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In vitro expansion and analysis of T lymphocyte microcultures obtained from the vaccination sites of cancer patients undergoing active specific immunization with autologous Newcastle-disease-virus-modified tumour cells

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Abstract. In order to understand further the effects of Newcastle-disease-virus(NDV)-modified tumour vaccines we investigated the feasibility of isolating lymphocytes from the site of injection of patients undergoing postoperative active specific immunization (ASI) with autologous NDV-modified tumour cells. Delayed-type-hypersensitivity(DTH)-like reactions from five cancer patients were surgically removed, minced and the tissue particles were digested with collagenase and DNase. Lymphoid cells recovered were expanded in a highly efficient limiting-dilution analysis system optimized for T cell growth [Moretta et al. (1983) J Exp Med 157: 743] and lymphocyte microcultures (clonal probability >0.8) could be grown for up to 1 year. Analysis of the microcultures for phenotype and function showed that the majority were positive for CD4 (92%) and TCRαβ (96%). Concanavalin-A-induced production of interleukin-2 (IL-2), IL-6, interferon γ and tumour necrosis factor α was detected in more than 70% of the microcultures. Lectin-dependent cytotoxicity was only very rarely observed. The general characteristics of the microcultures obtained support the notion of a DTH-like reaction taking place at the site of tumour cell challenge. The possibility of in vitro expansion and cultivation of T lymphocytes from ASI vaccination sites should help to elucidate further the role of these cells in active specific immunization against autologous tumour cells.

Key words: Active specific immunization – NDV-modified tumour cells – Microcultures – Tumour vaccines

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Introduction

The problem of metastasis is of paramount importance in cancer. This is reflected by the fact that, despite curative resection of the tumour, the clinical prognosis is finally determined by the presence of micrometastases, which are present but not therapeutically accessible at the time of surgery. Different approaches to reduce and cure micrometastases are under investigation at present [5, 13, 19]. The idea of enhancing the immunogenicity of cancer cells via modification with viruses or with biological response modifiers has led to the concept of active specific immunization (ASI). Work from our group has focused on elucidating the therapeutic effects of vaccination with cancer cells modified with Newcastle disease virus (NDV) [14, 16]. This experimental approach has yielded promising results in the murine ESb lymphoma system [6, 15], in the 3LL Lewis lung carcinoma [18] and in the guinea-pig L10 hepatocarcinoma [1]. In these systems about 50% of the animals bearing micrometastasis could be cured. Based on these results, standardized protocols for the preparation of human cancer vaccines have been developed [9]. In addition, clinical studies have been initiated investigating the effects of NDV-modified autologous cancer vaccines in patients with colon [2, 17], melanoma and ovarian cancer. Patients undergoing ASI with NDV-modified autologous tumour cells develop a delayed-type-hypersensitivity(DTH)-like reaction at the site of injection, consisting of an induration with a surrounding erythema [8]. The functional properties of the cells involved in this reaction have not been characterized. In this communication we describe for the first time the isolation, in vitro expansion and identification of the lymphoid cells infiltrating skin sites of vaccinated cancer patients. Such vaccination-site-infiltrating lymphocytes could be propagated for up to 1 year and consisted mainly of helper T lymphocytes expressing α,β T cell receptors.

Table 1. Characteristics of patient material[a]

Patient	Tumour	Status at time of surgery	Material used for vaccination[b]	Size of DTH reaction[c] (mm)	No. of cells recovered
1	Ovarian ca.	FIGO III	Tu-NDV	10	10000
2	Melanoma	pT4N1M1	Tu	13	20000
3	Melanoma	pT*x*N*x*M1	Tu	10	5000
4	Colon ca.	pT2N0M1	Tu-NDV	7	500
5	Colon ca.	pT3N0M0	Tu	16	5000

[a] Patients 3, 4 and 5 were vaccinated according to a regular schedule as described by Bohle et al. [2]; patients 1 and 2 were vaccinated out of schedule on a long-term immunization protocol. Patients 2, 3 and 5 were vaccinated with 10^7 irradiated tumour cells recovered from metastasis; patients 1 and 4 were vaccinated with 10^7 irradiated cells from the primary tumour.

[b] Either Newcastle-disease-virus(NDV)-treated or non-NDV-treated vaccines were used as indicated

[c] DTH, delayed-type hypersensitivity

Materials and methods

Reagents. Reagents were obtained as follows: concanvalin A (Pharmacia), phytohaemagglutinin M (PHA-M; Gibco); [^{3}H]thymidine, specific activity 2 Ci/mmol (Amersham-Buchler); ^{51}Cr (Amersham-Buchler); collagenase type Ia (Sigma); DNase I (Sigma). Human recombinant interleukin-2 (rIL-2) and human recombinant IL-6 (rIL-6) were generous gifts of Eurocetus (Frankfurt/Main, Germany) and Biotest (Dreieich, Germany) respectively.

Media. RPMI-1640 medium was prepared from powder (Gibco) and supplemented with 100 U/ml : 100 µg/ml penicillin/streptomycin, 2 mM glutamine, 50 µM 2-mercaptoethanol, 1 mM pyruvate and non-essential amino acids (Gibco). Heat-inactivated fetal calf serum (FCS) was from Gibco.

Characteristics of patients, material. Five patients who had volunteered to participate in the study received duplicate injections of autologous vaccine material. One of the developing DTH-like reactions was removed after 24 h. Details are given in Table 1.

Preparation of vaccination-site-infiltrating lymphocytes. DTH-like lesions of patients undergoing ASI were surgically removed and minced with a scalpel. After extensive washing the small pieces of tissue were digested with collagenase (2 mg/ml) and DNase (10 µg/ml) in medium with 20% FCS for 4–6 h at 37° C in a shaking waterbath. After that period cells were centrifuged, washed and incubated overnight in medium with 20% FCS. In some cases tissue was submitted to a second round of digestion. Lymphoid cells in the preparation were then counted and expanded in a limiting-dilution analysis system.

Culture of vaccination-site-infiltrating lymphocytes. Cells from DTH-like lesions could only be recovered in limited numbers (500–20000/site); therefore, cells had to be expanded in a highly efficient limiting-dilution analysis system originally described by Moretta et al. [12] that allows every T lymphocyte to give rise to a microculture. Briefly, lymphocytes at 10, 3 or 1 cells/well were stimulated in 96-well round-bottom microtitre plates (Costar) with 1% PHA-M in the presence of allogeneic human spleen feeder cells (50000 cells/well) irradiated with 50 Gy. After 48 h, 100 µl was removed and 60 IU rIL-2 in 100 µl was added per well. On day 7 and day 14 of culture 25000 irradiated human spleen feeder cells and 60 IU rIL-2 were added per well. Microcultures were subsequently grown by addition of rIL-2 (60 IU/well) every 7 days. When growth of the microcultures was slowing down (generally after 4–5 weeks of culture in rIL-2 only) cells were restimulated with rIL-2 and 25000 oxidized feeder cells. For oxidation, feeder cells were treated with 1 mM $NaIO_4$ for 20 min in phosphate-buffered saline (PBS) in the absence of serum. Restimulation with oxidized feeder cells seems to help to circumvent the "crisis phenomenon" and to allow for prolonged culture of human lymphocytes when compared to stimulation with rIL-2 only. A similar protocol, where the feeder cells were oxidized by treatment with neuraminidase and galactose oxidase, was described by Fleischer [4]. Human spleen for the preparation of feeder cells was obtained from the Department of Surgery, University of Heidelberg. All spleen cell preparations used in this study were pretested for high efficiency as feeders in our limiting-dilution analysis system. Using PBL from normal donors, frequencies of proliferating T lymphocyte precursors of 0.7–1.0 were constantly observed. Limiting dilution analysis was performed using the LIDIA computer program described in [20]. Only microcultures with a high clonal probability ($P > 0.8$) were included in the analysis.

Preparation of cytokine-containing supernatants. Microcultures were split and an aliquot was washed four times in order to remove exogenous rIL-2. Cells were then stimulated with 2.5 µg/ml concanavalin A and conditioned supernatants were harvested after 24 h. Microcultures of irradiated feeder cells stimulated equivalently served as controls.

Determination of IL-2. Titres of IL-2 were determined using the CTLL-2 cell line. Our variant of the CTLL-2 cell line does not respond to IL-6 and only minimally to IL-4. Briefly, 4000 cells/well were cultured in the presence of serial (threefold) dilutions of IL-2-containing supernatants in flat-bottom 96-well microtitre plates (Costar). After 20 h of culture 1 µCi [^{3}H]thymidine was added per well and the plates were harvested after a further 4 h of culture. A standard of 60 IU/ml rIL-2 was titrated in every assay. Titres of IL-2 are given in international units (5–6 IU are equivalent to 1 half-maximal unit or Cetus unit). The detection limit was 2 IU rIL-2.

Determination of IL-6. Titres of IL-6 were determined using the B9 cell line (a kind gift of Dr. L. Aarden, Amsterdam). Our variant of the B9 cell line does not respond to IL-2. Briefly, 3000–4000 cells/well were cultured in medium containing 10% FCS in the presence of serial (threefold) dilutions of IL-6-containing supernatants in flat-bottom 96-well microtitre plates. After 48 h of culture 1 µCi/well [^{3}H]thymidine was added per well and the plates were harvested after a further 18 h of culture. A standard of 10 U/ml rIL-6 was titrated in every assay (units as given by the supplier); half-maximal incorporation occurred at 3 U/ml. The detection limit was 1 U/ml.

Determination of TNFα. Titres of tumour necrosis factor α (TNFα) were determined as described by Männel and Falk [10]. L929 mouse fibrosarcoma cells (2×10^4 cells/0.2 ml culture, 2 µg/ml actinomycin D) were cultured in the presence of serial dilutions of TNFα samples. After 20 h of culture the surviving cells were fixed and stained with crystal violet (0.5% crystal violet, 3% by vol. formaldehyde, 0.17% NaCl, 22.3% ethanol) for 15 min. Excess dye was washed of with water, the remaining dye was solubilized with 33% acetic acid and the absorbance was measured at 540 nm. A standard of 5 ng/ml (kindly provided by Knoll AG, Ludwigshafen, Germany) was titrated in every assay. The detection limit was 0.25 ng/ml.

Determination of IFNγ. Human interferon γ (IFNγ) titres were determined in a standard cytopathic effect inhibition assay using cultures of human fibroblasts trisomic for chromosome 21 (GM02504G, obtained from the Human Genetic Mutant Cell Respository, Coriell Institute for Medical Research, Camden, N. J., USA). Cells were seeded into microtitre plates (5×10^3 cells/well) and cultured for 3 days to confluence. Twofold dilutions of test supernatants were then made in the plates, which were then incubated at 37° C for 18 h. Cell monolayers were washed twice and challenged with vesicular stomatitits virus (VSV, strain Indiana) at a multiplicity of 0.1 plaque-forming unit/well. The cytopathic effect was determined under an inverted microscop 72 h later. One laboratory unit is defined as the reciprocal of the last dilution of the test supernatant conferring 50% protection against lysis by VSV and corresponds to 0.5 international reference unit, on the basis of compara-

Table 2. Phenotype of T lymphocyte microcultures derived from active specific immunization (ASI) skin vaccination sites[a]

Phenotype	No. microcultures positive					Σ
	1[b]	2	3	4	5	(%)
Total no. of microcultures tested	23	8	5	18	15	69 (100)
$CD4^+$	20	7	4	17	15	63 (92)
$CD8^+$	1	0	1	1	0	3 (4.4)
$CD4^+CD8^+$	0	0	0	0	0	0
$CD4^-CD8^-$	1	1	0	0	0	2 (2.9)
Total no. of microcultures tested	21	6	5	15	15	62 (100)
$TCR\alpha\beta^+$	20	6	5	14	15	60 (96.7)
$TCR\alpha\beta^-$	1	0	0	1	0	2 (3.2)

[a] Lymphocytes from ASI skin vaccination sites were expanded in a highly efficient limiting-dilution analysis system. The T lymphocyte microcultures obtained were stained with phycoerythrin-labelled CD4 and fluorescein-isothiocyanate(FITC)-labelled CD8 in a double-immunfluorescence assay or with with mAb BMA031 and FITC-labelled goat anti-[mouse $F(ab')_2$]. Samples of 10 000 cells were analysed on a FACScan cytofluorometer

[b] Patient number

Table 3. Lymphokine production of T lymphocyte microcultures derived from ASI skin vaccination sites

Lymphokine level	No. microcultures positive					Σ
	1[b]	2	3	4	5	(%)
Total no. of microcultures tested	18	7	4	15	15	59 (100)
>2 IU IL-2	18	7	2	14	9	50 (84.8)
<2 IU IL-2	0	0	2	1	6	9 (15.2)
Total no. of microcultures tested	12	8	4	19	15	58 (100)
>0.25 ng/ml TNFα	9	3	2	16	11	41 (70.4)
<0.25 ng/ml TNFα	3	5	2	3	4	17 (29.3)
Total no. of microcultures tested	14	10	10	24	20	78 (100)
>1 U/ml IL-6	14	10	10	21	19	74 (94.8)
<1 U/ml IL-6	0	0	0	3	1	4 (5.2)
Total no. of microcultures tested	20	7	6	18	15	66 (100)
>3 IU/ml IFNγ	18	7	0	15	9	49 (74)
<3 IU/ml IFNγ	2	0	6	3	6	17 (26)

[a] IL, interleukin; TNF, tumour necrosis factor; IFN, interferon

[b] Patient number

ble titrations of human NIH IFNγ preparations (reference no. Gg 23-901-530, kindly provided by the Research Resources Section, NIAID, Bethesda, Md., USA).

Cytofluorometry. Phenotypic analysis of the microcultures was performed using the following monoclonal antibodies: phycoerythrin-labelled CD4 and fluorescein-isothiocyanate(FITC)-labelled CD8, obtained from Dako (Hamburg, Germany); the monoclonal antibodies BMA030 and BMA031, which recognize the human CD3 complex and the human α/β T cell receptor (TCRαβ) respectively, were generous gifts of Dr. Kurrle (Behringwerke, Marburg, Germany). Affinity-purified FITC-labelled goat anti-[mouse $F(ab')_2$] was from Jackson (Dianova, Hamburg, Germany). Aliquots of cells were stained in PBS supplemented with 5% FCS, 0.1% azide in the cold. A commercial preparation of human IgG and IgM (Pentaglobin, Biotest, Dreieich, Germany) was added to 3% during the whole staining procedure in order to block human Fc receptors. Samples of 10 000 cells were analysed on a FACScan fluorocytometer with appropriately set scatter gates.

Target cells. The following cells and cell lines were labelled with ^{51}Cr and used as target cells in assays of cytolytic activity: (a) P815 murine mastocytoma cells in the presence of 1% (v/v) PHA-M, i. e. lectin-dependent cytotoxicity (LDCC), which allows the detection of specific cytolytic activity irrespective of the antigenic specificity of the effector cells; (b) K562 human erythroleukaemia cells as a natural-killer(NK)-sensitive target.

Assays of cytolytic activity. For assay of cytolytic activity, cells were resuspended and an aliquot of 100 µl from each well was used to measure cytotoxicity. Target cells (1000 cells/well) labelled with ^{51}Cr were placed in wells of V-bottomed microtitre plates (Greiner, Nürtingen, Germany). In the LDCC assay PHA was added to the ^{51}Cr-labelled target cells immediately before these were placed in the microwells (in order to avoid the formation of cell clumps). A standard 4-h ^{51}Cr-release cytotoxicity assay was then performed. Specific lysis was calculated from the formula specific lysis (%) = (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. Spontaneous release was determined in microcultures containing ^{51}Cr-labelled target cells only. Maximum release was determined by lysing ^{51}Cr-labelled target cells with 1% sodium dodecyl sulphate.

Results

Phenotypic analysis of lymphocyte microcultures derived from ASI skin vaccination sites

Lymphocyte microcultures from ASI skin vaccination sites were obtained after expansion of cells in a highly efficient limiting-dilution analysis system [12] that allows every normal T lymphocyte to give rise to clonal progeny. Frequencies of proliferating T cell precursors in the vaccination-infiltrating lymphocytes ranged from 1/16 to 1/1. Immunofluorescence analysis revealed that 92% of the microcultures were positive for CD4; only 4.4% were positive for CD8; 96.7% of the microcultures were positive for TCRαβ (Table 2). All microcultures were positive for CD3 (data not shown).

Cytokine production of lymphocyte microcultures derived from ASI skin vaccination sites

Concanavalin A-induced supernatants derived from lymphocytes from DTH-like vaccination reactions were tested for IL-2, IL-6, TNFα and IFNγ content. As expected from the surface phenotype analysis, a high proportion of the microcultures produced IL-2 and IL-6 (84.8% and 94.8% respectively). The percentage of microcultures positive for TNFα and IFNγ was somewhat lower (Table 3).

Cytotoxic activity of lymphocyte microcultures derived from ASI skin vaccination sites

Lymphocyte microcultures were tested for LDCC against the murine mastocytoma line P815 in the presence of PHA and for NK-like activity against the erythroleukaemia line

Table 4. Cytotoxic activity of T lymphocyte microcultures derived from ASI skin vaccination sites

Cytotoxic activity	No. microcultures positive					Σ
	1[a]	2	3	4	5	(%)
A: Lecitin-dependent cytotoxicity						
Total no. of microcultures tested	22	7	4	16	15	64 (100)
Specific lysis >25%	0	0	0	0	1	1 (1.5)
25% > specific lysis >12.5%	2	5	1	6	2	16 (25)
Specific lysis <12.5%	20	2	3	10	12	47 (73.4)
B: NK-like cytotoxicity						
Total no. of microcultures tested	15	3	2	10	14	44 (100)
Specific lysis 10%–60%	2	0	0	0	2	4 (9)
No specific lysis	13	3	2	10	12	40 (91)

[a] Patient number

K562. Significant specific lysis in the LDCC system (>25%) could only be found in 1 out of 64 microcultures tested (1.5%). In the LDCC the magnitude of specific lysis was more homogeneously distributed with 16% of the microcultures showing intermediate lysis. In NK-like cytotoxicity the separation between positive and negative microcultures was very discrete; here the negative microcultures produced no specific lysis. Again, only 9% of the microcultures were positive for NK-like cytotoxic activity (Table 4).

Discussion

In active specific immunization, tumour cells are made more immunogenic by treatment with viruses or biological response modifiers. Vaccines made from patients' own tumours modified with NDV induce a DTH-like reaction at the site of injection in the skin [8]. We succeeded in isolating T lymphocytes from these lesions albeit in a low number. After expansion in a highly efficient limiting-dilution analysis system the majority of the lymphocyte microcultures were $CD4^+$. As $CD4^+$ and $CD8^+$ lymphocytes from peripheral blood lymphocytes grow equally well under the conditions we used for lymphocyte expansion [12], we believe that the preponderance of $CD4^+$ microcultures really reflects a preponderance of $C4^+$ lymphocytes in the infiltrate. Histological examination of the injection sites of NDV-modified autologous tumour cells, as reported by Bohle et al. [2], has also shown the majority of infiltrating T lymphocytes to be positive for CD4. These data together favour the notion that the immunological reaction at the site of injection is a DTH-like reaction, as the majority of T lymphocytes reactive in DTH are $CD4^+$ [3]. A high proportion of the microcultures produced the cytokines IL-2, IL-6, TNFα or IFNγ in response to stimulation with concanavalin A, the percentages ranging from 70.4% for TNFα to 94% for IL-6. It is interesting to note that IFNγ was also among the cytokines produced as this cytokine has macrophage-activating activity [7], which might play a role in the activation of macrophages in DTH. Cytolytic activity was not a prominent feature of the microcultures investigated.

Further research is needed for the evaluation of active specific immunization using NDV-modified tumour cells. The possibility of expansion and long-term culture should be of special value for the characterization of the lymphocytes infiltrating the injection site of the vaccine. It is important to note that restimulation at appropriate intervals with oxidized feeder cells was absolutely necessary for long-term culture as restimulation with IL-2 alone resulted in the demise of the microcultures. Given the existence of a tumour-specific antigen, ASI-vaccination-site-infiltrating lymphocytes might also be of value in the definition of these antigens. Through better proliferative behaviour they might offer an advantage over tumour-infiltrating lymphocytes, the proliferative capacities of which are usually pretty low [11, 21]. The problem of antigen specificity of course also demands techniques that allow for the reproducible establishment of human tumour cell lines from patient material; this, at the moment, is still a cumbersome task.

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