Peripheral Blood Lymphocytes From Patients With Cancer Lack Interleukin-2 Receptors

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When tumor cells develop in healthy adults, they activate the cellular immune system—natural killer (NK) cells, antigen-specific cytotoxic lymphocytes (CTL), and the synthesis of antigen specific cytotoxic antibodies. These are aimed at killing the intruding cells. However, in cancer patients the tumor continues to grow. As tumor cells proliferate, they were shown to release factors that mediate the inactivation of the host immune defense systems. The study documented in this article examined peripheral blood lymphocytes, mononuclear cells (MNC), NK cells, T-helper cells (THC). This study confirmed the interaction of the released inhibitor factors with these mononuclear cells. NULL* cells from healthy adults responding to interleukin-2 (IL-2) and NILL cells from patients with metastatic breast carcinoma nonresponsive to IL-2 were also isolated by the standard antibodies-pinning technique. The cells were obtained from age-matched subjects: ten healthy adults; ten patients each from Stage I, II, III, and IV metastatic breast carcinoma (BCa-I, BCa-II, BCa-III, and BCa-IV or MBCa); and ten patients with benign breast disease (BBD). The responsiveness of these THC, PBMNC, NK, NULL, and NILL cells *in vitro* to graded levels of phytohemagglutinin (PHA), Concanavalin A (Con A), and recombinant interleukin-2 (rIL-2) was examined. Responsiveness was monitored by ^{3}H-thymidine (^{3}H-TdR) uptake, production and release of IL-2, interleukin-2 receptor (IL-2R), and cytotoxic activities against K-562 cells and breast carcinoma short-term cell lines. A lack of functional IL-2R in peripheral blood lymphocytes from patients with metastatic breast carcinoma was confirmed by nonsignificant anti-Tac antibody binding. An elevation in the expression of cell surface antigen GP-120 has been observed to be associated with the activation *in vitro* of T-cells from healthy adults and from patients with benign breast disease, but not of T-cells from patients with breast carcinoma. Biochemical studies of the GP-120 using high performance liquid chromatography combined with nitrocellulose blotting confirmed that the glycoprotein was resistant to trypsin and chymotrypsin, but susceptible to pronase. It contained sialic acid and lactosaminoglycan as O-linked sugars. It could be labeled with pariodate/NaB($^3H_4$) and is recognized by MAbT-305 monoclonal antibodies. It contained sialic acid linked (2 - - - 3) to galactose. The ability of MAbT-305 to inhibit the proliferative response to mitogens and IL-2 in lymphocytes from healthy adults, but without any effect on PBL from patients with breast carcinoma at various pathologic stages confirms that the expression of the IL-2R function is defective.

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DURING TUMOR GROWTH, two events occur. Tumor cells acquire resistance to the host immune killing mechanisms, and as malignancy become disseminated, there is a decrease of immunity to tumor-associated antigens (TAA). Neither the cause nor the magnitude of variation of these events in patients with breast carcinoma of various pathologic stages are known. Tumor-mediated immunosuppression is a challenge in cancer treatment.[1] In cancer patients, despite the development of specific and nonspecific antitumor immunity, tumors continue to grow. It has been postulated that this "escape" from host immunosurveillance is associated with suppressive mechanisms that occur in cancer patients. Suppression could be mediated either by humoral factors released by the tumor and/or suppressor cells. There are two main types of suppressor cells. One is

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* NULL and NILL cell populations were obtained after panning of all known cell types. The identity of this cell population is heterogeneous and uncommitted. Because the type of the cell population from healthy adults could vary from that obtained from patients with BCa, in this study they are referred as NULL and NILL, respectively. To establish the exact identity of these cells, monoclonal antibodies to both NULL and NILL cell populations is in progress.

T-lymphocytes that interfere with specific antitumor immunity, and the other is the "nonspecific suppressor" macrophages that inhibit various immune responses.

In previous studies on the mechanisms of cellular immunoregulation in human cancer patients, we identified immunoregulatory factors that abrogate the host immune mechanisms. Specifically, there was a correlation between perchloric acid soluble serum sialoglycoproteins, immunity, and tumor cell burden.[2,3] The present study was initiated by the need to explain the observations on sensitivity differences of breast carcinoma cells to the killing effect of interleukin-2 (IL-2) (Cetus Corp., Emerville, CA)-activated killer cells from healthy adults and from patients with breast carcinoma.

Natural killer (NK) cells are the major host defense against tumor growth.[4] Unlike cytotoxic T-cells, little is known about the mechanism(s) by which NK cells recognize tumor cells.[4-6] The NK cells do not require sensitization, but can be activated.[7] In this context, plasma "cold insoluble globulin" protected cytotoxic peripheral blood lymphocytes (PBL) from adenosine-5'-triphosphate (ATP) inhibition.[8] Carcinoembryonic antigen (CEA), a tumor-associated glycoprotein, induced defective lymphocyte function.[9] There is also a correlation between human tumor cell membrane carbohydrate, protease release and the invasive metastatic capability of human cloned breast carcinoma cells.[10] Patients with metastatic disease frequently have reduced levels of serum antibody to autologous cell surface and cytoplasmic tumor-associated antigen (TAA).[11]

Using PBL from patients with breast carcinoma of various pathologic stages, the experiments described in this article showed that phytohemagglutinin (PHA) had a significantly reduced to no effect on cytotoxic lymphocytes (CTL) and NK cells. The results agree with previous reports, which showed that CEA, produced defective lymphocyte responsiveness to PHA. This cellular defect is directly related to reduced levels of IL-2 release, of interleukin-2 receptor (IL-2R) staining with anti-Tac antibodies, and a significantly reduced to a complete absence of cellular mRNA encoding the IL-2R.

Patients, Materials, and Methods

Patients

The research procedures in these investigations conform to the ethical standards of the Helsinki Declaration of 1975 and were approved by the Review Board for Protection of Human Subjects at Loyola University Medical Center. The pathologic staging of breast carcinoma patients was determined from a review of the surgical pathology reports and charts by an individual who had no knowledge of the work on immunosuppression in cancer. Staging was based on the criteria of the International Union Against Cancer (UICC). Ten patients each from Stage I, II, III, and IV with metastatic breast carcinoma (MBCa) supplied biopsies of BCa tissue. Metastatic tissue was also obtained from autopsy within 4 to 6 hours of death. Because benign breast diseases (BBD) has been considered a premalignant lesion, biopsies from five patients were included with five additional biopsies from noncancerous adults. All blood samples were obtained either prior to ay therapeutic manipulations (patients with BBD or Stage I) or 2 to 3 weeks after the last dose of chemotherapy, steroids, or radiation.

Materials

Recombinant Interleukin-2 (rIL-2) (Cetus Corp., Emerville, CA) had specific activity greater than 10^8 U/mg. The IL-2 titer in units per milliliter was determined as the reciprocal of the dilution required to sustain half of the maximal ^{3}H-thymidine incorporation into 3×10^3 CTLL-2 cells. The IL-2R (Tac antigen) was also obtained from Cetus Corp. It had a specific activity of 3 million Cetus U/mg.

Peripheral blood was drawn from ten of each of the age-matched subjects, from ten patients with metastatic breast carcinoma, benign breast disease, and healthy adults.

Preparation of Cells

Fifty milliliters of heparinized blood (20 U/ml) was obtained from each subject. Peripheral blood mononuclear cells (PBMNC) were separated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) sedimentation.[12] The monocytes in the PBC were quantitated by peroxidase cytochemistry. Adherent cells were prepared by suspending PBMNC (1×10^6/ml) in RPMI-1640 supplemented with 10% vol/vol heat inactivated pooled human serum. Five to 10 ml of the cell suspension were incubated in plastic dishes at 37°C for 1 hour. After nonadherent cells were removed, the adherent monolayers were separated by gentle scraping with sterile policeman. The resulting adherent cell population consisted of 85% to 95% monocytes, and 99% of these were viable by trypan blue exclusion. The nonadherent cells were washed onto 500-mg, acid-treated nylon-wool columns and incubated at 37°C for 30 minutes. The cells were then eluted. They were T-cell-enriched (90% erythrocyte rosetting) and 1% monocyte-depleted.

The nonadherent lymphocytes (1×10^7) were placed on the top of a seven-step discontinuous gradient of 40% to 50% Percoll (Pharmacia Fine Chemicals) in medium increased by 2.5% in 15-ml plastic tubes. The tubes were centrifuged at $550 \times g$ for 30 minutes.[13,14] The cells collected from low density fractions 2 and 3 consisted of more than 80% large granulated lymphocytes (LGL) as

judged by morphologic examination of Giemsa-stained smears. These are referred to as NK cells.

To remove most contaminating T-cells, the NK cells derived from the Percoll gradient (2 to 4 × 10^6 cells/ml) were mixed with an equal volume of fetal bovine serum and suspended with sheep erythrocytes at a ratio of 150 erythrocytes to one leukocyte. The cells were centrifuged at 100 × *g* and incubated for 1 hour at 20°C. They were then gently resuspended as described by Timonen *et al.*[14] Erythrocytes and rosetted cells were separated from NK cells on a one-step Percoll gradient (density 1.075) by centrifugation at 550 × *g* for 20 minutes, and the cells of the interface were washed once in the medium.

The cells collected from high density fractions 6 and 7 consisted mainly of small T-cells, as judged by R-rosette formation, with less than 2% LGL. The helper T-cells (THC) were further purified by panning using antibody-coated culture dishes as described by Engleman *et al.*[15]

The NULL (from healthy adults) and the NILL cells from patients with MBCa were obtained from nonadherent lymphocytes by panning sequentially onto OKT8, OKT4, OKT3, and B73.1 monoclonal antibody-coated culture dishes. (The nonadherent cells remaining after this sequential panning are hereafter referred to as either NULL or NILL cells depending on the donor.)

Measurement of Cytotoxicity

A 4-hour ^{51}Cr-release assay was used to monitor cytotoxicity of the effector cells against tumor cells. Target cells were labeled with 400 uCi of $Na^{51}CrO_4$ (Amersham Corp., Arlington Heights, IL) for 120 minutes in 0.5 ml of culture medium. The cells were then washed four times with culture medium and added at 1 × 10^3 cells/well to various numbers of the effector lymphocytes in round-bottomed, micro-titer plates (Limbo Chemical Co., Handen, CT). The plates were centrifuged at 500 rpm for 3 minutes and incubated for 4 hours at 37°C at 5% CO_2. The culture supernatants were harvested and counted in a gamma counter. Maximum isotope release was produced by incubating target cells with 0.1 N HCl. Spontaneous release was produced by incubating targets with the culture medium. The percentage of specific lysis was calculated by the formula: (Experimental CPM − Spontaneous CPM/Maximal CPM − Spontaneous CPM) × 100. All determinations were made in triplicate, and data are reported as the mean.[16]

Thymidine Incorporation and IL-2 Secretion

After incubation with PHA, Concanavalin A (Con A), or IL-2 at the indicated concentrations and for the defined period, the cells were washed and resuspended in RPMI 1640 medium (Gibco) supplemented with 10% heat inactivated pooled human serum (PHS), 100 U/ml penicillin, 25 μg/ml fungizone, and 100 μg/ml streptomycin at a concentration of 10^7 cells/ml.

For one-way mixed lymphocytes reactions (MLR), 10^7/ml PBMNC were mixed with 10^7/ml irradiated (3500 cGy) responsive, normal PBMNC. Samples of 100 μl were plated in triplicate into flat-bottomed plates and kept at 37°C in a humidified 5% CO_2 incubator for up to 5 days. Cultures of irradiated PBMNC were plated as controls for testing the effectiveness of irradiation.

The stimulation of DNA synthesis was assessed daily: the cells were pulsed for 1 hour with 1 uCi (methyl-^{3}H-thymidine (25 Ci/mmol, Amersham) and then harvested. The radioactivity incorporated into DNA was counted in 25% lumax/75% xylene in a liquid scintillation counter.

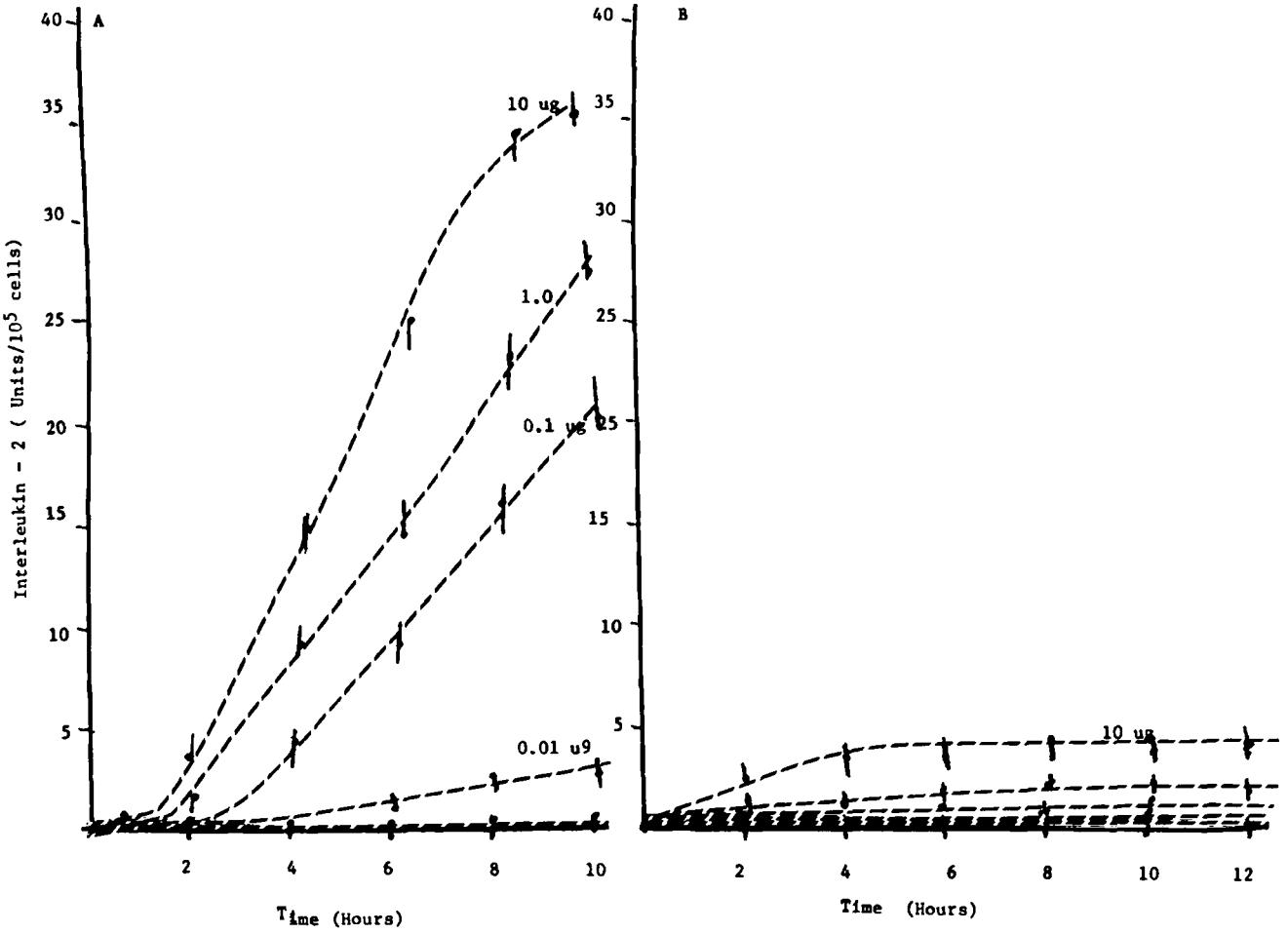
Aliquots of 500 μl from the various incubation mixtures were kept for 24 hours at 37°C, and the supernatants were collected and tested for IL-2 production. Microassays were conducted as follows: the IL-2-dependent T-cell line, CTLL-2, at a concentration of 5 × 10^4 cells/ml in the culture medium containing 50 μg/ml in the culture medium containing 10^{-6} mol/l mercaptoethanol. These were seeded in triplicate in 100 μl volumes in flat-bottomed, 96-well microtiter plates in the presence of double dilutions of putative IL-2 (100 μl) -containing samples. After 24 hours of culture, the cells were pulsed for 1 hour with ^{3}H-thymidine as described. To the IL-2 present in supernatants harvested from the PBMNC and other cell types, 100 μl of 5 × 10^4 cells were added. Proliferation rates was determined as described.

Generation of IL-2-Containing Supernatants

The PBMC, THC, NK, and NULL or NILL cells were incubated in 24-well Limbro pltes at 1 × 10^6/ml in RPMI 1640. This was supplemented with L-glutamine (2 mmol/l), penicillin (50 u/ml), gentamicin (50 μg/ml), and 2% v/v heat inactivated pooled human serum. Cell cultures were stimulated by graded levels of either PHA or Con A. After 48 hours of incubation at 37°C in 5% CO_2 atmosphere, supernatants were separated by centrifugation at 450 *g* for 10 minutes and stored at −20°C until assayed.

IL-2 Assay

The IL-2-dependent cytotoxic murine T-cell line (CTLL-20) was used. This cell line has been kept in culture with the IL-2-containing supernatant of Con A-activated rat spleenocytes for more than 2 years. These cells were extensively washed and suspended in minimal Eagle medium (MEM) supplemented with 4.5 g/liter glucose at 10^5 cells/ml. Aliquots of 100 μl of the cell



FIGS. 1A AND 1B. Time-dependent production of interleukin-2 (IL-2) by mitogenic stimulation of T-helper cells from (A) healthy adults and (B) patients with breast carcinoma (BCa). The bars at each reading indicate the variation between the subjects of each group of ten patients with Stage IV metastatic BCa and age-matched healthy adults.

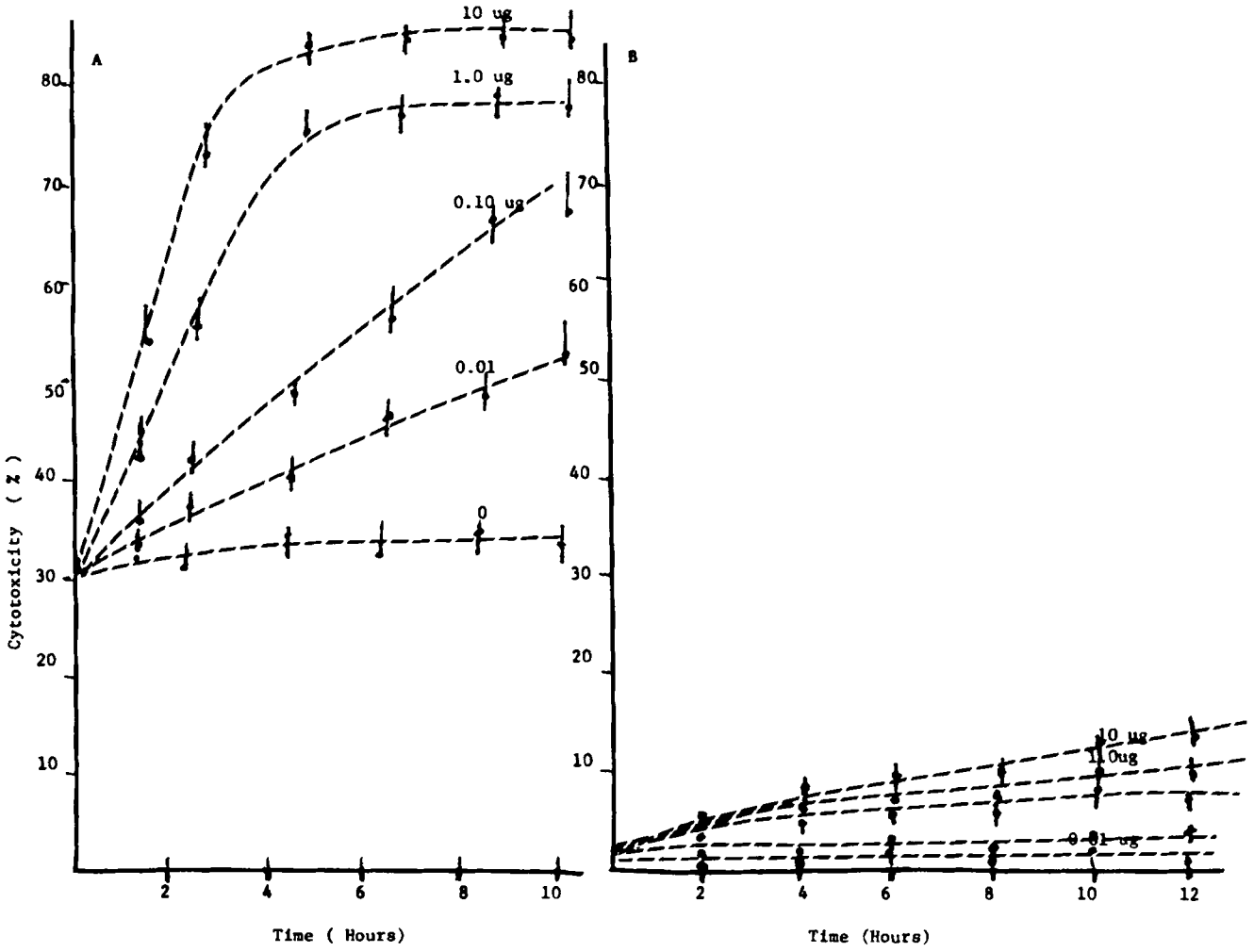
suspension were placed in flat-bottomed, micro-titer wells along with 100 μl of the supernatants described. The IL-2 determinations were performed in triplicate. The total duration of culture was 24 hours. One uCi of ^{3}H-thymidine sp. act. 6.7 Ci/mmol was added per well for the last 4 hours of culture. The cells were harvested, and ^{3}H-thymidine activity incorporated into the DNA was determined by liquid scintillation spectrometry. The IL-2 activity was expressed as the ^{3}H-thymidine incorporated into the CTLL-20 in culture with a 1:2 dilution of supernatants from stimulated cell cultures.

IL-2R Assay

The monoclonal antibody anti-Tac[12,17,18] assay was used for assessing IL-2R. The PBMC, THC, NK, NULL, and NILL cells in RPMI-1640 (10^5 cells/ml) were stimulated with PHA for 5 days, then incubated with 50 μl of a 1:100 dilution of anti-Tac antibody for 30 minutes at 4°C. Washed cells were further incubated with FITC-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) at a 1:20 dilution. The fluorescence of washed cells was then analyzed with a FACS cell sorter.

Preparation of mRNA Mediating In Vitro *Synthesis of IL-2R*

The PBMC and NK cells were obtained from ten healthy adults and age-matched patients with Stage IV metastatic breast carcinoma. The cells were cultured for 72 hours in mediums supplemented with either IL-2 or with Con A. The cells were harvested, washed, and Poly(A)-enriched mRNA was prepared by phenol extraction of total cellular RNA, followed by Oligo(dT)-cellulose chromatography. The mRNA was fractionated on an 8% to 25% sucrose gradient in 2 mmol/l EDTA, 100 mmol/l NaCl, and 10 mmol/l Hepes (pH 7.6), which was centrifuged at 29,000 rpm for 20 hours in a Beckman SW-40 rotor. Individual fractions were precip-



FIGS. 2A AND 2B. Time events of the action of phytohemagglutinin (PHA) on natural killer NK cells from (A) healthy adults and (B) patients with Stage IV metastatic breast carcinoma. The bars at each reading indicate the variation between the subjects of each group.

itated by the addition of 2 volumes of ethanol, resuspended in H_2O, and tested for IL-2R mRNA activity with a cell-free synthesizing system derived from wheat germ. After treatment with 200 μg/ml of RNase A for 30 minutes, the ^{35}S-methionine-containing products of translation were analyzed on sodium dodecyl-sulfate polyacrylamide gel electrophoresis.[19] The identity of IL-2R in each translation was confirmed by incubating the gel at 27°C for 1 hour in the presence of excess of fluorescein-labeled anti-Tac antibodies.[17,20]

All the data were statistically examined. Group means were compared using the student's *t* test.

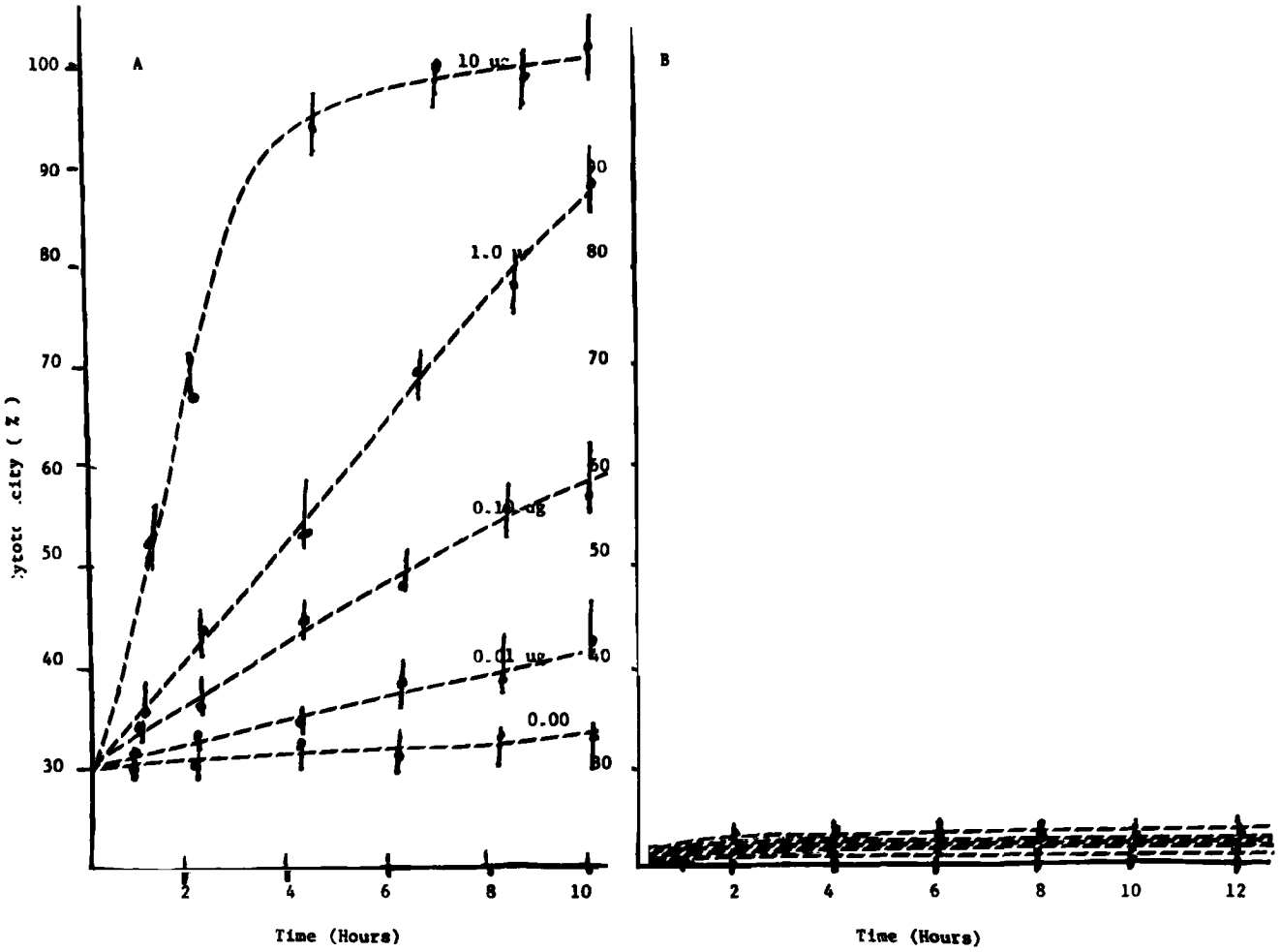
Results

Time-Dependent Secretion of IL-2 During T-Helper Cell Mitogenic Stimulation

T-helper cells (THC) were prepared from blood of ten patients with Stage IV metastatic breast carcinoma and ten age-matched healthy adults. The blood was drawn from six patients 2 to 3 weeks after the last dose of chemotherapy. Of the four patients who also had surgery, blood was drawn 4 weeks after the last dose of chemotherapy. The THC were incubated with PHA at the indicated concentration (0.01, 0.1, 1.0, and 10 μg/10^5 viable cells). At certain intervals supernatants were carefully withdrawn from triplicate wells, and IL-2 levels were determined. The data in the curves of Figure 1A show progressive release of IL-2 from the THC of healthy adults. During the first 4 hours, IL-2 levels increased in a straight line, then slowed to a plateau. The curves indicate that the IL-2 levels were PHA-concentration dependent. Supernatants from cultures of THC from patients with metastatic BCa contained nonsignificant IL-2 activity (Fig. 1B).

Time Events of PHA-Action on NK Cells

Three parameters, IL-2 secretion, cytotoxicity against K-562 target cells, and ^{3}H-thymidine, were used to



FIGS. 3A AND 3B. Time events of the action of interleukin-2 (IL-2) on natural killer (NK) cells (A) from healthy adults and (B) from patients with Stage IV metastatic breast carcinoma. The bars at each reading indicate the variation between the 10 subjects of each group.

monitor NK cell proliferation. During NK cell cultivation in a medium supplemented with graded PHA concentrations, cells of healthy adults showed that ^{3}H-thymidine uptake (CPM/min/10^5 cells) and the percent of cytotoxicity progressively increased; there was a sharp rise during the first 120 minutes (Fig. 3A). The IL-2 levels were nondetectable during the first 40 minutes, significantly increased for 4 hours, then leveled off and remained constant (Fig. 2A). When proliferating in a culture medium supplemented with PHA-graded concentrations, NK cells from patients with metastatic BCa (Fig. 2B) showed significantly lower cytotoxic activity than NK cells of healthy adults.

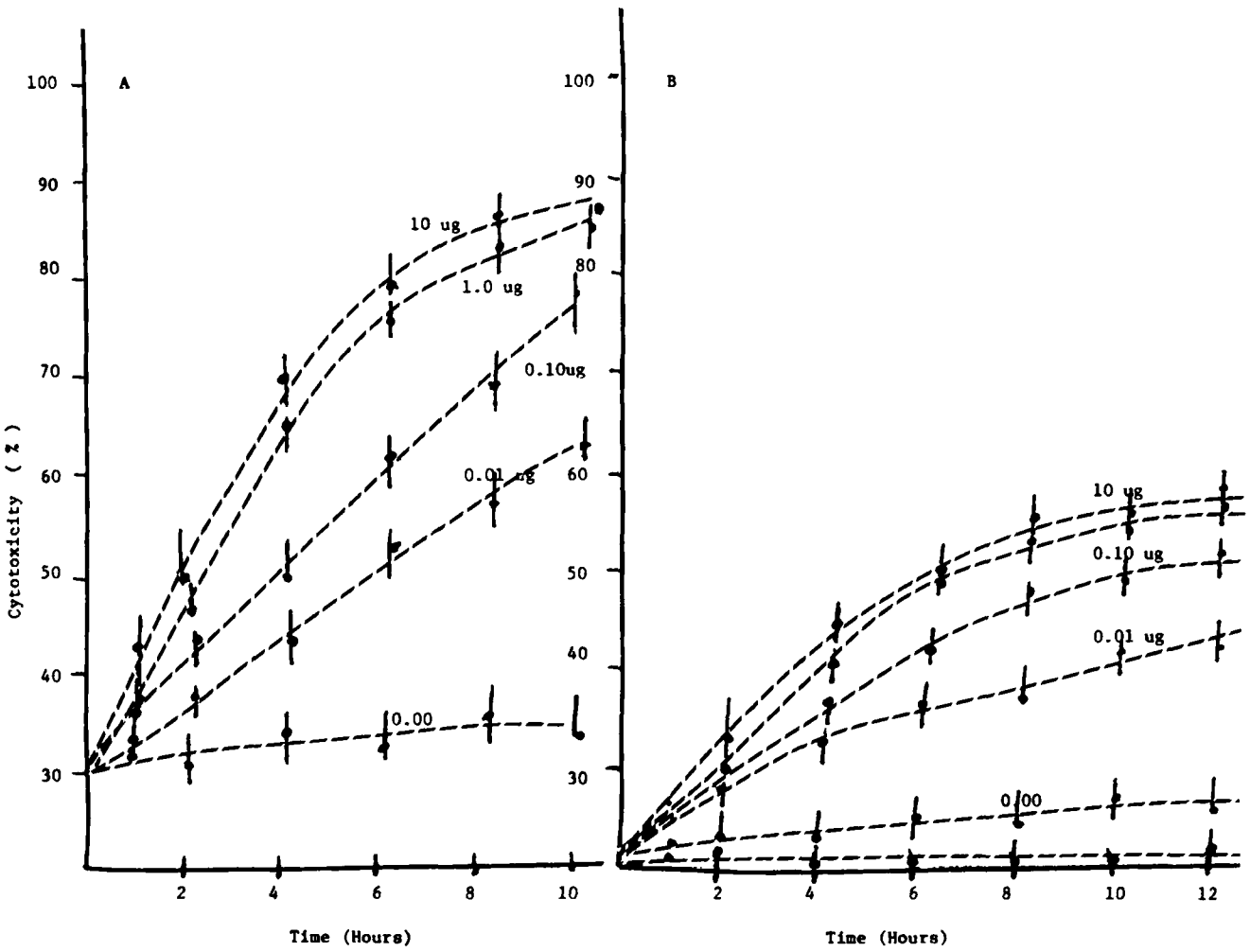
Time Events of IL-2 Action on NK Cells

The NK effector cells were first incubated at 37°C in a medium not supplemented (control), and then supplemented with 1, 5, 10, and 25 U IL-2/10^5 cells. Aliquots of the effector cells were harvested, washed, then reincubated with ^{51}Cr-K-562 target cells for an additional 4 hours. Cytotoxicity was monitored at effector to target cells at a ratio of 1:50. The NK cells of healthy adults showed a 28% to 35% inherent cytotoxicity prior to activation. If preincubated with 25 U of IL-2/10^5 cells for 4 hours at 37°C in 5% CO_2 humidified atmosphere, the cells showed a maximum of 93% to 98% cytotoxicity. This is a statistically significant increase ($P < 0.0001$).

The NK cells of patients with metastatic BCa had nondetectable inherent cytotoxicity before activation (Fig. 3B). When preincubated with 25 U IL-2/10^5 cells for 4 hours as described, the cells showed a maximum 5% to 10% cytotoxicity, a statistically nonsignificant response.

*Time Events of IL-2 Action on NULL Healthy Adults, and NILL From Patients With MBCa Cells**

The NULL cells as effector cells were first incubated at 37°C in a medium not supplemented (control) and then supplemented with 1, 5, 10, and 25 U IL-2/10^5



FIGS. 4A AND 4B. Time events of the action of interleukin-2 (IL-2) on (A) NULL cells from healthy adults and (B) NILL cells from patients with Stage IV metastatic breast carcinoma. Ten subjects per group were examined. The bars at each reading indicate the variation between the subjects of each group.

cells. Aliquots from each of these cells were harvested, washed, then reincubated with ^{51}Cr-K562 and ^{51}Cr-BCa cells as targets for an additional 4 hours. Activated or not, the NULL and NILL cells had nonsignificant cytotoxicity against K-562 cells, indicating that they are not NK cells. Using as the effector human BCa target cells at a 1:50 ratio, NULL cells from healthy adults showed a 25% to 32% inherent cytotoxicity prior to activation. When the NULL cells were preincubated with 25 units of IL-2/10^5 cells for 4 hours as described, a maximum of 83% to 85% cytotoxicity was obtained against the ^{51}Cr-BCa cells.

The NILL cells from patients with metastatic BCa had very weak, but significant 5% to 10% inherent cytotoxicity prior to activation. Preincubation with 25 units of IL-2/10^5 cells produced a significant sixfold to sevenfold increase in the cytotoxicity with a maximum of 50% to 55% cytotoxicity against the ^{51}Cr-BCa cells. These data indicate that IL-2 activated NULL and NILL cells from healthy adults and patients with MBCa (Fig. 4B).

Effect of Purified rIL-2 on Con A Blastogenesis and Lack of IL-2 Receptor in Cells From Patients With Metastatic Breast Carcinoma

The effect of purified rIL-2 on the responsiveness to Con A blastogenesis of PBMC, THC, and NK cells from ten healthy adults compared with ten age-matched patients with MBCa is summarized in Table 1. Purified rIL-2 was incorporated at 10 μg/ml of the culture medium, and cellular proliferation was monitored by ^{3}H-TdR uptake. The concentration of purified rIL-2 contains 20 U/ml of activity as determined by the described standard assay. There is an apparent increase in ^{3}HTdR uptake by Con A-stimulated PBMC, THC, and NK cells of patients to the presence of rIL-2. This increase is significantly lower than that obtained with cells from

TABLE 1. Effect of Interleukin-2 on Con A-Induced Blastogenesis of Cells From Healthy Adults and From Patients With Metastatic Breast Carcinoma

Experiment	IL-2	Con A	Blastogenesis*
PBMNC source			
Healthy adults (n = 10)	−	−	1.9 ± 0.5
	−	+	29.8 ± 3.8
	+	−	11.7 ± 1.9
	+	+	46.9 ± 6.8
BCa patients (n = 10)	−	−	0.9 ± 0.1
	−	+	3.9 ± 1.1
	+	−	2.7 ± 0.9
	+	+	9.8 ± 1.8
T-helper cells source			
Healthy adults (n = 9)	−	−	3.8 ± 1.2
	−	+	34.6 ± 4.9
	+	−	15.8 ± 2.2
	+	+	60.8 ± 7.2
BCa patients (n = 10)	−	−	1.2 ± 0.2
	−	+	4.3 ± 1.3
	+	−	3.1 ± 1.2
	+	+	10.5 ± 2.1
NK cells source			
Healthy adults (n = 10)	−	−	2.7 ± 1.1
	−	+	29.8 ± 4.7
	+	−	13.6 ± 1.9
	+	+	65.7 ± 7.9
BCa patients (n = 10)	−	−	1.1 ± 0.2
	−	+	3.9 ± 1.2
	+	−	2.4 ± 1.0
	+	+	9.8 ± 1.8

PBMNC: peripheral blood mononuclear cells; BCa: metastatic breast carcinoma; NK: natural killer; IL-2: interleukin-2.

* ^{3}H-TdR uptake in cell cultures. Results are presented as means ± standard deviation ($\times 10^{-3}$).

healthy adults. These results indicate a defective Con-A induced IL-2 production and the inability of exogeneous rIL-2 to correct the hyporesponsiveness of cells from patients with MBCa. This inability could be secondary to the lack of the development of functional IL-2R in patients with BCa. Accordingly, the expression of IL-2R was evaluated in the peripheral blood lymphocytes from the ten patients. The data in Table 2 show that the Tac expression is depressed. Therefore, the hyporesponsiveness to Con A in the four types of peripheral blood cells is caused by a defect in IL-2 production and a depressed Tac (that is, IL-2R expression).

^{125}I-IL-2-Binding to NK Cells From Various Subjects

A fixed number of NK cells were incubated with graded concentrations of ^{125}I-IL-2, and at a fixed incubation period the amount of bound and free radioactivity was determined and reported as a ratio. The Scatchard analysis in Figure 5 indicates statistically significant difference between ^{125}I-IL-2 binding profile to NK cells and the various groups of subjects. The NK cells from ten subjects in each group were examined. The variation between the various subjects within a group was between 7.6% and 15.9%. The plots indicate decreasing ^{125}I-IL-2 bindng sites on NK cells from patients with various stages of breast carcinoma. Although ^{125}I-IL-2 binds to NK cells in patients at Stages III and IV, the magnitude of binding is statistically nonsignificant.

Effect of Glycosylation Inhibitors on ^{125}I-IL-2 Binding to NK Cells

A fixed number of NK cells from healthy adults were first incubated with fixed concentrations of glycosyla-

TABLE 2. Expression of Interleukin-2 Receptor (Tac antigen) on Stimulated Cells From Healthy Adults and From Patients With Metastatic Breast Carcinoma

Experiment no.	BCa patients	Healthy adults
Percent of PBMNC reactive with anti-Tac		
1	3.76	29.86
2	8.65	42.65
3	9.73	47.97
4	11.43	58.56
Mean ± SD	8.39 ± 0.8	44.76 ± 3.6
$P < 0.005$		
Percent of T-helper cells reactive with anti-Tac		
1	3.25	28.78
2	4.63	25.87
3	6.85	36.23
4	7.39	39.85
Mean ± SD	5.53 ± 0.6	32.68 ± 2.9
$P < 0.01$		
Percent of NILL or NULL* reactive cells with anti-Tac		
1	2.19	19.37
2	3.85	30.17
3	5.69	44.96
4	8.32	66.82
Mean ± SD	5.01 ± 0.6	40.33 ± 3.5
$P < 0.005$		
Percent of natural killer cells reactive with anti-Tac		
1	4.12	34.65
2	5.45	45.73
3	6.83	55.64
4	9.75	79.63
Mean ± SD	6.54 ± 0.7	53.91 ± 3.9
$P < 0.001$		

Note: The cells were cultured with Con A for 5 days. Anti-Tac reactive cells were assessed by indirect immunofluorescence and FACS analysis.

BCa: metastatic breast carcinoma; PBMNC: peripheral blood mononuclear cells; SD: standard deviation.

* NILL cells are from patients with breast carcinoma, NULL cells from healthy adults.

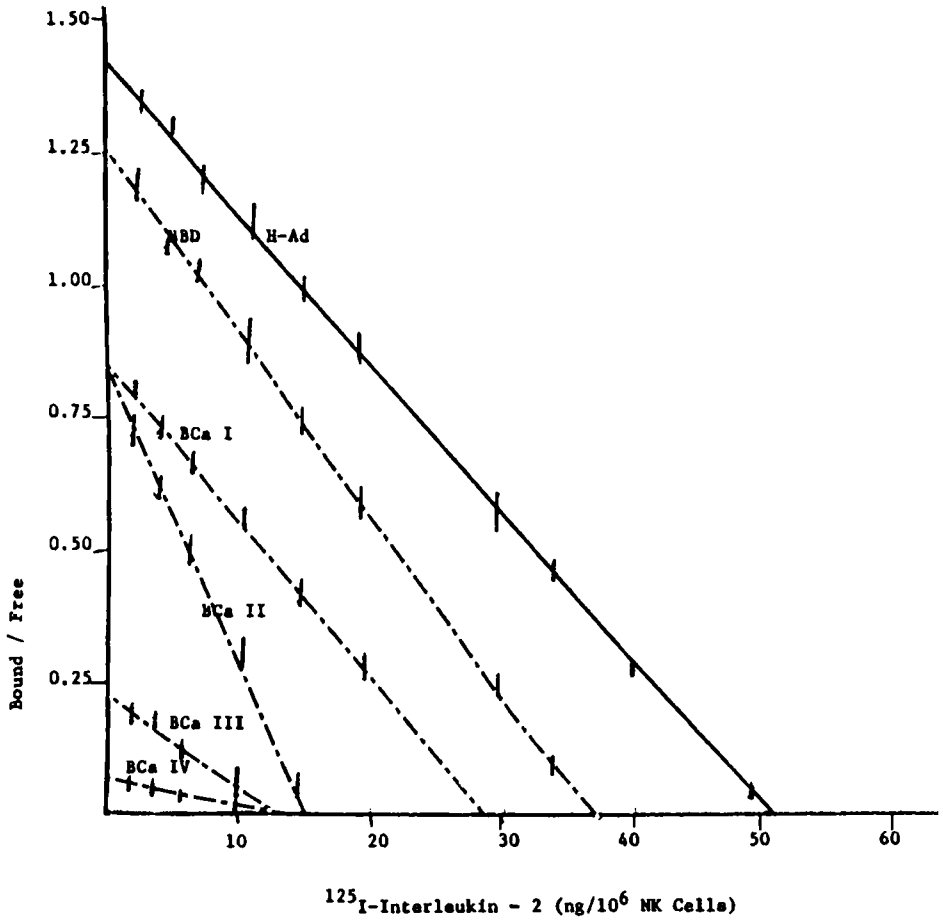


FIG. 5. Scatchard analysis of ^{125}I-interleukin-2 (IL-2) binding to natural killer (NK) cells from 10 healthy adults, ten patients with benign breast disease, and ten patients each with breast carcinoma Stages I, II, III, and IV. The cells were grown for 72 hours, then incubated in a medium supplemented with 1 to 200 μg/ml of ^{125}I-IL-2 for 3 hours at 4°C. Each cell type was processed in triplicate. Nonspecific binding was determined in the presence of at least a 50-fold excess of unlabeled IL-2. The curves represent the mean of binding ratio obtained with NK cells from ten patients in each group. The variation between the various subjects within a group was between 7.6% and 15.9%.

tion inhibitors. The cells were then harvested, washed, and then incubated with graded concentrations of ^{125}I-IL-2. The amount of bound and free radioactivity was determined and reported as a ratio. The Scatchard analysis in Figure 6 indicates that ^{125}I-IL-2 binding is increased by PBL preincubated with galactosyl transferase, but is decreased by NK cells preincubated with monensine, swansonine, glucosamine, and tunicamycin. Statistically nonsignificant ^{125}I-IL-2 binding occurred with NK cells preincubated with tunicamycin or glucosamine. These results indicate that inhibition of cellular glycosylation accompanies the inhibition of ^{125}I-IL-2 binding.

Effect of IL-2 on Peripheral Blood Lymphocyte Cytotoxic Activities

The host cytotoxic activities, cytotoxic lymphocytes (CTL), macrophages, and NK cells, play an important role not only in the protection of the host, but also in the proliferation and dissemination of tumor cells. The experiments summarized in Table 3 were conducted to examine the cytotoxic activities of PBMNC and NK cells in benign breast diseases (BBD) and in Stages I, II, III, and IV of disseminated breast carcinoma. The cells were obtained from ten patients of each group. Cellular cytotoxicity was monitored by the release of ^{15}Cr from labeled K-562 and BCa cells. The results are expressed as the mean ± standard deviation of the percent of radioactivity released. The result indicate a 5% to 7% variation in the cell cytotoxicity from one subject to another within a group.

There was a progressive decrease in the cytotoxicity of PBMNC and NK cells in patients with BBD, and in those with Stage I, II, III, or disseminated BCa against K-562 or BCa target cells. Parallel to these changes, there was a gradual decrease in the number of cells reactive with anti-Tac antibodies. Although the cells from patients with BBD had nonsignificant changes in effects (29.8%), cells from patients with Stage I BCa were reactive with anti-Tac antibodies. The number of cells reactive with these antibodies decreased significantly with advanced stages of BCa until no cells in patients with Stage IV or metastatic BCa were reactive. These data indicate that in early stages of cancer there is IL-2R production, which progressively decreases in the advanced stages.

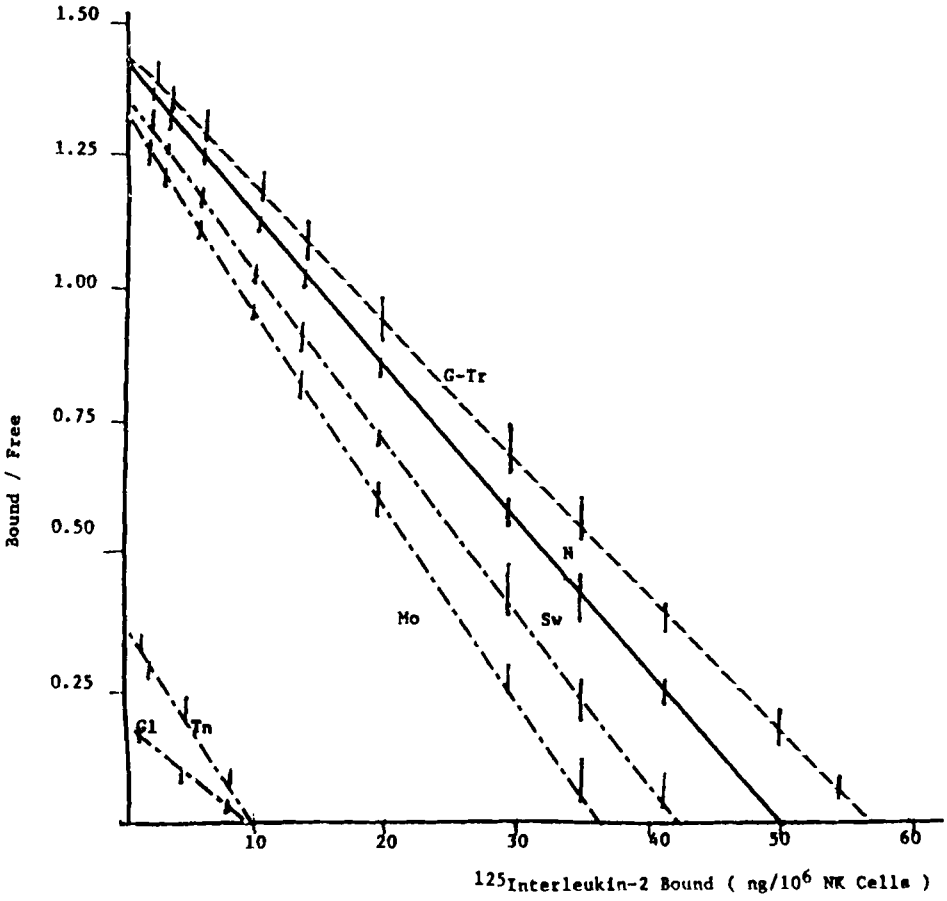


FIG. 6. Scatchard analysis of ^{125}I-interleukin-2 (IL-2) binding to inhibitor-treated natural killer (NK) cells. The NK cells from ten healthy adults were grown for 72 hours in the absence (N) of an in the presence of glycosyl transferase (G-Tr), swainsonine (Sw), monensin (Mo), tunicamycin (Tn), or glucosamin (GLC). Incubation of the cells with 1 to 200 μg/ml of ^{125}I-IL-2 for 3 hours at 4°C was done in triplicats. Nonspecific binding was determined in the presence of at least a 50-fold excess of unlabeled IL-2. The data represent the mean of ten separate assays with each of the inhibitors. The variation between each group of assays was between 5.3% and 11.3%.

This profile is confirmed by the cellular response to exogenous IL-2. Incubation of PBMNC or NK cells from patients with BBD or Stage I BCa in a medium supplemented with IL-2 produced a significant increase in cellular cytotoxicity. The IL-2 had no effect on the PBMNC or NK cells from patients with Stage IV BCa.

Peripheral Blood Lymphocyte Encoding IL-2R

Poly(A) RNA was prepared by phenol extraction of total cellular RNA followed by oligo(dT)-cellulose chromatography. The RNA was fractionated on an 8% to 25% sucrose gradient in 2 mmol/L EDTA, 100 mmol/L NaCl, and mmol/L Hepes (pH 7.6). The PBMNC from healthy adults and from patients with Stage IV metastatic BCa were used as a source of the RNA. The lymphocytes were preincubated with Con A prior to extraction. The resolution patterns in Figure 7 show significant differences in the RNA from the two sources. An assay of each fraction in a protein synthesis system *in vitro* indicated that fraction IV from PBMNC of healthy adults (Fig. 7, curve A) mediates the synthesis of proteins that bind anti-Tac antibodies. The PBMNC from patients with metastatic BCa (Fig. 7, curve B, solid line) contained very low amounts of peak IV.

When extracted with physiologic saline, PBMNC from healthy adults preincubated with Con A yielded determinants binding anti-Tac antibodies (Fig. 8, lane C). In addition, when extracted with physiologic saline, NK cells from healthy adults preincubated in a medium supplemented with IL-2 yielded determinants that bind IL-2 and/or anti-Tac antibodies (Fig. 8, lanes G and H).

The peak IV (Fig. 7) was examined for a protein synthesis mediated *in vitro*. Lane J (Fig. 8) is the product mediated by peak IV of the RNA from the PBMNC of healthy adults, Lane K (Fig. 8), however, represents similar products mediated by peak IV of the RNA from PBMNC of patients with metastatic BCa. After treatment with anti-Tac antibodies, lane L (Fig. 8) shows the solution of the precipitate from products J, whereas lane M shows the precipitate from K. Lane N (Fig. 8) shows the precipitate produced from product J, and lane O shows the precipitate from product K by IL-2. The autoradiogram shows the RNA-mediated determinants synthesized *in vitro* that precipitated with IL-2 or anti-Tac antibodies, that is, the synthesis of the IL-2 receptor.

A summary of the results of the study show that the T-helper and NK cells from cancer patients were significantly less responsive to PHA. NK cells from cancer patients were significantly less responsive to IL-2. The kinetics of the binding of ^{125}I-IL-2 to NK cells, unmodified and modified with glycosylation inhibitors, indicated that NK cells from cancer patients have reduced and even a lack of functional IL-2 levels. The analysis of Poly(A)-enriched messenger RNA profile from lymphocytes of patients with breast carcinoma indicated a reduction into a complete absence of an mRNA fraction encoding IL-2 receptor.

Discussion

The initial trigger for cell proliferation is the interaction of growth factors with the cell surface growth factor receptor. Activation of the growth factor leads to a cytoplasmic signaling system, which remain undefined. Under mitogenic or antigenic stimulation, resting T-lymphocytes in the presence of a T-cell growth factor, IL-2, enter proliferation cycles. The experiments described in this study examined the response to mitogenic stimulation of peripheral blood lymphocytes from healthy adults and patients with various stages of breast carcinoma in the presence and absence of IL-2. The expression of IL-2 receptors is the consequence of the interaction of the responding cells with the antigen- or mitogen-responding cells. The IL-2 receptor expression is a transient event, and repeated stimulation by the mitogen or antigen is required for continuous IL-2 receptor expression and, consequently, for long-term cell proliferation.

In contrast to activated normal T-cells in healthy adults, and because T-cells from cancer patients are associated with the autonomous growth of the tumor cells, T-cells in cancer patients must express the IL-2 receptor. They must thus demonstrate an enhanced response to exogeneous IL-2. The results obtained indicate that the lymphocytes from patients with breast carcinoma have a decreased responsiveness to exogenous IL-2. The magnitude of this decrease appears to correlate with the severity of the disease.

The experiments described in this article demonstrate that patients with metastatic breast carcinoma have a defect in PHA and Con A IL-2 production and lymphocyte blastogenesis. They also have peripheral blood lymphocytes (PBMC, T-helper, NULL, and NILL cells) that are hyporesponsive to IL-2 and to mitogenic stimulation. Proliferation and differentiation of B-cells are dependent on the accessory cell function of monocytes and the presence of functional T-helper cells. A functional defect in the PBMC and T-helper cells could result in reduced levels of serum antibody to autologous cell surface and cytoplasmic TAA frequently observed in patients with metastatic breast carcinoma.[18] During tumor growth, the tumor cells acquire resistance to the host immune killing mechanisms, and as the malignancy become disseminated, there is a decrease in immunity to TAA because of defective cellular immunoregulation.

TABLE 3. Cytotoxic Activities of Peripheral Blood Lymphocytes

Effector cells from subjects	Target cells: K-562 Percent cytotoxicity	Target cells: BCa cells percent cytotoxicity	IL-2 receptor: Percent of cells reactive with anti-Tac
Benign breast diseases			
PBMNC	25.6 ± 3.9	15.6 ± 1.2	1.2 ± 0.1
PBMNC + IL-2	78.5 ± 9.7	79.8 ± 10.7	42.2 ± 3.4
NK cells	47.8 ± 4.8	6.8 ± 0.5	2.8 ± 0.1
NK cells + IL-2	94.5 ± 9.6	80.5 ± 7.1	51.2 ± 3.9
Breast carcinoma (Stage I)			
PBMNC	24.9 ± 3.8	16.2 ± 1.1	None
PBMNC + IL-2	61.2 ± 9.6	65.2 ± 9.8	29.8 ± 2.1
NK cells	48.7 ± 4.9	7.9 ± 0.6	None
NK cells + IL-2	87.6 ± 8.9	75.6 ± 8.1	39.8 ± 1.2
Breast carcinoma (Stage II)			
PBMNC	9.8 ± 0.9	3.2 ± 0.2	None
PBMNC + IL-2	14.6 ± 1.2	5.1 ± 0.5	21.6 ± 1.9
NK	18.7 ± 1.8	2.1 ± 0.1	None
NK cells + IL-2	54.9 ± 5.7	2.7 ± 0.2	11.2 ± 1.2
Breast carcinoma (Stage III)			
PBMNC	1.1 ± 0.1	None	None
PBMNC + IL-2	1.9 ± 0.1	None	None
NK cells	2.6 ± 0.2	None	None
NK cells ± IL-2	1.9 ± 0.2	None	2.1 ± 0.1
Breast carcinoma (Stage IV metastatic)			
PBMNC	None	None	None
PBMNC + IL-2	None	None	None
NK cells	None	None	None
NK cells ± IL-2	None	None	None

PBMNC: peripheral blood mononuclear cells; NK: natural killer; BCa: metastatic breast cancer.

The PBL-mitogenic stimulation occurred with either Con A- or PHA generated lymphokine, that is, IL-2, which is released into the culture medium and an IL-2 receptor at the cell surface.[21] Interleukin-2 is a polypeptide that functions as a growth factors for T- and B-cells. When activated by an antigen or by a lectin, T-lymphocytes acquire the ability to proliferate in response to IL-2. The lymphokine also promotes the growth of activated B-cells. It acts by way of specific cell surface receptors that are up-regulated in PHA-stimulated human PBL.

When cultured with IL-2, normal human lymphocytes are capable of generating effector cells that lyse a wide spectrum of target cells. They can kill NK-sensitive as well as NK-resistant tumor cells.[22-24] The character-

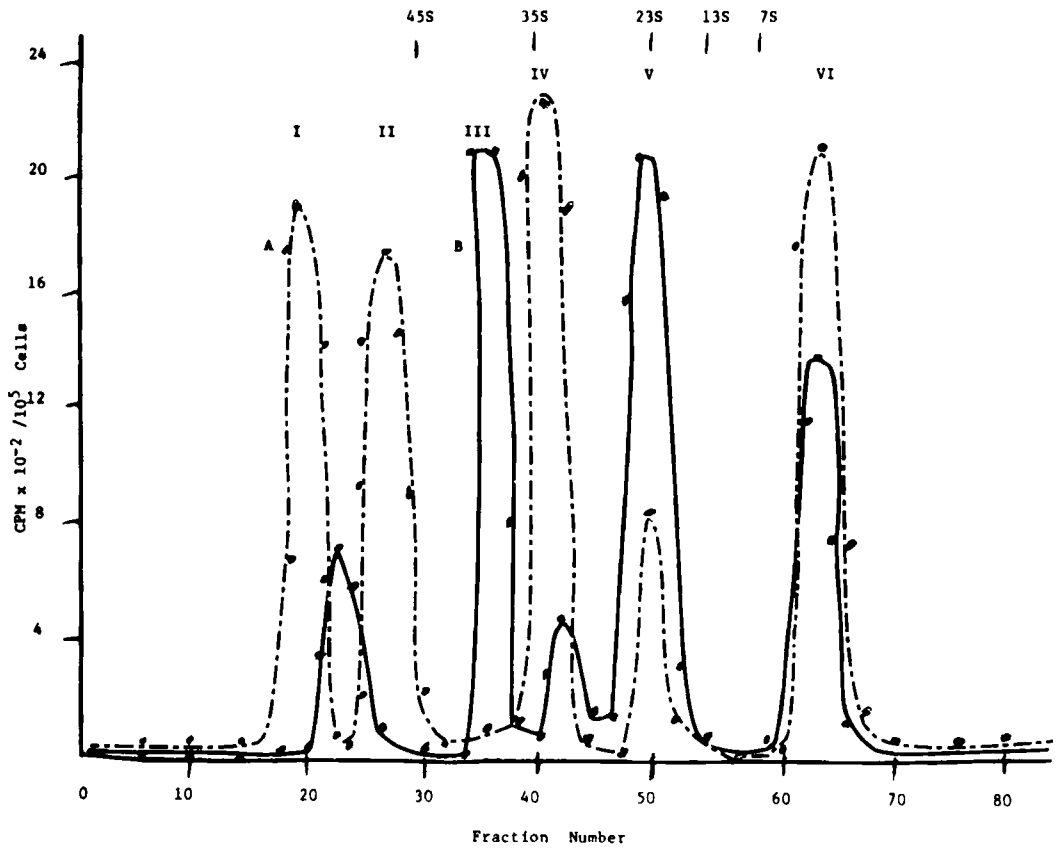


FIG. 7. Profile of Poly(A) RNA from peripheral blood lymphocytes on 8% to 25% sucrose gradient. Poly(A) RNA from Con A-treated peripheral blood mononuclear cells from (A) healthy adults, (B) from patients with Stage IV metastatic breast carcinoma.

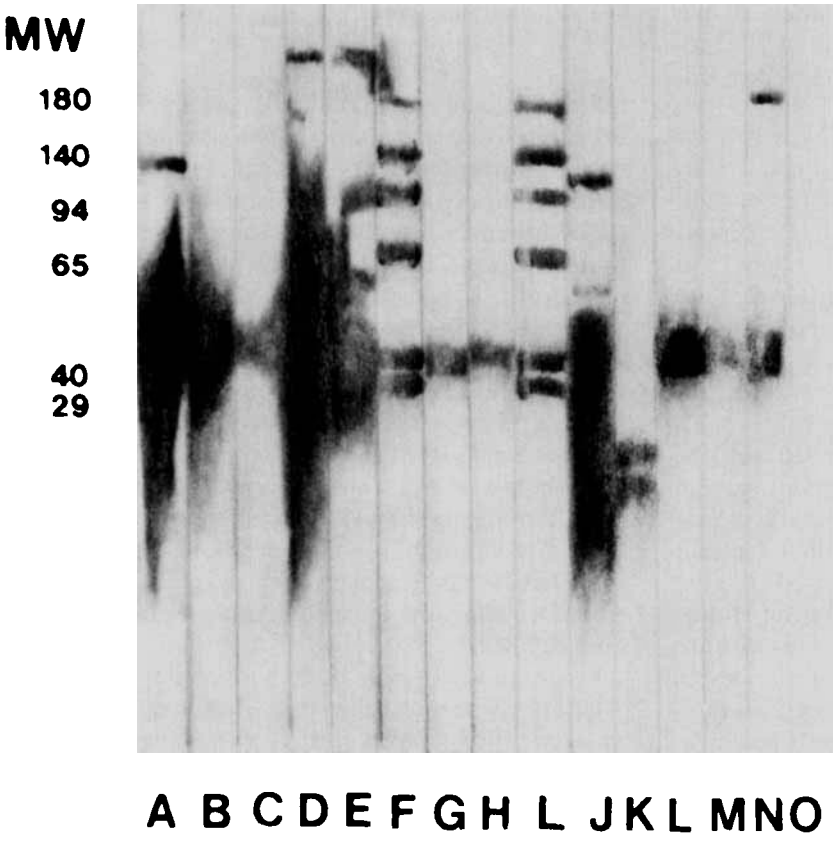


FIG. 8. Autoradiogram of SDS-polyacrylamide gel electrophoresis of RNA-mediated synthesis of interleukin-2 receptor (IL-2R) from peripheral blood mononuclear cells of healthy adults and patients with metastic breast cancer.

ization of these lymphokine-activated killer (LAK) precursors is a controversial issue. In analyzing the literature on LAK cell precursors, it becomes apparent that different tumor target cells were used in different reports, which indicates that LAK cell precursors are different depending on the target cell examined, that is, LAK cells are a heterogeneous population. More than one lymphocyte subset can generate LAK cells, and these subsets may express different surface markers. This is an immunoregulatory mechanism that is generated by culturing normal lymphocytes with IL-2 without the need for any additional antigenic stimulation. The results presented in this article demonstrate for the first time that the hyporesponsiveness of lymphocytes from patients with breast carcinoma to mitogenic or IL-2 stimulation is caused by a lack of functional IL-2R. These lymphocytes are shown to be unable to synthesize mRNA encoding IL-2R.

When mononuclear cells from healthy adults are exposed to IL-2, they develop cytotoxic reactivity against autologous and allogeneic cells.[23,24] Based on these findings, it was believed that immunotherapy with IL-2 and activated mononuclear cells circumvented the loss of host immunocompetence associated with advanced cancer and immunosuppressive cancer treatments.[25-31] Our findings that mononuclear cells from cancer patients with Stage IV metastatic BCa lack IL-2 receptor point to the need for other lymphokines that have receptors on the patient's mononuclear cells. Current experiments are being used to investigate methods to produce and select clones of more potent killer cells for potential use in adoptive transfer for tumor cell destruction.

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