Depressed Levels of Granular Lymphocytes with Natural Killer (NK) Cell Function in 247 Cancer Patients

CHARLES M. BALCH, M.D., F.A.C.S., ARABELLA B. TILDEN, PH.D., PATRICIA A. DOUGHERTY, M.S., GRETCHEN A. CLOUD, M.S., TORU ABO, M.D.

The HNK-1 (Leu-7) monoclonal antibody was used to enumerate and characterize the level of blood granular lymphocytes in 247 cancer patients. The results were compared to 146 control individuals. A fluorescence-activated cell sorter was used to purify blood HNK-1$^+$ cells from cancer patients. The monoclonal antibody identified a homogeneous population of granular lymphocytes with greater than 95% purity. Conversely, virtually 100% of HNK-1$^-$ cells from cancer patients were agranular lymphocytes. These results were the same as previously observed in normal individuals, where the HNK-1$^+$ cell fraction contained all the lymphocytes with spontaneous cytotoxicity in natural killer (NK) and killer (K) cell assays. The level of HNK-1$^+$ cells in cancer patients correlated significantly with the patient's age and sex, with older individuals having higher levels and male patients containing a higher proportion than female patients. The levels in the cancer patients were significantly lower than normal controls ($p = 0.04$). When the results were subdivided by the histologic type of cancer, additional differences were noted. Compared to age and sex-matched controls, significantly depressed levels of HNK-1$^+$ granular lymphocytes were observed in 49 patients with colon cancer (9.7% *vs.* 15.8%, $p = 0.0001$), 18 patients with lung carcinoma (11.7% *vs.* 27.0%, $p = 0.0001$), 24 patients with breast carcinoma (12.0% *vs.* 15.5%, $p = 0.04$) and 64 patients with head and neck carcinoma (15.9% *vs.* 19.1%, $p = 0.05$). However, there were no significant differences overall in the average HNK-1$^+$ cell level of 66 patients with melanoma (13.0% *vs.* 13.5%, $p = 0.75$) and nine patients with sarcomas (15.8% *vs.* 14.3%, $p = 0.71$). Thus, this important subpopulation of granular lymphocytes with NK and K cell function was significantly depressed in most cancer patients. Accounting for the patient's age and sex and the histologic type of cancer was critical to interpreting the results.

From the Departments of Surgery, Microbiology, and Biostatistics, the Cellular Immunobiology Unit, the Comprehensive Cancer Center, and the Veterans Administration Medical Center, The University of Alabama in Birmingham, The Medical Center, Birmingham, Alabama

NATURAL KILLER (NK) and killer (K) cells are named for their cytotoxic capability to lyse target cells without prior sensitization. NK and K cells are thought to be responsible for the initial immune surveillance against tumors and viruses,[1,2] until a pool of specifically sensitized cells becomes large enough to react with tumor or viral antigens. As evidence of this, genetic deficiencies of NK cell function in the beige mouse and in children with Chediak-Higashi disease are associated with a strikingly increased risk for developing malignant tumors.[3-7]

Two characteristics of human lymphocytes with classically defined NK and K cell cytotoxic function are: 1) they express receptors for the constant fragment of immunoglobulin (FcIgG) and 2) they are primarily granular lymphocytes found in the "null" cell fraction of human blood mononuclear cells.[8-10] However, NK cells are otherwise an operationally defined population of lymphoid cells, since no unique differentiation antigens have been identified previously on these cells. The operational definition has caused ambiguity about the types of cells performing NK and K cell function since, under certain circumstances, activated T cells and monocytes can also function as NK-like cytotoxic cells.[11,12]

We prepared the first monoclonal antibody (HNK-1) that reacts with classically defined human NK and K cells.[13] The HNK-1 differentiation antigen is expressed on virtually all human granular lymphocytes. Functional characterization of these purified cell populations demonstrated that they possess both NK and K cell function and that they exhibit little or no proliferative response to mitogens (PHA, Con A, and PWM) or to alloantigens in a mixed lymphocyte culture.[14] The cytotoxic efficiency of these HNK-1$^+$ cells can be boosted with interferon.[15] These granular lymphocytes reside primarily in the blood and spleen, with only a small proportion (1%–2%) in the lymph node, thymus, and

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Reprint requests: Charles M. Balch, M.D., Chief of Surgical Oncology, Department of Surgery, University of Alabama Medical Center, 320 Kracke Building, Birmingham, Alabama 35294.

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bone marrow.[16] At least three different subsets of HNK-1^+ cells, probably representing different stages of maturation, have been defined in fetal and adult tissues.[16,17]

In this study, we measured the levels of HNK-1^+ granular lymphocytes in 247 cancer patients and compared these to the levels in 146 age and sex-matched normal controls. The levels of these granular lymphocytes with NK and K cell function were depressed uniformly in patients with some types of cancer, while there was little variation from normal levels in patients with other types of cancer.

Materials and Methods

Cell Preparation

Human blood mononuclear cells were obtained using standard Ficoll-Hypaque density gradients, as previously described.[18] Blood samples were obtained from cancer patients who were treated at the University of Alabama in Birmingham and from normal controls with no significant medical illnesses or a history of cancer. All cell preparations were placed in RPMI 1640 medium, supplemented with 20% heat inactivated fetal calf serum and gentamicin (50 mg/ml).

Immunofluorescence Assay

Lymphocytes expressing the HNK-1 antigen on their cell membrane surface were enumerated by indirect immunofluorescence, as previously described.[13] A monoclonal IgM antibody, HNK-1 (Leu-7; Becton Dickinson and Co., Sunnyvale, CA), was used at a concentration of 10 μg/ml for indirect immunofluorescence. The secondary antibody was an FITC-conjugated goat anti-mouse IgM antibody purified by elution from a sepharose affinity column of mouse myeloma IgM.[19]

Fluorescence-Activated Cell Sorting

After immunofluorescence staining, the subpopulation of HNK-1^+ granular lymphocytes was separated from HNK-1^- cells using a fluorescence-activated cell sorter (FACS IV, Becton Dickinson and Co., Sunnyvale, CA), as previously described.[20]

NK Cell Function Activity

K-562 cells were labeled with ^{51}Cr by incubating 10^7 cells in 0.5 ml of medium and 0.5 ml of 1.0 mCi/ml $Na_2\ ^{51}CrO_4$ (New England Nuclear, Boston, MA) at 37 C for 1 hour. Target cells were then washed three times and resuspended at a concentration of 10^6/ml. $^{51}CrO_4$-labeled K-562 target cells (10^4) were incubated at 37 C in a humidified 5% CO_2 atmosphere with 10^5 effector lymphocytes in "U" bottom well plates containing a total volume of 200 μl/well. After overnight incubation, 100 μl of the upper supernatant were removed for counting. Triplicate samples were counted in a gamma counter for one minute. Chromium release was calculated by the following formula:

$$\%\ ^{51}Cr \text{ specific release} = \frac{2(S - R)}{M - 2R}$$

where M is the CPM of maximum release by target cells, S is the CPM of test supernatants, and R is the CPM of supernatants from cultures containing target cells only. Spontaneous ^{51}Cr release of K-562 without effector cells was always less than 10% of the total counts.

Statistics

The data were compared initially by analyzing each cancer type *versus* the entire normal population. Univariate statistics for these groups were determined for each of the descriptive variables. Analysis of variance was utilized to analyze the significant factors relating to the level of HNK-1^+ cells.[21]

Since age and sex had such a significant correlation with HNK-1 levels, the computer was programmed to select a cohort of normal individuals that would match the age range of patients with each type of cancer. When a patient group contained an individual with an age extreme, this patient was eliminated, so that results would not be biased by an outrider. The gender was also used as a selection criteria for choosing each control group. All analyses were performed on an IBM Model 4341 computer using the Statistical Analysis System (SAS).

Results

Correlation of HNK-1 Levels and NK Cell Function in Normals

Blood lymphocytes from 35 healthy individuals were analyzed for numbers of HNK-1^+ cells by immunofluorescence with HNK-1 monoclonal antibody and NK cell functional activity against K-562 target cells. These experiments were performed to demonstrate a correlation between levels of HNK-1^+ cells and NK cell activity. HNK-1^+ cells previously have been shown to contain the majority of NK effector cells in normal blood lymphocyte preparations.[13] As shown in figure 1, the correlation between HNK-1^+ level and NK function was highly significant ($r = 0.62$, $p < 0.001$).

Isolation of HNK-1^+ Granular Lymphocytes from Cancer Patients

Blood lymphocytes from cancer patients were first purified with the HNK-1 monoclonal antibody using

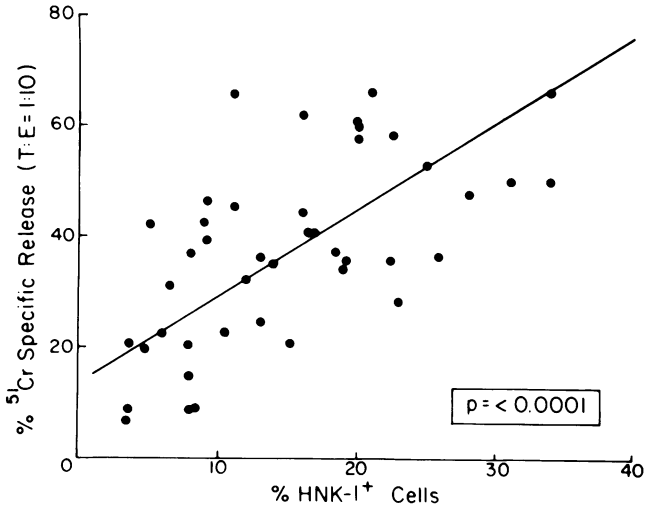

FIG. 1. Correlation between HNK-1$^+$ cell level and NK cell function. HNK-1$^+$ cells were enumerated from blood mononuclear cells and compared with NK cell function against K-562 target cells in 35 healthy adult donors. HNK-1$^+$ cells were detected by direct immunofluorescence using FITC-conjugated HNK-1 antibody. There was a significant correlation of HNK-1$^+$ levels and NK cell function in these normal individuals ($r = 0.62$, $p < 0.001$).

the fluorescence-activated cell sorter. Monocytes were excluded from the separation procedure by eliminating the large cells (using light scatter criteria). These experiments were necessary to prove that the HNK-1$^+$ cells from cancer patients had the same morphologic characteristics as those previously defined for normal individuals.[13,16,17] More than 95% of the HNK-1$^+$ cells from cancer patients were medium-sized lymphocytes with abundant cytoplasm containing azurophilic granules (Fig. 2a). In contrast, virtually all HNK-1$^-$ cells were small to medium-sized lymphocytes with a narrow cytoplasmic rim and no cytoplasmic granules (Fig. 2b).

Correlation of HNK-1$^+$ Levels with Age and Sex

We have previously demonstrated in normal humans that the HNK-1$^+$ granular lymphocyte population expands as a function of age and sex.[22] In the present experiments, we confirmed this observation with a threefold larger population of normal adults. Levels of HNK-1$^+$ cells in normal human blood increased with age, with men having a consistently higher proportion of HNK-1$^+$ cells compared to women (Fig. 3). The levels of HNK-1$^+$ cells in cancer patients had the same age and sex correlations observed for normal individuals, but the actual proportion of HNK-1$^+$ cells was significantly lower for the cancer patients compared to controls ($p = 0.04$) (Fig. 4).

The above observations were critical in this data analysis, since the levels of HNK-1$^+$ cells for the entire group of cancer patients was very similar to the entire group of normal controls (13.6% *vs.* 15.4%). However, a closer examination of the data showed that the control population was younger as a group than the cancer patients (mean age, 42 years for the 146 normal subjects *vs.* 56 years for the 247 cancer patients). Furthermore, the lev-

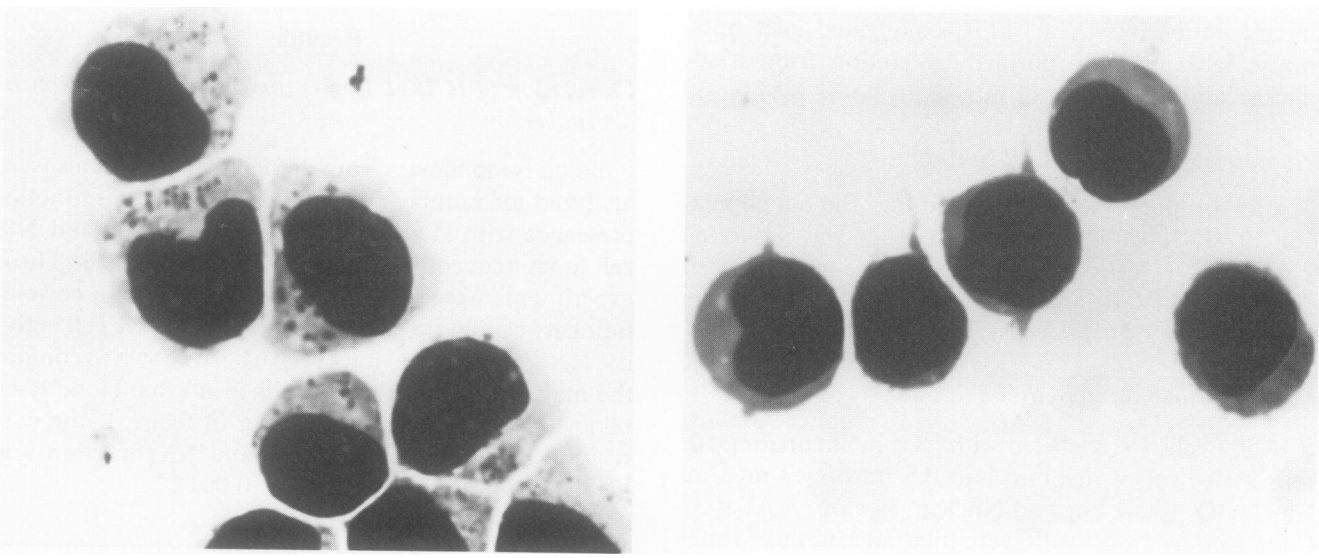

FIG. 2. PBMC from a colon cancer patient were isolated with a cell sorter into HNK-1$^+$ and HNK-1$^-$ fractions of lymphocytes and their morphologic appearance examined by light microscopy (magnification ×600) after staining with May-Grünwald-Giemsa. HNK-1$^+$ cells were a homogeneous population of medium to large lymphocytes with abundant cytoplasm containing azurophilic granules (a). HNK-1$^-$ cells were small to medium sized lymphocytes with narrow cytoplasmic rims and no cytoplasmic granules (b).

els of HNK-1$^+$ and the mean age and sex ratio were different for each type of cancer. The HNK-1$^+$ levels were lowest for colon and breast carcinomas but were essentially normal for melanoma, sarcoma, lung carcinoma, and head and neck carcinoma (Table 1). Because of these findings, the levels of granular lymphocytes for each histologic type of cancer were analyzed separately and the results compared to an age- and sex-matched cohort of normal individuals.

HNK-1$^+$ Levels in Colon Carcinoma

The HNK-1$^+$ levels were depressed more significantly in colon carcinoma patients than for any other cancer type. The 49 patients had a mean age of 58.8 years (range, 32–82 years). Sixty-Five per cent of the patients were men. The mean level of HNK-1$^+$ cells was significantly depressed in the colon cancer patients compared to the total group of 146 controls (9.7% *vs.* 15.5%, $p = 0.0001$). An age-matched cohort of 74 normal individuals was defined by computer selection for comparison. Their mean age was 49.4 years (range, 32–88 years). When the colon patient data was compared to this control subgroup, the difference became even more significant ($p < 0.0001$) (Table 2). There was no difference in the HNK-1 levels within stages of disease ($p > 0.10$). The individual patient data is displayed in a scattergram (Fig. 5).

HNK-1$^+$ Levels in Breast Carcinoma

The 25 breast carcinoma patients had a mean age of 57.6 ± 2.9 years (range, 28–85 years). All but one were women. The HNK-1$^+$ cell level for each individual patient is displayed on the scattergram (Fig. 5). The HNK-1$^+$ levels in the patients were significantly lower than controls (11.8% *vs.* 15.5%, $p = 0.05$). There was no difference in the results within the stages of disease ($p > 0.10$). For the purpose of matching the appropriate control group, the single male patient was excluded and the female patient data was compared with a cohort of 68 normal women who were selected to encompass the age range of the patient group. The HNK-1$^+$ levels in the female breast cancer patients was still significantly lower than age-matched normal women (12.0% *vs.* 15.5%, $p = 0.04$).

HNK-1$^+$ Levels in Lung Carcinoma

The 22 lung carcinoma patients were the oldest patient group examined, with a mean age of 61.5 years (range 41–72 years). All but two were women. The HNK-1$^+$ levels in this group were not significantly different from the HNK-1$^+$ levels for the total normal control group of 146 individuals (Table 2). However, the control group was younger and had more women than the patient group. Therefore, the levels of HNK-1$^+$ cells in these patients were compared to the levels in a cohort

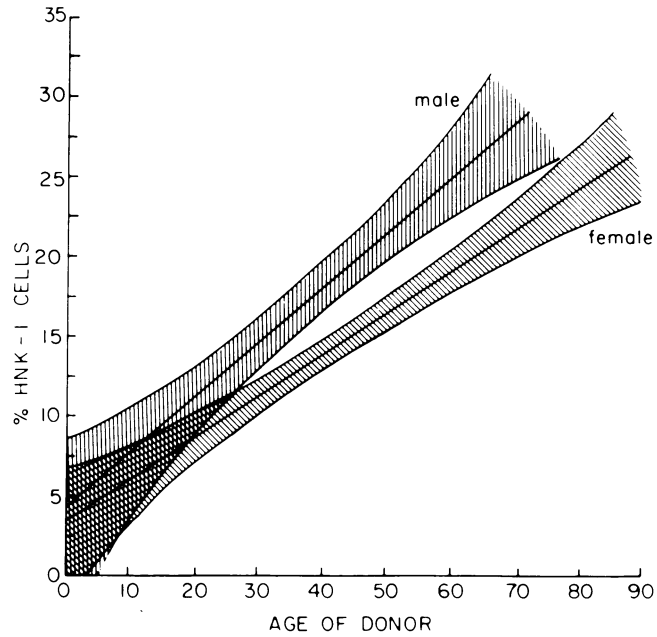

FIG. 3. HNK-1$^+$ lymphocytes in 146 normal subjects (mean levels ± 1 SEM) when analyzed by fluorescent microscopy. HNK-1$^+$ levels increase as a function of age and were higher in men than in women.

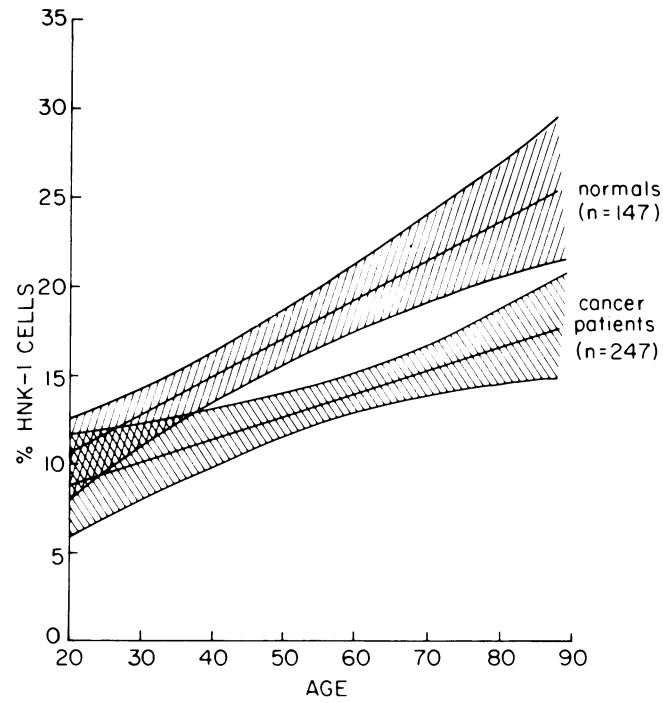

FIG. 4. Depressed levels of HNK-1$^+$ blood lymphocytes in 247 cancer patients compared to that in 146 normal individuals (mean levels ± 1 SEM). The differences are statistically significant ($p = 0.04$).

TABLE 1. *Levels of HNK-1$^+$ Granular Lymphocytes in Cancer Patients and Normal Controls*

			Male Patients			Female Patients		
	HNK-1$^+$ Levels in Patients	Mean Age ±1 SEM	No.	HNK-1$^+$ Levels	Mean Age ± SEM	No.	HNK-1$^+$ Levels	Mean Age ±1 SEM
Colon carcinoma (49)*	9.7 ± 0.9%	58.8 ± 1.9 years	32	10.1%	61.6 years	17	8.8%	53.3 years
Lung carcinoma (22)	13.4 ± 2.1%	61.5 ± 1.4 years	20	13.7%	61.1 years	2	11.0%	64.5 years
Breast carcinoma (25)	11.8 ± 1.1%	57.6 ± 2.9 years	1	7.0%	47 years	24	12.0%	58.0 years
Head and neck (70)	16.1 ± 1.1%	60.5 ± 1.1 years	61	16.0%	59.5 years	9	17.1%	66.8 years
Melanoma (72)	13.8 ± 1.0%	46.9 ± 1.8 years	42	14.7%	46.9 years	30	12.1%	46.2 years
Sarcoma (9)	15.8 ± 2.7%	58.1 ± 5.3 years	6	18.8%	62.3 years	3	9.7%	49.7 years
Normals (146)	15.5 ± 0.7%	42.0 ± 1.3 years	52	18.2%	42.2 years	94	14.0%	41.9 years

* Numbers of individuals studied are shown in parentheses.

of age- and sex-matched normal individuals. This cohort had a mean age of 64.6 years (range, 55–72 years). The HNK-1$^+$ levels for the male lung carcinoma patients were significantly lower than the age- and sex-matched controls (11.7% *vs.* 27.0%, p = 0.0001). There were not a sufficient number of patients to analyze within disease stage. The actual data is shown in Fig. 5.

HNK-1$^+$ Levels in Head and Neck Carcinoma

The 70 patients with head and neck carcinoma had a mean age of 60.5 ± 1.1 years (range, 35–85 years) with 87% being men. HNK-1$^+$ levels in this group of patients were actually higher than the average level of HNK-1$^+$ cells in the entire control population (Table 1). A cohort of normal individuals was selected who had a mean age of 60.6 years (range 45–76 years). The mean HNK-1$^+$ level for these patients was depressed compared to the age-matched control group (15.9% *vs.* 19.1%, p = 0.05) (Table 2). There was no difference in the results within disease stages (p > 0.10). The individual data are displayed on a scattergram (Fig. 5).

HNK-1$^+$ Levels in Melanoma

The 72 melanoma patients were the youngest patients as a group, with a mean age of 46.9 years (range, 21–79 years) and 58% being men. The average HNK-1$^+$ level was slightly less in the patient group compared to the control group (13.8% *vs.* 15.5%, p = 0.68). The patient levels were then compared to an age-matched cohort of 92 normal individuals (67% male) with a mean age of 40.5 years (range, 21–70 years). The average HNK-1$^+$ level was virtually the same in the patient and the age- and sex-matched control group (13.0% *vs.* 13.5%, p = 0.75). Because of the broad spectrum of ages for the melanoma patients, we examined a number of patient subgroups that were categorized by age and sex for comparison with control levels. The only significant differences were seen in 14 male melanoma patients over 55 years of age, whose HNK-1$^+$ levels were significantly depressed compared to that of 12 normal men over 55 years of age (16.0% *vs.* 27.0%, p = 0.008). The individual patient data is displayed in a scattergram (Fig. 5).

HNK-1$^+$ Levels in Sarcoma

The levels of HNK-1$^+$ lymphocytes were analyzed in nine sarcoma patients who had a mean age of 58 years (range, 29–76 years). The mean level of HNK-1$^+$ cells in these patients was essentially the same as the mean level in normal individuals (15.8% *vs.* 15.5%). The number of sarcoma patients was too low to permit an analysis of subgroups. The individual data are shown in Fig. 5.

Discussion

This report demonstrates that the HNK-1 monoclonal antibody specifically identifies a unique popula-

TABLE 2. *Levels of HNK-1$^+$ Granular Lymphocytes in Cancer Patients Compared to Matched Controls*

	HNK-1$^+$ Level in Patients	Mean Age ± SE	HNK-1$^+$ Level in Matched Controls	Mean Age ± SE	p Value*
Colon (49)†	9.7%	58.8 ± 1.9 years	15.8%	49.4 ± 1.4 years	0.0001
Lung (18)	11.7%	62.8 ± 0.9 years	27.0%	64.6 ± 1.6 years	0.0001
Breast (24)	12.0%	58.0 ± 3.0 years	15.5%	48.4 ± 1.9 years	0.04
Head and Neck (64)	15.3%	60.6 ± 0.8 years	18.8%	60.5 ± 1.1 years	0.05
Melanoma (66)	13.0%	44.8 ± 1.7 years	13.5%	40.5 ± 1.5 years	0.75

* Significance of difference of HNK-1$^+$ level from matched controls. † Numbers of individuals studied are shown in parentheses.

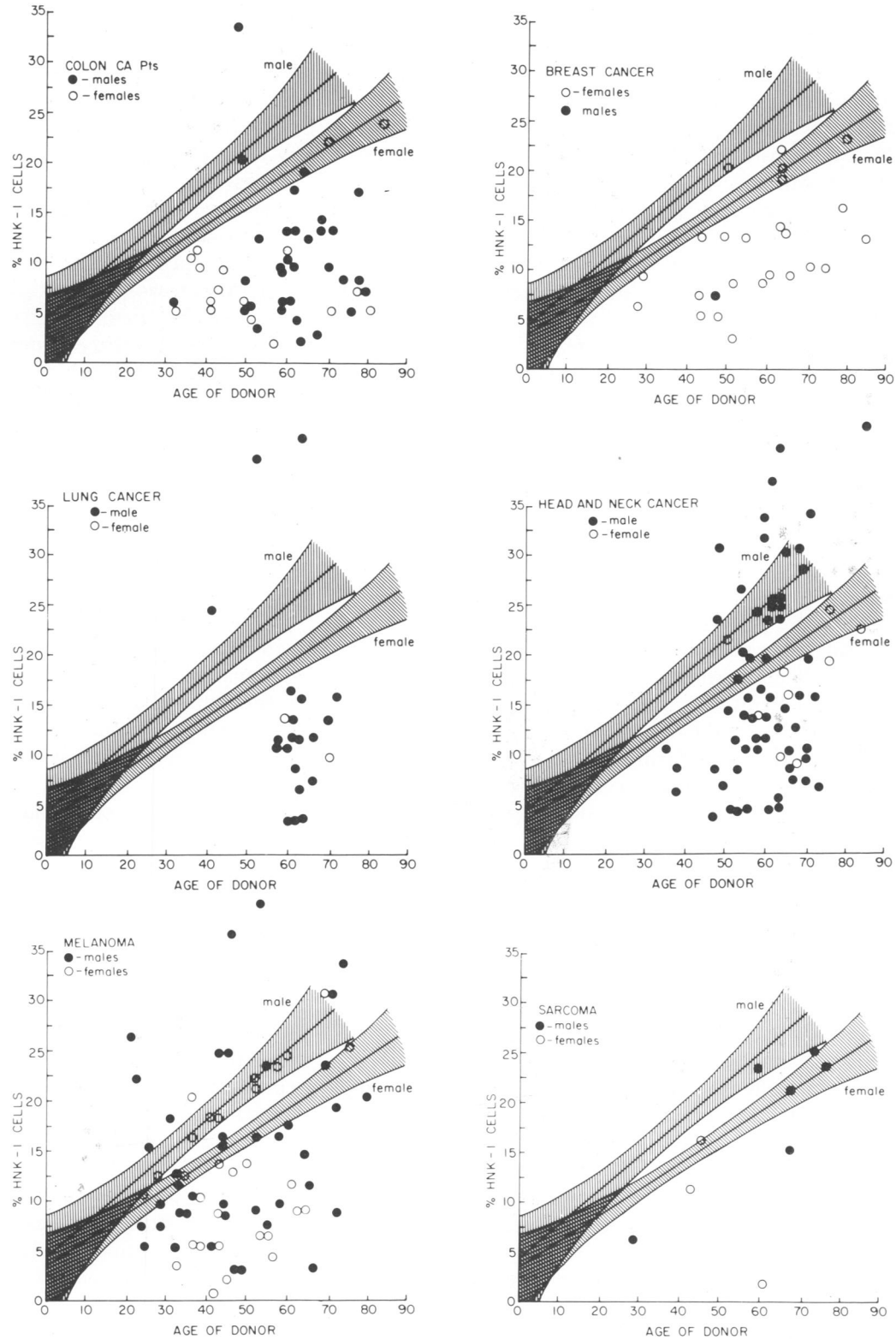

FIG. 5. The levels of blood HNK-1$^+$ cells for male and female cancer patients compared to the mean levels in 146 normal subjects (shaded values).

tion of granular lymphocytes in cancer patients, confirming similar findings in normal individuals.[13,22] The HNK-1$^+$ subpopulation in normal individuals contains virtually all the cells with classically defined NK and K cell function,[13,14] and there is an excellent positive correlation between HNK-1$^+$ levels and NK cell activity. The data demonstrates the diversity of HNK-1$^+$ levels among various types of cancer and the importance of

accounting for the patient's age and sex when analyzing the results.

The evidence suggesting an important role for NK cells in tumor immuno-surveillance is largely derived from laboratory studies in mice.[2] Several investigators have demonstrated that a strain of mutant mice (beige mouse) with low NK cell activity develop tumors faster and at a higher incidence than do mice of the same strain with normal NK activity.[3,4,7] A direct correlation has also been shown between the levels of NK cell activity in mice and their ability to reject transplanted lymphoma cells.[23-25]

In humans, some indication that low NK activity might predispose to cancer comes from studies of patients with Chediak-Higashi disease who have little or no NK cell activity; these individuals often die with infections or lymphoproliferative malignancies.[5] However, other studies have been conflicting regarding NK and K cell function in cancer patients, since some investigators identified profound deficiencies of NK or K cell function,[26-33] while others observed no differences or even elevated levels compared to normal controls.[34-36] Since there is such a large number of variables to be considered in analyzing data about NK and K cell function, it is not surprising that there is ambiguity in the literature about their role in cancer patients. Some of the variables that should be accounted for include: 1) the type of cell performing NK and K cell function (classical NK, activated T cells or macrophages); 2) the experimental methods used; 3) the stage of maturation of NK cells; 4) the histologic type of tumor; and 5) the patient's age and gender.

Conflicting results involving NK cell studies are due, in part, to the definition of NK cells in terms of their functional property and the multiple cell types that can spontaneously kill different types of tumors without prior sensitization. Human NK and K cells are defined classically as granular lymphocytes that express FcIgG receptors and lack the usual T- and B-cell markers.[9,10,20] However, cells other than granular lymphocytes can also exhibit NK-like killing *in vitro*. For example, blood macrophages can kill tumor cells by both direct and IgG dependent cytotoxicity.[12,37] Furthermore, activated T lymphocytes have also been shown to have NK-like cytotoxic function.[11,14] Although the granular lymphocytes are the primary source of NK cells in normal individuals, it is entirely possible that the other cell types might contribute to NK cell function in disease states.

Discrepancies may also arise due to the variations in the actual NK and K cell assays among different laboratories. The choice of target cell, effector to target cell ratio, and other experimental parameters can alter the results of such analyses.[33,37,38] By determining the actual number of HNK-1$^+$ cells, many problems associated with the operational definition of NK and K cells can be obviated. Furthermore, the ability to purify HNK-1$^+$ cells with this monoclonal antibody enables more accurate comparisons of NK cytotoxicity between cancer patients and normal individuals, since any contributions by other cell types (*e.g.*, activated T cells and macrophages) in the cytotoxicity assays can be accounted for. In fact, we have observed a substantial contribution of HNK-1$^-$ cells (presumably T cells) to NK cell function in some cancer patients but not normal individuals.*

Another reason for conflicting data may relate to differences in the maturational stages of the NK cells being studied. At least three maturational stages of granular lymphocyte development have been identified, with immature HNK-1$^+$ cells having low levels of NK and K cell function, while mature HNK-1$^+$ cells have the most efficient cytotoxic function.[16] This observation is important, for some cancer patients have an increased proportion of immature HNK-1$^+$ cells and minimal cytotoxic functional capability, suggesting a block in their NK cell differentiation.*

Another reason for the disparity of NK cell results is that many previous investigations examined patients with a variety of histologic types of cancer and they did not analyze their results within each group or compare them with an age- and sex-matched control population. As shown in this study, the level of HNK-1$^+$ cells varies greatly among patients with different histologic types of cancer.

The HNK-1 monoclonal antibody has several important advantages for detailed analysis of NK cell function. First, it delineates the major population of cytolytic NK cells and permits their purification. Second, it can be used to identify the maturational stage of NK cells to determine whether abnormalities in certain cancer patients might be due to an arrest in NK cell development. Third, it enables more detailed studies of the cytotoxic capability of T cells and macrophages, since the contribution by classically defined NK cells can be accounted for while the other types are being examined. This may be particularly helpful in determining what compensating mechanisms for classical NK cell function are available for host defense when there are abnormalities of classically defined NK cells. The results of the present study also clearly demonstrate the importance of accounting for the patient's age, sex and histologic type of cancer when performing these studies.

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