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NATURAL CYTOTOXICITY AND INTERFERON PRODUCTION IN HUMAN CANCER: DEFICIENT NATURAL KILLER ACTIVITY AND NORMAL INTERFERON PRODUCTION IN PATIENTS WITH ADVANCED DISEASE[1]

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Natural cytotoxicity was measured in 51 adult patients with solid epithelial malignant tumors and in 27 normal subjects. Peripheral blood leukocytes (PBL) from 31% of the patients and 7% of the controls failed to kill target cells (K562) in a short-term chromium-release assay. When patients were classified according to clinical stage, PBL from 12% of patients with localized cancers, but 50% of patients with advanced disease, failed to exhibit cytotoxicity within the normal range. Pretreatment of PBL with interferon$_\alpha$ (IFN$_\alpha$) or with Newcastle Disease Virus (NDV), a potent inducer of IFN$_\alpha$, enhanced cytotoxicity from all normal subjects. Of patients whose PBL lacked spontaneous cytotoxicity, half were able to kill normally after pretreatment of PBL with IFN$_\alpha$ or NDV. Virtually all the patients whose PBL were unable to kill despite pretreatment with IFN$_\alpha$ or virus had disseminated malignancies. IFN$_\alpha$ production by PBL exposed to NDV and to K562 cells was normal in all the patients regardless of stage of disease or ability to kill K562 cells. The observed defect in natural cytotoxicity is thus unlikely to be due to a failure of PBL to produce IFN$_\alpha$.

Lymphocytes from normal experimental animals and human subjects have been shown to be capable of killing a variety of virus-infected or tumor-derived target cells *in vitro* (1–3). This spontaneous cytotoxicity has been demonstrated by using peripheral blood leukocytes (PBL)[4] from normal healthy donors and has been called "natural cytotoxicity". Human natural killer (NK) cells appear to be distinct from conventional T cells, B cells, and macrophages (4) and have been identified as large, granular lymphocytes (5) with a weak affinity for rosetting with sheep red blood cells and with receptors for the Fc portion of IgG (6, 7). At least a portion of murine and human NK cells may be related to the T lymphocyte lineage by virtue of their reactivity with antisera against T cell-associated surface antigens (8–10). NK cells are capable of lysing a variety of target cells, but little is known about the nature of target recognition or about the mechanism of killing in this system. It is unclear at present whether NK cells are truly nonspecific, or whether multiple clones of effector cells exist, each with its own target specificity.

Natural cytotoxicity has been shown to be stimulated by interferons (IFN) and by IFN inducers *in vivo* and *in vitro* (11, 12). The molecular mechanism of IFN activation of NK cells is unknown. The phenotype of the lymphocyte that produces IFN on exposure to viruses or tumor cells (IFN$_\alpha$) and that of the cytotoxic effector cell appear to be the same (4, 13), but it has not yet been formally established that the same cell is responsible for both functions.

It has been suggested that natural cytotoxicity may play an important role in immune surveillance against tumors and viral infection in experimental animals (14, 15) and in man (16). In the nude mouse, which lacks competent T effector cells, there is evidence to suggest that natural cytotoxicity and interferon are critically involved in the regulation of the growth and metastasis of virally infected cells and heterologous tumors (13, 17). In the beige mouse, an analogue of the human Chediak-Higashi syndrome, defective NK activity has been shown to predispose to enhanced growth of transplanted tumors (18, 19). Diminished NK activity has been found in tumor-bearing animals (20) and humans with malignant disease, especially those with widely disseminated tumors (16), but the mechanism for this defect is as yet unclear. Low NK activity has also been described in patients with familial malignant melanoma and their close relatives, suggesting that defective natural cytotoxicity may predispose to the development of malignant disease (21). Recently, deficient natural cytotoxicity was found in 2 patients with Chediak-Higashi syndrome, a disease entity associated with an increased incidence of lymphoproliferative disorders (22).

Because of the possible role of the natural cytotoxicity-interferon system in immune surveillance against cancer, we have studied NK activity and IFN$_\alpha$ production in patients with malignant disease. We present here evidence that: 1) NK activity is markedly deficient in some patients with cancer, and the defect can be correlated with the clinical stage of disease, as has been reported by others; 2) the abnormality in cytotoxicity in some, but not all, of these patients can be corrected by pretreatment of PBL with IFN$_\alpha$ or an IFN$_\alpha$ inducer; and 3) IFN$_\alpha$ production induced from PBL by virus or K562 cells is normal in cancer patients regardless of clinical stage of disease or ability to lyse K562 cells.

MATERIALS AND METHODS

Population studied. Control subjects included 27 normal individuals aged 26 to 57 yr. The patient group consisted of 51 adults aged 32 to 74 yr with

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[3] Dr. Steinhauer is supported by Training Grant 09173 from the National Cancer Institute.
[4] Abbreviations used in this paper: IFN, interferon; L.U., lytic unit; NDV, Newcastle Disease Virus; NK, natural killer; PBL, peripheral blood leukocytes.

a variety of solid epithelial malignant tumors (17 genitourinary, 13 breast, 9 gastrointestinal, 6 head and neck, 5 lung carcinomas, and 1 malignant melanoma). Patients were studied from 2 wk to 4 yr after surgical resection or biopsy of their tumors. All diagnoses were histopathologically confirmed. Three patients with metastatic tumors had received courses of chemotherapy 2 to 3 yr before study. Two of these patients had also undergone radiotherapy 2 yr before testing. Two patients with prostatic cancer had received hormonal therapy within 6 mo of study. The remaining patients were untreated. All patients were ambulatory at the time they were tested; none had evidence of other intercurrent illness. Patients whose tumors were confined to the primary site with or without regional lymph node involvement were considered to have "localized" disease. Patients with distant metastases, recurrent, or extensive unresectable tumors were classified as having "advanced" cancers. Patients were age and sex matched with control subjects whenever possible.

Preparation of lymphocytes. Twenty-five to 30 ml of heparinized blood were obtained by venipuncture. PBL were isolated by sedimentation in Ficoll-Hypaque (Pharmacia, Piscataway, NJ), washed in Hanks' BSS and resuspended in RPMI 1640 (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum (Flow Laboratories, Rockville, MD), glutamine, and antibiotics. In some experiments PBL were further fractionated by removal of adherent cells on nylon wool columns (23). PBL were cultured in round bottomed 17 x 100-mm tubes at 5×10^6 cells/ml (2 ml/tube). PBL were cultured overnight in medium alone, in medium containing 100 U/ml of IFN_α, and, in some cases, with inactivated Newcastle Disease virus (NDV) before cytotoxicity assay. There was no significant difference in NK activity between cytotoxicity assays performed on the day of venipuncture and those done after overnight incubation.

Cytotoxicity assay. The assay used was a short-term chromium-release assay using K562 cells derived from a patient with chronic myeloid leukemia (24) as target cells. K562 cells were labeled with ^{51}chromium (sodium chromate, 50 to 400 mCi/ml, Amersham Corp., Arlington Heights, IL), washed, and dispensed into round bottomed microwells (Linbro Scientific, Hamden, CT) at 10^4 cells/well in 0.1 ml RPMI 1640 with 10% FCS. Effector PBL were washed, counted, and added to target cells in triplicate (0.1 ml of lymphocyte suspension/well) at lymphocyte to target cell ratios of 80, 40, 20, and 10 to 1. Target cells were incubated in medium without PBL as controls for spontaneous release and total label. Plates were incubated at 37°C for 5 hr. Thirty minutes before harvesting, detergent (NP 40) was added to half of the target control wells to lyse target cells for measurement of total label. One-tenth milliliter of culture supernatant was carefully removed from spontaneous release control wells and from each test well and expelled into glass tubes. The entire contents of detergent-treated control wells were also removed. Samples were counted in a gamma counter (LKB 80,000 Gamma Sampling Counter, LKB, Rockville, MD). Spontaneous release was less than 10% of total label in all experiments.

Percent cytotoxicity was calculated with the following formula:

$$\% \text{ Cytotoxicity} = 100 \times \frac{\text{Test cpm minus spontaneous release cpm}}{\text{Total cpm/2 minus spontaneous release cpm}}.$$

Cytotoxic activity was expressed as lytic units (L.U.)/10^7 PBL. A L.U. was defined as the number of PBL needed to effect 30% cytotoxicity of 1×10^4 target cells. L.U. were calculated from the cytotoxicity curve for each test by linear regression analysis. The use of L.U. calculated in this manner as a measure of cytotoxicity has been shown to be inaccurate when the data points obtained are not in the linear portion of the cytotoxicity curve (25). To minimize such error, we have used data points graphically determined to be on the linear portion of the curve to calculate L.U. for the normal donors. The mean cytotoxicity for the normal control group was calculated in $\log_{10}$ L.U./10^7 cells (geometric mean). The normal killing range was defined as the mean ± 2 SD (see Fig. 1). Individuals whose NK activity was below the defined normal range (i.e., less than 2 L.U./10^7 PBL, see Fig. 1) were considered to be nonkillers. For use in calculations of mean cytotoxicity for the different test groups, L.U. values below 1/10^7 cells were arbitrarily assigned the value of 1 (0.00 log L.U.). This assignment was necessary since values calculated for such poor killers are highly inaccurate and require enormous extrapolation from the experimental killing curves.

Interferon preparation. IFN_α was induced from PBL of normal individuals by using β-propiolactone-inactivated NDV as previously reported (26). The preparation used in all experiments contained 3170 U of antiviral activity per milliliter. IFN_α was added to PBL cultures at a final concentration of 100 U/ml.

Treatment of PBL with NDV and K562 cells. β-Propiolactone-inactivated NDV was added to PBL at 5×10^5 PFU/ml. After an overnight incubation at 37°C, supernatants were removed for assay of antiviral activity and PBL were used for cytotoxicity assay. In some experiments 5×10^5 K562 cells were added to 1×10^6 PBL in 1 ml and 24 hr later supernatants were harvested and assayed for IFN.

Interferon assay. Antiviral activity was measured by reduction of cytopathic effect of vesicular stomatitis virus (VSV) on human 21 trisomic fibroblasts (Detroit 532, American Type Cell Culture Collection, Rockville, MD) as previously reported (26). Each assay included a reference human leukocyte IFN preparation (G-023-901-527, National Institutes of Health). One unit of IFN in our assay system corresponded to 0.6 reference units.

RESULTS

NK activity in normal controls. PBL from 27 normal subjects were tested for cytotoxicity against K562 cells. The mean cytotoxicity in 65 separate experiments was 28 L.U./10^7 PBL (1.44 ± 0.62 log L.U.). When the mean cytotoxicity for each subject who was tested on multiple occasions was used, the mean cytotoxicity for the 27 normal individuals was 22 L.U. (1.35 ± 0.54 log L.U.), not significantly different when using the Student *t*-test. It should be noted that the relative ranking of cytotoxicities for normal donors tested repeatedly remained constant throughout the experimental period, as has been reported by others (24). Two of the normal subjects repeatedly exhibited cytotoxicity below the normal range. The distribution of mean cytotoxicities for the normal subjects is shown in Figure 1. The normal killing range was defined as the mean ± 2 SD. There was no significant difference in killing between males and females, or subjects under age 40 and those over 40.

NK activity in cancer patients. Mean cytotoxicity for the 51 cancer patients was 15 L.U./10^7 PBL (1.17 ± 0.89 log L.U.), not significantly different from the mean for normal subjects. Of these, 16 (31%) exhibited killing below the normal range (Fig. 1, Table I). The remainder of the patients exhibited normal killing. When patients were divided into either localized or advanced disease groups, 13 (81%) of the 16 patients who

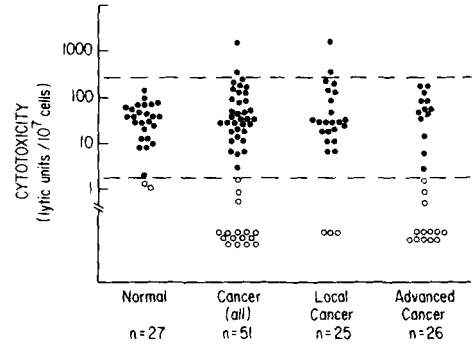

Figure 1. Cytotoxicity of PBL from normal subjects and cancer patients against K562 cells. The dashed lines delineate the normal range of cytotoxicity as defined by the (geometric) mean ± 2 SD. A L.U. is defined as the number of PBL per target cell needed to effect 30% cytotoxicity. Open circles represent individuals exhibiting killing below the normal range ("nonkillers", <2 L.U./10^7 PBL).

TABLE I

Natural cytotoxicity against K562: Effect of pretreatment of PBL with IFN_α or NDV

	Medium:[a]		IFN_α:[a]		NDV:[a]	
	Mean L.U.[b]	% killers[c]	Mean L.U.[b]	% killers[c]	Mean L.U.[b]	% killers[c]
Normals	22	93 (25/27)	52	100 (26/26)	83	100 (20/20)
Cancer (all)	15	69 (35/51)	53	84 (38/45)	66	89 (25/28)
Localized[d]	27	88 (22/25)	79	96 (23/24)	170	100 (13/13)
Advanced[d]	6	50 (13/26)	12	76 (16/21)	29	80 (12/15)

[a] PBL incubated overnight in medium, 100 units/ml of NDV-induced IFN_α, or β-propiolactone-inactivated NDV.

[b] Mean cytotoxicity expressed as L.U./10^7 PBL, calculated as the geometric mean for each group.

[c] Killers are defined as having ≥2 L.U./10^7 PBL (log L.U. ≥ 0.27). The results show the percent of killers in each patient group. The numbers in parentheses represent the number of killers/patients tested for each group.

[d] Localized disease includes patients with resectable tumors confined to the primary site with or without regional lymph node involvement. Advanced disease includes patients with distant metastases, extensive unresectable local tumors, or recurrent tumors.

failed to exhibit normal natural cytotoxicity were in the group with advanced disease. Mean cytotoxicity for 25 patients with localized cancers was 27 L.U. (1.43 ± 0.78 log L.U.), not significantly different from the mean for the normals. Mean cytotoxicity for 26 patients with advanced disease was 6 L.U. (0.81 ± 0.89 log L.U.), significantly lower than the means for both normals and patients with local disease, probability less than 0.01 and 0.05, respectively. Three of 25 patients with localized disease were nonkillers, whereas 13 of 26 patients with advanced cancers failed to kill (p less than 0.01). Two patients with localized cancers killed above the normal range.

When patients were grouped according to type of carcinoma, there was no difference in the results obtained; for all tumor types studied, the majority of nonkillers had advanced disease. Of the patients with metastatic tumors, those with visceral metastases exhibited a greater deficit in killing in that 7 of 10 of these patients were nonkillers, whereas only 1 of 7 patients with metastases to bone alone failed to kill. The frequency of killers and nonkillers did not change with increasing age. Of the 10 patients aged 70 and over, 3 were nonkillers; all of these had advanced disease. Similarly, there was no difference between male and female patients.

In an attempt to determine whether adherent suppressor cells might be responsible for the deficient NK activity in patients with advanced malignancy, cytotoxicity assays were done in a number of patients before and after nylon wool depletion of adherent cells. Mean cytotoxicity in a group of 6 patients with advanced cancers was 7 L.U. before depletion of adherent cells and 13 L.U. after nylon wool filtration. For a comparable group of normal donors tested on the same days, mean cytotoxicity was similarly increased approximately 2-fold (from 40 to 85 L.U.) by depletion of adherent cells. Those patients whose unfractionated PBL failed to kill within the normal range still failed to kill normally after depletion of adherent cells. These results suggest that adherent suppressor cells are not likely to be responsible for the deficient NK activity seen in these patients.

Pretreatment of PBL with IFN$_\alpha$. IFN are known to enhance the natural cytotoxicity of PBL from normal donors. In order to determine whether the NK activity of cancer patients' PBL could be augmented by IFN, PBL were incubated overnight with 100 U of virus (NDV)-induced IFN$_\alpha$ before cytotoxicity assay. NK activity of PBL from normal subjects was boosted by IFN$_\alpha$ in all experiments. Addition of a sheep anti-human IFN$_\alpha$ (26) to IFN$_\alpha$-treated PBL abrogated the NK boosting activity of our IFN$_\alpha$ preparation in several experiments, suggesting that the NK activation was indeed due to IFN$_\alpha$ and not to other lymphokines that might be present in our relatively crude IFN$_\alpha$ preparation. The mean cytotoxicity of PBL from 26 normal individuals after IFN$_\alpha$ treatment was 52 L.U. (1.72 ± 0.28 log L.U.) compared to 22 L.U. (1.35 ± 0.54 log L.U.) without IFN$_\alpha$ pretreatment, significantly different at p less than 0.005. When PBL from the cancer patients were pretreated with IFN$_\alpha$, cytotoxicity increased in most, but not all cases. The results are shown in Figure 2 and Table I. Some of the patients whose PBL failed to kill spontaneously demonstrated NK activity within the normal range (greater than or equal to 2 L.U./10^7 PBL) after IFN$_\alpha$ pretreatment of PBL, others showed enhanced cytotoxicity but still failed to kill normally, and some showed no measurable augmentation of cytotoxicity. Cytotoxicity curves for 3 cancer patients and 1 normal control tested on the same day are shown in Figure 3. Mean cytotoxicity of IFN$_\alpha$-treated PBL from patients with local cancer was 79 L.U. (1.90 ± 0.71 log L.U.), not significantly different from that of IFN$_\alpha$-treated

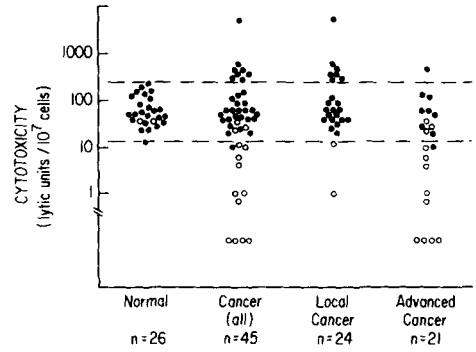

Figure 2. Cytotoxicity of PBL preincubated with IFN$_\alpha$. The dashed lines delineate the mean cytotoxicity for normal subjects after IFN$_\alpha$ pretreatment of PBL ± 2 SD. A L.U. is defined as the number of PBL per target cell needed to effect 30% cytotoxicity. Open circles represent cytotoxicity of individuals who exhibited NK activity below the normal range when PBL were cultured in medium alone (see Fig. 1).

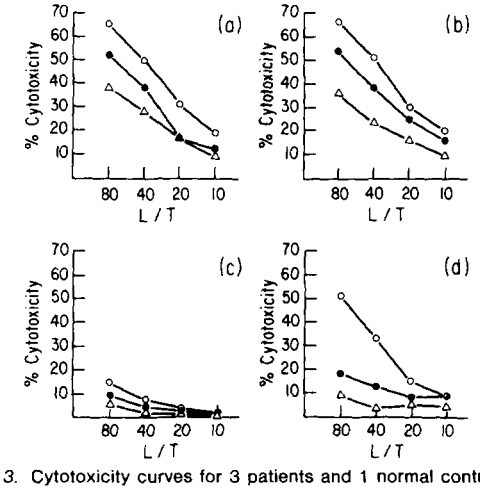

Figure 3. Cytotoxicity curves for 3 patients and 1 normal control tested on the same day. PBL incubated overnight in medium (△), or in medium containing 100 μ/ml IFN$_\alpha$ (●), or β-propiolactone-inactivated NDV (○). *a*) 28-yr-old normal male; *b*) 66-yr-old male with localized bladder cancer; *c*) 58-yr-old female with unresectable carcinoma of the esophagus; *d*) 67-yr-old female with advanced breast cancer.

PBL from normal individuals. Eight patients exhibited cytotoxicity above the normal range after IFN$_\alpha$ pretreatment of PBL (Fig. 2); all but one of these individuals had localized cancers. Mean cytotoxicity for IFN$_\alpha$-treated PBL from advanced cancer patients was 12 L.U. (1.08 ± 0.84 log L.U.), significantly lower than values for both normals and local cancer patients, p less than 0.005. Of 14 patients whose PBL failed to exhibit normal spontaneous cytotoxicity, 7 killed normally after IFN$_\alpha$ pretreatment of PBL; 6 of these had advanced disease. Six of the 7 patients who remained nonkillers after IFN$_\alpha$ treatment of PBL had metastatic disease. Thus, pretreatment of PBL with IFN$_\alpha$ corrected the defect in NK activity in some, but not all cancer patients. The vast majority of patients who failed to kill despite IFN$_\alpha$ stimulation were in the group with advanced disease.

Induction of IFN$_\alpha$ from PBL. IFN has been shown to be critically involved in the generation of mature, functional cytotoxic effector cells. A possible explanation for the failure of some cancer patients to exhibit natural cytotoxicity might be an inability of their PBL to produce IFN. We therefore incubated PBL from normal subjects and cancer patients overnight with β-propiolactone-inactivated NDV, a potent inducer of IFN$_\alpha$, before NK assay. When supernatants from these cultures were assayed for antiviral activity, there was no significant difference between IFN$_\alpha$ titers obtained from normal subjects and those

from cancer patients, regardless of stage of disease or ability to kill K562 target cells (Fig. 4). Most culture supernatants from normals and patients contained from 1000 to 10,000 U of IFN_α/ml. From these results we can conclude that there is no correlation between the NK activity of PBL and their ability to produce IFN_α *in vitro* on exposure to virus. We have also measured IFN_α production by PBL co-cultured overnight with K562 cells and have found no difference between normal controls and cancer patients regardless of their ability to kill. Mean IFN_α titers induced by K562 cells from PBL of 6 normal donors, 6 cancer patients who killed normally, and 4 cancer patients who failed to kill were not significantly different (log IFN titers of 2.18 ± 0.97, 2.57 ± 0.61, and 2.67 ± 0.58, respectively). The observed defect in NK activity is thus not likely to be due to a failure of PBL to produce IFN_α.

NK activity of PBL exposed to NDV. PBL from all normal subjects pretreated with NDV showed markedly enhanced NK activity. Mean cytotoxicity for NDV-treated normal PBL was 83 L.U. (1.92 ± 0.45 log L.U.). When PBL from the patients were cultured with NDV, cytotoxicity was measurably enhanced in *most, but not all cases* (Fig. 3). After treatment of PBL with NDV, all patients with localized disease and 80% of the patients with advanced disease exhibited normal natural cytotoxicity (Table I). The results of NK assays on 12 patients with advanced cancers whose PBL were treated with both IFN_α and NDV are shown in Table II. Of 6 patients whose PBL failed to kill spontaneously or after pretreatment with IFN_α (100 μ/ml), 3 were boosted into the normal range after exposure to NDV; the other 3 patients failed to kill despite treatment of PBL with NDV and despite the presence of 1000 to 3170 U of IFN_α in supernatants of the same PBL used for NK assay. All patients *whose PBL failed to kill after treatment with virus had advanced* disease. Of the patients with deficient NK activity, half could be corrected *in vitro* by treatment of PBL with IFN_α or an IFN_α inducer.

TABLE II

Natural cytotoxicity in patients with advanced cancer: Effect of pretreatment with IFN_α and virus (NDV)

Patient No.	Medium[a, b]	IFN[a, b]	NDV[a, b]
P71	<1	1	7
P73	38	50	63
P75	<1	<1	<1
P79	<1	<1	30
P86	1	36	83
P87	<1	<1	5
P88	47	59	79
P89	14	11	33
P92	<1	<1	<1
P93	<1	<1	<1
P94	63	170	477
V18	3	27	33
Killers/total tested[c]	5/12	6/12	9/12

[a] PBL incubated overnight in medium in 100 units/ml NDV-induced IFN_α, or with β-propiolactone-inactivated NDV.

[b] Numbers shown are L.U./10^7 PBL for each patient. L.U. below 1/10^7 PBL are designated as <1

[c] Killers are defined as individuals with ≥2 L.U./10^7 PBL (Log_{10} L.U. ≥ 0.27). The fractions shown represent the number of killers/total number of patients tested.

DISCUSSION

We have found a significant defect in natural cytotoxicity against a tumor-derived target cell line (K562) in a group of patients with solid epithelial cancers. When patients were classified according to clinical stage, the majority of patients whose PBL failed to kill normally had advanced disease (metastatic, recurrent, or extensive unresectable local tumors). Pretreatment of PBL with IFN_α or an IFN_α inducer (NDV) corrected the defect in half of the patients studied. Essentially all patients whose PBL failed to kill even after treatment with IFN_α or virus were in the group with advanced disease. PBL from patients with cancer, regardless of their clinical stage of disease or their ability to kill K562 cells, produced normal amounts of IFN_α after exposure to NDV and to K562 target cells. Thus, the failure of PBL from patients with advanced cancer to exhibit natural cytotoxicity appears unlikely to result from an inability to produce IFN_α.

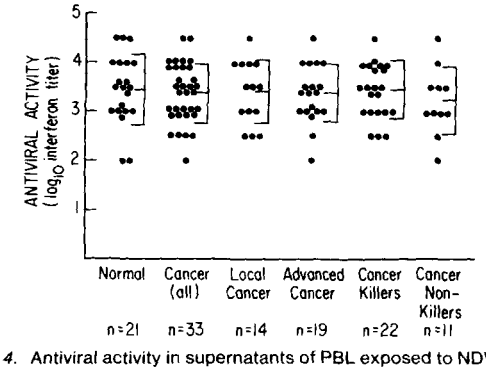

Figure 4. Antiviral activity in supernatants of PBL exposed to NDV. PBL were cultured overnight with β-propiolactone-inactivated NDV. For each group of individuals the bars represent the mean IFN_α titer ± 1 SD.

Numerous attempts have been made to identify defects in immune mechanisms in tumor-bearing humans and experimental animals that could predispose them to the development of cancer. Our finding of defective natural cytotoxicity in patients with cancer, in particular in patients with advanced malignancies, confirms the findings of other investigators and suggests a role for NK activity in the control of tumor growth and spread, but does not answer the critical question of whether deficient NK activity predisposes to the development of malignancy, or is a consequence of the cancer. Preliminary observations on a small group of cancer patients followed over a period of several months suggest that defective NK activity may correlate with a large tumor burden and that the defect may be at least partly reversible with tumor regression. We do not know as yet whether those patients with localized tumors who exhibited deficient NK activity are at increased risk of developing metastatic disease.

The finding that some of our patients with disseminated malignancies exhibited normal cytotoxicity raises questions about the role of NK cells in regulating tumor growth. The ability of an individual to kill allogeneic target cells *in vitro* may not, in fact, correlate with effector-target interactions *in vivo*. Vanky and co-workers (27) have recently reported that many cancer patients were able to kill allogeneic tumor target cells *in vitro*, but were unable to kill autologous tumor cells. These investigators also observed that most freshly explanted tumor cells were resistant to natural cytotoxicity immediately after explantation, but became sensitive after several days in culture, suggesting that some tumors may be rendered NK cell-resistant *in vivo* by factors yet to be defined. It has also been demonstrated that NK activity in the peripheral blood of cancer patients may be normal, whereas lymphocytes isolated from resected tumor specimens from the same patients may not exhibit natural cytotoxicity (28). It is conceivable that in some patients immunoselection for NK-resistant tumor cells may occur, leading to active tumor growth despite normal NK activity as measured *in vitro* against allogeneic target cells. Some tumor lines are resistant to NK cell-mediated cytotoxicity, despite the ability of some of these lines to induce IFN and to enhance cytotoxicity against sensitive targets (12).

IFN$_\alpha$ is induced from lymphocytes by viruses and by tumor cells *in vitro* (4) and *in vivo* (29) and is a potent stimulator of natural cytotoxicity. Minato and co-workers (13) reported that, in the nude mouse, IFN acts by causing the differentiation of a precursor NK cell into a mature cytotoxic effector cell with distinct surface markers. In the human, IFN appears to act by inducing the differentiation of precursor cells into mature, functional cytotoxic effectors and also by activating preexisting mature NK cells (30). Functionally mature human NK cells can be distinguished from the precursor cells recruited by IFN by virtue of differential adhesion to tumor cell targets and differences in surface markers such as FcR (5, 31).

Our observation that NK activity in some, but not all, cancer patients with defective spontaneous cytotoxicity could be boosted into the normal range by pretreatment of PBL with IFN$_\alpha$ or an IFN$_\alpha$ inducer suggests that some patients may have decreased numbers of mature functional NK cells in the peripheral blood, but may have normal numbers of pre-NK cells that can be activated by exposure to IFN. Still other patients may lack both mature and precursor cytotoxic effector cells. Alternatively, decreased NK activity measured in a chromium-release assay such as we have employed may reflect decreased killing efficiency or inability of effector cells to recycle after the first round of target cell lysis (32), rather than changes in numbers of NK or preNK cells. Preliminary results of effector-target cell-binding assays in a small number of patients suggested that target binding was not measurably diminished in those patients who failed to kill. Similarly we have found no consistent difference in the number of large granular lymphocytes identified in smears of fractionated and unfractionated PBL from individuals who killed normally and those who failed to kill.

The ability of PBL from some patients to be boosted with low doses of IFN$_\alpha$ (100 U/ml) and of others to be boosted only with the higher levels of IFN$_\alpha$ obtained in virus-treated cultures (1,000 to 10,000/ml), suggests that there may be quantitative as well as qualitative differences in the numbers of activatable NK cells in different individuals or that patients may differ in their sensitivity to the immunoregulatory effects of IFN on NK cells. It is possible that this type of study might provide a useful screening test in the selection of patients for the therapeutic administration of IFN and in the determination of optimal dose levels of IFN for different individuals. Alternatively, the lack of response to IFN$_\alpha$ might reflect a delayed response to IFN$_\alpha$ rather than an absolute lack of response. In several experiments (data not shown) we cultured PBL in the presence of IFN$_\alpha$ for 48 hr in addition to the standard overnight incubation and found no difference in the results obtained, making this possibility unlikely.

It has been shown that the cytotoxicity effected in the first few hours of lymphocyte-target cell co-culture is IFN independent and that only later does the IFN induced from lymphocytes by the tumor cells stimulate additional NK activity (3, 13). Our cytotoxicity assay was a short-term assay and was thus most likely measuring IFN-independent killing. PBL from cancer patients produced normal levels of IFN$_\alpha$ on exposure to K562 cells and virus, regardless of their ability to kill K562 cells. It has been suggested that the same lymphocyte subset is responsible for IFN$_\alpha$ production induced by tumor cells and for natural cytotoxicity (4). Our results indicate that different lymphocyte subsets, or different functional states of the same lymphocyte population, may be responsible for the 2 activities. IFN$_\alpha$ induced by virus may be produced by lymphocyte subsets other than NK cells as well as by macrophages and may thus be largely independent of the contribution of NK cells. The failure of some patients to exhibit measurable cytotoxicity despite normal IFN$_\alpha$ titers induced by virus in the same cultures suggests that the defect was not due to an inability of PBL to produce IFN$_\alpha$.

It is conceivable that NK activity might be regulated by subsets of suppressor lymphocytes or macrophages. Our results in a small number of patients suggest that adherent cells do not appear to be responsible for the defective cytotoxicity observed in patients with advanced malignancy. We have employed Percoll gradients (5, 7) in 2 cancer patients and several normals to fractionate adherent cell-depleted PBL into populations enriched for NK cells (data not shown). One of the patients exhibited no demonstrable killing when either unfractionated or adherent cell-depleted PBL were tested. Although we were able to enrich for NK activity in the expected Percoll fractions in this patient, the level of cytotoxicity obtained was still markedly lower than that obtained in comparable Percoll fractions of individuals exhibiting normal NK activity. These experiments do not lend support to the hypothesis that functional NK cells in the peripheral blood of these patients are suppressed by populations of regulatory cells that can be separated from NK cells by these techniques.

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NON-MHC-LINKED GENETIC CONTROL OF MURINE CYTOTOXIC T LYMPHOCYTE RESPONSES TO HAPTEN-MODIFIED SYNGENEIC CELLS

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Spleen cells from unimmunized inbred mice were sensitized *in vitro* to autologous spleen cells modified with different concentrations of either trinitrobenzene sulfonate (TNBS) (TNP-self) or fluorescein isothiocyanate (FITC) (FTC-self). The resulting hapten-specific cytotoxic T lymphocyte (CTL) responses were assayed on hapten-self Con A blast target cells. A number of mouse strains that expressed the *H-2*^{d}, *H-2*^{k}, or *H-2*^{b} haplotypes were studied to determine whether non-H-2-linked genes could affect CTL response potential. Among the 3 *H-2*^{d} strains tested, BALB/c was a high responder, whereas B10.D2 and DBA/2 were low responders for CTL generated against TNP-self and FTC-self. Studies using (BALB/c × B10.D2)F$_1$ responding cells and F$_1$ or parental-modified stimulating cells indicated that the F$_1$ cells generated cytotoxic activity equivalent to that of the high responder strain BALB/c. Among 3 *H-2*^{k} strains studied, B10.BR mice were relatively low responders to TNP-self at all concentrations of TNBS stimulation. Among 4 *H-2*^{b} strains compared, C57BL/6J, C3H.SW, and A.BY were all observed to be relatively high responders, whereas C57BL/10J was the low responder strain for CTL responses to TNP-self. Further comparisons of the C57BL/6J and C57BL/10J strains indicated that these non-H-2-linked differences could be detected for TNP-self CTL responses at 4, 5, or 6 days of culture and that these differences appeared to be attributable to 1 or more functional cell types in the responding cell pool and were not found to be due to differences either in the TNP-modified stimulating or target cells. These findings indicate that non-H-2-linked genetic factors can contribute appreciably to modified-self cytotoxic T lymphocyte responses and provide a system for investigating whether the Ir phenotype of T cells can be influenced by non-MHC genes in the thymic environment.

Although considerable interest in immune response (Ir) gene control of immunity has been concerned with major histocompatibility complex (MHC)[2]-linked genes, a number of Ir genes have been described that are not linked to the MHC. These have been classified according to their linkage to heavy chain allotype (1), the X chromosome (2, 3), or to other, unknown,

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[2] Abbreviations used in this paper: MHC, major histocompatibility complex; CTL, cytotoxic T lymphocytes; TNBS, trinitrobenzene sulfonate; FITC, fluorescein isothiocyanate; ACK, ammonium chloride lysing buffer; TNP-self, TNBS-modified syngeneic spleen cells; FTC-self, FITC-modified syngeneic spleen cells; Ir, immune response.