Abstract. It has been demonstrated repeatedly that a high proportion of tumours derived from MHC class I+ precursors are MHC class I-. Since a major task in immunotherapy strategies for treatment of malignancies is to develop polyvalent tumour vaccines efficient against a broad spectrum of tumours, we have examined whether MHC class I+ cell-based tumour vaccines can cross-protect against homologous MHC class I tumour challenge and vice versa. For these purposes, we have used two oncogenic cell lines induced independently by co-transfection of murine H-2b cells with E6/E7 HPV16 and activated Ha-ras oncogenes, the tumours TC-1 (MHC class I+, HPV16 E7+) and MK16/1/IIIABC (MHC class I-, HPV16 E7+). Surprisingly, it was found that these two tumours do not cross-react, although both of them contain the crucial HPV16-coded tumour rejection antigen E7. Preimmunization with the MHC class I+ tumour did not protect against a subsequent challenge with the MHC class I- tumour and vice versa; however, immunization with the TC-1 tumour could protect syngeneic mice against the TC-1 tumour challenge and, similarly, immunization with the MK16/1/IIIABC tumour could protect mice against the MK16/1/IIIABC tumour challenge. If this finding can also be confirmed as a more general phenomenon with other MHC class I+ and class I- tumours, it could have serious implications for design of immunotherapeutic vaccines and protocols.

The major problem in the development of cancer vaccines is to produce vaccines that could be used against a broad spectrum of tumours belonging to the same type ("homologous tumours") originating in different tumour-bearing individuals. Such polyvalent tumour vaccines should express a variety of tumour-associated antigens and MHC alleles shared by a high percentage of tumour-bearing individuals. Since a high proportion of tumours derived from MHC class I+ precursors are MHC class I-, we have attempted here to investigate whether the "homologous" MHC class I+ and MHC class I- tumours sharing identical tumour-associated rejection antigens do cross-react with each other, i.e. whether a polyvalent MHC class I+ tumour vaccine can protect individuals with homologous MHC class I- tumours and vice versa. We have used MHC class I+ (TC-1, Lin et al., 1996) and MHC class I- (MK16/1/IIIABC, Šmahel et al., 2001) tumours derived from murine cells of H-2b haplotype by in vitro co-transfection with E6/E7 HPV16 and activated (G12V) Ha-ras oncogenes. Both tumours were shown to be specifically immunogenic; as can also be seen in this paper, repeated immunization with irradiated TC-1 cells inhibited growth of the transplanted TC-1 tumour inocula. Similarly, preimmunization with irradiated MK16/1/IIIABC cells protected mice against MK16/1/IIIABC tumour challenge (Bubeník et al., 1999). Both oncogenic cell lines expressed the E7 HPV16 gene, the product of which has previously been shown to specifically activate T-cell immunity and to induce tumour resistance (Chen et al., 1991; Lin et al., 1996; Ji et al., 1998; Šmahel et al., 2001; Zwaveling et al., 2002; Jinoch et al., 2003). The most convincing evidence for this has come out from experiments in which cell-free immunogens like DNA vaccines and recombinant viruses have been used (Lin et al., 1996; Bubeník et al., 1999; Šmahel et al., 2001, 2003; Němečková et al. 2002). However, despite these findings, the TC-1...
and MK16/1/IIIABC tumours did not cross-react in the immunization/challenge experiments reported here. If these results could also be reproduced with other homologous MHC class I+ and class I− HPV16-associated tumours, they might be of crucial importance for the construction of effective tumour vaccines against HPV16-associated tumours.

Material and Methods

Mice

C57BL/6 males, 2−4 months old, were obtained from AnLab Co., Prague, Czech Republic.

Tumour cell lines

The MHC class I+, non-metastasizing TC-1 cells were prepared by transformation of C57BL/6 primary mouse lung cultures with HPV 16 E6/E7 oncogenes and activated Ha-ras (Lin et al., 1996). The MHC class I+, metastasizing MK16/1/IIIABC cells were developed by in vitro co-transfection of murine kidney cells with a mixture of activated Ha-ras oncogene and HPV16 E6/E7 genes (Šmahel et al., 2001). Both cell lines were maintained in RPMI 1640 medium supplemented with 10% foetal calf serum (BioClot Ltd., Aidenbach, Switzerland), 2 mM L-glutamin (Sevac, Prague, Czech Republic), penicillin (Biotika, Slovenská Lupča, Slovakia) and streptomycin (Sigma, Steinheim, Germany). The expression of the E7 gene in the TC-1 cells was previously determined by reverse transcriptase polymerase chain reaction and the expression of HPV16 E7 protein by immunofluorescent staining and Western blotting (Lin et al., 1996). The transcript of E7 HPV16 was found in the MK16/1/IIIABC cells and in derived sublines by reverse transcriptase-polymerase chain reaction (Jinoch et al., 2003); however, the content of the E7 oncoprotein in MK16 cells as determined by Western blotting was considerably lower (about ten times) than in TC-1 cells (Ludvíková et al., unpublished results). Furthermore, the two cell lines differ in several other characteristics. TC-1 cells possess fibroblastoid morphology, do not express cytokeratins and they carry co-stimulatory B7.1 molecules at their surfaces (Janoušková et al., 2003). On the other hand, MK16/1/IIIABC cells are epitheloid, express cytokeratins (Šmahel et al., 2001) and do not carry B7.1 molecules; they are approximately 10 times less oncogenic than the TC-1 cells.

Flow cytometry

The expression of MHC class I molecules on the transplanted cells was determined by cytofluorometric analysis with FITC-anti-mouse H-2Kb/H-2Db monoclonal antibody (clone 28-8-6, Pharmingen, CA). As an isotype control, FITC-labelled antibody of irrelevant specificity (clone 155-178, Pharmingen, CA) was used. As can be seen in Fig. 1, the TC-1 cells which were used here (in vitro 20th generation) were MHC class I positive (A), whereas the MK16/1/IIIABC cells which were used here (in vitro 14th generation) were MHC class I molecules negative (B). MHC I formation has been induced in MK16/1/IIIABC cells by interferon gamma and cells treated in this way may serve as targets in cytotoxicity tests (Indrová et al., 2002).

Design and evaluation of the immunization/challenge experiments

Groups of C57BL/6 males were immunized s.c. on days 0 and 21 with irradiated (100 Gy) 1 x 10^7 TC-1 or MK16/1/IIIABC cells and challenged s.c. on day 35 with 1 x 10^4 TC-1 or 1 x 10^5 MK16/1/IIIABC cells, respectively. Tumour-bearing mice were observed twice a week, and the numbers of tumour-bearing mice and size of the tumours were recorded. On day 55, the mice were sacrificed and autopsied. Lungs were removed and the macroscopically detectable metastatic nodules were counted under a stereoscopic microscope. For statistical analyses, Student’s t-test and χ^2 comparison test from NCSS, Number Cruncher Statistical System (Kaysville, UT), statistical package were used.

Results and Discussion

Groups of mice were immunized on days 0 and 21 with irradiated MHC class I+ TC-1 or MHC class I− MK16/1/IIIABC cells. On day 35, the mice were challenged with the TC-1 or MK16/1/IIIABC cells. Preimmunization with the TC-1 tumour cells protected syngeneic mice against the TC-1 tumour challenge and, similarly, preimmunization with the MK16/1/IIIABC tumour cells protected mice against the MK16/1/IIIABC tumour challenge (Table 1). However, preimmunization with the MHC class I+ tumour cells TC-1 did not protect against a subsequent challenge with the
Cervical cancer affects nearly 500,000 women per year worldwide and is in almost all cases associated with high-risk HPV types. HPV16 DNA has been found in approximately 60% of these tumours (Zur Hausen, 2001; Munoz et al., 2003; Waggoner, 2003), clearly indicating that this HPV type is the most important one from the viewpoint of public health. Based on the results of numerous experimental studies, the immunotherapy of HPV-associated tumours has attracted a particular interest and is prospective as a new therapeutic modality for treatment of this type of cancer (Bubeník, 2000a).

These efforts have been strongly boosted by the recent success with a virus-like particles (VLP)-based prophylactic vaccine (Koutsky et al., 2002). For the development of therapeutic vaccines in human HPV-associated tumour systems, animal models remain to be of particular importance (Bubeník, 2002b). The only HPV proteins expressed in cervical carcinomas are the non-structural virus proteins E6 and E7. These oncoproteins are involved in malignant transformation of cervical carcinoma cells and their presence is required for the maintenance of the malignant phenotype of the cells. Therefore, the HPV E6/E7 oncoproteins are considered as a target of choice for immune reactions against cervical carcinoma and they are being used for the construction of therapeutic vaccines. Although the immune reactions against various HPV16 E7+ MHC class I+ and HPV16 E7+ MHC class I− tumours have been intensively studied, surprisingly little is known about immunological cross-reactivity of the MHC class I+ and class I− tumours. It is not clear whether the immune response directed against a tumour with a certain level of the MHC class I expression can result in a protective immunity against another tumour with a different MHC class I expression level.

The role of therapeutic vaccines is particularly important for patients with recurrence or metastases of a recognized tumour, since the MHC class I+ cells are not recognized by NK cells (Table 1). A negligible, non-significant inhibition of the number of MK16/1/IIIABC lung metastases was observed after preimmunization of mice with TC-1 cells (Table 2).

Table 1. Lack of cross-reactivity between TC-1 and MK16/1/IIIABC tumours in immunization/challenge experiments: subcutaneous tumour transplants

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Challenge</th>
<th>TC-1</th>
<th>MK16/1/IIIABC</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC-1</td>
<td>2/8*</td>
<td>7/7</td>
<td></td>
</tr>
<tr>
<td>MK16/1/IIIABC</td>
<td>8/8</td>
<td>3/7**</td>
<td></td>
</tr>
<tr>
<td>Untreated controls</td>
<td>15/15</td>
<td>13/13</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.001 as compared to untreated controls; **P < 0.01 as compared to untreated controls. The experiment was repeated three times and representative results are shown here.

Table 2. The effect of preimmunization with irradiated TC-1 or MK16/1/IIIABC cells on the development of MK16/1/IIIABC lung metastases

<table>
<thead>
<tr>
<th>Immunization with</th>
<th>No. of mice with MK16/1/IIIABC lung metastases/total</th>
<th>No. of MK16/1/IIIABC lung metastases/mouse (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC-1</td>
<td>4/5</td>
<td>3.2 ± 2.7</td>
</tr>
<tr>
<td>MK16/1/IIIABC</td>
<td>0/7*</td>
<td>0</td>
</tr>
<tr>
<td>Untreated controls</td>
<td>5/13</td>
<td>6.0 ± 11.3</td>
</tr>
</tbody>
</table>

P < 0.01 as compared to the group of mice immunized with TC-1. Groups of mice were immunized s.c. on days 0 and 21 with 100 Gy-irradiated 1 x 10⁷ TC-1 cells or MK16/1/IIIABC cells. On day 35, the experimental groups together with untreated controls were challenged s.c. with 1 x 10⁵ MK16/1/IIIABC cells. Fifty-five days after challenge, the mice were sacrificed, autopsied and the incidence of lung metastases per group, as well as the number of lung metastatic nodes per mouse were recorded.
this might have been conditioned by the significantly lower content of the E7 oncoprotein in MK16 cells (see above). On the other hand, insertion of the IL-2 gene into the MK16/1/IIIABC cells can apparently enhance the activity of the vaccine, since the genetically modified vaccine was capable of reducing the TC-1 tumour recurrence rate in mice suffering from surgical minimal residual TC-1 tumour disease (Bubeník et al., 2003). It has been found that immunization with the MHC class I+ TC-1 cells can protect mice against the TC-1 tumour, but not against the MK16/1/IIIABC tumour challenge. Apparently, due to the MHC class I restriction, the MK16/1/IIIABC tumour cells are resistant to the cytolytic lymphocytes induced by immunization with the TC-1 tumour vaccine. Such interpretation is supported by our previous finding that spleen cells from the TC-1 tumour-immunized mice were in vitro not cytolytic when allowed to react with the MK16/1/IIIABC targets. However, when the MK16/1/IIIABC cells were grown in vitro in the IFNγ-containing medium, the MK16/1/IIIABC cells acquired, together with the MHC class I expression on their surface, the sensitivity to the cytolytic effect of spleen cells from the TC-1 tumour-immunized mice (Indrová et al., 2002).

Downregulation of MHC class I expression is an important mechanism by which a substantial percentage of various tumour types, including HPV16-associated carcinomas, evade classical T-cell-dependent immune responses. However, even MHC class I- tumours can respond to the immunotherapy with tumour vaccines. Our results indicate that the vaccines matched in the MHC class I expression with the treated tumour will probably have a higher therapeutic efficacy than the MHC class I unmatched vaccines. Moreover, genetic modification of the vaccines by insertion of IL-2, IL-12, IFNγ, GM-CSF, or adjuvant administration of these cytokines with the vaccines (Levitsky et al., 1994; Bubeník et al., 1999; Indrová et al., 2002; Bubeník, 2002a; Bubeník et al., 2003; Bubeník et al., 2003; Bubeník, in press) can substantially enhance the effect of the vaccines. Still, the present results have to be interpreted with some caution, since also other factors might be involved in addition to the MHC class I presence or absence. The lower content of the E7 oncoprotein in MK16 cells as compared with the TC-1 cells could influence the results. In addition, it has been shown that MHC class I- MK16/1/IIIABC cells upregulate MHC class I molecule expression in vivo, similarly as does the MHC I- subline derived from TC-1 cells (Mikyšková et al., 2003; Šmahel et al., 2003). Thus, one has to consider the MK16/1/IIIABC tumour cell populations as a dynamic system, the MHC I profile of which can gradually change in the course of tumour growth. Consequently, the susceptibility of the respective tumours to the different effector immune reactions may also be altered. It may be objected, however, that the tendency of reversion of the MHC I+ will be rather suppressed in the TC-1 immunized animals. Last but not least, it is possible that, in addition to the viral oncoproteins, other tumour antigens generated by mutation or overexpression of cell proteins might be present and involved in the monitored immune reactions. These tumour antigens may be different in the two cell lines and, theoretically, may be of significance. Additional experiments are needed to clarify this point.

References


