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An Experimental Study on the Cytolytic Effects of Natural Killer cells on Various Types of Lung Carcinoma Cell Lines with Augmented HLA Expressions by Gamma Interferon

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=Abstract=**Lung carcinoma cell lines (NCI-N417, NCI-N146, NCI-H69, PC-14) were evaluated on their expression of HLA-class I with W6/32 antibody and HLA-class II with L243 by flow cytometry. Also examined were the killing and/or cytolytic effects of allogeneic NK cells and T lymphocytes as effector cells to the lung carcinoma cell lines by chromium release assay, before and after treatments with gamma interferon.**

HLA class I expressions significantly increased after treatment with gamma interferon (P 〈 0.05), whereas HLA class II expressions showed no differences (P 〉 0.05). The killing and/or cytolytic effects of allogeneic NK cells very significantly decreased after treatment with gamma interferon (P 〈 0.001), while those of the T lymphocytes showed either no change or a tendancy to decrease (P 〉 0.05).

Key Words: *Lung carcinoma cell line, Major histocompatibility complex, Gamma interferon, NK cell*

INTRODUCTION

Natural killer cells, cytotoxic T lymphocytes, and mononuclear cells are well-known defensive cells of neoplasms in humans. Natural killer cells are known to be independent cells on major histocompatibility complex receptor, but cytotoxic T lymphocytes are dependent cells. Morphologically, natural killer cells are slightly larger than small lymphocytes and contain fine granules in the cytoplasm. Cytotoxic T lymphocytes are well-known to have T5 or T8 antigen on their surface membranes. All the nucleated cells are known to express HLA antigens on their surfaces. However, the neoplastic cells, especially malignant cells, show diminished expressions (Doyle *et al.*, 1985). In pulmonary neoplasms, the expressions of HLA class I antigens are reported to be significantly diminished in the small cell carcinoma but diminished to a lesser degree in adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (Markman *et al.*, 1984; Ball *et al.*, 1986). The killing effects of the effector cells on the neoplastic cells are varied, and there are individual exceptions. The killing effects of such effector cells on neoplastic cells in vitro are reported to be augmented after the treatment with either gamma interferon or interleukin-2 (Weyants *et al.*, 1988; Robinson & Morstyn, 1987). However, in clinical situations, the killing effects were lower than expected. To find out the reason for this, we attempted, in this experiment, the target cells (lung carcinoma cell lines) were

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treated with gamma interferon and observed the killing and/or lytic effect of NK cells.

MATERIALS AND METHODS

Lung carcinoma cell lines NCI-N417, NCI-N146, NCI-H69, and PC-14 were used for the target cell lines, and for the effector cells, NK cells and T cells were harvested from young healthy volunteer donors.

As the culture media, RPMI 1640 with antibiotics was used after the addition of 10% fetal calf serum.

W6/32 mouse monoclonal antibody for HLA class I and L243 mouse monoclonal antibody for HLA class II from Becton-Dickinson Laboratories and rabbit FITC labeled anti-mouse antibody (IgG class) were used to evaluate their expressions by flow cytometry (FACS scanner, Becton-Dickinson Laboratories).

To harvest NK and T cells, Leu-19 and OKT 3 monoclonal antibodies with rabbit polyclonal anti-mouse antibody as the secondary antibody were used.

Recombinant gamma interferon was from the Genzyme Company.

1. Kinetics of HLA class I expressions of lung carcinoma cell lines:

Freeze-thawed lung carcinoma cell lines were cultured for the duration of 0,1,2,3, and 4 days after treated with gamma interferon and tested for HLA class I expression. From cultured cell lines 0.2 x 10^6 cells/ml were prepared and measured HLA class 1 expressions by flow cytometry. Maximum results were obtained after 1 day (Table 1). To be sure, 2-day cultured cell lines were chosen. Since 200 units of gamma interferon had been widely used and recommended by the company, it was decided that the concentration of gamma interferon used in this experiment would be 200 units.

Table 1. Results of kinetics of HLA class I expressions of lung carcinoma cell lines by cultured days: After 1 day culture, the expressions were increased and remained high.

(% positive cells)

	Day 0	Day 1	Day 2	Day 3	Day 4
NCI-N417	17	54	76	62	20
NCI-H69	14	77.5	69	76.5	53
PC-14	95	98	95	93	73

2. Changes of HLA class I and II antigen expressions before and after gamma interferon treatment:

Of the tumor cells 5 X 10^5 cells were treated with 200 unit/ml of gamma interferon and measured the HLA class I and II expressions by flow cytometry and compared to the untreated groups previously measured. As for the primary antibody, W6/32 of anti-HLA class I and L243 of anti HLA class II were used and for the secondary antibody FITC labeled anti-mouse immunoglobulin G, composed only of Fab without Fc component.

3. Separations of NK cells and T lymphocytes of peripheral blood:

1) Separation of mononuclear cells: The blood from healthy volunteer donors was collected, and the mononuclear cell layers were separated with Ficoll-Hypaque and diluted with heparin-treated RPMI 1640 media in a ratio of 1 : 1. The collected mononuclear cell layer was washed three times with 1 x phoshate buffered saline (PBS) containing 2% fetal calf serum. From this, 5 ml of 5 x 10^6 cells/ml of mononuclear cells was poured into a petri dish (10cm in diameter) and incubated in a 5% CO_2 incubator at 37℃ for 1 hour. Then the nonadherent cells were harvested and used in the next steps.

2) Separation of NK cells: 10ul of 10^7 cells were mixed with L243 (anti-HLA-DR), markers of B cell and monocytes, and reacted to B cells, monocytes, and OKT3 antibody for T cells. It was then poured into a plastic plate previously coated with goat anti-mouse antibody and incubated at 4℃ for 90 min. A supernatant was obtained, which contained only NK cells that were used in this experiment.

3) Separation of T cells: The procedure was almost the same as above except for using leu-19 for the NK marker in place of OKT3 pan-T-antibody. The supernatant contained both T4 and

T8 cells.

4. Chromium release assay to evaluate the lytic effect of the target cell lines-the lung cell lines by the effector cells; NK cell and T cell:

1) Labeling of chromium to target cell lines: 100 uCi/ml of $Na^{2}Cr^{51}O^{4}$ were added to the lung carcinoma cell lines and incubated at 37℃ in a 5% CO^{2} incubator for 1 hour.

2) The above chromium labeled target cell lines were divided into 3 groups (Group 1 for spontaneous release, Group 2 and 3 for experimental releases) of 2 pairs (experimental groups and control groups) of 5 x 10^{3} cells.

3) 1 x 10^{5} effector cells were added to the wells previously prepared in the above procedure and incubated at 37℃ for 4 hours.

4) Then, to the other pairs of chromium-labeled lung carcinoma cell lines, 10% sodium dodecyl sulphate was added to induce the complete lysis of the carcinoma cell and released all the chromium from the carcinoma cells that had been labeled (maximum release).

5) % lysis was calculated as follows:

$$\frac{\text{experimental chromium}^{51}\ \text{release-spontaneous release}}{\text{maximum chromium}^{51}\ \text{release-spontaneous release}} \times 100 = \%\ \text{lysis}$$

5. Statistical analysis:

Utilized student "t" test.

RESULTS

1. Gamma interferon enhanced the expression of HLA class I antigen in lung carcinoma cell lines (P 〈 0.05) (Table 2). However, it did not influence the expression of HLA class II antigen (P 〉 0.05) (Table 3).

2. The lytic effect of the NK cells significantly decreased on the lung carcinoma cell lines that had been treated with gamma interferon (P 〈 0.001) (Table 4, Figure 1), whereas the T cells showed either no significant lytic effect or a tendency to decrease the lytic effect on the gamma interferon-treated group (P 〉 0.05) (Table 5, Figure 2).

Table 2. Results of HLA-class I expressions of lung carcinoma cell lines before and after gamma interferon treatments, assayed by flow cytometry (FACS): HLA class I expressions were significantly increased after gamma interferon treatments than before treatments (P 〈 0.05).

		No interferon (%)	With interferon (%)	Difference (%)
NCI-N417	1*	24.5	36.4	+11.9
	2˙	36.1	55.2	+19.1
NCI-N146	1	31.6	51.8	+20.2
	2	47.9	76.8	+28.9
NCI-H69	1	28.2	37.9	+ 9.7
	2	ND˙˙	52.7	ND
PC-14	1	41.5	43.3	+ 1.8
	2	57.2	80.6	+23.4

* : the result of the first experiment
˙ : the result of the second experiment
˙˙: not determined due to cell death

Table 3. Results of HLA class II expressions of lung carcinoma cell lines before and after gamma interferon treatments: there were no significant difference (P 〈 0.05)

		No interferon (%)	With interferon (%)	Difference (%)
NCI-N417	1*	33.0	25.7	− 7.3
	2⁺	22.8	24.6	+1.8
NCI-N146	1	32.9	31.5	− 1.4
	2	29.9	68.3	+38.4
NCI-H69	1	19.9	21.1	+ 1.2
	2	19.1	18.1	− 1.0
PC-14	1	17.6	20.9	+ 3.3
	2	11.6	ND⁺⁺	ND

* : the result of the first experiment
⁺ : the result of the second experiment
⁺⁺: not determined due to cell death

3. The NK cells seemed to have no relationship to HLA expression of the lung carcinoma cell lines but rather to have a reverse relationship be-

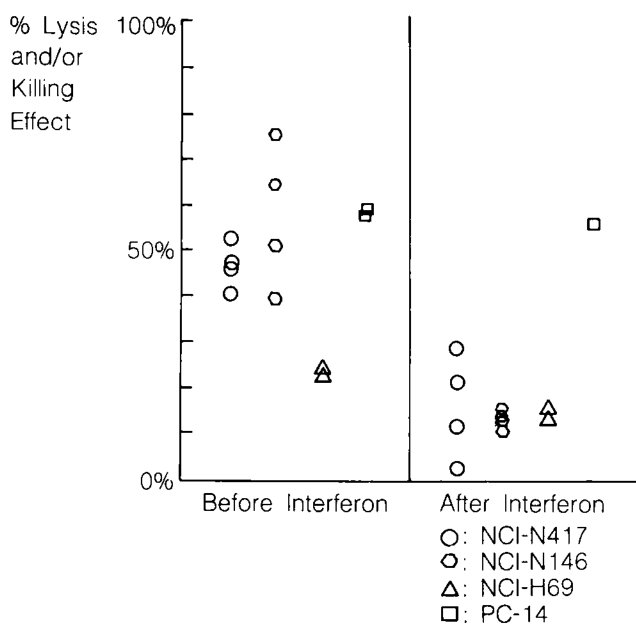

Fig. 1. Graph of NK cell killing effects on lung carcinoma cell lines, assayed by chromium release assay

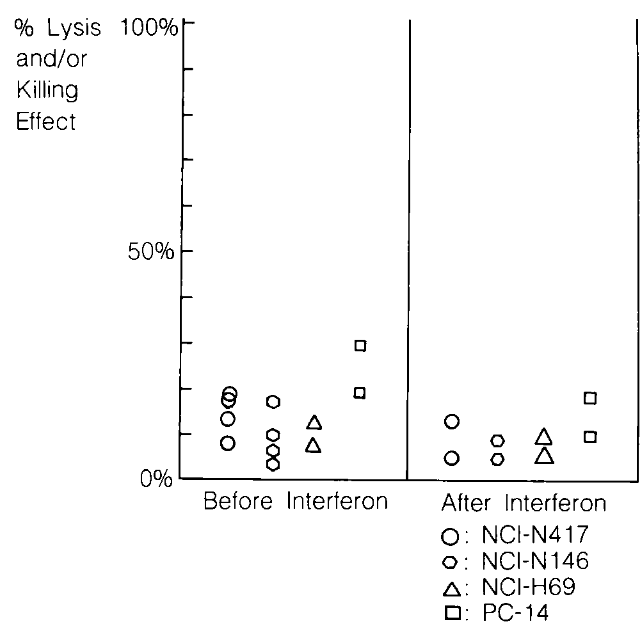

Fig. 2. Graph of T cell killing/cytolytic effects on lung carcinoma cell lines, assayed by chromium release assay

Table 4. Results of NK cell killing effects to lung carcinoma cell lines, assayed by chromium release assay: the killing effects were very significantly increased after gamma interferon treatments than before treatments ($P < 0.001$)

		No interferon (%)	With interferon (%)	Difference (%)
NCI-N417	1a*	46.0	20.7	−25.3
	1b'	48.0	29.3	−18.7
	2a''	52.6	12.7	−39.9
	2b §	40.7	2.5	−38.2
NCI-N146	1a	75.4	11.6	−63.8
	1b	64.3	15.8	−48.5
	2a	50.9	12.3	−38.6
	2b	39.2	13.4	−25.8
NCI-H69	1a	24.3	13.1	−11.2
	1b	24.0	15.7	− 8.3
	2a	ND‖	ND	ND
	2b	ND	ND	ND
PC-14	1a	58.8	56.3	− 2.5
	1b	57.9	ND	ND
	2a	ND	ND	ND
	2b	ND	ND	ND

* : the result of the first assay of the first experiment
' : the result of the repeated assay of the first experiment
'': the result of the first assay of the second experiment
§: the result of the repeated assay of the second experiment
‖ : not determined due to cell death

Table 5. Results of T cell killing/cytolytic effects to lung carcinoma cell lines, assayed by chromium release assay before and after gamma interferon treatments: the killing effects showed no significant difference ($P < 0.05$)

		No interferon (%)	With interferon (%)	Difference (%)
NCI-N417	a*	7.9	4.7	− 3.2
	b+	13.1	12.4	− 0.7
NCI-N146	a	16.0	4.6	−11.4
	b	3.5	8.6	− 5.1
NCI-H69	a	6.4	9.2	+ 2.8
	b	ND	4.8	ND
PC-14	a	29.3	18.0	−11.3
	b	19.3	10.6	− 8.7

* : the result of the first assay of the experiment
' : the result of the repeated assay of the experiment
++: not determined due to cell death

tween the expression and the lytic effect of the NK cells.

DISCUSSION

A cell line is usually obtained through serial cultures of fresh biopsied minced specimen being

cultured in nonselective media with 10% fetal calf serum. From this method the success rate is usually 10%. However, Carney *et al.* (1988) report that in the case of small cell carcinoma of the lung, if hormones are added to this media, the success rate could be improved to approximately 75%. The hormones used include hydrocortisone, insulin, transferrin, estradiol, and selenium. In nonsmall cell carcinoma, the culture is usually done in an attached monolayer culture, and the success rate from the fresh biopsed specimen is very low. However, if the hormone ACL-3 and the attachment factor were added and the biopsy was obtained from the metastatic lesion, Knowles (1986) reports the success rate improves to 40%.

Small cell carcinoma cell lines of the lung are largely divided into 2 separate groups: classical and variant. The classical cell line contains more L-dopa, decarboxylase, bembesin/gastrin releasing peptide, neuron specific enolase, creatine kinase BB, relatively long doubling time, low cloning efficiency and sensitivity to radiation, and morphologically shows the characteristics of the intermediate cell type. The variant group shows low or absent L-DOPA, absent bombesin/gastrin-releasing peptide but increased NSE and CK-BB and shows a faster growth than the classical cell line, is resistant to radiation, and morphologically has similar characteristics to the large cell undifferentiated carcinoma and shows 4 to 60 times increased c-myc of DNA (Knowles, 1986). In the nonsmall cell carcinoma cell lines of the lung are not well known, but Knowles (1986) reports that 15-20% of these cell lines show an increased L-dopa and DDC and the characteristics of neuroendocrine tumor.

MHC class I antigen expression of small cell carcinoma of the lung, Knowles (1986) reports, was absent or much lower than that of nonsmall cell carcinoma of the lung, but in the molecular study there was no damage in the molecular level, and recovered its expression of HLA class I if treated with gamma interferon, that agrees with our result, and he observed the same phenomenon in vivo. Currently the absent or diminished MHC antigen expression of the tumor cell lines was in part believed to be related to the early metastasis of this tumor.

Basham *et al.* (1983) and Houghton *et al.* (1984) report that with gamma interferon, HLA-DR expression could be induced in the cell lines with HLA-DR negative. Masuyama *et al.* (1986) report that with gamma interferon they could enhance the HLA-DR expression of endothelial cells, and Pfizenmaier *et al.* (1985) report that with gamma interferon, in 7 human colonic cell lines, they could enhance the expressions of HLA-A, B, C in all the cell lines as well as HLA-DR expression in 3 out of 7 cell lines. Carrel *et al.* (1985) report that in the melanoma cell line, with recombinant gamma interferon, IL-2 and other lymphokine were also the enhancing substances to MHC expressions. Cohen *et al.* (1987) report that, in 1 case of follicular lymphoma, 6 cases of melanoma, 1 case of breast carcinoma, 1 case of renal cell carcinoma, IL-2 could increase the HLA-DR expression and showed no therapeutic effect, but in the case of combined therapy IL-2 and other lymphokine could increase the lytic effect.

Meuer *et al.* (1982) report that the allosensitized T4 lymphocytes gained the ability to kill MHC class II antigen expressed cells and the allosensitized T8 lymphocyte antigen gained the ability to kill MHC class I antigen. It has been known that the NK cells are independent to MHC antigen. Weyants *et al.* (1988) report that the lymphocytes sensitized by the cultured variant cell lines of the small cell carcinoma of lung did not have the ability to kill the small cell carcinoma cell lines of the lung, but after treatment with gamma interferon the killing effect was observed as well as the increased expression of HLA class I antigen. However, Ball *et al.* (1986) report the opposite result. In their experiment when small cell carcinoma cell lines of the lung treated by gamma interferon were cultured with allogeneic peripheral lymphocytes, the killing effect decreased. This result is similar to the result of our experiment. Pfizernmaier *et al.* (1985) report that they observed an increased expression of HLA class I and class II of colon carcinoma cell lines after being treated by gamma interferon, as well as a resistance to allogeneic NK cells and cytotoxic T lymphocytes. Gomi *et al.* (1986) report that they observed in melanoma cell lines that

recombinant gamma interferon increased the expression of HLA A, B and was positively related to the resistance of the tumor cell lines. Harel-Ballan *et al.* (1986) report that the increased expression of HLA antigen and the sensitivity of NK cells, in Epstein Barr virus transformed B lymphocyte cell lines were of a reverse relationship. Lobo *et al.* (1989) report that after the HLA antibodies reacted to the lymphoid system and to various solid tumor cell lines, and masked the antigenic sites of the surfaces of tumor cells, the killing effect of the NK cells increased.

In our experiment, gamma interferon enhanced the expression of HLA class I ($P < 0.05$) but did not significantly increase the expression of HLA class II ($P > 0.05$). The NK cells showed a decreased killing effect in the group of the increased HLA expression treated by gamma interferon ($P < 0.001$). The T lymphocytes did not show any significant changes of the killing effect in the tumor cell lines treated by gamma interferon but showed a tendancy to decrease the killing effect ($P > 0.05$).

In conclusion, gamma interferon appears to enhance the resistance of the tumor to being killed. The result of this experiment may explain the reason why the killing effects on cancer with gamma interferon in vivo trials, in case that it is expected that enhance the killing effect of only T and/or NK cells, show the different and/or decreased effect of the result compared with that in vitro experiments.

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=국 문 초 록=

인체 폐종양 세포주에서 MHC 항원 표현 정도와 자연살세포(NK)의 세포 살해 효과와의 상관관계에 관한 연구

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이갑노 · 박성회 · 함의근

종양을 살해 또는 융해시키는 인체내의 세포로서 자연살세포(Natural killer cell)와 세포상해성 살해세포(cytotoxic killer cell)가 알려져 있고 이 세포들은 감마 인터페론으로 처리하면 그 기능이 항진되는 것으로 알려져 있으나, 실제로 임상에 적용시 그 효과가 기대치보다 저하되거나 전혀 효과가 없는 경우가 있는데 그 이유에 대하여는 알려진 바 없다. 주요 조직적합복합체(Major Histocompatibility Complex) 항원은 세포상해성 T 세포의 수용체로서 그 역할이 알려져 왔으나, 자연살세포에 대한 역할은 확실치 않고, 자연살세포의 살해기전에 대하여도 정확히 알려진 바 없다. 이에 저자들은 종양 살해효과에 대한 주요 조직복합체 항원의 역할과 자연살세포의 역할과의 관계를 이해하고자, 인체 폐종양세포주 4종(3종의 미분화 소 세포암종 ; NCI-N417, NCI-N146, NCI-N69와 1종의 선암종 ; PC-14)을 대상으로, 감마 인터페론으로 처치하기 전, 후에 주요 조직적합복합체 항원의 표현정도를 유세포측정법으로 측정하였고, 이어서 이들을 자연살세포와 혼합배양한 후 자연살세포의 세포 살해효과를 방사선 동위원소 크로뮴 측정법으로 관찰하여 다음과 같은 결론을 얻었다.

1. 4종의 인체 폐종양 세포주에서의 HLA class I과 class II 항원의 표현을 관찰하여, 감마 인터페론은 HLA class I 항원의 표현을 항진시키나 ($P < 0.05$), HLA class II 항원의 표현에는 영향을 주지 않음을 알아냈고 ($P > 0.05$),

2. 종양세포의 살해에 작용하는 효과 세포들을 관찰함에 의하여, 자연살세포는 T 림프구보다 살해효과가 우수하였으며, 감마 인터페론으로 처치 하였을 경우에는 자연살세포의 살해효과가 현저히 감소하였고($P < 0.001$), T 림프구의 살해효과에는 별다른 영향을 주지 않거나 다소 감소하는 경향이 있음이 관찰되었다($P > 0.05$).

3. 자연살세포는 주요 조직적합복합에 항원 특히 종양 세포의 HLA class I의 표현이 증가하면 종양 살해효과가 감소하는 것이 관찰되어, 자연살세포의 종양 살해효과와 주요 조직적합복합체 항원 표현정도는 역상관 관계에 있는 것이 아닌가 추리되었다. 이는 감마 인터페론의 임상적용시의 문제점인 종양세포의 저항성에 대한 한 설명이 될 수 있지 않을까 사료된다(Key words ; 폐 종양세포주, 주요 조직복합체 항원, 감마 안터페론, 자연살세포).