Immune Response Against Genetically Modified Tumour Cells

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Introduction

Theoretically, the immune system should be able to recognize and destroy spontaneously arising malignantly transformed cells. This hypothesis has been formulated as immunosurveillance. Assuming that the concept of immunosurveillance is valid, immune effector cells such as cytotoxic T lymphocytes (CTL) and natural killer (NK) cells must be able to recognize tumour antigens and mediate the killing of malignant cells. In order to be recognized by the immune system tumour cells must express specific tumour antigens (1). Unfortunately, most tumour cells do not express any potent antigens, which could be able to induce efficient anti-tumour immune response (2). Although many malignant cells are weekly immunogenic, there are numerous examples of tumour antigens that can stimulate strong immune response. Despite potential immunogenecity of some tumour cells, they are still able to evade immune destruction because many mechanisms can lead to the so called "tumour escape". In many experimental systems different approaches have been used for immunotherapy of cancer. Nonspecific stimulation of immune response against tumour cells can be achieved with adjuvants such as the bacilli Calmette-Guerin (BCG) mycobacterium injected at the site of tumour growth. Specific stimulation of anti-tumour response has been tried by immunisation with modified malignant cells. The rationale for this specific approach is based on the assumption that immune response to the modified tumour cells will then be effective on the unaltered tumour cells. Tumour cells can modified by different ways in order to increase their

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immunogenecity. Infections of the cells with nonpathogenic viruses, treatment with mutagens or other chemical agents and hybridization with allogeneic cells are among the approaches used. In most cases, the combination of tumour antigens with antigens foreign to the host, stimulates anti-tumour response that exceeds the one induced by inactivated tumour cells alone. In the last years, transfer gene technology has been used to generate modified tumour cells that are able to induce potent immune response. In cancer gene therapy the immunogenecity of tumour cells can be increased by transfer of genes encoding for major histocompatibility complex (MHC) antigens or genes for various cytokine and their receptors (3). Most tumour cells express very low level of MHC antigens and thus these cells are not able to induce efficient specific anti-tumour immune response. Transfer of cytokine genes resulted in the production of biologically active molecules which can augment local or systemic immune response (4). Cytokines may enhance both specific and non-specific immune response (5). In specific immune response usually CTL directed to cancer cells is generated. In addition to CTL, specific anti-tumour antibodies can also be produced. Enhancement of non-specific immune response by various cytokines resulted in an increased activity of NK cells, macrophages, production of other cytokines and various inflammatory active substances directed non-specifically against tumour cells (6).

In the first part of the paper the experiments dealing with the decreasing tumorogenecity by the transfer of heterologous melanoma associated antigen (MAA) gene into mouse melanoma cells is briefly summarised. Next part of the paper is devoted to the problem of enhancing tumour immunogenecity by transfer of cytokine genes, namely IL6 and sIL6R genes into melanoma cells. This includes preclinical study in the animal experimental system and some preliminary results of the clinical trial.

2. Material and methods

Mice

The following strains have been used: C57BL/6, C57BL/6xCH3 and SCID CB17.

Cells

The following tumour cell lines were used: B78H1 a subclone of B16 melanoma cell line (kindly provided by L.H. Graf Chicago Ill,USA), Ui11-transfected B78H1 cells expressing human ICAM 1, WM9 and WM239-human melanoma cell lines, K562 — human chronic myelogenous leukaemia cell line, YAC1-mouse lymphoma cell line.

Transfection of B78H1 mouse melanoma cells with genomic DNA from human melanoma cells and selection of transfectans (Ui11 cells) expressing a human ICAM 1 antigen has been described elsewhere (7,8). Transfection of B78H1 cells with human IL6, murine sIL6R and human LIF as described earlier (9).

Analysis of lymphocyte surface antigens and receptors has been carried out by direct immunofluorescence using a panel of monoclonal antibodies (Dako, Immunotech) labelled with FITC and specific to CD4, CD8, CD16, CD19, CD25, HLA DR; and labelled with PE and specific to CD3 and CD56. PBL were stained with monoclonal antibodies by standard technique. Evaluation of stained cells was performed using Ortho Cytoron analyzer (Ortho Diagnostics).

Cell mediated cytotoxicity and natural killer (NK) cell assay. A standard 4 h — specific ^{51}Cr-release assay was performed using ^{51}Cr-labelled target cells and varying numbers of mouse spleen cells or human peripheral blood lymphocytes as effector cells. The results were expressed as cytotoxicity indices calculated as follows:

$$\frac{\text{test } ^{51}\text{Cr release} - \text{spont. } ^{51}\text{Cr release}}{\text{max } ^{51}\text{Cr release} - \text{spont. } ^{51}\text{Cr release}} \times 100\%$$

As the so called "anti-cancer-vaccine", both melanoma cell lines transfected with IL6 and sIL6R genes as well as autologous melanoma cells were used as described earlier (10).

3. Results and discussion

3.1. Immune response and tumour genecity of genetically modified mouse melanoma cells

The crucial point was to answer the question how genetic modification of the tumour cells can enhance host immunity. Human MAA, which is an intercellular adhesion molecule 1 (ICAM 1), was transferred into B78H1 mouse melanoma cells. The effect on tumourgenecity and immune response against transfected melanoma cells was studied.

Human ICAM 1 expressed by mouse melanoma cells appeared to be highly immunogenic leading to the rejection of the modified mouse melanoma cells. To investigate which immunological mechanism is responsible for the rejection of the mouse melanoma cells expressing human ICAM 1, cytotoxic activity of spleen lymphocytes from the mice immunised with transfected Ui11 cells expressing human MAA (ICAM 1) toward B78H1 mouse melanoma cells has been assessed in 4 h chromium release assay. The representative results of cytotoxicity toward mouse melanoma are presented in Figure 1.

Cytotoxicity was measured on ^{51}Cr-labelled Ui11 target cells 7 days after 3rd injection. Ui11- mouse melanoma cells expressing human ICAM 1; B78H1-nontransfected mouse melanoma cells. Spleen lymphocytes from unimmunised mice were used as control.

Cytotoxic activity of spleen lymphocytes immunised with transfected or nontransfected cells was higher when transfected cells were used as targets.

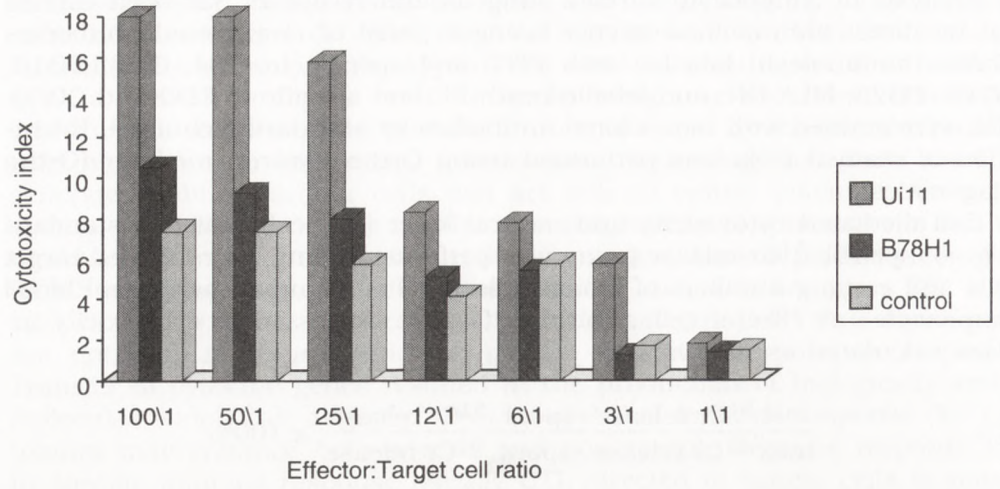

Fig. 1. Cytotoxic activity of spleen lymphocytes of C57BL/6 mice immunised with Ui11 or B78H1 cells against Ui11 cells as target. Effect of various effectors: target cell ratio on cytotoxic activity.

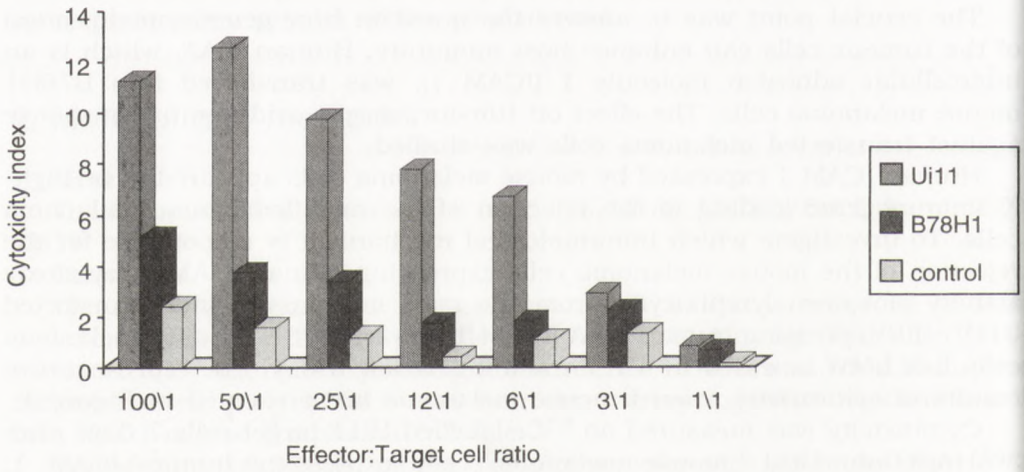

Fig. 2. Cytotoxic activity of spleen lymphocytes of C57BL/6 mice immunised with Ui11 or B78H1 cells against B78H1 cells as target. Effect of various effectors: target cell ratio on cytotoxic activity.

Using the same chromium release assay it was found that mouse melanoma cells modified by human ICAM 1 were strongly immunogenic in contrast to the non transfected B78H1 cells. The representative results of cytotoxicity directed against transfected or non-transfected B78H1 cells are given in Figure 2.

Cytotoxicity was measured on ^{51}Cr-labelled B78H1 target cells 7 days after 3rd injection. Ui11- mouse melanoma cells expressing human ICAM 1; B78H1-nontransfected mouse melanoma cells. Spleen lymphocytes from un-immunised mice were used as control.

Much higher cytotoxicity indices were observed when transfected Ui11 cells were used. This finding suggests that transfected Ui11 cells are more susceptible to NK lysis.

This part of the study showed that melanoma cells expressing human MAA (ICAM 1) completely lost tumourgenecity. Human ICAM 1 expressed on mouse melanoma cells appeared to be highly immunogenic augmenting anti-tumour specific (CTL killing) and non-specific (NK killing) response. Unfortunately, in this experimental system, the generated specific and non-specific immune response against heterologous antigens was not sufficient to prevent the growth of parental tumour cells.

3.2. The effect of IL6 and sIL6R gene transfection of mouse melanoma cells on specific and nonspecific antitumour immunoresponse

In many preclinical and clinical studies enhancing tumour immunogenecity leading to the induction of specific CTL or NK cells may be enhanced by the transfer of HLA genes into tumour cells. In human gene therapy the transfer of various genes for cytokines and their receptors into tumour cells can be used for enhancing immune response against tumour. One of the aims of the preclinical study was to find out the way in which genetic modification of tumour cells can be applied to human cancer gene therapy. The B78H1 mouse melanoma cells were transfected with IL6, sIL6R or LIF genes. The transfected and nontransfected cells were injected subcutaneously and the tumour growth and immune response were monitored. Specific cytotoxic T lymphocyte activity as assessed in 4 h chromium release assay has was relatively low in all animal group studied (Tab. 1). Non-transfected B78H1 cells and various transfected cells were used as targets.

In another series of experiments, effector cells isolated from mice immunised with sIL6R gene transfected B78H1 melanoma cells tested at various effector:target cell ratio also showed relatively low level of specific cytotoxicity against parental B78H1 cells as well as against target cells modified by transfection of sIL6R and LIF genes. Representative results are given in Table 2.

TABLE 1
CTL ACTIVITY OF SPLEEN CELLS FROM MICE INJECTED WITH NONTRANSFECTED AND TRANSFECTED B78H1 MELANOMA CELLS

Effector cells	Target cells			
	B78H1	B78H1-IL6	B78H1-sIL6R	B78H1-LIF
B78H1	1.4	1.2	2.1	2.9
B78H1-IL6	2.3	3.1	2.2	5.1
B78H1-sIL6R	5.1	3.7	6.4	6.8
B78H1-LIF	6.9	3.1	5.8	8.2

TABLE 2
CTL ACTIVITY OF SPLEEN CELLS FROM MICE INJECTED WITH SIL6R GENE TRANSFECTED MELANOMA CELLS

Effector cells: Target cells	Target cells		
	B78	sIL6R	LIF
50:1	9.4	11.2	6.4
25:1	3.0	4.8	1.6

On the basis of the obtained results it can be postulated that genetic modification of mouse melanoma cells with IL6, sIL6R and LIF genes transfection gives a moderately augmenting effect on specific anti-tumour immune response mediated by CTL.

TABLE 3
NK ACTIVITY OF SPLEEN CELLS FROM MICE IMMUNISED WITH TRANSFECTED MELANOMA CELLS

Effector cells	Effector: target cell ratio					
	3:1	6:1	12:1	25:1	50:1	100:1
B78H1-IL6	4.3	5.8	11.2	17.0	20.5	23.0
B78H1-sIL6R	1.3	5.9	12.8	14.3	17.2	24.4
B78H1-IL6/sIL6R	1.6	3.9	8.5	12.9	17.0	20.2
B78H1-IL6+sIL6R	3.7	8.5	18.0	23.7	26.0	32.0
B78H1	0.6	4.0	5.6	7.9	9.1	14.9

In 4 h chromium release assay YAC1 cells were used as targets. Relatively high NK cytotoxicity was exerted by spleen lymphocytes from mice immunised with mouse melanoma cells transfected with IL6 or sIL6R.

As compared to the control, significantly increased natural killer activity was observed in mice immunised with melanoma cells transfected with IL6 and sIL6R genes.

From the above described studies it can be concluded that IL6 and sIL6R gene transfected melanoma cells are more immunogenic than parental cells; besides they induce the increase of NK activity.

The results obtained from preclinical studied justified the undertaking of the phase I clinical trial. In the clinical trial HLA-A1 and/or HLA-A2-positive patients with melanoma were immunised with an admixture of autologous tumour cells and allogeneic melanoma cells genetically engineered to secrete IL6 and sIL6R in order to elicit or enhance specific and non-specific anti-melanoma immune responses to autologous tumour cells (10). The HLA A-1 and HLA A-2 positive patients were selected, because these antigens are well known as being very good presenters of specific, melanoma antigen to the T lymphocytes. As the so called "anti-cancer-vaccine", both melanoma cell lines transfected with IL6 and sIL6R genes as well as autologous melanoma cells were used.

3.3. Surface immunophenotype of lymphocytes of patients treated with modified melanoma cells

A panel of specific monoclonal antibodies labelled with FITC or PE was been applied for the analysis of surface immunophenotype in the treated patients. Analysis was carried out by direct immunofluorescence technique using flow cytometer (Ortho Diagnostics).

No difference was observed between the studied patients and the healthy control group in regard to the content of T (CD3+) and B lymphocytes (CD19+) as well as NK cells (CD56+) was observed. Patients treated with "anti-cancer vaccine" showed only slight variation in the percentage of T, B and NK cells in the peripheral blood after subsequent injections (Fig. 3A and 3B).

Control median values for 13 healthy individuals for T, B and NK cells were 70.3%, 12.4% and 20.1% respectively.

In two of the treated melanoma patients, a significant increase of peripheral blood lymphocytes expressing activation markers was noticed. The lymphocyte activation markers include HLA-DR and CD25 antigen, i. e. an interleukin 2 receptor. The frequency of T lymphocytes (CD3+) expressing activation markers (CD3+CD25+, CD3+HLA DR+) along with antigens characteristic for the NK cells (CD56+CD16+) is presented in the Figures 4A and 4B.

It is worthy of note that increase of NK cell number was paralleled to the peak of T cells expressing activation markers in the treated patients. In the healthy control (n=13), the median percentage of T lymphocytes (CD3+) expressing CD25 was 7.5% and expressing HLA DR — 4.9%.

Complete analysis of surface immunophenotype of peripheral blood lymphocytes was performed before and after each injection of "anti-cancer vaccine". Representative results of selected markers expressed by peripheral blood lymphocytes of the melanoma patients are presented in Table 4.

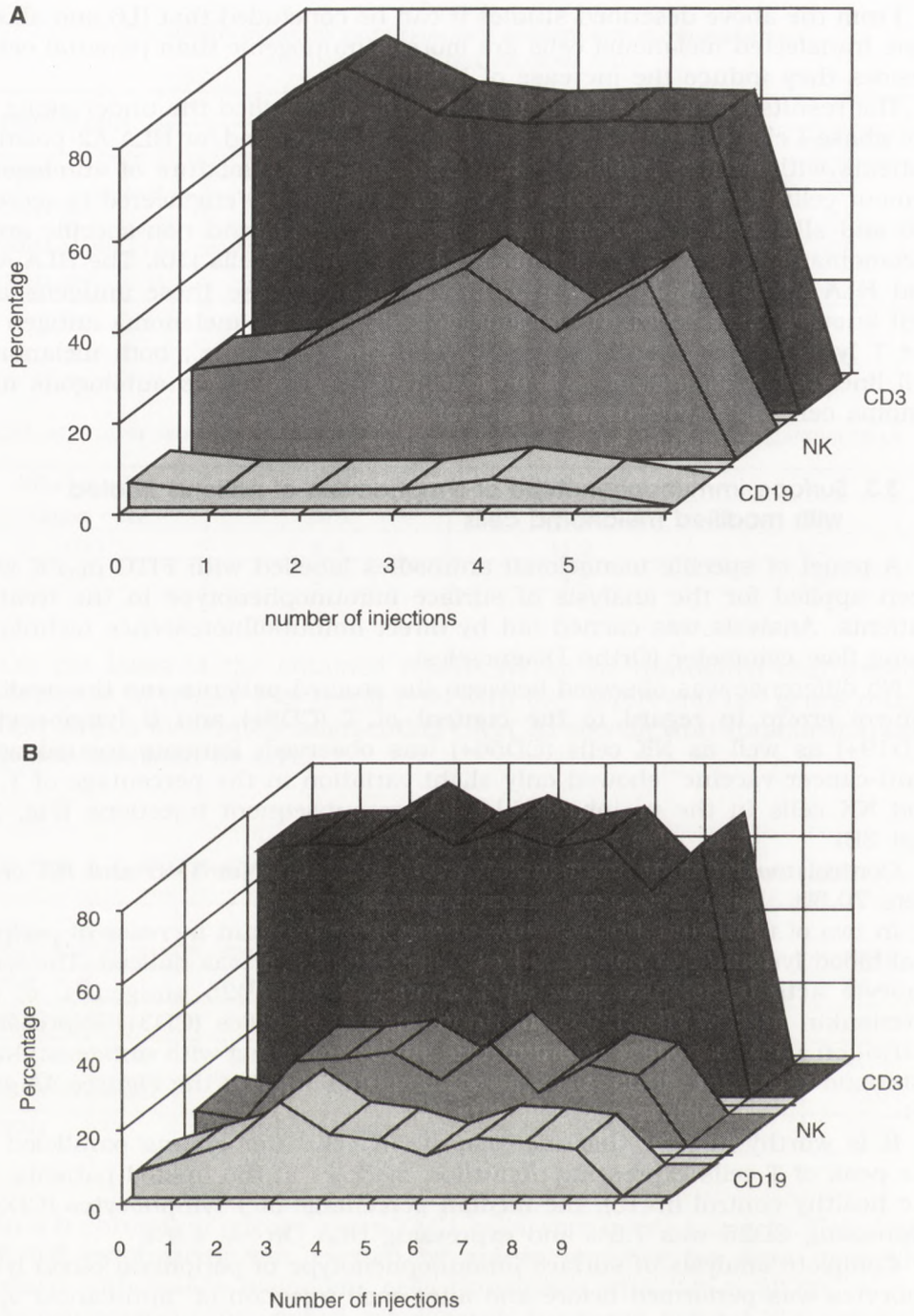

Fig. 3. The percentage of T and B lymphocytes and NK cells in peripheral blood of tw melanoma patients (A and B) receiving multiple "anti-cancer vaccine" injections.

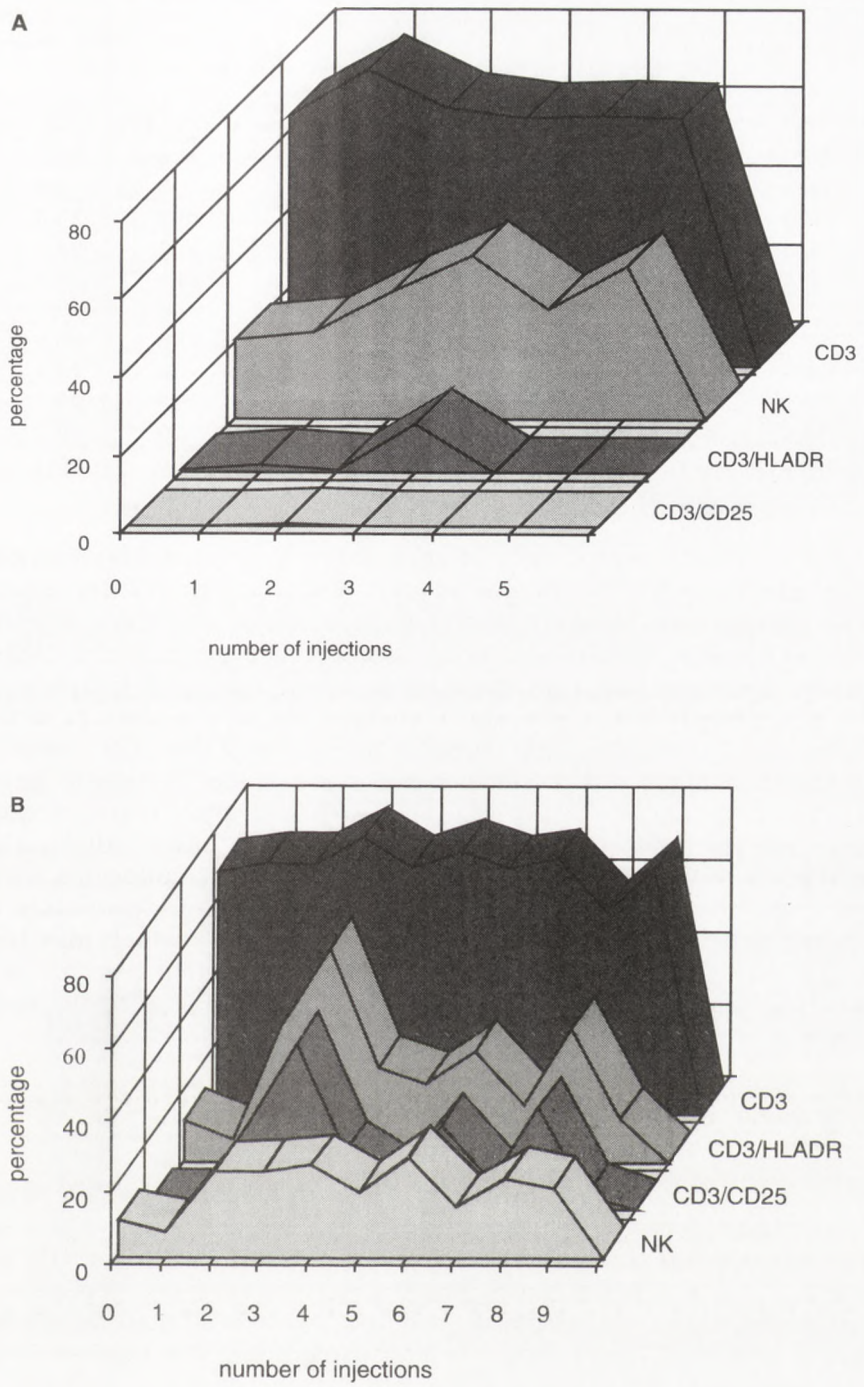

Fig. 4. Expression of activation markers on T lymphocytes along with NK cells in peripheral blood of two patients (A and B) treated with multiple "anti-cancer vaccine" injections.

TABLE 4
SURFACE IMMUNOPHENOTYPE OF PERIPHERAL BLOOD LYMPHOCYTES OF MELANOMA PATIENT TREATED WITH MULTIPLE "ANTI-CANCER VACCINE" INJECTIONS

No. inj.	CD3	CD4/CD3	CD8/CD3	CD4:CD8	HLADR/CD3	CD25/CD3	NK	CD19
0	67.2	49.3	56.4	0.9	11.6	2.6	10.1	6.4
1	69.3	36.5	32.1	1.1	6.4	2.6	7.4	10.3
2	68.8	42.7	51.5	0.8	37.2	23.7	23.6	14.4
3	76.4	62.4	37.5	1.7	59.2	46.2	24.0	25.5
4	68.4	41.5	52.6	0.8	26.2	16.1	25.5	16.8
5	72.9	34.3	59.6	0.6	21.5	7.6	18.1	14.8
6	68.1	38.1	50.8	0.8	30.7	23.6	28.0	11.0
7	70.5	44.2	39.0	1.1	15.9	7.9	14.4	15.8
8	56.2	36.7	21.0	1.7	37.2	28.3	22.1	14.7
9	68.3	38.2	41.2	0.9	11.9	4.0	19.3	12.9
control	70.3	59.1	30.0	1.9	4.9	7.5	20.1	12.4

Figures represent percentages of cells expressing particular markers.

Exceptionally high level frequency of T lymphocytes (CD3+) expressing HLA DR antigen was noticed in the patients after the third injection of "anti-cancer vaccine". Precise analysis revealed that the majority CD3+ cells with HLA DR+ were large, as opposed to small resting T lymphocytes. It may be speculated that "anti-cancer vaccine" is very potent in activating peripheral blood T lymphocytes. The high frequency of the CD3+ with CD25+ interleukin 2 receptor also confirmed the observation. It should be added that the increased level of T lymphocytes with activation markers was only transient and fell down to the values observed in healthy individuals after subsequent (4^{th}-9^{th}) injections. Great variations of T lymphocytes with HLA DR and CD25 markers may be due to some inflammatory processes taking place in the patients treated with "anti-cancer vaccine" , which may function mostly by non-specific activation of the immune system. As far as other analysed markers are concerned, only slight variations were noticed after each injections.

3.4. Immune response of melanoma patients treated with "anti-cancer vaccine"

One of the aims of the clinical trials was to evaluate specific and non-specific immune response by assessing CTL and NK cell activity. Non-specific immune response in treated patients was measured by NK activity against K562 cells. The peripheral blood lymphocytes isolated on Ficoll/Uropolina gradient according to the standard procedure, were evaluated for NK activity in patients receiving "anti-cancer vaccine" treatment. The representative results obtained after first and third injections are presented in Table 5.

TABLE 5
NK ACTIVITY OF PBL FROM MALIGNANT MELANOMA PATIENTS TREATED WITH IL6 — AND sIL6R — ALLOGENEIC MELANOMA CELLS

Effector: Target		1W	2P	Control
before treatment	50:1	25.8	20.0	53.5
	25:1	26.7	14.3	61.7
	12:1	22.8	11.8	41.3
	6:1	18.4	9.8	31.1
1st injection	50:1	24.2	35.5	52.7
	25:1	23.6	34.8	53.3
	12:1	16.1	27.1	49.2
	6:1	12.0	22.8	42.5
3rd injection	50:1	83.4	76.2	82.2
	25:1	77.1	76.2	78.0
	12:1	81.2	70.3	82.6
	6:1	59.5	44.1	62.7

The results are presented as cytotoxic indices in 4 h chromium release assay with K562 cells as targets.

In the patients 1W and 2P (Tab. 5) NK activity showed increasing tendency after subsequent vaccine injections and reached the level seen in the control group. It should be added, however, that in other patients relatively low level of NK activity was observed and the treatment had no effect on the NK activity.

Encouraging results were obtained in some of the treated patients, who showed increased activity of specific cytotoxic T lymphocytes (CTL). In Fig-

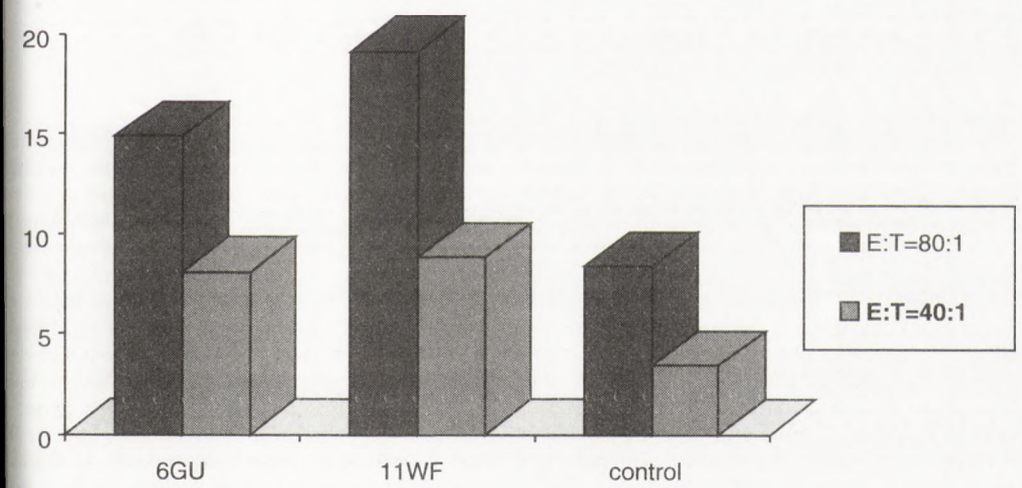

Fig. 5. CTL activity of malignant melanoma patient treated 8 times with IL6 and sIL6R transfected allogeneic melanoma cells.

ure 5 representative results of CTL activity of the melanoma patient treated 8 times with the vaccine are presented.

WM239 cells of established human melanoma cell line were used as targets in 4 h chromium release assay. In the cytotoxicity test two different effector:target cell ratios e.i. 80:1 and 40:1 were applied.

The preliminary conclusion from testing various immunological parameters of treated melanoma patients is that the so called "anti-cancer vaccine" may enhance specific and non-specific anti-tumour immune response in some of the malignant melanoma patients.

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Summary

Most of the tumour cells are too weakly immunogenic to be destroyed by the immune system. There are many ways to augment the specific immune response against malignant cells. In the present study the weakly immunogenic B78H1 mouse melanoma cells were transfected with genomic DNA from a line of human melanoma cells expressing a 96kD melanoma associated antigen (MAA) i.e. intercellular adhesion molecule 1 (ICAM 1). The transfected cells expressed fivefold higher quantities of the melanoma associated antigen from which the DNA was obtained. Human ICAM 1 expressed by mouse melanoma cells appeared to be highly immunogenic leading to the rejection of the modified mouse melanoma cells. The immune response against modified mouse melanoma cells appeared to be mediated by specific cytotoxic T lymphocytes (CTL) as evidenced by killing ^{51}Cr-labelled targets in 4 h cytotoxic assay. The transfected cells were also more prone for natural killer (NK) cytotoxicity. Unfortunately, in this experimental system generated specific (CTL) and non-specific (NK) immune response against human ICAM 1 expressed by mouse melanoma cells was not sufficient to prevent growth of parental tumour cells. In other studies B78H1 murine melanoma cells were stable transfected with cDNAs coding for human IL6, murine sIL6R (soluble IL6 receptor) and human leukaemia inhibitory factor (LIF). The parental and transfected melanoma cells were i.v. or s.c. injected into C57BL/6xC3H and SCID CB17 mice. Transfection of IL6, sIL6R or LIF reduced tumour growth and metastases formation. The

transfected cells enhanced NK killing with only minimal effect on CTL generation. The results obtained from animal experiments might indicate that augmented nonspecific immune response induced by sIL6R gene transfected melanoma cells, is responsible by inhibition of tumour growth and its ability to metastasize. In the clinical trial HLA-A1 and/or HLA-A2-positive patients with melanoma were immunised with an admixture of autologous tumour cells and allogeneic melanoma cells genetically engineered to secrete IL6 and sIL6R in order to elicit or enhance specific and non-specific anti-melanoma immune responses to autologous tumour cells. Immunophenotyping of peripheral blood lymphocytes with panel of specific monoclonal antibodies showed elevated level of CD3+ lymphocytes expressing activation markers, i.e. HLA-DR and CD25 (interleukin 2 receptor) which corresponded with increase number of NK cells. In the cytotoxicity assay using K562 cells as well as melanoma cell lines, it was shown that "anti-cancer vaccine" may result in enhancement of NK and CTL responses after subsequent immunisation of some melanoma patients. The provisional conclusion is that "anti-cancer vaccine" may enhance unspecific and specific anti-tumour immune response in some of the treated malignant melanoma patients.

Key words:
tumour cells, immune response, genetical modification.

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