumor growth in the colons of rodents treated with carcinogens (*5-18*). The effect may be related to the NSAID dose (*17,18*) and may be reversible after discontinuation of treatment (*11*). The rodent colon is considered a good model for the study of human colon cancer because the pathologic and biologic features of chemically induced colon cancer in rats resemble those of human colon cancer (*19*).

To assess the influence of NSAID use on human cancer risk, we analyzed data collected in a case-control drug surveillance study designed to assess the relation between prescription and nonprescription drug use and the risk of various illnesses (*20*). In initial analyses, we examined data on cancers of the lung, breast, large bowel, endometrium, ovary, testis, and bladder as well as lymphomas, leukemias,

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