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In vitro and *in vivo* effects of reovirus on HPV16-transformed mice cells

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Oncolytic viruses are examined to serve as anticancer therapeutics. It is expected that in addition to direct oncolytic effect their action will also help eliciting a solid antitumor immunity. In presented series of experiments we have employed two HPV16-transformed mouse (strain C57/B6) cell lines, TC-1 and MK16/III/ABC (MK16), and reovirus type 3, strain Dearing (RV). Both cell lines are highly susceptible to RV and produce large amounts of infectious virus *in vitro* while normal human are not susceptible to RV. Still, some differences were encountered. TC-1 cells produced moderately lesser amounts of infectious virus, but, paradoxically, were more efficient producers of δ1 antigen of RV and as a consequence of virus infection died more rapidly than simultaneously infected MK16 cells. Minor differences between the cell lines were observed in the percentage of cells arrested in theG2/M phase of the cell cycle and in some markers of apoptosis. When inoculating high doses ($5x10^6$) of infected cells (MOI 10 PFU/cell) into syngeneic animals their oncogenic activity was strongly suppressed, nearly completely in the case of MK16 cells and somewhat less efficiently in the case of more oncogenic TC-1 cells. Immunizing experiments in which non-oncogenic doses (10^6) of RV infected TC-1 cells were tested in parallel with the same doses of irradiated cells brought surprising results. When immunized animals were challenged with TC-1 cells, the irradiated cells proved to be a much better immunogen that the infected cells. However, when challenged with MK16 cells the opposite was true. It is believed that this difference was associated with the different biological properties of the cell lines tested.

Key words: reovirus type 3, HPV16-transformed mouse cell lines, apoptosis, cell cycle, immunization/challenge experiments.

Despite the advances in clinical oncology that help to decrease patients´ mortality, cancer remains one of the main causes of death in developed countries. Current efforts to improve cancer therapy are aimed at enhancing drug efficacy while maintaining acceptable degree of toxicity. In order to succeed, innovatory therapeutic modes have been designed. One of these is represented by oncolytic viruses that infect, replicate in, and lyse tumor cells, but do not grow at all, or at a limited extent, in non-tumor cells [1–3].

Oncolytic viruses can cause the destruction of tumor cells not only by direct lysis resulting from virus replication. They may also effectively induce antitumor immunity that comprises both the antibody and T cell responses targeting tumor-associated antigens [4–6]. Schulz et al. showed that cells infected with viruses were more effective at delivering non-viral antigens for cross-priming of dendritic cells *in vivo* [7]. Furthermore, viral infection may induce cytokine production which support infiltration of tumor microenvironment by cytotoxic cells such as macrophages, neutrophils, and NK cells. Experiments with melanoma cell lines showed that reovirus infection of tumor cells induces lymphocyte expansion, IFN-**γ** production, specific anti-tumor cytotoxicity, and activates CD8+ T cells specific against the tumor [8–11].

However, immune reactions are likely to influence the efficiency of oncolytic virus therapy in various ways and, apparently, at various levels [12, 13]. For example, immunosuppresion decreased efficiency of oncolytic herpesvirus G207 in transplanted tumors [14]. On the other hand, Hirasawa et al. [15] found an increased efficiency of reoviral therapy after application of cyclosporine A or anti-CD4 and anti-CD8 antibodies in immunocompetent-mice model. However, it still remains to be determined which of the different immune mechanisms are involved in suppression of viral growth and which contribute to the establishment of anti-cancer immunity.

Reoviruses (acronym for **R**espiratory **E**nteric **O**rphan viruses) are viruses that, thanks to their natural properties, selectively

replicate in a wide spectrum of tumor cells [16]. Research into the mechanism of reovirus tumor selectivity has revealed that they replicate well in cells with activated *ras* signaling pathway, which is an attribute shared by many cancer cells [17]. *In vitro* studies, animal experiments and, subsequently, clinical studies suggested that reovirus type 3, strain Dearing /RV/, may be an efficient and safe anticancer agent [for review see 2, 3, 18].

Based on the results of experimental studies, the immunotherapy of HPV-associated tumors seems to be an effective, highly perspective therapeutic modality for treatment of these cancers and animal models remain of particular importance in this respect [19–24]. The only HPV proteins expressed in cervical carcinomas are the non-structural proteins E6 and E7. Therefore they are considered targets for immune reactions. In addition to the papillomaviral oncoproteins, other viral antigens generated by RV in infected cells might enhance anticancer immune reactions.

In the previous paper we reported on the efficacy of RV inoculation into tumors induced by HPV16 and H-ras- transformed cells [24]. The aim of this study was to compare the effects of RV on two HPV16 and H-ras-transformed cell lines and to determine whether RV potentiates the efficiency of tumor vaccines expressing HPV16 E6 and E7 in syngeneic mouse model.

Materials and methods

Cell lines. TC-1 and MK16/IIII/ABC (MK16) cells were described in previous papers [24]. In brief, MK16 cells were established in our laboratory by transformation of C57/B6 primary kidney cells by co-transfection with E6/E7 genes of HPV16 and activated H-ras oncogene [21]. They have an epithelioid morphology and downregulated MHC class I expression. However, MHC class I molecules are formed after exposure to interferon γ *in vitro* [25] and in the course of tumor growth *in vivo,* apparently due to endogenous interferon γ production [26]. These cells do not express B7.1 molecules at their surfaces but synthesize relatively large amounts of IL-1α [27]. One TID_{50} corresponds to approximately $5x10^4$ MK16 cells. Subcutaneous tumors induced by MK16 cells do metastasize to lymph nodes and lungs. TC-1 cells were derived by co-transfection of the C57/B6 mouse lung cells with E6/E7 genes of HPV16 and activated H-ras oncogene [28]. They are of fibroblastoid morphology and express MHC class I molecules at their surfaces. Furthermore, they express co-stimulatory B7.1 molecule at surfaces [29] but do not produce appreciable amounts of IL-1α [27]. One TID_{50} corresponds to approximately $5x10^3$ TC-1 cells. Subcutaneous tumors induced by these cells do not metastasize. Vero cells and normal human fibroblasts (NHF) were kindly provided by J. Cinatl Jr (J.W. Goethe University, Frankfurt/M, Germany). All cells were cultivated in IMDM medium or RPMI-1640 medium (both Sigma Aldrich Corp., St.Louis,Mo) supplemented with 10% fetal calf serum, 1% L-glutamine and antibiotics (all PAA Labs., Linz, Austria), at 37°C either in plastic culture flasks or in Petri dishes kept in humidified atmosphere with 5%CO_2.

Reovirus. Reovirus type 3, strain Dearing, was kindly provided by J. Cinatl Jr.. The virus was propagated in Vero cells. Virus stocks were kept frozen at –80˚C. Their titres were determined by a standard plaque assay using agar overlay. RV growth curves in Vero, MK16 and TC-1 cells were constructed after infecting the cultures at a MOI of 5 PFU/ cell. Samples were taken at 0 h (i.e. at the time of withdrawing the unattached virus, washing the cultures with PBS and adding media) and then at 6, 20, 26 and 48 hours post infection. After repeated freezing and thawing, the suspensions were spun down and the supernatants were titrated in Vero cells grown in 96-well plates. The final titres were determined 7 days after inoculation.

MTT test. The cytotoxicity of RV was determined using MTT test. For constructing the dose-response curves, the cultures of Vero, TC-1, MK16 and NHF cells were infected with different doses of stock RV diluted in the growth medium. Briefly, 10^4 cells in 0.1 ml of cultivation medium harvested in their exponential growth phase were seeded into a 96-well plates. Twenty-four hrs later, equal volumes of decreasing dilutions (10^{-3} to 10^{-12}) of RV were added. After 4 day incubation, the MTT solution was added. After additional 4 hr incubation, 50% N,N-dimethylformamide containing 20% SDS was added to disolve blue formazan crystals formed in functional mitochondria. The absorbance of emerged violet solution was measured at 570 nm for every single well by ELISA reader Versamax (Molecular Devices, CA, USA). The mean absorbance of wells containing only medium was subtracted from each measurement. The mean absorbance of control wells (containing cells and medium but not RV) was considered as 100% viability and the values determined for the infected cells were calculated as the proportion of this control. Each value represented the mean of 8 wells with corresponding standard deviation (SD).

DNA analysis. NHF, TC-1 and MK16 cells were incubated as described above for 24 hrs. Then RV was added at the MOI of 5 PFU/cell. Cell cycle was monitored in infected and control non-infected cultures by measuring the DNA contents at 24, 48 and 72 hrs after infection. Cells were stained by DNA Prep Reagent Kit (Beckmann Coulter, Fullerton, CA, USA) according to manufacturer´s instructions. The fluorescence intensity of 30,000 cells was measured by FACS Calibur (BD, San Jose, CA, USA) flow cytometer and list mode data were analyzed by ModFitLT software (Verity Software House, Topsham, ME, USA). The tests were repeated four times.

Apoptosis detection. For detection of apoptosis 24, 48 and 72 hrs after infection at the MOI 5 PFU /cell, we used cytometric detection of cells with subdiploid DNA content after extraction of low molecular weigh DNA [30]. Cells were harvested by trypsinization, washed and fixed in 70 % ethanol overnight at 4°C and washed again. The cell pellets were resuspended in phosphate-citrate buffer (pH 7.8) and incubated for 30 min at room temperature. After one more centrifugation, the cell pellets were resuspended in PBS and stained for DNA content by propidium iodide with RNAse (DNA Prep Stain solution from DNA Prep Reagents Kit, Beckmann Coulter). Cells were tested using FACSCalibur flow cytometer. The analysis of cellular DNA content revealed apoptotic cells as the cells represented by a „sub-G1 peak" on DNA content frequency histogram.

Detection of reovirus antigen in infected cells. We determined RV antigen producing cells by indirect immunofluorescence measured by flow cytometry after permeabilisation of the cells using Fix & Perm kit (An der Grub, Kaumberg, Austria) according to manufacturer´s protocol. As the primary antibody we used MAB994 monoclonal antibody reactive with RV type 3 σ1 hemaglutinin (Millipore, Billerica, MA, USA) and as the secondary antibody the FITC-Conjugated Goat Anti-mouse Immunoglobulin Polyclonal Antibody (BD, San Jose, CA, USA). Non-infected cells cultivated under the same conditions were used as a negative control.

Animals and oncogenicity tests. C57BL/6 female mice, 7-8 week old, were obtained from Charles River, Germany. All work with animals was done according to the Guidelines for Animal Experimentation valid in the Czech Republic. In different experiments the mice were inoculated subcutaneously (s.c.) with either $5x10^6$ RV-infected MK16 cells, or 10^5, $5x10^5$, 10^6 or $5x10^6$ of RV-infected TC-1 cells suspended in 0.2 ml PBS. In all instances the MOI was 10 PFU/cell. Groups of mice were simultaneously inoculated with non-infected cells. Animals were inspected twice a week for up to 113 days. Tumor size was expressed as the area index (AI), as described previously [24].

Cell vaccines TC-1 cells grown in culture flasks were infected at the MOI of 5 PFU/cell. After 3 hr incubation at 37°C, the unattached virus was removed by three washes with PBS. Cells were trypsinized, washed two times with PBS and 10^6 cells were administrered s.c. to mice. The second dose was administered two weeks later. In parallel, groups of mice were inoculated with the same doses of irradiated (100 Gy) TC-1 cells. Two weeks after the second dose the animals were challenged with either $5x10^5$ MK16 cells or $5x10^4$ TC-1 cells administered at a site different from the immunization sites. Thus, approximately 10 TID_{50} of the cells were used in both instances. Non-immunized mice served as controls.

Statistics Numerical data were presented as mean and SD and analysed using Student t-test**.** Tumor formation was analysed in 2x2 contingency tables by two-tailed Fisher's exact test. Analysis of tumor growth curves was performed by two-way analysis of variance. Calculations were done using GraphPad Prism version 3 (GraphPad Software, San Diego, CA, USA). A difference was considered significant if $p<0.05$.

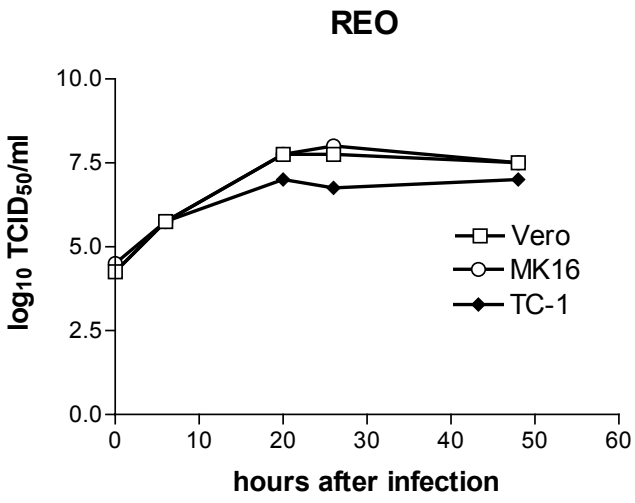


Fig.1. Growth curves of reovirus in Vero, TC-1 and MK16 cells

Results

Growth of reovirus in Vero, TC-1 and MK16 cells. Fig 1 presents the growth curves of RV in Vero, MK-16 and TC-1 cells. It can be seen that they were nearly identical in all three cell lines, but the production of infectious virus was somewhat diminished in TC-1 cells. The results of MTT are shown in Fig 2. They suggest that infected TC-1 were dying more rapidly than MK16 cells infected at the same MOI. As expected, RV did not disturbe the viability of NHF cells.

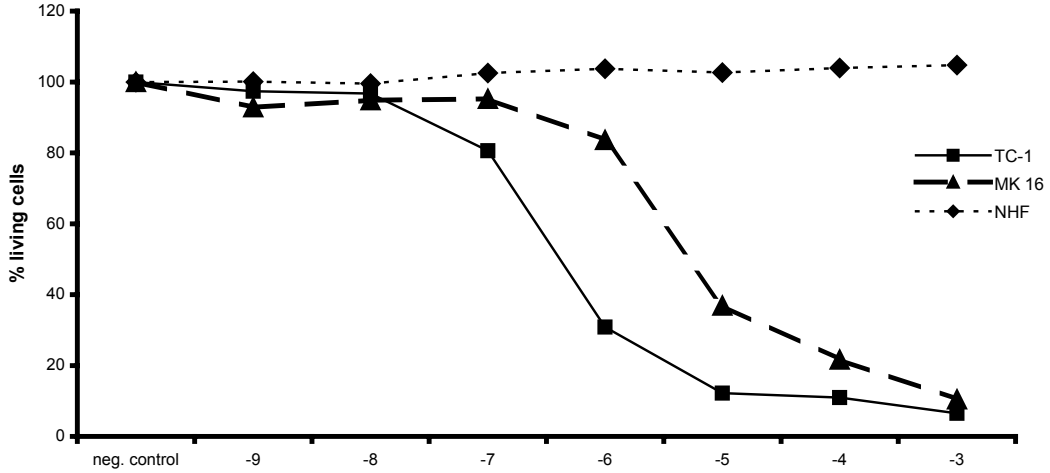


Fig.2. Results of MTT tests.

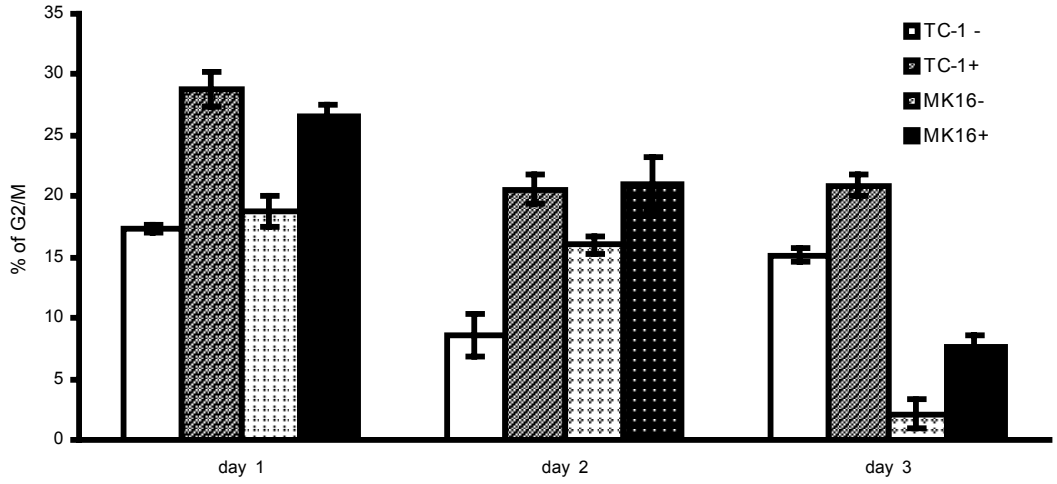


Fig.3. Percentage of cells in G2/M phase detected by flow cytometry DNA analysis: TC-1 and MK-16 cells infected (+) or not (–) with RV. (NHF not shown because percentage of G2/M phase was < 1% in all NHF samples)

Influence of reovirus on cells. As could be expected, TC-1 and MK16 cells but not NHF cells infected with RV had significantly increased proportion of cells in G2/M phase compared to noninfected cells 48 and 72 hrs ($p< 0.01$) after infection. The increase of percentage of cells in G2/M phase was higher in TC-1 cells than in MK16 at 24 as well as at 48 hrs after infection, however 72 hrs after infection, the contrary was observed (Fig 3).

As indicated in Fig. 4 we detected increased percentage of apoptotic cells 48 and 72 hrs after infection of TC-1 and 72 hrs after infection of MK16 ($p<0.01$). Percentage of apoptotic NHF in infected culture was only slightly increased.

Interesting results were obtained when the production of RV antigens in the infected cells was followed. Results expressed as means of three experiments shows Fig. 5. There were marked differences between TC-1 and MK16 infected cells. On the first day more than 75% of TC-1 cells and on the second and the third day more than 90 % cells produced detectable amounts of RV antigen. On the other hand, only 15 %, 26.6 % and 44.5 % of MK16 cells were positive on the first, second and third day, respectively. As expected, there was no marked increase of RV positive cells with time in the cultures of nonpermissive NHF cells (16.5% on the first; 11.5% on the second and 20.2% on the third day, respectively). This

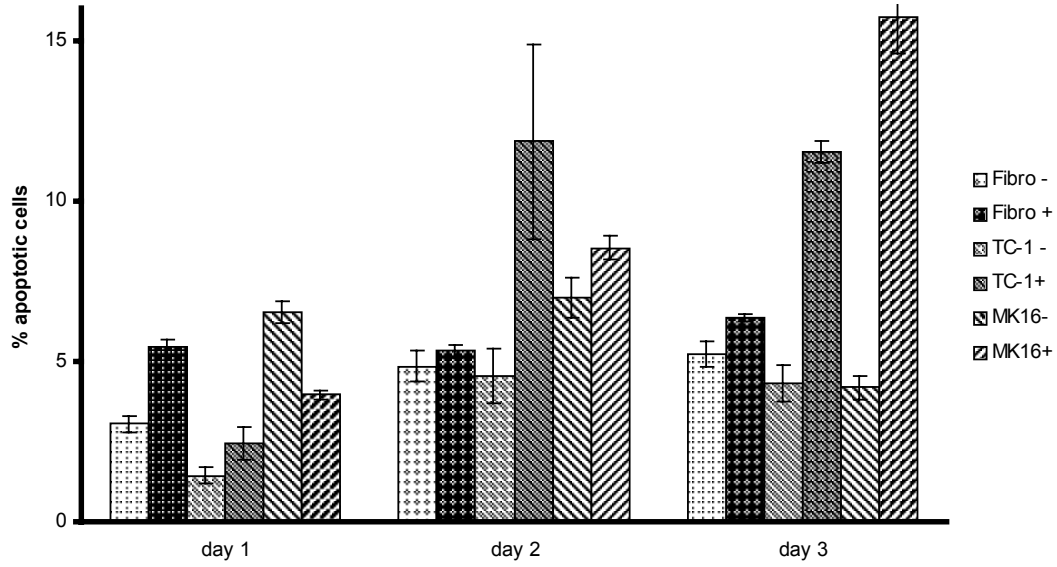


Fig.4. Percentage of apoptotic cells detected by flow cytometry as subdiploid peak: NHF, TC-1, MK16 without and with RV (– and + respectively) .

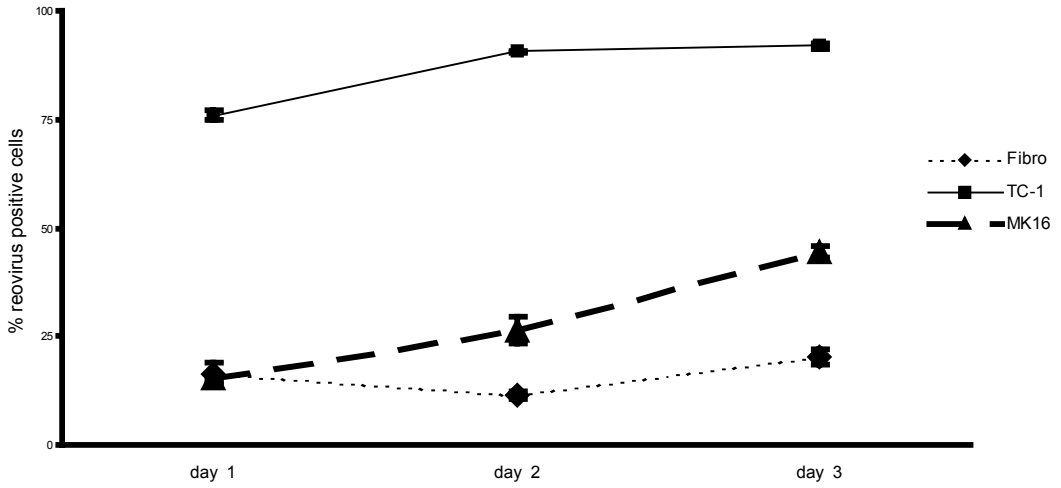


Fig.5. The production of reovirus antigens in the infected cells. Indirect immunofluorescence measured by flow cytometry - primary antibody MAB994 monoclonal antibody anti reovirus type 3 σ1 hemaglutinin.

implies that an abortive virus infection occurred in a fraction of the NHF cell population

. *Pathogenicity of reovirus-infected cells.* The oncogenicity of $5x10^6$ TC-1 and MK16 cells which were either infected with RV (at the input MOI of 5 PFU/cell) or remained non-infected is shown in Fig 6. It can be seen that all animals inoculated with non-infected cells developed tumors. In agreement with previous experiments, in these mice the tumors were detected earlier in the TC-1-inoculated mice than in those inoculated with MK16 cells. Five of 6 animals to which RV-infected TC-1 cells had been administered developed tumors. However, these tumors appeared significantly later than in mice inoculated with non-infected cells, and one of these regressed. The other tumors grew progressively. In the case of MK16 cells the tumor suppressive effect of infection with RV was nearly complete. Although tumors developed in four animals around day 50 after inoculation, they were of small size (1 - 2 mm^3) and regressed completely in the course of subsequent weeks and all animals remained tumor-free until the end of the observation period. In the subsequent experiment lower doses, 10^6, $5x\ 10^5$ and 10^5 of RV-infected TC-1 cells were tested. In this experiment all animals remained tumor-free till the end of the observation period (results not shown).

Immunogenicity of reovirus-infected cells. To find out whether the RV-infected HPV16-tranformed cells were capable of inducing immunity in the inoculated animals, mice were immunized with either RV-infected or irradiated TC-1 cells and challenged with 10 TID_{50} of either TC-1 or MK16 cells. The results of the challenge experiment shows Fig 7. It can be seen that both the infected and irradiated cells induced significant protection against TC-1 cells. However, this was much more pronounced when irradiated cells were used. Four out of six animals immunized with irradiated cells remained tumor-free. On the other hand, all animals immunized with the RV-infected cells developed tumors, though at a slower rate. The difference between these two groups was highly significant ($p<0.001$) (see Fig 7A).

The results of the challenge with MK-16 cells were different (see Fig 7B). In this case half of the mice immunized with the infected cells developed tumors while nearly all the animals immunized with the irradiated cells did so, although

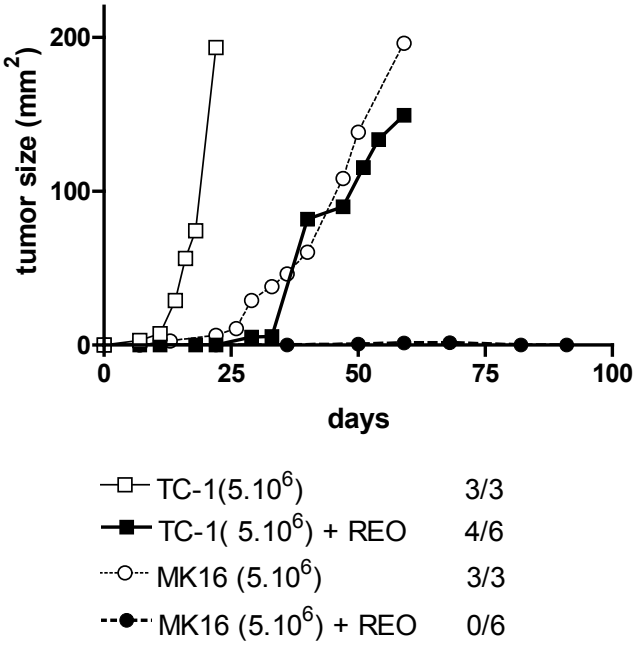
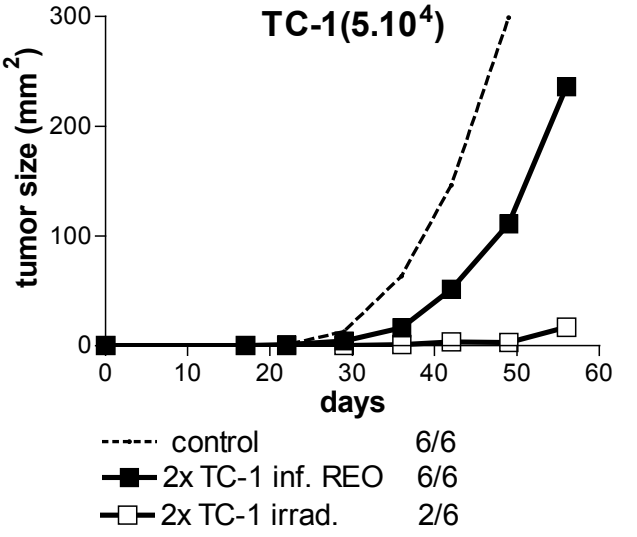


Fig.6. Tumor growth of reovirus infected and non-infected TC-1 and MK16 cells.
TC-1 + REO *vs.* TC-1 ($p<0.0001$)
MK16 + REO vs. MK16 ($p<0.0001$)



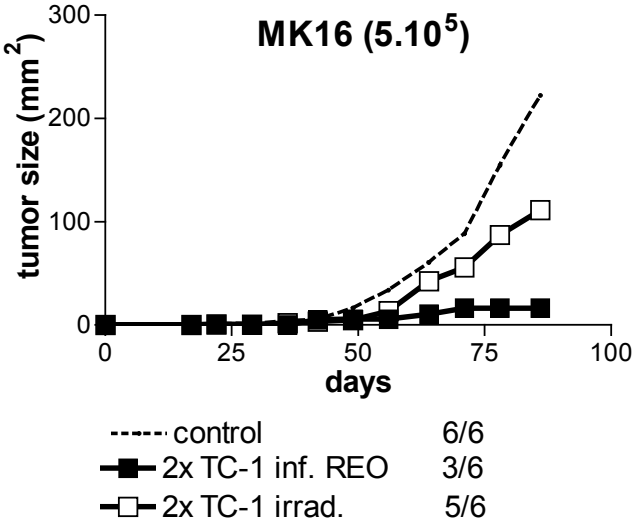


Fig.7A. Tumor growth in immunized and nonimmunized mice after challenge with TC-1 cells.
2x TC-1 inf. REO *vs.* nonimmunized (TC-1) p<0.02
2x TC-1 irrad. *vs.* nonimmunized (TC-1) p<0.001
2x TC-1 inf. REO *vs.* 2x TC-1 irrad. p<0.001

Fig.7B. Tumor growth in immunized and nonimmunized mice after challenge with MK16 cells.
2x TC-1 inf. REO *vs.* nonimmunized p<0.0001
2x TC-1 irrad. *vs.* nonimmunized p<0.02
2xTC-1+ REO *vs* 2x TC-1 irrad. p<0.01

the tumors in these animals grew more slowly than in the non-immunized controls. The difference between the controls and immunized animals was more significant with the virus infected than with the irradiated cells (p<0.0001 vs. p<0.02) .The difference between animals immunized with the infected cells and those immunized with irradiated cells was significant (p<0.01).

Discussion

In the present study we demonstrated that RV grew well in both HP16- and H-ras- transformed mouse cell lines (TC-1 and MK16 cells), but not in the control NHF cells. However, some differences between the two HPV16-transformed cell lines were apparent. Although TC-1 cells appeared to be somewhat less efficient producers of infectious RV, they were more susceptible to the killing effects of the virus and produced RVσ1 antigen in a higher percentage of cells than the MK16 cells . In accordance with the observations made in mouse L929 cells and other cell lines susceptible to RV [31, 32], the virus induced apoptosis and cell cycle arrest in G2/M phase in both TC-1 cells and MK16 cells. Although some differences between the two cell lines were encountered in repeated tests, they were not very marked. Still, they indicated that the onset of G2/M arrest and apoptosis was fairly quicker in TC-1 cells. This seems to be in consent with the findings mentioned above. Such difference was observed in all repeated tests.

The influence of RV on the viability of both the MK16 and TC-1 cells was manifested by a decreased oncogenic ability in syngeneic mice. When using high dose ($5x10^6$) of cells infected at the MOI 10 PFU/cell, 4 out of 6 mice inoculated with the infected TC-1 cells developed progressively growing tumors while all animals inoculated with similarly infected MK16 cells were tumor-free still at the end of the observation period. This definitely does not mean that TC-1 cells were less susceptible to the killing effects of RV than the MK16 cells. Such a conclusion would be in disagreement with the results of the *in vitro* tests. Two other factors might be involved. First, TC-1 cells are more oncogenic than the MK16 cells. Thus, the dose inoculated corresponded to approximately $5x10^3$ TID_{50} in the case of TC-1 cells but only to $5x10^2$ TID_{50} in the case of MK16 cells. Should the same fraction of the cells survive in both the TC-1 and MK16 cells, one would expect a residual oncogenic activity in the former rather than in the latter cells. This is what actually happened. Second, the incubation period between cell inoculation and tumor development is longer in the latter than in the former cells. Thus, in the case of MK16 cells there was more time for establishing immunity elicited by the infected cells present in the inoculum.

The most important aim of our study was to find out whether the presently used tumor cells when infected with RV would represent a more potent immunogen than the non-infected irradiated cells. In the immunization experiment TC-1 cells were used and both the TC-1 cells and MK16 cells were used for the challenge. The results were rather surprising. When the homologous TC-1 cells were used, then the irradiated cells were clearly a better immunogen than the virus-infected cells. However, the situation was opposite, when the immunized animals were challenged with MK16 cells: the immunity induced by the infected TC-1 cells appeared to be much more solid than that

induced by the irradiated cells. The reasons for this difference are not clear at this moment. One can only speculate that it reflects the differences in the biological properties of the two cell lines as described above and, possibly, some other not yet known. Although both cells share HPV16 E6 and E7 antigen, it is likely that the two cell lines differ in their antigenic make-up [33] which may, in addition, be influenced by the virus infection. Experiments are under way to clarify the mechanisms involved. Without respect to their nature, it seems clear from the present data that the assumed potentiation of the immunogenicity of the tumor virus-infected cells may indeed come into force in some (MK16) but not in the other systems (TC-1).

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