Effect of Various Interferons on the Spontaneous Cytotoxicity Exerted by Lymphocytes from Normal and Tumor-bearing Patients

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ABSTRACT

Natural killer cell activity, which represents the spontaneous cytotoxicity of lymphocytes toward tumor cells, has been measured in 173 tumor-bearing patients and 25 healthy volunteers; no significant difference was found in mean natural killer cell activity between the two groups. The parameters of interferon-induced activation of natural killer cells were studied in order to provide a suitable test for monitoring the effect of interferon in clinical trials.

The three interferons tested (leukocyte, lymphoblastoid, and fibroblast) were equally active in inducing spontaneous cytotoxicity of lymphocytes from all healthy individuals and tumor-bearing patients studied. Incubation for one hr with 100 units of interferon was sufficient to increase spontaneous cytotoxicity activity, the maximum effect being obtained when lymphocytes were incubated with 1000 units of any of the interferons used. This effect was blocked with the appropriate antiinterferon sera. The target cells for interferon seem to be positive Fcγ receptor lymphocytes.

INTRODUCTION

The role of the immune system in host defense against neoplastic growth is still open to question. For years, it has essentially been viewed in terms of T-cells reacting with specific tumor transplantation or with tumor-associated antigens (3). However, with time, it appeared that, while this mechanism was certainly effective in virus-induced tumors in which T-cells recognize viral-coded antigens in association with histocompatibility products (8), there was little evidence that they directly limit the growth of chemically induced or spontaneous tumors. It has been suggested that other effector mechanisms also play a role in antitumor surveillance against these 2 types of tumors, especially NK cell cytotoxicity (17, 22).

NK cells have been found both in human (11) and in experimental (18, 20) systems. These cells, apparently devoid of antigen specificity, kill tumor cells (11, 18, 22), transformed cells (21, 28), and under certain conditions normal cells (21). NK cells seem to be good candidates for effector cells in a primitive defense against spontaneous tumors. Large numbers of NK cells are found in athymic nude (nu/nu) mice, and their presence may explain the low incidence of spontaneous tumors in these animals (25). A correlation has been observed between the in vivo resistance of mice to the transplantable YAC lymphoma and the in vitro level of NK cell cytotoxicity (9, 23). A similar correlation was found between the NK cell level and the rejection of small tumor inocula (24).

IF was shown recently to enhance NK cell activity in vivo and in vitro (7, 28), and it has been suggested that such enhancement may partly account for its antitumor effects (26). Since several therapeutic assays using IF for treatment of human tumor-bearing patients are already in progress (1, 14, 16, 19, 27), it appeared of interest to study carefully the parameters for activation of human NK cells by IF, as well as the NK cell activity of lymphocytes from tumor-bearing patients.

The results reported here show that natural cytotoxicity of human lymphocytes from normal and tumor-bearing patients is enhanced in vitro by the same doses and kinetics by fibroblast, leukocyte, and lymphoblastoid IF's. This effect is blocked by the relevant anti-IF sera and is directed towards Fcγ receptor-positive lymphocytes. When lymphocytes from 173 patients bearing various types of tumors were tested for the level of NK cell activity, no significant difference was found compared to lymphocytes from 25 healthy donors.

MATERIALS AND METHODS

Healthy Donors

The 25 healthy donors were laboratory workers at the Institut Curie, Paris, France. They included 14 women and 11 men, 22 to 60 years old.

Tumor-bearing Patients

The 173 patients studied before or several months after treatment comprised 98 women and 75 men, ages 20 to 70 years. Tumor type distribution among patients was as follows: 43 lymphomas; 42 malignant melanomas; 25 lung tumors; 24 breast adenocarcinomas; 17 squamous cell carcinomas of the upper respiratory and digestive tract; 5 cervical carcinomas; 3 ovarian carcinomas; 1 fallopian tube epithelioma; 1 vulvar epidermoid carcinoma; 2 pancreatic adenocarcinomas; 2 rectal carcinomas; 1 colon carcinoma; 1 malignant glioma; 1 testicular dyssembryoma; 1 leiomyosarcoma; 1 neurinoma; 1 Ewing’s sarcoma; 1 kidney carcinoma; 1 osteogenic sarcoma.

Lymphocyte Preparation and Identification of Lymphocyte Subpopulations

Peripheral blood was collected into a syringe containing heparin (Calciparin; Laboratories Choay, Paris, France), and lymphocytes were prepared on a Ficoll gradient (Ficoll Radio...
Selectan; Sigma Chemical Co., St. Louis, Mo., and Schering, West Germany) according to the technique of Boyum (2). The lymphocytes were resuspended in RPMI 1640 (Eurobio, Paris, France) supplemented with 20% FCS (Grand Island Biological Co., Glasgow, Scotland) at a final concentration of $10^7$ cells/ml.

Spontaneous erythrocyte-forming rosettes detecting T-cells were formed with lymphocytes and sheep RBC (Sorga, Paris, France), using a previously described technique (22).

EA$_2$ rosettes detecting FC$_7$ receptor-positive cells were performed according to the method of Peter et al. (22).

In some experiments, lymphocyte suspensions were either depleted or enriched in RFC or EAG-RFC by centrifugation in Ficoll gradient after rosette formation (22). The cells recovered at the interface were used when completely free of RFC.

**Interferons**

Human fibroblast IF, obtained from Hem Research, Inc. (Rockville, Md.), was prepared on HFS No. 30 normal foreskin fibroblasts. Each lyophilized vial contained $1.89 \times 10^5$ units of purified IF (2.37 $\times 10^6$ units/mg of protein). After solubilization in distilled water, IF samples at $10^5$ units/ml were stored at $-70^\circ$.

Human leukocyte IF, supplied by Dr. K. Cantell (Helsinki, Finland) (4), was obtained after treatment of human leukocytes with Sendai virus. Each vial contained $6 \times 10^6$ units/ml (4 $\times 10^6$ units/mg of protein). IF samples were processed as described above.

Human lymphoblastoid IF, provided by Dr. N. Finter (Wellcome Research Laboratory, London, England), was prepared by infecting Namalwa lymphoid cells with Sendai virus. Each lyophilized bottle contained $4.67 \times 10^6$ units (1.57 $\times 10^6$ units/mg of protein). IF samples were processed as described above.

In some experiments, lymphocytes from healthy donors and cancer-bearing patients were incubated with different doses of human fibroblast and leukocyte IF's ($10^1$, $10^2$, $10^3$, or $10^4$ units) prior to the spontaneous cytotoxic activity assay for different incubation periods (1, 4, or 24 hr). Cells ($10^7$) were incubated in 0.9 ml of RPMI 1640 supplemented with 20% FCS and 100 µl of IF solution for the required time at $37^\circ$. At the end of the incubation, 4 ml of medium were added, and cells were centrifuged and washed twice.

**Anti-human IF Sera.** The following sera were obtained from the National Institute of Allergy and Infectious Diseases (Bethesda, Md.). For antiserum to human leukocyte IF, 1 ml of serum contained $10^4$ neutralizing units against 10 units of IF; i.e., a $10^{-4}$ dilution of this serum neutralizes 10 units of human leukocyte IF. Antiserum to human fibroblast IF was prepared against fibroblast IF. It contained $10^2$ neutralizing units against 10 units of IF.

In some experiments, IF was preincubated with anti-IF serum before the NK cell activity test. For this purpose, sufficient antiserum to neutralize 100 units of IF as determined by subsequent titration (final antiserum dilution: 1/1000 for anti-leukocyte IF and 1/100 for anti-fibroblast IF) was added to the IF preparation which was then incubated for 1 hr at $37^\circ$. Of this solution, 100 µl were then added to 900 µl of cell suspension containing $10^7$ lymphocytes. The cells were incubated for 1 hr at $37^\circ$, and the cytotoxicity assay was performed. Control tubes were prepared by incubating lymphocytes with IF and antiserum separately. In other experiments designed to study the kinetics of IF-mediated enhancing SC, lymphocytes were incubated with $10^3$ units of leukocyte or fibroblast IF, and the appropriate antiserum were added at different times thereafter.

Before use or after neutralization by antiserum, IF titers were determined by the cytopathogenic inhibition test (10) on GM 2504 human fibroblasts, provided by Dr. A. Greene (Institute of Medical Research, Camden, N. Y.). The vesicular stomatitis virus was used as challenge. The IF titer was expressed in international units with human leukocyte IF as reference (NIH, Bethesda, Md.).

**Lymphocyte Cytotoxicity Assay.** Cells of the K 562 line (kindly provided by Dr. M. Fellous, Hôpital Saint-Louis, Paris, France) were used as targets. They were grown in the laboratory in RPMI 1640 supplemented with 10% FCS and Gentalline (40 mg/liter; Unilabo, France).

The cytotoxicity assay was performed according to the method of Cerottini and Brunner (5). $^{51}$Cr (C.E.A., Saclay, France)-labeled target (K 562) cells ($10^4$) in 100 µl of RPMI 1640 supplemented with 10% FCS were mixed with 100 µl of increasing numbers of lymphocytes at effector/target cell ratios varying from 1/1 to 100/1. The cell mixtures were incubated for 4 or 16 hr at $37^\circ$. At the end of incubation, 100 µl of the supernatant were transferred into vials and counted in a gamma counter (C.G. 4000; Intertechnique, France).

The percentage of specific $^{51}$Cr release was determined according to the formula

$$\frac{E - S}{T - S}$$

where $E$ is the amount of $^{51}$Cr released from the target cells in the presence of lymphocytes, $S$ is the spontaneous release of $^{51}$Cr measured in control cultures (target cells mixed with culture medium), and $T$ is the maximum release when control cultures are treated with 2 n HCl. Spontaneous release did not exceed 30%. All the cultures were set in triplicate. Variability for the triplicate determination did not exceed 10%.

**RESULTS**

**Kinetics of IF-activated SC.** In a first set of experiments designed to establish the kinetics of NK cell activity enhancement by IF's, lymphocytes from healthy donors were incubated for various periods with IF and tested at different effector/target cell ratios for their SC activity on K 562 target cells.

Chart 1 shows the results obtained when $10^7$ lymphocytes were incubated with 100 units of leukocyte or fibroblast IF's for 1, 4, and 24 hr. Their SC on K 562 was measured in 4- or 16-hr assays (Chart 1) at an effector/target cell ratio of 100/1.

Both IF's enhanced NK cell activity in the same proportions. The level of enhancement was similar, whether cells were incubated with IF for 1, 4, or 24 hr. It is noteworthy that incubation of lymphocytes for 4 hr in the absence of IF enhanced their SC. Incubation with IF produced the same effect, but the level of SC was higher than in lymphocytes incubated for 4 hr in IF-free medium. Finally, the effect of IF was clearly detectable in both the 4-hr and 16-hr cytotoxic assays.

**Comparative Effects of Different Doses of Leukocyte,
Lymphoblastoid, and Fibroblast IF. To compare the effects of various IF's on SC, lymphocytes from a healthy donor were incubated with graded doses of leukocyte, lymphoblastoid, and fibroblast IF and tested for their SC on K 562 cells.

Chart 2 shows the results obtained with an effector/target cell ratio of 100/1, for which no quantitative differences, were observed between the 3 IF's tested. For all 3 IF's, 100 units of IF were sufficient to induce a significant increase in NK cell activity.

We tested various effector/target cell ratios from 1/1 to 100/1, and the enhancing effect of IF's on NK cell activity was highly significant for ratios of 6/1 and more (data not shown).

Effect of Anti-IF Sera. To confirm that IF was indeed the active molecule in enhancing SC, 100 units of fibroblast IF, or anti-leukocyte IF, were incubated with the relevant anti-fibroblast or anti-leukocyte IF sera, at a dilution at which all viral activity was neutralized (data not shown), and then tested for their effect on NK cell activity. Table 1 (Experiment 1) shows that neutralization of IF's by the relevant antisera completely abolished its enhancing activity. In no case did the incubation of effector cells with anti-IF sera in the absence of IF lead to a decrease in cytotoxicity (Table 1).

In another set of experiments, anti-IF sera were used to analyze the kinetics of IF mediation in enhancing NK activity. In the experiments shown in Chart 4, lymphocytes were incubated with 1000 units of leukocyte or fibroblast IF, and the relevant antisera were added at different times. SC was tested 1 hr after incubation. When the antiserum and IF were added simultaneously, addition of the antiserum completely abolished the enhancing activity of IF in both cases, whereas addition of anti-IF serum 30 min after IF had almost no effect. This showed that a very brief contact between IF and lymphocytes was sufficient to induce the events leading to the expression of cytoxicity. In no case did the incubation of effector with anti-IF sera in the absence of IF lead to a decrease in cytotoxicity (data not shown).

Characterization of the Lymphocyte Subpopulation Reacting with IF. To determine whether T-cells were necessary for IF-induced enhancement of NK cell activity, lymphocytes were deprived of T-cells by depletion of erythrocyte RFC by centrifugation in a Ficoll gradient and then incubated with 1000 units of leukocyte IF.

Chart 3 shows that incubation of IF for 1 hr with a lymphocyte population deprived of T-cells leads to enhanced SC activity identical to that obtained by incubating IF with an undepleted lymphocyte preparation. By contrast, depletion of Fcγ receptor-positive cells by Ficoll sedimentation of EAg-RFC abolished SC (Chart 3). Treatment of the Fcγ receptor-negative cells with IF did not induce NK activity on K 562 cells (Chart 3).

Since in humans NK cells have been shown to be contained in the Fcγ receptor-positive population (22), these experiments suggest that IF may act directly on the effector NK cells. In another set of experiments, where IF was incubated either with Fcγ-RFC-depleted cells or with unseparated lymphocytes, no synergistic effect could be observed (data not shown), suggesting that no cell-to-cell cooperation is needed for the expression of IF activity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (units/ml)</th>
<th>Specific % of specific 5’Cr release at different anti-IF added</th>
<th>Specific 5’Cr release at different effector/target ratios</th>
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<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
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<tr>
<td>No IF</td>
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<tr>
<td>Leukocyte</td>
<td>10^2</td>
<td>5.6, 10.8, 16.7</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Leukocyte</td>
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<td>6.3, 11.6, 15.6</td>
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</tr>
<tr>
<td>No IF</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Fibroblast</td>
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<td>5.4, 11.8, 15.9</td>
<td></td>
</tr>
<tr>
<td>Fibroblast</td>
<td>10^2</td>
<td>8.4, 19.4, 34.2</td>
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<td>4.1, 10.6, 13.8</td>
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<tr>
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<tr>
<td>Leukocyte</td>
<td>10^2</td>
<td>4.7, 13.8, 21.3</td>
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<tr>
<td>Leukocyte</td>
<td>10^2</td>
<td>4.5, 12.4, 14.9</td>
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<tr>
<td>Leukocyte</td>
<td>10^2</td>
<td>8.6, 22.3, 27.7</td>
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<tr>
<td>Leukocyte</td>
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</tr>
<tr>
<td>Leukocyte</td>
<td>10^2</td>
<td>13.9, 26.2, 41</td>
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<td>Fibroblast</td>
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<td>7.6, 15.3, 23</td>
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<td>16.2, 31.2, 41</td>
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<tr>
<td>Fibroblast</td>
<td>10^2</td>
<td>14, 33.6, 40.6</td>
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</table>

* Specific antisera were incubated with or without the indicated IF for 1 hr before addition to the lymphocyte suspension.

** Specific antisera were added directly to the lymphocyte suspension at the indicated time.
Comparison of NK Cell Activity in Cancer-bearing Patients and Healthy Donors. We measured SC toward K 562 target cells of lymphocytes from 173 patients and 25 healthy controls.

As shown in Table 2, the overall mean of the spontaneous cytotoxicity exerted by lymphocytes from tumor-bearing patients and healthy donors was similar (33.6 ± 23.3 (S.D.) compared to 32.5 ± 14.5).

Taking into account the considerable scatter of the values obtained for the percentage of specific 51Cr release and in order to enable comparison between cancer patients and healthy donors, our cases were arbitrarily divided into 3 groups: low responders (less than 20%); medium responders (20 to 40%); and high responders (more than 40%) at an effector/target cell ratio of 50/1. Table 1 also shows that, in each group, mean SC values and the respective numbers of healthy donors and cancer patients were similar. Moreover, when SC was compared in lymphocytes from tumor subgroups, no significant difference was found.

Reactivity to IF of Lymphocytes from Tumor-bearing Patient. We have tested the in vitro enhancement of NK cell activity of lymphocytes from 74 tumor-bearing patients. As a general rule, incubation of their lymphocytes with IF led to an enhancement of NK cell activity comparable to the enhancement obtained for IF incubation with lymphocytes from healthy donors.

These data can be schematically summarized as following 3 patterns of reactivity: in patients exerting low spontaneous NK cell activity (Chart 4a), in vitro treatment with IF brought the response to normal levels; in those presenting normal NK cell activity, IF enhanced the response with dose and kinetics similar to those observed when testing healthy individuals (Chart 4b); finally, in those patients presenting spontaneous high NK cell activity, such as patients receiving Bacillus Calmette-Guérin immunotherapy, IF could not enhance the cytotoxicity to K 562 above the already maximum reactivity (Chart 4c).

**DISCUSSION**

The data reported here analyze the parameters of IF-induced enhancement of NK cell activity in humans. We showed that there was no detectable difference in the effects of NK cell activity of 3 different types of IF (leukocyte, fibroblast, and lymphoblastoid). We further established that IF is the active moiety in enhancing SC by neutralizing its effect with the relevant antisera.

Consequently, from the viewpoint of therapeutic application, all these IF's act equally well on the immunological compartment considered. The kinetics of the effects of IF under our experimental conditions shows that a short period of incubation (30 min) was sufficient to induce the events leading to enhancement of NK cell activity. These results are in agreement with data from the mouse showing that a few min incubation of effector lymphocytes with IF enhanced NK cell activity (26).

To analyze the kinetics of IF effects, we also neutralized free IF by adding the relevant antisera at different times. In contrast to a previous report (13), we did not find that antisera when added to effector cells in the absence of IF reduced their SC under our experimental conditions. Production of free IF by cells being tested is therefore unlikely to play an important part of promoting natural cytotoxicity. The role of immune IF produced by the cells under test cannot be ruled out by our experiments since immune IF would not react with the antisera used. Finally, the enhancement of NK cell activity was as good in a 4-hr 51Cr release assay as in a 16-hr assay, indicating that maturation of NK cells after their induction by IF is a short-period event which probably does not involve cell division.

In an attempt to define the cells which react with IF in the system considered, we showed that IF does not induce SC activity in Fcγ receptor-negative T-cells (EAγ-RFC-depleted population), or does it render T-cells able to induce NK cells. By contrast, IF acts directly on human Fcγ receptor-positive cells containing mature NK cells or their immediate precursors, as suggested previously (22, 29). Finally, no synergistic effect could be obtained when an IF-treated cell population was added to untreated cells, which argues against possible cell-to-cell cooperation in mediating the effects of IF.

Our observations confirm and extend previous findings (13, 28) which showed that IF may enhance NK cell activity in

**Table 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>% of specific 51Cr release</th>
<th>Overall mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy donors</td>
<td></td>
<td></td>
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<tr>
<td>&lt;20%</td>
<td>16.5 ± 2.2 (9)</td>
<td>32.5 ± 14.5</td>
</tr>
<tr>
<td>20-40%</td>
<td>28.6 ± 6.3 (9)</td>
<td>33.6 ± 13.3</td>
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<tr>
<td>40%</td>
<td>55.3 ± 5.6 (8)</td>
<td>34.3 ± 13.3</td>
</tr>
<tr>
<td>&gt;40%</td>
<td>61.1 ± 10.8 (60)</td>
<td>31.7 ± 13.8</td>
</tr>
<tr>
<td>All cancer patients including</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy donors</td>
<td></td>
<td>32.5 ± 14.5</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>12.5 ± 4.7 (52)</td>
<td>33.6 ± 13.3</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>27.2 ± 5.6 (61)</td>
<td>35.6 ± 11.7</td>
</tr>
<tr>
<td>Breast adenocarcinoma</td>
<td>60.5 ± 6.2 (15)</td>
<td>34.3 ± 13.3</td>
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<td>Breast adenocarcinoma</td>
<td>67.3 ± 6.5 (12)</td>
<td>31.7 ± 13.8</td>
</tr>
<tr>
<td>Malignant melanoma</td>
<td>25.1 ± 4.1 (7)</td>
<td>31.7 ± 13.8</td>
</tr>
<tr>
<td>Malignant melanoma</td>
<td>54.5 ± 5.3 (10)</td>
<td>35.6 ± 11.7</td>
</tr>
<tr>
<td>Lung tumors</td>
<td>45.6 ± 5.6 (9)</td>
<td>33.6 ± 12.8</td>
</tr>
<tr>
<td>Lung tumors</td>
<td>24.9 ± 3.3 (6)</td>
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</tr>
<tr>
<td>Malignant melanoma</td>
<td>57.2 ± 5.7 (10)</td>
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</tbody>
</table>

* Mean ± S.D. at lymphocyte/target cell ratio of 50/1. Cytotoxicity assay 16 hr. p not significant when SC in lymphocytes from each tumor group is compared with that of healthy donors.

* Numbers in parentheses, number of individuals tested.

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human lymphocytes. In the mouse, in vivo injection of IF was shown to enhance NK cell activity (7). In humans, for monitoring of patients treated with IF in clinical trials, it seems necessary to establish the kinetics of the in vivo effects of IF on SC. Such a trial is currently in progress, and preliminary results indicate that IF does indeed enhance NK cell activity in vivo (6, 15).

For the purposes of clinical trials, it appeared to be a necessary prerequisite to measure SC in tumor-bearing patients and to compare it to a control population.

We did not find any significant difference in NK cell activity between patients and controls, which argues against the possibility of a relationship between low NK cell activity and host defense against cancer. NK cells from nearly all tumor-bearing patients, except those with already high NK cell activity at the time of our assays, can be stimulated by IF, as can be NK cells from healthy individuals. It is therefore not likely that this assay could be used to choose between IF-responsive- and IF-non-responsive patients but rather, in the course of a clinical trial, to assess the presence of a biological effect of IF. Regardless of its relevance to tumor evolution, enhanced NK cell activity demonstrates an active interaction of IF and the immune system allowing monitoring of patients. However, the important issue may be the sensitivity of various tumor cells to destruction by the autologous NK cells. In this respect, the results of preliminary experiments in our laboratory suggest that the sensitivity to NK cells of various tumor cells, especially lymphoma cells, differed from one individual to another. Since all tumor-bearing patient lymphocytes were responsive to IF, it may well be that screening of sensitive tumors serve as a guide in applying IF treatment.

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