

Immunization with MHC class I-negative but not -positive HPV16-associated tumour cells inhibits growth of MHC class I-negative tumours

MILAN REINIS, JANA SÍMOVÁ, MARIE INDROVÁ, JANA BIEBLOVÁ, HANA PRIBYLOVÁ, SIMONA MORAVCOVÁ, TÁNA JANDLOVÁ and JAN BUBENÍK

Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Received October 26, 2006; Accepted December 22, 2006

Abstract. Loss or downregulation of MHC class I molecules on tumour cells is a common mechanism by which tumours can escape from T-cell mediated immune responses. In this study we have investigated the immunologic crossreactivity between murine tumour cell lines expressing human papilloma virus (HPV) 16-derived E6/E7 oncoproteins with distinct surface expression of MHC class I molecules. The aims of this study were to demonstrate whether immune responses capable of coping with MHC class I-positive tumours can also be effective against their MHC class I-deficient derivatives and whether it is possible to induce immunity against MHC class I-deficient tumours by cellular vaccines based on MHC class I-deficient tumour cell lines. Our data showed that immunization with MHC class I-deficient but not with MHC class I positive tumour cells inhibited the growth of MHC class I-deficient tumours. *In vivo* depletion studies revealed that the mechanisms underlying effective immune responses against MHC class I-negative tumours in animals immunized with MHC class I-deficient tumour cells involved natural killer cells. The presented findings are of particular clinical relevance in the sense of construction of vaccines directed against a broad spectrum of HPV-associated tumours.

Introduction

One of the major problems in the search for effective anti-cancer vaccines is the development of vaccines that could be used against a broad spectrum of tumours belonging to the same histological type, but originating from different patients or against tumours at different stages of their development. Partial and total loss of the MHC class I expression on tumour cells are frequent events in the course of tumour development, including cervical carcinoma (1-4). MHC class I down-modulation is considered to be one of the principal mechanisms of tumour escape from the immune surveillance mediated by cytotoxic T lymphocytes (CTLs) (5-8). On the other hand, complete loss of MHC class I surface expression on tumour cells makes them susceptible to the lysis mediated by natural killer (NK) cells due to 'missing self' recognition (9,10). This NK-mediated immune response is considered to be weaker than the CTL-mediated responses, although this conclusion is not universal (11,12).

A question is whether it is possible to induce immunity against MHC class I-deficient tumours by immunization with cellular vaccines and whether such vaccines would be active exclusively against MHC class I-negative tumours (13). Another important factor in understanding the mechanisms of the tumour escape from immune surveillance is the efficacy of the possible MHC class I-unrestricted immune responses elicited by MHC class I-positive tumour cells against MHC class I-deficient tumour derivatives.

It is known that MHC class I-deficient tumours can be effectively controlled by the immune system (13,14) and that they are susceptible to immunotherapeutic intervention with cytokines (15), unmethylated CpG oligonucleotides (16) or cellular vaccines based on tumour cells engineered to express immunoactive molecules (17,18). The mechanisms underlying immunocytokine therapy can differ according to the level of MHC class I expression on tumour cells (19). The effects of specific vaccinations are much less understood. So far, the results of the limited number of studies using well-defined experimental tumours in respect to their MHC class I status indicate that it is possible to induce effective immunity against MHC class I-deficient tumours at least with immunization protocols using cytokines or costimulatory molecules as adjuvants (17,20-23). The mechanisms underlying this

Correspondence to: Professor Jan Bubeník, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20, Prague 4, Czech Republic
E-mail: bubenik@img.cas.cz

Abbreviations: HPV, human papilloma virus; T_{reg} cells, T-regulatory cells; CTL, cytotoxic T lymphocyte; Th, T helper lymphocyte; NK, natural killer; MK16 cells, MK16/1/IIIABC cells; MFI, mean fluorescent intensity; E/T, effector/target cell ratio; TAP, transporter associated with antigen processing; LMP, low-molecular mass polypeptide

Key words: HPV16, MHC class I-deficient tumours, immunologic crossreactions, anti-tumour vaccines

immunity are not fully understood; they may include for example NK, Th cells and IFN γ production (21,24). In addition, the role of CD8⁺ cells cannot be excluded (25). The specificity of immunization protocols may be questionable as protective immunity, e.g. after DNA immunization, was achieved also with control vectors (24). More studies on the differences in immune responses against tumours of the same aetiology, but different in expression of MHC class I molecules, as well as experiments focused on immunologic crossreactivity between parental tumour cells and their MHC class I-deficient derivatives are required.

Most cervical neoplasms can be attributed to infection with high-risk human papilloma virus (HPV), HPV16 and HPV18 being the most prevalent types. Viral oncoproteins E6 and E7 are expressed in all tumour cells and crucial for maintaining a malignant phenotype. Therefore, they have been selected as targets of choice for specific immunotherapy promoting the response against HPV-associated tumours. Several MHC class I-deficient sublines derived from model murine tumour cell lines expressing E6/E7 viral oncogenes have been established and characterized (26).

Preliminary data from our laboratory revealed that two distinct cell lines TC-1 (MHC class I-positive) and MK16/IIIABC (MK16, MHC class I-negative) expressing E6/E7 antigens did not immunologically crossreact *in vivo* (27). In this study, we have analyzed the immune crossreactivity between parental tumour cells and their immune escape variants, expressing E6/E7 antigens but deficient in their MHC class I expression. Moreover, we have investigated the crossreactivity between two distinct MHC class I-deficient tumour cell lines expressing the same E6/E7 oncoproteins. We have used a model of tumour cells with MHC class I down-modulation due to defects in APM. This model is of clinical relevance since the MHC class I-deficient tumour derivatives originating from coordinated downregulation of APM components (28,29) are frequently observed in HPV-associated cervical carcinomas in humans (30,31).

Materials and methods

Cell lines. Moderately immunogenic MHC class I-positive cell line TC-1 was obtained by *in vitro* co-transfection of murine lung C57BL/6 cells with HPV16 E6/E7 and activated human Ha-*ras* (G12V) oncogenes (32). TC-1/A9 (MHC class I-negative) (33) and TC-1/P3C10 (with reduced expression of MHC class I molecules) (21) cell lines represent tumour cell derivatives which escaped from the selection pressure mediated by the specific immune response due to concomitant tumour immune response since they were obtained from TC-1 tumours developed in immunized mice. Another E6/E7-expressing MHC class I-deficient cell line MK16, moderately immunogenic in syngeneic mice derived from kidney C57BL/6 cells, was described in detail elsewhere (34). MHC class I downmodulation in all cell lines was caused by defects in expression of genes of antigen-presenting machinery (TAP-1, TAP-2, LMP-2, LMP-7). The RVP3 cell line (35) has been used as an indifferent MHC class I-deficient control, not associated with HPV16. All cell lines were cultured in RPMI-1640 medium supplemented with 10% of foetal calf serum, L-glutamine and antibiotics.

Mice. C57BL/6 (B6) mice, 2-4 months old, were obtained from Anlab, Prague, Czech Republic. The mice were housed in the animal facility of the Institute of Molecular Genetics. Experimental protocols were approved by the Institutional Animal Care Committee of the Institute of Molecular Genetics, Prague.

***In vivo* immunization-challenge experiments.** Mice were twice immunized with 1×10^7 irradiated tumour cells (days -35 and -14, subcutaneous injection, irradiation dose 150 Gy) and challenged on day 0. All tumour cells were injected subcutaneously. The experimental and control mice were observed bi-weekly, and the numbers of tumour-bearing mice and the size of the tumours were recorded. Two perpendicular diameters of the tumours were measured with a calliper and the tumour size was expressed as the tumour area (cm²).

Peptides and peptide immunization. The HPV16-derived, high affinity H-2D^b binding peptide E7₄₉₋₅₇ (RAHYNIVTF) (36) and the control peptide, H-2K^b binding, ovalbumin-derived OVA₂₅₇₋₂₆₄ (SIINFELK), both of them CTL epitopes, were synthesized at the Institute of Organic Chemistry and Biochemistry, AS CR, Prague, or custom-synthesized by Sigma Genosys (Cambridge, UK). For immunizations, peptides were s.c. injected 25 days and 11 days prior to the challenge with tumour cells. Unmethylated CpG oligodeoxynucleotide 1826 (CpG ODN 1826) (37) was used as an adjuvant. The dosage for immunization was 100 μ g peptide and 50 μ g CpG ODN 1826 in 150 μ l PBS per dose.

Flow cytometry. Cell surface expression of MHC class I (H-2K^b/H-2D^b), CD80, CD86 and CD54 on the cell lines was determined by cytofluorometric analysis using anti-mouse monoclonal antibodies: PE hamster IgG1 λ 1 isotype control, PE anti-H-2D^b (KH95), PE anti-H-2K^b (AF-88.5), PE anti-CD80 (B7-1) (16-10A1), PE anti-CD54 (ICAM-1) (3E2), FITC rat IgG_{2a} κ isotype standard, PE anti-CD86 (GL1), FITC anti-I-A^b (A β ^b) (AF6-120.1), purified anti-mouse Qa-1^b (6A8.6F10.1A6) and FITC-conjugated goat anti-mouse Ig antibody. Cells were preincubated with anti-CD16/CD32 antibody to minimize non-specific binding and the analysis was performed using a FACScan Elite cytometer (Coulter, Miami, FL). For analysis, 10,000 cells were counted. All antibodies utilized including the isotype-matching controls were purchased from Pharmingen, San Diego, CA.

RT-PCR. Total RNAs were isolated from cell lines using the RNA isolation kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. Oligo (dT)-primed reverse transcription was performed with GeneAmp RNA Core Kit (Applied Biosystems, Foster City, CA). cDNAs were amplified by PCR using the same kit and specific primers for β -actin, H-2D^b, H-2K^b, TAP-1, LMP-2, HPV16 E6, HPV16 E7 (33,34), TAP-2 (38), LMP-7 (28), B7-H1 and B7-H2 (39).

Determination of the relationship between the levels of MHC class I expression on tumour cells and their sensitivity to lysis by spleen cells from immunized mice. MHC class I expression on TC-1/A9 or MK16 cells was induced by culturing for 72 h in

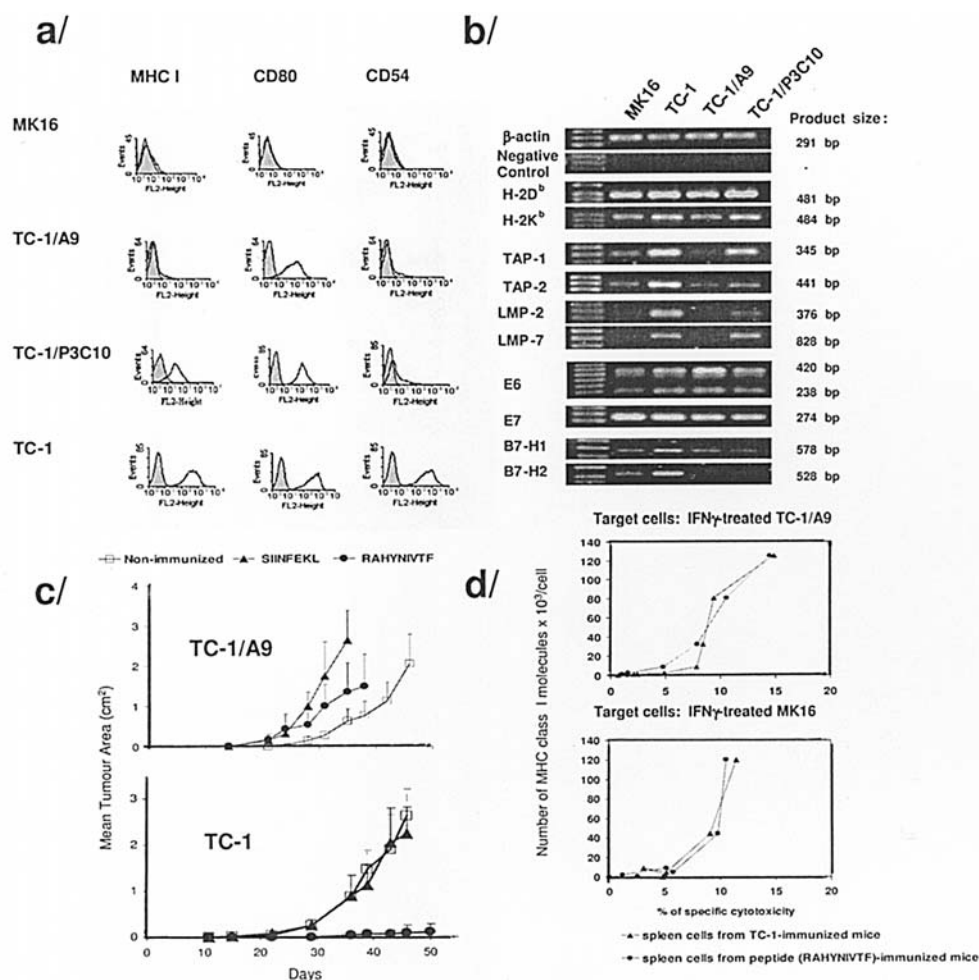


Figure 1. Phenotypic characterization of the tumour cell lines. (a) Distinct expression of MHC class I (H2-D^b and H2-K^b), CD80 and CD54 molecules on the tumour cell lines was determined by flow cytometry. (b) Analysis of mRNA expression in the cell lines by RT-PCR revealed defects in the expression of TAP-1, TAP-2, LMP-2 and LMP-7 and different expression of B7-H1 and B7-H2 in distinct cell lines. Expression of E6 and E7 oncoproteins was proved in all cell lines. Samples with no addition of reverse transcriptase in the cDNA preparation step served as negative controls. (c) Peptide immunization. Mice (8 per group) were immunized with E7₍₄₉₋₅₇₎ peptide or with control peptide OVA₂₅₇₋₂₆₄ and challenged with TC-1/A9 or TC-1 cells. Controls were non-immunized mice. Significant inhibition of the TC-1 but not of the TC-1/A9 tumour growth in E7₍₄₉₋₅₇₎-immunized mice compared to non-immunized and control peptide-immunized mice was recorded. The experiments were performed twice with similar results. (d) IFN_γ-treated TC-1/A9 and MK16 cells were lysed by spleen cells from mice immunized both with TC-1 cells and with E7₍₄₉₋₅₇₎ peptide. The lysis efficacy is related to the number of the MHC class I molecules per cell.

complete RPMI-1640 medium supplemented with 0-50 ng/ml IFN_γ (40), and sets of cell populations with distinct surface expression of MHC class I molecules were obtained. The numbers of MHC class I molecules (H2-D^b and H2-K^b) were assessed by flow cytometry using specific antibodies and a QuantiBRITE PE beads (Becton Dickinson) quantification kit for fluorescence quantitation. The cell populations characterized in respect to the level of MHC class I expression were subjected to ⁵¹Cr-microcytotoxicity assays (41) and the relationship between levels of MHC class I expression on target cells and their susceptibility to lysis were determined.

In vivo depletion studies. *In vivo* depletion of NK1.1⁺, CD4⁺ and CD8⁺ cells was performed using monoclonal antibodies PK 136, GK 1.5, and 2.43, respectively (40-44). To deplete the effector cells, 0.1 mg of the antibody was i.p. injected into mice, during the first week injections were given three times and in the following two weeks, mice received injections once a week. Depletion was verified by the staining of spleen cells with labelled antibodies and FACS analysis. To efficiently

diminish the number of NKT cells without activation of NK cells, 10 μg of β-galactosylceramide (C12) (45) were repeatedly i.p. injected into mice at the same time-points as depletion antibodies.

Statistical analysis. For statistical analyses of the numbers of tumour takes in experimental and control groups, Peto-Wilcoxon and log-rank tests from NCSS, Number Cruncher Statistical System (Kaysville, UT) statistical package and the χ² test were utilized. Analysis of variance by Newman-Keuls and Tukey-Kramer tests was used for statistical evaluation of the tumour growth curves. The differences between the curves were considered significant if the p-value was <0.05. For evaluation of chromium release microcytotoxic assays, the Student's t-test was utilized.

Results

Characterization of the cell lines. The tumour cell lines used in this study have been characterized in detail (Fig. 1). The parental

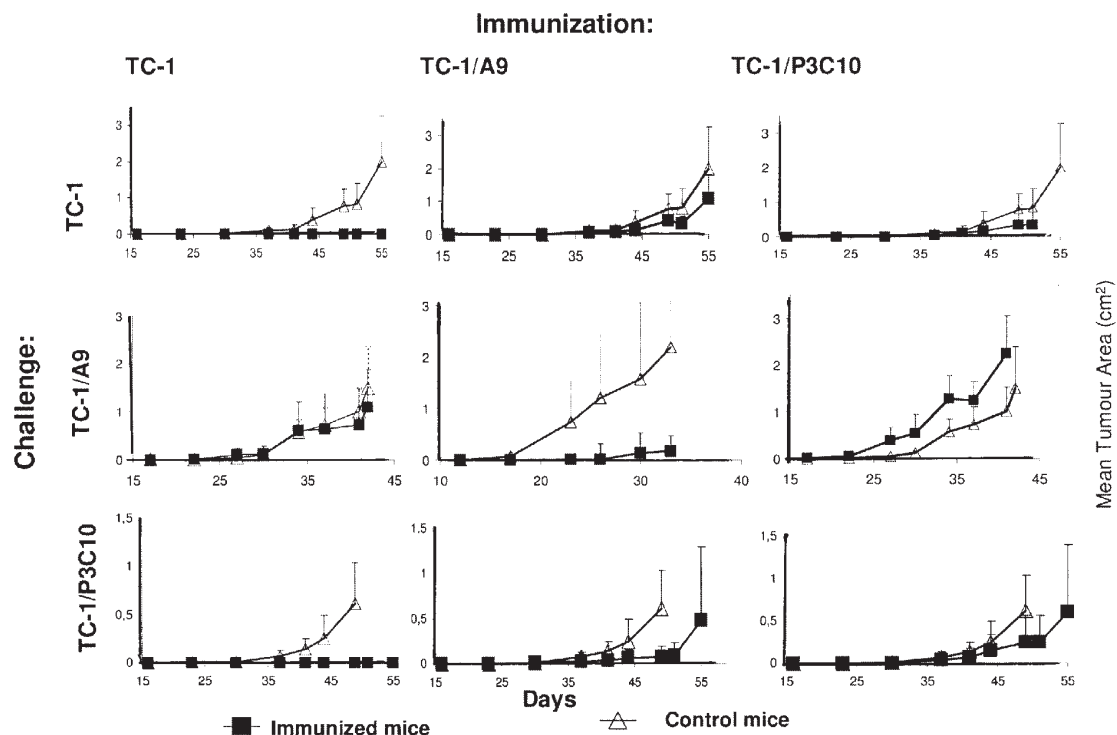


Figure 2. Immunologic crossreactivity between TC-1 cells and their MHC class I-deficient variants *in vivo*. Mice (7-8 per group) were immunized with TC-1, TC-1/A9 or TC-1/P3C10 tumour cells 5 weeks and 2 weeks prior to challenge (day 0) with 1×10^4 TC-1 cells, TC-1/P3C10 and TC-1/A9. All experiments were performed at least twice with similar results. Significant inhibition of the tumour growth in immunized mice compared to the controls (determined by Newman-Keuls and Tukey-Kramer tests, $p < 0.05$) was recorded in mice immunized with the TC-1 cells and challenged with the TC-1 and the TC-1/P3C10 cells, in mice immunized with the TC-1/A9 cells and challenged with all tested cell lines and in mice immunized with the TC-1/P3C10 cells and challenged with the TC-1 and TC-1/P3C10 cells. Immunization with P3C10 did not block but significantly accelerated the growth of the TC-1/A9 tumours.

TC-1 cell line was MHC class I (H2-D^b and H2-K^b)-positive and subline TC-1/A9 MHC class I-negative, as determined by flow cytometry. The TC-1/P3C10 cell line displayed decreased but still detectable cell surface expression of MHC class I. The MK16 cell line was MHC class I-negative. Differences in the cell lines were also noted in the expression of other selected immunoactive molecules (CD80, CD54, B7-H1 and B7-H2). All cell lines expressed HPV16-derived E6 and E7 oncogenes (determined by RT-PCR), as well as H-2K^b and H-2D^b mRNAs. Expression of genes representing the antigen-presenting machinery (APM), namely TAP1, TAP2, LMP2 and LMP7, was suppressed in MHC class I-deficient derivatives. All cell lines were MHC class II-, non-classic MHC class I Qa-1- and CD86-negative (data not shown). The efficacy of the specific immune response mediated by CTLs after immunization with the immunodominant peptide RAHYNIVTF against TC-1 but not TC-1/A9 was demonstrated *in vivo* by immunization-challenge experiment. Finally, we have demonstrated that both TC-1/A9 and MK16 cells can be lysed by specific CTLs after induction of MHC class I expression on their surface with IFN γ and the efficacy of the lysis was related to the MHC class I expression level on the target cells. MK16 cells were less susceptible to the lysis compared to TC-1/A9 cells. Significant specific lysis of TC-1/A9 and MK16 cells by spleen cells from TC-1-immunized mice was observed in our experimental setting when the target cells expressed $\sim 10,000$ MHC I molecules and 45,000 molecules of MHC I per cell, respectively. Similarly, when the spleen cells from the mice

immunized with the E7₄₉₋₅₇ peptide were used as effectors, the lysis became significant when TC-1/A9 cells and MK16 cells expressed 3000 and 10,000 molecules per cell, respectively.

Crossreactivity in vivo. The first goal was to assess the immunologic crossreactivity *in vivo* between parental MHC class I-positive TC-1 tumour cells and TC-1/A9 and TC-1/P3C10 cell lines (Fig. 2). We have demonstrated that immunization with MHC class I-deficient TC-1/A9 cells inhibited the growth of TC-1/A9 tumour while immunization with MHC class I-positive TC-1 and TC-1/P3C10 cells with reduced MHC class I expression did not result in protection against challenge with the TC-1/A9 MHC class I-deficient cell line. On the other hand, immunization with the MHC class I-deficient tumour cell lines inhibited the growth of the MHC class I-positive TC-1 tumours, albeit this protection was significantly weaker as compared to the TC-1 cell immunization. While immunization with the TC-1 cells prevented the growth of the TC-1 tumours (on day 49, no mice out of 8 was tumour bearing compared to 7 out of 7 in the control group), immunization with the TC-1/A9 and the TC-1/P3C10 cell lines inhibited the tumour growth but did not influence the number of tumour takes. These results demonstrate that cross-priming, not dependent on MHC class I expression, is weaker than the direct priming. Immunization with the TC-1/P3C10 cells induced protective immunity against challenge with the same tumour cells as well as with the TC-1 cells, but not with MHC class I-negative TC-1/A9 cells. Furthermore, we assessed the crossreactivity between two

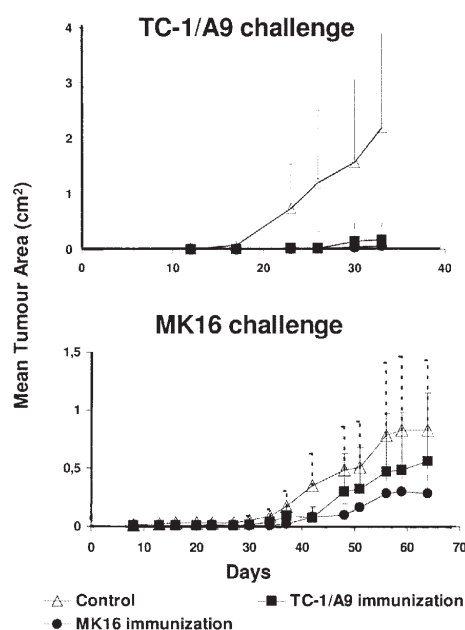


Figure 3. Immunologic crossreactivity between two MHC class I-negative tumour cell lines. Mice (8-10 per group) were immunized with TC-1/A9 or MK16 tumour cells 5 weeks and 2 weeks prior to challenge (day 0) and challenged with either 1×10^4 TC-1/A9 or 1×10^5 MK16 cells. Significant inhibition of the TC-1/A9 and the MK16 tumour growth compared to controls was observed in mice immunized with both cell lines (determined by Newman-Keuls and Tukey-Kramer tests, $p < 0.05$). The experiments were performed three times with similar results. Control experiments demonstrated that MHC class I-deficient cell line RVP-3, which did not express E6/E7 oncoproteins, had no effect on the growth of TC-1/A9 cells (data not shown).

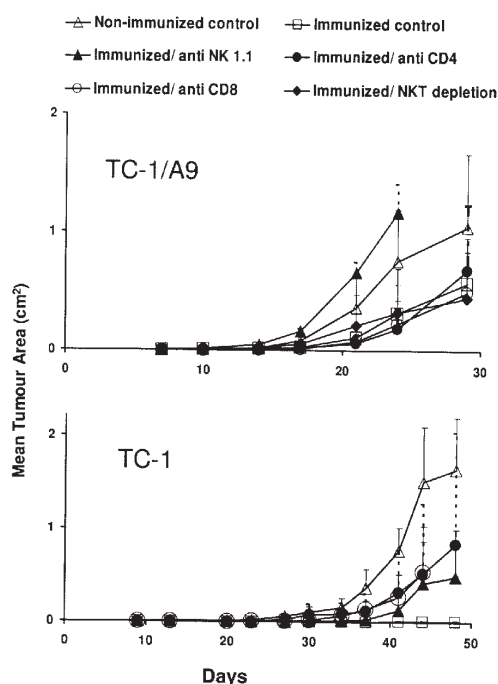


Figure 4. *In vivo* depletion of NK 1.1⁺ cells but not of CD4⁺ or CD8⁺ cells accelerates TC-1/A9 tumour growth. The growth of the TC-1/A9 tumours in mice immunized with the TC-1/A9 cellular vaccine was significantly accelerated ($p < 0.05$) after the depletion of NK1.1⁺ cells but not of CD4⁺, CD8⁺ or NKT cells. In the control experiment, the growth of the TC-1 tumours was significantly accelerated after the depletion of CD4⁺ and CD8⁺ cells. Each experimental group comprised at least 7 mice; the experiments were performed twice with similar results.

E6-, and E7-expressing cell lines TC-1/A9 and MK16, which were phenotypically MHC class I-negative, but of different origin and, moreover, they expressed distinct sets of selected immunomodulatory molecules (CD80, CD54, and B7-H1). Crossreactivity between these two cell lines was determined since the immunization with these tumour cell lines inhibited the growth of both TC-1/A9 and MK16 tumours (Fig. 3). Importantly, no immunity was induced by control MHC class I-deficient cell line RVP3. These results strongly suggest the significance of MHC class I expression in immunologic cross-reactivity between various tumour cell lines of the same aetiology.

In vivo depletion experiments. The role of CD4, CD8, NK and NKT cells was assessed by *in vivo* depletion experiments. These experiments revealed that the growth of TC-1/A9 tumours in immunized, similarly to non-immunized mice, depended on NK cells; only the depletion of the NK1.1⁺ cells but not of the CD4⁺, CD8⁺ or NKT cells significantly accelerated tumour growth (Fig. 4). In the control experiment, depletion of CD8 and CD4 cells, as expected, significantly accelerated TC-1 tumour growth in TC-1-immunized animals. These results indicate that even in the immunized mice the NK cells play a crucial role in the protection against the TC-1/A9 tumour challenge.

Discussion

We have demonstrated that immunization with a particular tumour cell line (MHC class I-positive or -deficient) induced immunity against the homologous tumours. These results suggest that immunization with cellular vaccines based on MHC class I-deficient tumour cells can be an effective tool against MHC class I-deficient tumours which escaped from the original immune response mediated mainly by CD8⁺ CTLs. Such vaccines can also be effective against parental MHC class I-positive tumours due to activation of the CTLs by cross-priming. On the other hand, the lack of protective immunity against MHC class I-negative tumours after immunization with MHC class I-positive tumour cells indicate that effective MHC class I-unrestricted responses are not elicited by immunization with cellular vaccines based on the MHC class I-positive tumour cells. Notably, TC-1/P3C10 cells, with ~10-fold down-regulated MHC class I cell surface expression compared to the level on the TC-1 cells, did not elicit protective immunity against the challenge with TC-1/A9 cells and thus could be considered as 'MHC class I-positive'. The model used for our studies, the tumour cell sublines established from immunized animals, is relevant to the *in vivo* situation. On the other hand, it was not possible to exclude other factors which could, besides the MHC class I down-modulation, influence the mutual immunologic crossreactivity between two cell sublines. Therefore, the experiments analyzing the crossreactivity between the MK16 and the TC-1/A9 cell lines were performed. These two tumour cell lines were selected because they expressed the same tumour rejection antigens (E6 and E7), both were phenotypically MHC class I-negative and they were distinct in the expression of molecules important for interaction with T lymphocytes (CD54, CD80, B7-H2), an expression level of which could also contribute to

the immune escape (46). Importantly, these cell lines were different in their origin (lungs vs. kidney). The crossreactivity between these two cell lines suggested a key role for the lack of MHC class I expression in the immune response and documented that immunization with a cellular vaccine based on the tumour cells deficient in MHC class I expression could elicit protective immune responses against tumours with a similar MHC class I status.

Distinct mechanisms of the immune responses against MHC class I-positive and -deficient tumours were further demonstrated by the *in vivo* depletion experiments. Our results document that the NK cells play an important role in the immune control of tumour growth not only in the treatment-naïve but also in immunized animals, albeit the involvement of other mechanisms, e.g. those dependent on antibodies, cannot be excluded. The possible mechanisms by which immunization can influence the NK-mediated immunity are not clear and will be further investigated.

Unlike in the control immunization-challenge experiments with the TC-1 cells, *in vivo* depletion studies with mice immunized with and bearing TC-1/A9 tumours did not provide a role for the CTLs in the immune control of the tumour growth. Mechanisms leading to the MHC class I downmodulation, its reversibility and the level of downmodulation may be of particular importance in this context and CTLs could theoretically contribute to the anti-tumour immunity. MHC class I re-expression can be induced during experimental tumour growth by local IFN γ secretion (40), making the originally MHC class I-negative tumour cells again sensitive to the CTLs. Further, MHC class I-deficient tumours with impaired antigen processing machinery can be targeted by CTL clones recognizing novel epitopes derived from proteins processed in APM-independent manners, presented in the context of the 'residual' MHC class I molecules and different from those being immunodominant on MHC class I-positive tumour cells (25). However, so far we have found no evidence regarding the role of CD8⁺ CTL inhibition of TC-1/A9 tumour growth in our experimental settings.

Finally, the CD4⁺ cell depletion did not significantly accelerate the tumour growth in the immunized mice. These results suggest either no role of Th CD4⁺ cells in the immune responses or that the possible effect of CD4⁺ Th cell depletion was eliminated by the effect of simultaneous T_{reg} (CD4⁺/CD25⁺) depletion.

Recently, prophylactic vaccines comprising structural proteins derived from the four most common high-risk HPV types preventing establishment of persistent virus infection have been developed (47,48). On the other hand, no therapeutic vaccine is available against tumours expressing HPV viral oncoproteins. The data presented in this study are of clinical relevance since they document the possibility of vaccination against MHC class I-deficient tumours. Our results also strongly support the idea that the optimal immunotherapeutic strategy should vary according to the distinct MHC class I expression.

Acknowledgements

We are grateful to Dr T.C. Wu, who kindly provided the TC-1 cells, to Dr M. Smahel for making available the TC-1/A9

cells and to Dr Chien-Fu Hung for the TC-1/P3C10 cells. This study was supported by grant No. NR 7807-3 from the Grant Agency of the Ministry of Health of the Czech Republic, by the League against Cancer, Prague, by EU-FP6-NoE Clinigene Project No. 018933 and by grant No. AVOZ50520514 from the Academy of Sciences of the Czech Republic. The authors are grateful to Jasper Manning and Sarka Takacova for editorial help.

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