

Volume 12, issue 1, March 2001 | Articles originaux

Retrovirus-mediated gene transfer of the cytokine genes interleukin-1b and tumor necrosis factor-a into human neuroblastoma cells: consequences for cell line behavior and immunomodulatory properties.

Abstract

We have investigated the value of a gene therapy approach for neuroblastoma (NB), based on retroviral transduction of the IL-1b or TNF-a cytokine genes into

human NB lines. Secretion of the corresponding cytokine, was demonstrated in all lines, although with considerable quantitative variations. Cytokine gene expression significantly reduced the proliferation index ($p = 0.0001$); this effect was associated with either terminal neuronal (one TNF-a line) or fibroblast-like differentiation (two IL-1b lines), leading to growth arrest after a few weeks. Cell surface levels of CD54 and HLA class II remained unaffected, but HLA class I ($p < 0.001$) and CD58 expression ($p = 0.01$) increased on SKNSH after TNF-a gene transfer. Mononuclear cells from normal allogeneic donors cocultured with both IL-1b ($p < 0.001$) and TNF-a lines ($p < 0.01$), showed a significant increase in the proportion of activated T cells (CD3+DR+); however, their cytotoxicity and proliferation rate remained unchanged. Immunotherapy of neuroblastoma will require identification of transduced lines in which cytokine secretion induces phenotypic changes in such a way as to augment their likely immunomodulatory properties without impeding cell growth. Because of the limited efficacy of IL-1b or TNF-a gene transfer alone, further studies should focus on combination with other immunomodulatory agents, to improve their potential efficacy in neuroblastoma.

Full text

INTRODUCTION

Despite the most intensive therapies, the prognosis of patients with neuroblastoma (NB), the most common extracranial solid tumor of childhood, remains dismal [1-4]. Most patients achieve a complete remission, but tumor recurs frequently. Disease-free survival is less than 30% at five years for children above one year, with metastatic disease, emphasizing the need for new strategies to eliminate residual disease.

Several phase I and II studies have reported the use of adoptive immunotherapy mainly in adults [5-10], but also in pediatric solid tumors including neuroblastoma [11-16] However, both in children and adults, systemic administration of cytokines has so far produced few responses and major toxicity. Genetically modified tumor cells expressing cytokine as well as other immunomodulatory genes have been shown to be effective in several animal tumor models including neuroblastoma [17-23]. Therefore, local activation of the immune system could provide an alternative approach while decreasing systemic effects, leading to the approval of clinical trials in adult solid tumors; so

far a few results have been reported in adults, showing that both partial local responses as well as systemic immune activation may be achieved through an *in situ* gene therapy approach [24-27].

We have previously reported transfer and expression of the IL-2 or IFN-gamma genes in human neuroblastoma, in both primary cultures and cell lines [28-30]. We report here the feasibility of transferring other cytokine genes such as IL-1beta and TNF-alpha into human neuroblastoma cells to generate allogeneic cytokine-secreting tumor cell lines for use as *in vivo* immunogens. In particular, we have investigated whether expression of any cytokine can directly accelerate or retard neuroblastoma cell growth, effects which would predict the safety and feasibility respectively of projected clinical studies. We have also looked to see whether expression of transferred cytokine genes can augment their likely immunomodulatory properties *in vitro*.

MATERIALS AND METHODS

Neuroblastoma cell lines

Five human NB cell lines were studied: SKNSH and SKNMC were obtained from the American Type Culture Collection (Rockville, MD, USA), NB4, NB5 and NB14 were initially established and characterized at our institution. All cell lines were maintained in culture flasks using RPMI 1640 medium supplemented with 10% bovine calf serum, L-glutamine and antibiotics (Biowhittaker).

Retroviral vectors

Helper-free retroviral vector-containing supernatants, the kind gift of Y Chiang (Genetic Therapy, Inc., Gaithersburg, MD, USA) were used in all experiments. Both neoR and cytokine genes were inserted into the same Moloney murine leukemia virus retroviral vector backbone either G1Na (neoR), G1il1SvNa (neoR + IL-1beta) or G1T2SvNa (neoR + TNF-alpha). Cytokine genes were expressed under the control of the 5' LTR, the neoR gene being driven from the SV40 early promoter. In the IL-1beta construct, the human growth hormone secretion signal has been added.

Transduction conditions

NB cells were usually plated in 24-well tissue culture plates and allowed to attach overnight. Retroviral supernatant was added at a multiplicity of infection of 1 to 10 in the presence of 4 mug/ml protamine sulfate (titers of retroviral supernatants were 1.4 x 10^5 vp/ml for IL-1beta and 2.9 x 10^3 vp/ml for TNF-alpha). Cells were incubated for 6 hours at 37° C with 5% CO_2. For TNF-alpha gene transfer, two 6 hour rounds of transduction were used. Forty-eight hours after transduction, G418 (GIBCO BRL, Grand Island, NY, USA) was added at a final active concentration of 1 mg/ml, a concentration which has been previously shown to kill all non-transduced NB cells [31]. Once established, the transduced cell lines were maintained continuously in selective medium.

Evaluation of cytokine production

Enzyme-linked immunosorbent assays (ELISA, R&D Systems, Minneapolis, MN, USA) were used to quantify cytokine production. To allow comparison between the levels of secretion by different cell lines, cells were trypsinized after supernatant collection, counted with an hemocytometer and results expressed in pg/24 hours/10^6 cells.

Determination of cell surface phenotype

Flow cytometry analysis (FACScan, Becton Dickinson) was performed in parallel on parental and transduced cell lines using the following antibodies: HLA class I ABC (Olympus, Lake Success, NY, USA), HLA class II DR (Becton Dickinson, San Jose, CA, USA), ICAM I (CD54) and LFA3 (CD58) (AMAC Inc., Westbrook, ME, USA). Isotype-matched antibodies were used as negative controls.

Proliferative characteristics of transduced lines

H^3thymidine incorporation was used to compare the proliferation of transduced and parental lines. Briefly, NB cells were plated in triplicate at 10^4 cells/well. After 72 hours, 1 muCi of H^3thymidine (Amersham, Arlington Heights, IL, USA) was added per well. After an 18 hour exposure, cells were trypsinized, harvested and counted in a beta-counter (1205 Betaplate, LKB).

Activation of peripheral blood mononuclear cells (PBMC) through coculture with transduced lines

NB cells were plated at 0.5 x 10^6 cells/well in 6 well tissue culture plates and allowed to attach overnight. PBMC from normal volunteers were obtained through ficoll hypaque separation of heparinized blood. While the number of NB cells in coculture was kept constant in all experiments, the PBMC to tumor ratio varied from 15/1 to 72/1 depending on the PBMC recovery after ficoll. After 2 to 7 days, suspension cells were harvested and adjusted to the desired cell concentration depending on the assay.

Evaluation of lymphocyte subpopulations

PBMC phenotype was determined before and at day 2 and 7 after coculture by flow cytometry analysis. CD45/CD14 (Dako A/S, Denmark) was used to define the lymphoid gate ($CD45^+/CD14^-$ > 85%); within this gate, the percentage of the different lymphocyte subpopulations were calculated using the following combinations: CD3/CD4, CD3/CD8 (Dako A/S, Denmark), CD3/CD16, CD3/DR (Becton Dickinson, San Jose, CA, USA), CD3/CD45RO (AMAC, Inc., Westbrook, ME, USA).

Cytotoxicity assay

A standard ^{51}Cr release assay was used to quantify cytotoxicity of PBMC before and after 48 hours of coculture with fresh NB cells used as targets [30]. To remove contaminating NB cells, PBMC harvested from coculture as above were submitted to immunomagnetic separation using the NB specific anti-GD2 antibody (IgM, from hybridoma cell line HV 8568, ATCC) and IgM-coated beads (Dynabeads M-450, Dynal AS, Oslo, Norway) except for cocultures with SKNSH-derived cell lines which do not express this antibody. Specific lysis (%) was defined as experimental CPM - spontaneous CPM/maximum CPM - spontaneous CPM x 100; spontaneous release was obtained by incubating targets with complete medium, and maximum release with 1% Triton X100 (Sigma).

Mixed lymphocyte-tumor reaction (MLTR)

10^4 NB cells/well were seeded in 96-well flat bottom plates and irradiated at 100 Gy (dose rate 7,14 Gy/min, cobalt source), a dose shown to inhibit neuroblastoma proliferation and maintain an effective cytokine production at the same time [28]. 2.10^5 fresh PBMC were then added to each well. After a 72 hour incubation

at 37° C, 5% CO_2, 1 muCi of H^3thymidine was added. Following an 18 hour exposure, cells were trypsinized, harvested and counted in a beta-counter (1205 Betaplate, LKB). All experiments were done in quadruplicate and results were analyzed as mean cpm.

Statistics

ANOVA and chi-squared testing were used to assess levels of statistical significance.

RESULTS

Transduced cell lines and cytokine production

All neuroblastoma cell lines were transduced using retroviral supernatant; G418 selection (1 mg/ml active concentration) was started 48 hours after transduction and maintained throughout the study period. None of the NT or neoR cell lines had greater than background secretion of cytokine in any ELISA assay. Both neoR and cytokine transduced - G418 resistant lines were successfully established from all five parental lines.

All cytokine-transduced lines secreted the corresponding protein, however considerable quantitative variations in cytokine synthesis were seen as shown in Figure 1. Cells were maintained in continuous culture in the presence of 1 mg/ml of G418 for a median of 5 months (IL-1beta, 2 lines) or 6 months (TNF-alpha, 3 lines) and then reassessed for cytokine production. A high, although variable, level of secretion was maintained throughout the study period at between 1.19 and 7.36 times the initial level for IL-1beta and between 0.47 and 1.17 times for TNF-alpha. For clinical trials, cytokine secretion also has to be maintained *in vivo* without the use of any selective agent. Transduced cells reassessed by ELISA, 2 and 4 weeks after removal of G418 from the culture medium show persistence of cytokine production ranging from 29 to 166% of the levels obtained in selective medium (Table 1).

Effects of gene expression on NB cells morphology and growth

All neoR-transduced cell lines were morphologically identical to their parental cell line. However, one TNF-alpha transduced cell line (NB4-TNF-alpha) displayed

a terminal neuronal differentiation, and two IL-1beta lines (derived from SKNSH and NB4) a differentiated fibroblast-like appearance as shown in Figure 2. Both led to growth arrest after a few weeks in continuous culture. All remaining lines were morphologically identical to their parental control.

A standard H^3thymidine incorporation assay was used to quantify cell proliferation (Figure 3). The proliferation of cell lines was unaffected by neoR gene transfer ($p = 0.25$). However, all cytokine-transduced lines displayed a significant reduction in their proliferation rate compared to their parental control ($p = 0.0001$). Of the cytokines, the two lines transduced with IL-1beta which developed a fibroblast-like morphology displayed the lowest proliferation index (0-4% of their parental controls). Culture with or without G418 did not influence further the growth rate of neoR- or cytokine-transduced cell lines (data not shown).

Effects on neuroblastoma cell surface phenotype

To further characterize the effects of cytokine gene transduction on the cell lines, the adhesion molecules ICAM I (CD54) and LFA3 (CD58), as well as HLA class I and II molecules have been studied by flow cytometry analysis and the percentage of positive cells was determined for each cell line for the four antibodies (Table 2).

No difference was demonstrable after neoR or cytokine gene transfer for any of the four markers for SKNMC, NB14 and NB5 (Table 2). However, as a consequence of TNF-alpha gene expression, the SKNSH cell line displayed a significant increase in the percentage of cells expressing HLA class I ($p < 0.001$) and CD58 ($p = 0.01$).

Distribution of lymphocyte subpopulations after coculture with transduced cell lines

In order to assess the immunomodulatory properties of cytokine-transduced lines, we first compared the phenotype of PBMC after coculture with cytokine-transduced or control lines. The following antibody combinations were used to label fresh (day 0) and cocultured PBMC (day 2 and 7): CD3CD4 (T helper), CD3CD8 (T cytotoxic), CD16 (NK cells), CD3DR (activated T cells), CD3CD45RO (memory T cells). No phenotype changes were seen at day 2 of coculture (data

not shown). The most dramatic changes in the lymphocyte phenotype were seen after 7 days of coculture, as illustrated by Table 3 for the $CD3^+DR^+$ (activated T cells) subpopulation in both IL-1beta and TNF-alpha lines (65 ± 3% and 47 ± 11% respectively *versus* 24 ± 12% for the parental control, $p < 0.001$ and $p < 0.01$).

Cytotoxicity and proliferative responses of PBMC after coculture

To evaluate functional properties of PBMC after coculture with cytokine-transduced neuroblastoma cell lines, both cytotoxicity and proliferation assays were performed. After 2 days of coculture with the parental and transduced cell lines, suspension cells containing PBMC were harvested, sorted with anti-GD2 antibody and immunomagnetic beads to remove contaminating neuroblastoma cells, and assayed in a 4 hour Cr^{51} assay for their ability to lyse the fresh parental cell line. As shown in Figure 4, none of the PBMC obtained after coculture with IL-1beta or TNF-alpha displayed any significant increase in cytotoxicity against the parental line compared to cocultures with the NT or NeoR lines.

Proliferative responses of allogeneic PBMC stimulated by 72 hours coculture with irradiated transduced lines were studied using H^3thymidine incorporation (Table 4). Coculture with neoR lines did not induce a significant increase in PBMC proliferation compared to parental control. An increase in PBMC proliferation was seen in two out of three cocultures with either IL-1beta or TNF-alpha. However, this effect did not reach statistical significance ($p = 0.16$).

DISCUSSION

Because of the poor results of conventional therapy in disseminated neuroblastoma, we have been studying the value of immunotherapy with cytokine-transduced tumor cells. Previous reports have shown that both primary human NB cells and established lines can be genetically modified to produce either IL-2 or IFN-gamma [23, 28-30, 32]. However, other cytokines, either alone or in combination, may be required for optimal therapeutic benefit. Therefore, transduction of either IL-1beta or TNF-alpha genes was investigated based on the known pleiotropic biological activities of both cytokines, including immunoregulation, induction of inflammatory responses, as well as their antiproliferative and/or differentiation-promoting properties in different tumor models including neuroblastoma [5, 6, 13, 33-39]. In this study, we showed that

after IL-1beta or TNF-alpha gene transfer, most NB cell lines were able to produce large quantities of cytokines over several months in selective medium (Figure 1). As required for the use of transduced lines for gene therapy, cytokine synthesis was stable over several weeks after the selective agent was removed (Table 1).

Two morphologically distinct neuroblastoma subtypes, deriving from the same precursor cell, have long been described in neuroblastoma cell lines [40-42]. The neuroblastic (N) type generally predominates, showing a small cell body with neurite-like processes, while the substrate-adherent (S) type displays either a fibroblast or an epithelial-like aspect and grows more slowly. More recently, a third "I" (intermediate) type has been described from which subclones preferentially display S-type or N-type morphology. Agents such as BrdU, or cytokines such as TNF-alpha or IFN-gamma can either produce phenotypic conversion between subtypes or induce morphological differentiation resulting in the appearance of long neurite extensions [43-45]. In this study, both S-type and N-type differentiations were observed in two IL-1beta lines and one TNF-alpha line respectively (Figure 2). Terminal differentiation of the neuroblasts led to complete growth arrest after less than five passages. Although we cannot totally eliminate the possibility of random subclone selection during the transduction process, due to the low transduction efficiency of neuroblastoma cells, the reproducibility of the same phenotypic modification in two cell lines transduced with the same cytokine suggests that the transferred cytokine gene product has a direct effect on maturation. Since it has been shown that the growth of neuroblastoma cell lines can be increased by exogenous TNF-alpha [46], we were also concerned that cytokine gene transfer might stimulate autocrine tumor growth. In fact, we never found any increase in the growth rate of our transduced cell lines: both IL-1beta and TNF-alpha gene transfer substantially slowed the proliferative rate of the transduced line even in cell lines without detectable morphological changes (Figure 3). While acceleration of tumor cell growth following expression of the transferred cytokine gene would be undesirable from a safety standpoint, profound growth retardation or induction of differentiation are also undesirable since these effects would compromise efforts to expand and validate the transduced lines for use as vaccines, and might lead to the loss of differentiation stage-specific tumor antigens. Establishing autologous tumor cell lines from individual patients at presentation of neuroblastoma is a time consuming procedure with a success rate of 20% or less [29]. Our data show that this problem may be aggravated when the intent is to use tumors transduced with

cytokine genes. Therefore, as reported for IL-2 gene transfer [47], an alternative approach relying on the use of established, partially HLA matched lines chosen on the basis of their ability to grow rapidly even after cytokine gene transfer will be of interest for most clinical trials using cytokine gene-transduced neuroblastoma cells.

One important mechanism by which gene transfer may render tumor cells immunogenic is the modification of the immunoreactive or adhesion molecules they express. The absence or low expression of HLA class I molecules has been observed in a number of tumors including neuroblastoma [48] and may play a major role in preventing an effective presentation of tumor-specific antigens. Indeed, tumor specific immunity has been induced in a number of animal models (including neuroblastoma) following upregulation of HLA molecules expression [11, 44, 48, 49]. We have previously shown that IFN-gamma gene transfer regularly induced a dramatic increase in HLA class I and to a lesser extent in class II expression at the cell surface of NB lines [28]. In this report we demonstrate that a significant increase in both HLA class I and CD58 expression could also be obtained through TNF-alpha gene transfer. However, this effect was limited to one of the transduced cell lines (Table 2).

To evaluate the immunomodulatory properties of cytokine-transduced lines, PBMC from random allogeneic donors were co-cultured with transduced NB lines. By studying the different lymphocyte subpopulations after co-culture with both IL-1beta and TNF-alpha lines we were able to show, after 7 days, a significant increase in the percentage of activated T cells ($CD3^+Dr^+$) whereas NK cells ($CD16^+$), T helper ($CD3^+CD4^+$), T cytotoxic ($CD3^+CD8^+$) subpopulations remained similar to controls (Table 3). In contrast with our previous results with the same IL-2-transduced NB lines using identical experimental conditions [28], no difference in cytotoxicity (Figure 4) or proliferative responses of PBMC against parental cell line (Table 4) could be demonstrated after coculture with either IL-1beta and TNF-alpha transduced lines. Recent studies in different animal models including neuroblastoma have suggested that effective immunotherapy would not only require the transfer of a single cytokine gene but also a second activation step that could be provided through gene transfer of an additional cytokine or a costimulatory molecule such as B7.1 [18-20]. Because of the limited immunomodulatory effects shown in this study after IL-1beta or TNF-alpha gene transfer alone into human neuroblastoma cell lines, alternative strategies should be considered using combination with other

immunomodulatory molecules or, as successfully observed in other tumor models with other gene therapy approaches such as the use of suicide genes [50]. Moreover, comparison of the results obtained in the two clinical trials involving IL-2 gene transfer for neuroblastoma seem to favor the use of adenovirally-transduced autologous tumor cells regarding both the induction of a systemic immune response and the clinical effects [47, 51]. Whether those results reflect an intrinsic conceptual problem of allogeneic vaccines or the influence of many other factors (type of vector, irradiation of cells) remains to be addressed with additional studies. To address this issue and avoid selection of transduced cells, optimization of efficient retroviral vector production strategies to allow stable transduction of NB cell lines and fresh tumor samples is currently under investigation.

CONCLUSION

Acknowledgments. The authors gratefully acknowledge Dr. Malcolm K. Brenner for his help when performing the laboratory work within his program at St Jude Children's Research Hospital, Memphis. We also thank Y. Chiang formerly of Genetic Therapy Inc., Gaithersburg, for providing the retroviral supernatants. This work was supported in part by l'Association pour la Recherche sur le Cancer (C. Coze) and by NIH grants CA21765 (CORE) and CA58211 and by the American Lebanese Syrian Associated Charities (ALSAC).

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Figures

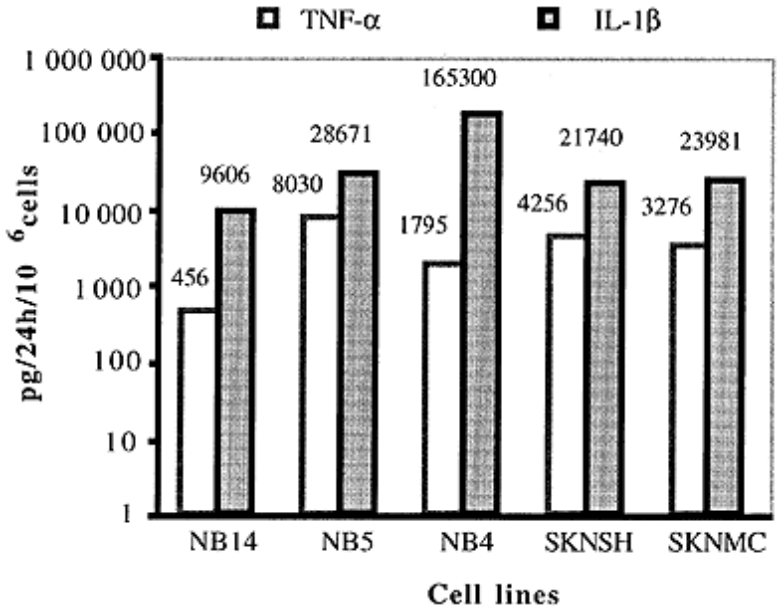


Figure 1

Cytokine production by transduced cell lines. Enzyme-linked immunosorbent assays were used to quantify cytokine production.

To allow comparison between the levels of secretion by different cell lines, cells were trypsinized after supernatant collection, counted with an hemocytometer and results expressed in pg/24 hours/10^6 cells.

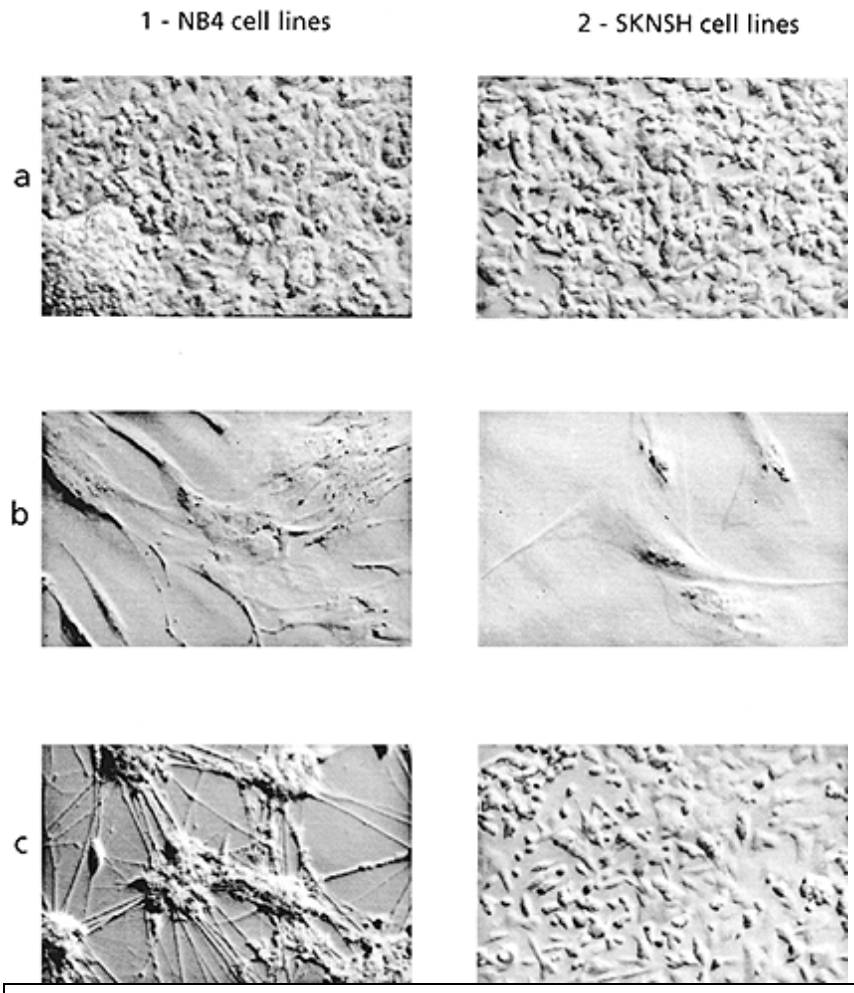


Figure 2

Consequence of cytokine gene transfer on cell line morphology.

1) NB4 cell lines, 2) SKNSH cell lines a) parental non-transduced lines, b) IL-1beta-transduced cell lines, c) TNF-alpha-transduced cell lines. NB4 shows a terminal neuronal differentiation after TNF-alpha gene transfer, and both SKNSH and NB4 show a differentiated fibroblast-like appearance after IL-1beta gene transfer.

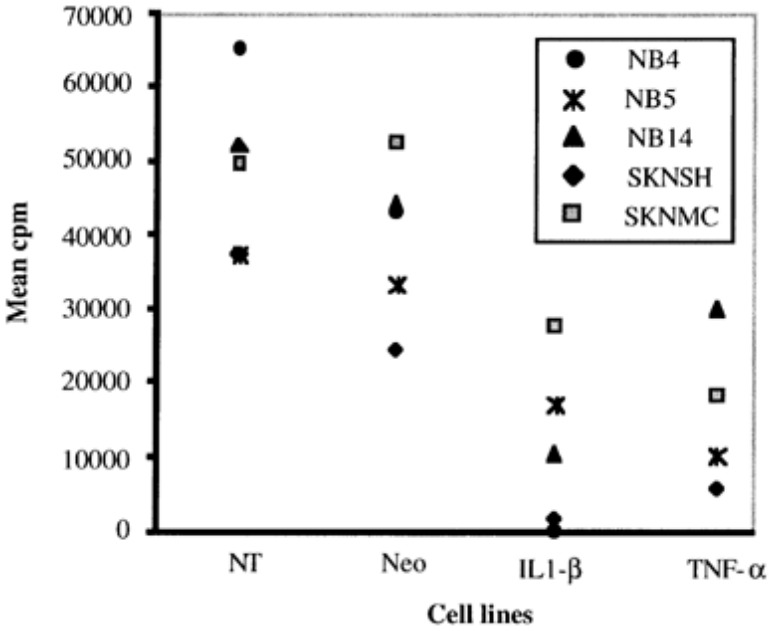


Figure 3

Anti-proliferative effects of IL-1beta and TNF-alpha gene transfer.

NB cells were plated at 10^4 cells/well and 1muCi of H^3thymidine was added after 72 hours; cells were trypsinized, harvested and counted in a beta-counter after an 18 hour exposure. All experiments were done in triplicate and results analyzed as mean cpm. NB4/TNF-alpha not done.

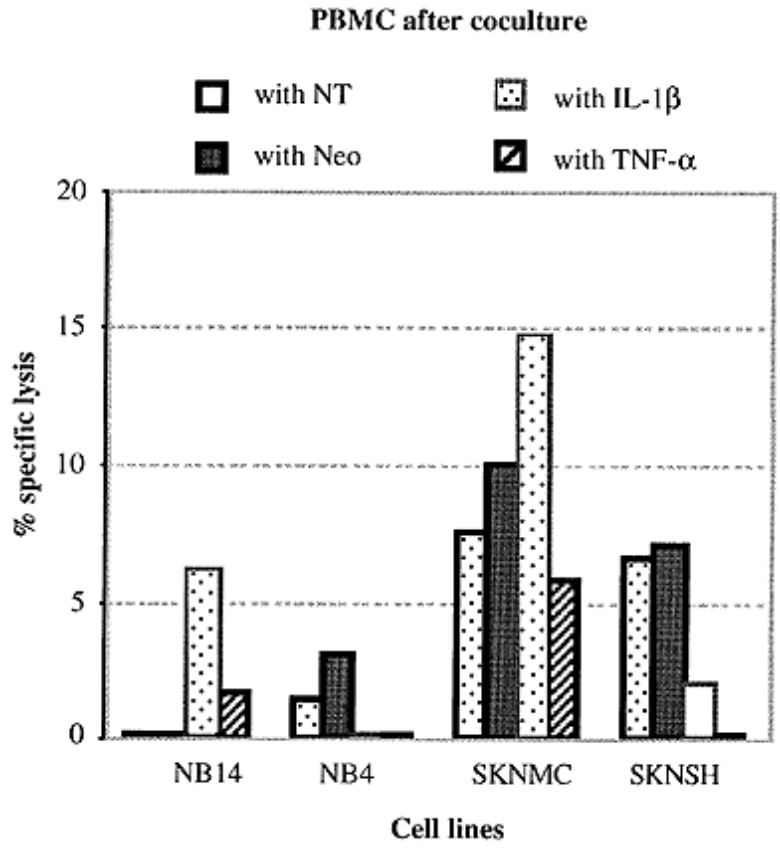


Figure 4

Cytotoxicity of PBMC against parental line after 2 days of coculture with transduced or control lines A standard ^{51}Cr release assay was used to quantify cytotoxicity of PBMC after 48 hours of coculture with fresh parental cells as targets.

Specific lysis (%) is defined as experimental CPM - spontaneous CPM/maximum CPM - spontaneous CPM x 100; spontaneous release was obtained by incubating targets with complete medium, and maximum release with 1% Triton X100. Results are presented as % specific lysis for an effector to target ratio of 40/1.

Table 1
Influence of G418 selection on cytokine production

Cell lines	IL-1 beta		TNF-alpha	
	2 weeks	4 weeks	2 weeks	4 weeks
SKNMC	96%	73%	44%	39%
NB14	46%	29%	166%	69%
SKNSH	ND	ND	50%	103%

ELISA assays were performed 2 and 4 weeks after removal of G418 from the culture medium. Results are expressed as percentage cytokine synthesis compared to a reference supernatant obtained the same day from the corresponding line kept under selective pressure. ND: not done.

Table 2
Effect of cytokine gene expression on cell surface phenotype of neuroblastoma cell lines

Cell lines		CD58	HLA ABC	HLA DR
SKNMC	NT	99	84	0
	Neo	99	86	0
	IL-1beta	100	85	0
	TNF-alpha	99	94	0
SKNSH	NT	54	16	0
	Neo	57	24	0
	TNF-alpha	75[1]	45[2]	1
NB14	NT	50	1	1
	Neo	48	0	0
	IL-1beta	68	4	2
	TNF-alpha	64	1	2
NB5	NT	97	81	0
	Neo	98	84	0
	IL-1beta	98	82	0
	TNF-alpha	ND	72	2

Results obtained by flow cytometry analysis are expressed as percentage of positive cells. Significant difference in chi-squared test: [1] $p = 0.01$ and [2] $p < 0.001$. ND: not done.

Table 3
Distribution of lymphocyte subpopulations after coculture with cytokine-transduced lines

	CD3	CD3CD4	CD3CD8	CD16	CD3DR	CD3CD45RO
Day 0	71	45	21	12	20	45
Day 7						
NT	84	58	25	5	24	55
Neo	82	57	25	5	28	56
IL-1beta	82	72	18	3	65[1]	69
TNF-alpha	85	68	20	4	47[2]	56

PBMC from different normal donors (IL-1beta: 3 experiments, TNF-alpha: 4 experiments) have been assayed and the percentage positivity obtained from all experiments averaged. Significant difference in chi-squared test: [1] $p < 0.001$ and [2] $p < 0.01$.

Table 4
Mixed lymphocyte-tumor reaction against irradiated transduced cell lines after 3 days of coculture

	H^3thymidine incorporation (mean cpm)			
	NB14	SKNSH	NB5	NB4
PBMC + NT	861 ± 89	658 ± 169	644 ± 303	1,977 ± 386
PBMC + Neo	558 ± 93	464 ± 237	470 ± 125	1,063 ± 436
PBMC + IL-1β	1,411 ± 137	ND	1,710 ± 803	1,249 ± 390
PBMC + TNF-α	707 ± 126	1409 ± 1075	1,380 ± 497	ND

All experiments were done in quadriplicates and results expressed as mean cpm ± standard deviation. ND: not done.

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IL-10, recombinant, rat, cytokines.

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