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Somatic gene therapy for cancer: the utility of transferrinfection in generating 'tumor vaccines'*

(Recombinant DNA; immune surveillance; cytotoxic T-cells; nude mice; adenovirus; cytokines; xenogenization)

Kurt Zatloukal[a], Walter Schmidt[a], Matthew Cotten[a], Ernst Wagner[a], Georg Stingl[b] and Max L. Birnstiel[a,1]

[a] *Research Institute of Molecular Pathology, I.M.P., Dr. Bohr-Gasse 7, A-1030 Vienna, Austria; and* [b] *Department of Dermatology, Div. Imm. All. and Infect. Dis., Univ. of Vienna Med. School, Währinger Gürtel 18–20, A-1090 Vienna, Austria. Tel. (43-222) 404005844*

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SUMMARY

The last few years have seen the development of a branch of somatic gene therapy which aims at strengthening the immune surveillance of the body, leading to eradication of disseminated cancer tumor cells and occult micrometastases after surgical removal of the primary tumor. Such a tumor vaccination protocol calls for cultivation of the primary tumor tissue and the insertion of one of three types of genes into the isolated cultured tumor cells followed by irradiation of the transfected or transduced cells to render them incapable of further proliferation. The cells so treated constitute the 'tumor vaccine'. A review of the literature suggests that for mouse models, in the initial period after inoculation, rejection of the tumor cells is usually effected by non-T-cell immunity, whereas the long-term systemic immune response is based on cytotoxic T-cells. High expression of the gene inserted into the tumor cells may be critical for the success of the vaccination procedure. Examples are given which indicate that transferrinfection, a procedure to introduce genes by adenovirus-augmented receptor-mediated endocytosis, meets some important prerequisites for successful application of this type of gene therapy.

INTRODUCTION

The major obstacle to immunotherapy of cancer is the poor immunogenicity of tumors, especially in man. This is somewhat surprising since the large number of genetic alterations found in advanced cancers should give rise to peptide neo-epitopes capable of being recognized in the context of MHC-I molecules by cytotoxic lymphocytes (Lurquin, 1989). Indeed, tumor-associated (Groen, 1987) and tumor-specific antigens (Van der Bruggen and Van den Eynde, 1992) which should be targets for immunological attack have recently been identified. It is therefore possible that the immune system fails to eliminate tumors not because neoantigens are absent, but rather because the response to these neoantigens is inadequate (Fearon et al., 1990).

Correspondence to: Dr. M.L. Birnstiel, Research Institute of Molecular Pathology, Dr. Bohr-Gasse 7, A-1030 Vienna, Austria. Tel. (43-222) 79730500; Fax (43-222) 7989390.

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[1]Co-chairman of this session.

Abbreviations: Ad, adenovirus; CD, cluster designation; CMV, cytomegalovirus; CTL, cytotoxic T-lymphocytes, G-CSF, granulocyte colony stimulating factor; GM-CSF granulocyte-macrophage colony stimulating factor; IFN, interferon; IL, interleukin; LAK, lymphokine-activated killer cells; NK, natural killer cells; nt, nucleotide(s); RENCA, renal carcinoma; TNF, tumor necrosis factor; u, unit(s).

(a) Introduction of certain genes elicits a systemic immune response against tumor cells

In recent years it has been discovered that transfection of certain genes into tumor cells can render them more immunogenic and, therefore, less tumorigenic. These genes fall into three categories.

(1) Genes expressing proteins which are foreign to the repertoire of tumor cells

Such xenogenization of tumor cells can be brought about, for example, by expressing syngeneic MHC-I antigens in MHC-I deficient tumor cells, expressing allogeneic MHC-I or MHC-II antigens or expressing viral proteins such as hemagglutinin (for references see Table I). Xenogenization can also be brought about by mutagenesis of tumor cells (Van Pel and Boon, 1982) to yield Tum^- cell clones which elicited a rejection response in syngeneic mice (reviewed in Van der Bruggen and Van den Eynde, 1992).

The properties of xenogenized tumor cells have been explained by the concept of associative recognition (Lake and Mitchison, 1976; 1977; Kenne and Foreman, 1982) which proposes that minor cell surface antigenic differences (as in the case of cancer cells) are insufficient for the induction of an immune response. The addition of more powerful antigens to the cell surface not only provides for a response to the added antigens but also induces immunorecognition of the original tumor-associated cell-surface antigens (Fearon et al., 1988) and systemic protection against the tumor cell (see Table I).

The precursor of these xenogenization experiments utilizing gene transfer is the systemic protection elicited against tumor cells by infecting them with virus expressing new antigens on tumor cells or yielding viral oncolysates (Lindenmann and Klein, 1967; Wallack et al., 1977; Ito et al., 1990; Lehner et al., 1990; Liebrich et al., 1991).

(2) Cytokine-encoding genes

The goal of this operation is to activate the immune system so that it recognizes the tumor cells as foreign and rejects them. The insertion of cytokine-encoding genes such as interleukin, colony stimulating factor and interferon-encoding genes, into tumor cells nearly uniformly reduces the tumorigenicity of injected tumor cells with the known exceptions so far of IL-6, which actually leads to an accelerated growth of injected cells, and of TNF-α or IFN-γ, where only partial reduction of tumorigenicity is obtained (see Table II). In most instances, where tested, a systemic protection against the parental tumor has been observed. However, no such systemic protection was detected after IL-4 (exception see Golumbek et al., 1991), G-CSF or TNF-α transfection of tumor cells (see Table II).

(3) Genes encoding accessory proteins

The aim of this operation is to impart features of antigen presenting cells to the tumor cells. The CD28 antigen on $CD4^+$ and $CD8^+$ T-cells is the primary receptor for the activating antigen B7. CD28 binds to B7 in the absence of other accessory molecules. Interaction between these two antigens is co-stimulatory for T-cell activation (Linsley et al., 1991). The two-signal model (Janeway, 1989; Nossal, 1989; Schwartz, 1989) postulates that for optimal activation lymphocytes require both an antigen-specific signal delivered through the T-cell receptor and a second signal, a non-specific co-stimulatory signal such as B7.

This model suggests that immunogenic tumors which do not express B7 can escape destruction by the immune response because tumor-targeted T-cells receive inadequate co-stimulation. This leads to the proposition that transfer of a gene encoding the co-stimulatory ligand B7 into melanoma cells, when introduced into a syngeneic mouse, would induce rejection of these cells in vivo. Recently two groups (Chen et al., 1992; Townsend and

TABLE I

Genes used to xenogenize tumor cells

Tumor model	Xenogizing gene	Tumorigenicity	Systemic protection	Reference
Lewis lung carcinoma	allogeneic H-$2L^d$ MHC-I	ab.	yes	Itaya et al. (1987)
Colon adenocarcinoma CT26	hemagglutinin	ab.	yes	Fearon et al. (1988)
Facs 3	hemagglutinin	ab.	yes	Fearon et al. (1988)
Lung carcinoma 3LL-D122	syngeneic H-$2K^b$ MHC-I	ab.	yes	Plaksin et al. (1988)
Sal sarcoma	A^k_α and A^k_β MHC-II	ab.	yes	Ostrand-Rosenberg et al. (1990)
Lung carcinoma 3LL-D122	c-*fos*	ab.	n.d.	Kushtai et al. (1990)

ab. = absent; n.d. = not done.

TABLE II

'Tumor vaccines' produced by the transfer of cytokines into tumor cells

Ctk[a]	Tumor model	Mode of generation	Transf. cells	Cytokine production	Tumorigenicity[b]	Systemic protection[c]	Reference
IL-2	Colorectal carcinoma CAT26	$Ca \cdot PO_4$	pooled clones	≤ 300 u/10^6 cells/24 h	ab.	yes	Fearon et al. (1990)
	Melanoma B16					yes	
	Sarcoma CBA-SP_1					yes	
	Dunning prostate carcinoma					yes	
	Fibrosarcoma CMS-5	transduction	cell line	≤ 30 u/ml	ab.	yes	Gansbacher et al. (1990a,b)
	Rat sarcoma HSNLV	transduction	cell line	≤ 2500 u/10^6 cells/24 h	ab.	n.d.	Russell et al. (1991)
	Mastocytoma P815	$Ca \cdot PO_4$	cell line	≤ 3000 u/10^6 cells/24 h	ab.	yes	Ley et al. (1991)
	Mastocytoma P815	$Ca \cdot PO_4$	cell line	≤ 3500 u/10^6 cells/24 h	ab.	n.d.	Roth et al. (1992)
	Rat medullary thyroid carcinoma	$Ca \cdot PO_4$	pooled clones	50 u/10^6 cells/24 h	ab.	n.d.	
	Lewis lung carcinoma LLC	lipofection	cell line	≤ 100 u/10^6 cells/24 h	ab.	(yes)	Ohe et al. (1993)
	Lewis lung carcinoma 3LL-D122	transduction	cell line	≤ 7 u/ml	ab.	yes	Porgador et al. (1993)
	Melanoma M3	receptor-mediated gene transfer	pooled cells	$\leq 10^5$ u/10^6 cells/24 h	ab.	yes	this paper
IL-4	Plasmacytoma J558	electroporation	cell line	$\leq 29\,000$ u/10^6 cells/24 h	ab.	no	Tepper et al. (1989)
	Mammary adenocarcinoma K485	$Ca \cdot PO_4$	cell line	≤ 2800 u/10^6 cells/24 h	ab.	no	Tepper et al. (1992)
	Renal carcinoma RENCA	$Ca \cdot PO_4$	cell line	$\sim 15\,000$ u/10^6 cells/24 h	ab.	(yes)	Golumbek et al. (1991)
	Lewis lung carcinoma LLC	lipofection	cell line	35 ng/ml	ab.	no	Ohe et al. (1993)
IL-6	Plasmacytoma J558	electroporation	cell line	500 u/ml	acc.	n.d.	Blankenstein et al. (1991)
IL-7	Ependymoblastoma 203 glioma	electroporation	cell line	≤ 250 u/ml	ab.	yes	Akoi et al. (1992)
	Plasmacytoma J558L	electroporation	cell line	4–65 u/ml	ab.	n.d.	Hock et al. (1991)
	Mammary adenocarcinoma TS/A			50 u/ml	ab.	n.d.	
G-CSF	Colon adenocarcinoma C26	transduction	cell line	≤ 450 pg/ml	ab.	(no)	Colombo et al. (1991)
M-CSF	Plasmacytoma J558L	electroporation	cell line	≤ 100 u/ml	part.	n.d.	Dorsch et al. (1993)
GM-CSF	Melanoma B16	transduction	pooled cells	1500 ng/10^6 cells/24 h	ab.	yes	Dranoff et al. (1993)
IFN-γ	Neuroblastoma C1300	transduction	cell line	≤ 50 u/ml	ab.	yes	Watanabe et al. (1989)
	Fibrosarcoma CMS-5	transduction	cell line	≤ 25 u/ml	ab.	yes	Gansbacher et al. (1990a,b)
	Squamous cell carcinoma Pam-T	transduction	cell line	≤ 200 u/ml	part.	n.d.	Maraguchi et al. (1991)
	Adenocarcinoma SP1	$Ca \cdot PO_4$/lipofection	pooled clones	256 u/ml	ab.	(no)	Esumi et al. (1991)
	CT26		cell line	≤ 64 u/ml	part.	n.d.	
TNF-γ	Plasmacytoma J558	transduction	cell line	≤ 40 pg/ml	part.	n.d.	Blankenstein et al. (1991)
	Sarcoma MCA-205/WP-4	transduction	cell line	420 pg/10^6 cells/24 h	part.	no	Asher et al. (1991)
JE/	Duk × B-11	polybrene	pooled clones	–	ab.	n.d.	Rollins and Sunday (1991)

[a] Ctk = cytokine.

[b] ab. = absent or strongly reduced, depending on the level of cytokine production; part. = partial tumorigenicity; acc. = accelerated.

[c] n.d. = not done.

Allison, 1993) showed that this concept is indeed valid for melanoma cells. Rejection of B7-transfected tumor cells was primarily mediated by $CD8^+$ T-cells and anti-CD4 antibodies ablating CD4 cells had no effect. Moreover, a systemic protection against the parental $B7^-$ tumor cells was obtained (Chen et al., 1992; Townsend and Allison, 1993). These results open the door for experimentation with other accessory, co-stimulatory molecules which impart certain features of antigen presenting cells to tumor cells. The introduction of syngeneic MHC-II antigen (Table I) may also change the properties of tumor cells so that they present tumor antigens in the MHC-II context which can be recognized by T-helper cells, resulting in an immunological cascade and eventual eradication of the tumor cell.

(b) In some instances initial rejection of the tumor cells is based on non-T-cells

Interestingly, tumorigenicity of tumor cells during the 'vaccination' step (initial injection) is also strongly diminished for IL-4 and G-CSF-secreting tumor cells when tested in nude mice which are deficient for T-cells (Colombo et al., 1991; Golumbek et al., 1991). This suggests that the initial rejection of tumor cells is supported by a non-T-cell response. By contrast, no reduction of tumorigenicity is seen in nude mice in the case of IL-7 and IFN-γ-secreting tumor cells (see Table III). This is an indication that IL-7 and IFN-γ induce a T-cell mediated immune response in immune competent mice.

Results obtained for IL-2 varied according to the tumor model chosen, the number of cells injected and levels of cytokine secretion. Thus a whole spectrum of results has been reported, from no reduction of tumorigenicity in nude mice (Ohe et al., 1993) to partial and full rejection of IL-2-producing tumor cells, depending on the level of IL-2 secretion (Russell et al., 1991; Porgador et al., 1993). These findings have been interpreted as meaning that for low secretors, reduction in tumor growth in immune competent mice is mostly due to T-cell activity, whereas for high IL-2 secretors a non-T-cell dependent mechanism is at work (Porgador et al., 1993). This is in accordance with the finding that T-cells are stimulated via high-affinity heterodimeric IL-2 receptors which are expressed following antigen activation. NK cells, LAK precursors and macrophages are stimulated initially via constitutively expressed intermediate-affinity IL-2 receptors and thus require a higher concentration of IL-2 (Porgador et al., 1993, and references therein).

TABLE III

Tumorigenicity of tumor cells transfected or transduced with cytokines

Cytokine	Tumorigenicity in nude mice	Reference
IL-2	abolished	cited in Bubenik et al. (1990)
	abolished	Hock et al. (1993)
	abolished[a]	Porgador et al. (1993)
	no reduction[b]	Russell et al. (1991)
IL-4	abolished	Tepper et al. (1989)
		Golumbek et al. (1991)
IL-7	no reduction	Hock et al. (1991; 1993)
G-CSF	abolished	Colombo et al. (1991)
IFN-γ	no reduction	Watanabe et al. (1989)
		Esumi et al. (1991)
	abolished	Hock et al. (1993)
TNF-α	weakened	Blankenstein et al. (1991)
	abolished	Hock et al. (1993)

[a] In high IL-2 producers.
[b] In low IL-2 producers.

The results tabulated in Table III can be correlated with histological investigations of the injection sites in immunocompetent mice. The initial injection sites after inoculation of IL-4-producing RENCA cells are infiltrated by activated macrophages and granulocytes (Golumbek et al., 1991). Similarly IL-4-producing plasmacytoma cells evoke infiltrated eosinophiles and macrophages (Tepper, 1992). G-CSF-producing tumor cells show infiltration of neutrophilic granulocytes. Thus, in the case of IL-4 and G-CSF the presence of non-T-cells at the injection sites correlates with the rejection of the tumor cells in the nude mouse test system. Moreover, NK cells are not likely to be involved, since NK-depleted mice are nevertheless protected from tumor development (Columbo et al., 1991). In the same vein, IFN-γ-producing tumor cells show infiltration of $Thy\ 1^+$ small lymphocytes (Watanabe et al., 1989) whereas IL-7-producing, and in some cases IL-2-producing, cells (Pardoll, 1992) are marked by infiltration of T-cells as well as macrophages (Hock et al., 1991). This again correlates with the data in Table III where the nude mouse system fails to reject IFN-γ- and IL-7-producing tumor cells. As already mentioned the behavior of IL-2 producers is not uniform. In some systems the inocula are infiltrated by T-cells (Pardoll, 1992) while in others the presence of macrophages and mast cells has been noted (Fearon et al., 1990).

A recent study is particulary informative (Hock et al., 1993) because the behavior of a single tumor cell line (J558) secreting a series of different cytokines has been investigated. At early times after injection of the inocula, IL-2-, IL-4-, TNF-α- and IFN-γ-producing J558 cells showed suppressed tumorigenicity in the immunodeficient nude, SCID, beige and NIHIII mice, but tumors eventually arose with a delay of 3 weeks in comparison

with mice injected with the parental cell line. IL-7-producing tumor cells were not rejected. This is confirmatory evidence that most cytokines can elicit a T-cell-independent mechanism capable of suppressing tumor growth in a first, early phase after injection. The late outgrowth of tumors noted above was shown to be correlated with a partial or complete loss of cytokine production in cytokine-producing cells presumably through a selection process for a non-cytokine-producing population of cells.

A correlation of the behavior of the tumors with the immunohistology of the injection site after 3–13 days in immunocompetent mice has been made (Hock et al., 1993): IL-2-, IL-7- and TNF-α-producing inocula were already infiltrated by T-cells at early time points. T-cells were also observed in IL-4-producing tumors but not until 1 week after injection. In the case of IL-2 producers, $CD8^+$ but not $CD4^+$ infiltrates could be found. IL-7- and IFN-γ-secreting tumors were densely infiltrated with both $CD4^+$ and $CD8^+$ cells. However, the predominant cell type found in the infiltrates of all cytokine producers excepting IL-7 producers were macrophages, which presumably accounts for the rejection of these tumors in the nude mouse at early times.

(c) Systemic long-term tumor rejection is based on CTLs

Mice which have been immunized with transfected tumor cells producing a variety of cytokines such as IL-2, IL-4, GM-CSF, or IFN-γ, develop a systemic immune response which protects the animals from tumor growth after challenge with non-transfected tumor cells at a site distant from the primary immunization site (see also Table II). This protective activity is maintained for a prolonged period of time. For instance, Gansbacher et al. (1990a,b) observed complete protection in a CMS-5 fibrosarcoma model, even when the challenge was performed 6 weeks after the immunization with IFN-γ-secreting tumor cells. Ley et al. (1991) demonstrated partial prevention of tumor growth when the challenge was made 90 days after immunization with IL-2-producing mastocytoma cells. Fearon et al. (1990), however, found a less persistent protection in a colon carcinoma model where the immune response induced by IL-2-transfected CT26 cells lost 50% of its efficacy within 4 weeks.

There are several lines of evidence that this prolonged systemic immune response is mediated by CTLs. Many groups (e.g., Fearon et al., 1990; Gansbacher et al., 1990a,b; Golumbek et al., 1991; Ley et al., 1991; Dranoff et al., 1993) have demonstrated an increase of CTL activity upon immunization with cytokine expressing tumor cells. The actual contribution of CTLs to tumor rejection has been confirmed in that depletion of $CD8^+$ cells in immunized mice abolishes the ability to suppress tumor growth. In the case of GM-CSF-producing B16 melanoma cells, $CD4^+$ T-cells are required in addition to $CD8^+$ cells for systemic protection (Dranoff et al., 1993). The observation that the systemic immune response is specific to the tumor used for immunization, and that the growth of non-cross-reactive syngeneic tumors is not affected, underlines the role of CTLs for the generation of a tumor-specific, MHC-I-restricted, systemic immune response against the parental tumor in these models (Fearon et al., 1990; Gansbacher et al., 1990a,b).

(d) Transferrinfection of tumor cells meets several requirements for efficient vaccine production

The proposed immunization procedure in man calls for the following steps: (*i*) Operative removal of the primary tumor tissue; (*ii*) culture of the tumor cells in vitro; (*iii*) transfection or transduction of tumor cells with the chosen gene or combination of genes; (*iv*) irradiation of the tumor cells to prevent their proliferation; (*v*) subcutaneous or intradermal injection of the treated tumor cells into the patient.

Since the tumor tissue may be heterogeneous due to tumor progression it may be desirable not to select for transfected or transduced cell clones, as has been done in most experiments reported up to now (see Table II). This procedure, besides being time consuming and delaying unnecessarily the application of the 'tumor vaccine', may inadvertently lead to selection of an unrepresentative cell clone. Furthermore, extensive culturing and expansion of cells may lead to a loss of the tumor antigen. A procedure by which the bulk of the tumor cells can be transfected soon after removal and culture would seem desirable.

We believe that our recently developed transferrinfection procedure (Cotten et al., 1992; Curiel et al., 1992; Wagner et al., 1992; Zatloukal, 1992) has many advantages over retroviral transduction or DNA transfection followed by clonal expansion of the genetically modified cells (see Table II).

Transferrinfection is a new transfection protocol in which the plasmid DNA to be transported into the tumor cell is reacted with transferrin-polylysine to form highly condensed round particles with a diameter of approx. 100 nm, referred to as 'donuts' (Zatloukal et al., 1992). These 'donuts' are linked to Ad5dl312 or Ad5dl1014, which, owing to its endosomolytic property, greatly enhances the receptor-mediated transfer of genes into cells and ultimately into the cell nucleus (Cotten et al., 1992; Curiel et al., 1992; Wagner et al., 1992; Zatloukal et al., 1992). We show below that transferrinfection of tumor cells with IL-2-producing plasmids leads to secretion of cytokines at high levels (cf. Table II), eradicates tumori-

TABLE IV

Loss of tumorigenicity of transfected M3 melanoma cells with DBA/2 mice

Gene transfer	Appearance of tumors [weeks (w) after tumor cell implantation[a]]						
	1w	2w	3w	4w	5w	6w	7w
No gene transfer	0/6	6/6	6/6	6/6	6/6	6/6	6/6
IL-2	0/6	0/6	0/6	0/6	0/6	0/6	0/6
IFN-γ	0/6	0/6	1/6	1/6	1/6	1/6	1/6

[a] To generate the transfer complexes, 2×10^9 biotinylated Ad5d1312 in 100 ml HBS (20 mM Hepes/150 mM NaCl) was mixed with streptavidinylated polylysine (0.5 μg) in 100 ml HBS and incubated for 30 min at room temperature. Thereafter, 6 μg of plasmid DNA (IL-2 or IFN-γ) in 150 ml HBS were added, mixed thoroughly and incubated for 30 min. Finally, polylysine-modified human transferrin (7.5 μg) in 150 μl HBS were added, mixed thoroughly and incubated for a further 30 min. For transfection the complexes were applied to M3 cells (3×10^5) in a total volume of 2 ml culture medium. After 4 h of incubation at 37° C the complex-containing medium was removed and fresh medium containing serum was added. The cells were then trypsinized and washed twice in Ham's F10 culture medium without serum. 10^5 M3 cells not transfected or transfected with IL-2 or IFN-γ plasmids were applied subcutaneously into the backs of each of six anesthesized DBA/2 mice. Tumors were scored at weekly intervals.

genicity and yields systemic protection against a challenge with live tumor cells.

(e) Loss of tumorigenicity in transferrinfected murine melanoma M3 cells

The murine melanoma cell line Cloudman S91 (clone M3) was obtained from ATCC (No. CCL53.1). M3 cells which were established from a spontaneously developed melanoma in DBA/2 mice (Cloudman, 1941)** express low levels of MHCI antigens (unpublished observation) and are only moderately immunogenic. The cells were cultured in 0.1% gelatin-coated 6-cm plastic dishes or T25 culture flasks in Ham's F10 medium containing 12.5% horse serum/2.5% fetal calf serum/2 mM glutamine and antibiotics. In order to demonstrate loss of tumorigenicity of the M3 cells upon transfection with *IL-2* or *IFN-γ* genes driven by CMV promoter cells were applied subcutaneously into the backs of six anesthesized DBA/2 mice. IL-2 production prior to injection but after irradiation was at 2400 units/10^6 cells per 24 h and IFN-γ production was 865 ng/10^6 cells per 24 h. Tumors were scored at weekly intervals.

As can be seen in Table IV the control mice all developed tumors within 2 weeks after inoculation. All mice challenged with IL-2-producing M3 cells remained free of tumors, while in the case of IFN-γ 5/6 remained tumor-free. The results obtained show that the highly tumorigenic M3 cells lose their tumorigenicity completely when producing IL-2 and nearly completely when producing IFN-γ. Therefore, the secretion of cytokines can abolish tumorigenicity of the M3 cells.

(f) Moderate levels of IFN-γ afford no systemic protection, high levels of IL-2 afford complete systemic protection against a challenge with tumorigenic M3 cells

In another set of experiments mice were immunized with high-IL-2-expressing M3 cells (3×10^4 units/10^6 cells per 24 h); another group was immunized with cells producing IFN-γ at moderate levels. Ad5dl1014 was used as endosomolytic agent in these experiments. Injection of the immunizing dose (10^5 cytokine-producing irradiated M3 cells) was into the left flank on day 0 and day 7 followed by a challenge with 10^5 live tumorigenic M3 cells injected into the right flank on day 14. As can be seen from Table V, non-immunized mice developed tumors very quickly. They were killed after week 7. The IL-2 producers were fully protected. Although moderate IFN-γ producers nearly completely abolished tumorigenicity of (non-irradiated) M3 cells (see Table IV), no systemic protection against the challenge with live M3 cells was obtained as the values did not differ from those obtained with non-transferrinfected irradiated M3 cells (unpublished results). This contrasts with the findings of Watanabe et al. (1989) and Gansbacher et al. (1990a,b) who used neuroblastomas and fibrosarcomas and found a systemic protection in both of these tumor types after application of IFN-γ-producing cells.

(g) Only M3 cells expressing high levels of IL-2 establish complete systemic protection against challenges with tumorigenic M3 cells

A striking feature of the data in Table II is that generally low levels of cytokine secretion are obtained. A notable exception to this is the recent work of Dranoff et al. (1993) in which generation of novel retroviruses allows high expression levels of all cytokines tested. The claim of these authors that in most previous studies vaccination with irradiated tumor cells alone would have generated systemic antitumor immunity at levels comparable to those induced by live transduced cells has serious implications.

High levels of IL-2 secretion of between 2×10^4 and 10^5 units/10^6 cells per 24 h can be obtained in vitro by transferrinfection of murine M3 melanoma cells. The desired levels of IL-2 production can be adjusted simply by mixing the IL-2 expression vector with plasmid devoid of the *IL-2* cDNA.

That the high level of IL-2 secretion is an important

**Note that the information given by ATCC on CCL 53.1 Clone M3 (Cloudman S 91 melanoma) is erroneous with respect to the syngeneic host.

TABLE V

Protection of immunized DBA/2 mice from M3 melanoma development[a]

Immunizations (2×) (1×10^5 cells, irradiated)	Appearance of tumors [weeks (w) after tumor cell]								
	1w	2w	3w	4w	5w	6w	7w	8w	9w
No immunization	6/6	6/6	6/6	6/6	6/6	6/6	6/6		
IL-2 100% (dl1014)	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
IFN-γ 10% (dl1014)	4/6	5/6	5/6	5/6	5/6	5/6	5/6	5/6	5/6

[a]Transfer complexes were generated as shown in Table IV for IL-2 100% and IFN-γ 10%. IFN-γ plasmids were diluted out with parental plasmid pSP in a ratio of 10:90. IL expression was: IL-2 100% = 3×10^4 u/10^6 cells per 24 h; IFN-γ 10% = 800 ng/10^6 cells per 24 h.

feature for eliciting systemic protection can be seen from Table VI. M3 melanoma cells were transferrinfected with the *IL-2* gene to reach secretion levels of 3×10^4 units/10^6 cells per 24 h in vitro. In order to reduce the level of IL-2 production, the IL-2 plasmid was mixed with the parental plasmid pSP which did not contain the CMV *IL-2* gene construct (in a ratio of 4:96). The secretion rate obtained was 400 units IL-2 per 10^6/24 h. For vaccination, groups of mice, usually six, were injected with irradiated (2000 rad) IL-2-producing M3 cells at the two different levels indicated above. Injection was into the left flank on day 0 and day 7, as above, followed by a challenge with 10^5, 3×10^5 and 10^6 live tumorigenic M3 cells injected into the right flank on day 14. As shown in Table VI, all non-immunized mice developed tumors within 3 weeks. Three out of six mice vaccinated with irradiated cells which had been transfected with the parental plasmid devoid of the *IL-2* gene developed tumors, suggesting that transfected and irradiated M3 tumor cells were partially immunogenic. Mice vaccinated with irradiated high-level IL-2 producers afforded complete and lasting protection even with challenges as high as 3×10^5 cells, but at 10^6 cells 1/4 mice developed a small tumor. By contrast, low IL-2-producing cells did not withstand a challenge with 3×10^5 cells, 3/5 mice developing tumors. The high IL-2 producers did not protect against a challenge with a low dose of KLN205 cells showing that the protection in the above experiments was tumor-specific.

TABLE VI

Protection of immunized DBA/2 mice from M3 melanoma development after increasing challenge with tumorigenic cells[a]

Challenge with	10^5	3×10^5	10^6 cells
No immunization	6/6	–	–
pSP	3/6	–	–
IL-2 (3×10^4 u)	0/6	0/6	1/4
IL-2 (4×10^2 u)	0/5	3/5	4/5
Challenge with	10^5 KLN205 cells		
IL-2 (3×10^4 u)	6/6	–	

[a]The transfer complexes were generated as shown in Table IV except that Ad5dl1014, 8-methoxypsoralen (8-MOP)/UV-irradiated, was used. For high-producer M3 cells 6 μg IL-2 plasmid DNA was used. For the low IL-2 producers the 6 μg DNA consisted of IL-2 plasmid diluted out with the parental plasmid without insert (pSP) in a ratio of 4:96. Tumor load was scored 3 weeks after tumor challenge.

(h) Conclusions

The examples given in Tables IV–VI clearly demonstrate that transferrinfected M3 cells express cytokines at high levels and over a period long enough to suppress tumorigenicity of their cells and in the case of high IL-2 secretors impart systemic and lasting protection against a challenge of highly tumorigenic cells. Our results reinforce the concerns of Dranoff et al. (1993), and suggest that high levels of cytokine production may be necessary for high efficacy of 'tumor vaccines'.

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