

p53 as a target for cancer vaccines: Recombinant canarypox virus vectors expressing p53 protect mice against lethal tumor cell challenge

(tumor antigens/immunotherapy)

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ABSTRACT The p53 protein is an attractive target for immunotherapy, because mutations in the p53 gene are the most common genetic alterations found in human tumors. These mutations result in high levels of p53 protein in the tumor cell, whereas the expression level of wild-type p53 in nonmalignant tissue is usually much lower. Several canarypox virus recombinants expressing human or murine p53 in wild-type or mutant form were constructed. Immunization with these viruses protected BALB/c mice from a challenge with an isogenic and highly tumorigenic mouse fibroblast tumor cell line expressing high levels of mutant p53. The tumor protection was equally effective regardless of whether wild-type or mutant p53 was used for the immunization, indicating that the immunologic response was not dependent on any particular p53 mutation and that immunization with this live virus vaccine works effectively against mutant p53 protein expressed in a tumor cell. In tumors escaping immunologic rejection, the expression of the p53 protein was commonly down-regulated.

A highly sought goal in experimental oncology is the ability to increase the specific immunologic response against malignant tumors. Current approaches based on the use of tumor cells modified to express immunostimulants have yielded encouraging results (1, 2). However, these forms of immunotherapies can only be applied on an individual basis since they are directed against antigens expressed uniquely by the targeted tumor. For practical and economic reasons, it would be highly desirable to develop a vaccine that could increase the specific immunologic response against a broad range of tumors from many individuals. This requires the targeting of defined tumor-associated antigens, which are expressed both in multiple tumor types and are capable of eliciting a specific immune response. Promising candidates shown to be recognized by cytotoxic T-cells include viral gene products (3), melanoma-associated antigens such as MAGE1 (4), and oncoproteins such as HER-2/neu (5). However, because relatively few tumor types express these antigens, the range of tumors against which these vaccination strategies might prove to be promising is still quite narrow.

Mutations in the p53 tumor suppressor gene are the most commonly found genetic alterations in human malignancies (6, 7). These mutations usually result in the expression of a mutant p53 protein that, due to a prolonged half-life, accumulates to high levels as compared with the wild-type p53 protein in normal cells (8–10). Because such overexpression may result in the presentation of peptide epitopes by tumor cell major histocompatibility complex (MHC) molecules in quantities sufficient to elicit a specific immune response in cancer patients,

the p53 protein may present an effective target for a broad-based immunotherapy strategy. It has been reported that a cytotoxic T-cell response can be obtained against tumors harboring a mutant p53 protein following vaccination with a synthetic peptide designed to correspond to a novel MHC class I epitope generated from the particular mutant p53 protein (11, 12). However, vaccination against wild-type p53 protein might have much broader applications since it could work against any tumor overexpressing p53 without the need to precisely assess the p53 mutation and the HLA type of a patient. Therefore, experiments were designed to determine whether epitopes derived from the wild-type p53 protein are immunogenic when overexpressed and whether the immune response against such epitopes is protective against tumors harboring mutant p53. To test this and compare the effectiveness of vaccination with wild-type versus mutant p53 protein, both wild-type and mutant p53 expressing viruses were engineered and used as vaccinating agents.

For the immunizations, recombinant canarypox viruses (ALVAC) were constructed to express p53 protein. Although ALVAC is restricted to avian species for productive replication, it has been shown to function effectively as an immunization vehicle since it infects mammalian cells nonproductively and expresses foreign antigens (13, 14). Moreover, ALVAC has already been demonstrated to work safely and efficiently in the vaccination of both animals and humans (15–17). BALB/c mice were immunized with several ALVAC-p53 recombinant viruses, and the tumor-protective effect of the vaccination was assessed by challenging the immunized mice with a transformed BALB/c fibroblast cell line expressing high levels of human mutant p53 (Arg²⁷³ → His) but lacking endogenous murine p53 (18). The results of these experiments indicate that the vaccination against p53 with both mutant and wild-type p53 recombinants protected animals equally well from tumor challenge. Therefore, this approach represents a promising concept for a generally applicable immunotherapy against cancer.

MATERIALS AND METHODS

Cell Lines. The 10(3)273.1NT24 cell line was obtained from a nude mouse tumor after injection of 10(3)273.1 cells. 10(3)273.1 cells (18) were derived from 10(3) cells, a murine (BALB/c) fibroblast cell line of H-2^d haplotype lacking endogenous murine p53 (19). A cDNA expression clone was introduced into these cells encoding a human mutant p53 (Arg²⁷³ → His) (10) linked to the gene conferring resistance to G418 and the cell line was termed 10(3)273.1NT24. The cells were cultivated in DMEM plus 10% fetal bovine serum

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Abbreviations: MHC, major histocompatibility complex; ALVAC, canarypox virus vector.

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(GIBCO/BRL) supplemented with 500 $\mu\text{g}/\text{ml}$ G418 (GIBCO/BRL). The 10(3)Tx4BT87 cell line was derived from a tumor caused by injection of 10^7 cells of a spontaneous focus of 10(3) cells into BALB/c mice and does not express any p53 protein. Chicken embryo fibroblasts (ATCC) were grown in DMEM plus 10% fetal bovine serum.

ALVAC-p53 Recombinant Viruses. The ALVAC-based recombinant viruses expressing p53 were generated by *in vitro* recombination using standard techniques (13, 14, 20). All recombinants contained the p53 coding sequences under the control of the early/late vaccinia virus H6 promoter (21). ALVAC-hup53/wt contains the human wild-type p53 coding sequence. ALVAC-hup53/175 expresses human p53 with an amino acid change at position 175 (Arg to His). ALVAC-hup53/273 expresses human p53 with an amino acid change at position 273 (Arg to His). ALVAC-mup53/wt expresses the

wild-type murine p53 protein. ALVAC-mup53/KH215 contains a mutant murine p53 coding sequence with an alteration of nucleotides 643–646 so that the wild-type sequence is changed from GTAC to CCAAGCTTGG and the predicted amino acid sequence is changed from Val²¹⁵-Pro²¹⁶ to Pro-Ser-Leu-Ala (8).

Immunoprecipitation. Cells were labeled with [³⁵S]methionine, soluble protein extracts were prepared, and p53 protein was analyzed by immunoprecipitation of 5×10^6 cpm from whole cell lysate using the anti-p53 monoclonal antibody PAb421 (22) as described (23). Each cell lysate was immunoprecipitated in parallel using antibody PAb419 (22) as negative control. The immunoprecipitates were separated by SDS/PAGE and visualized by autoradiography.

Immunizations and Tumor Challenge. Female BALB/c mice were obtained from The Jackson Laboratory. Six- to

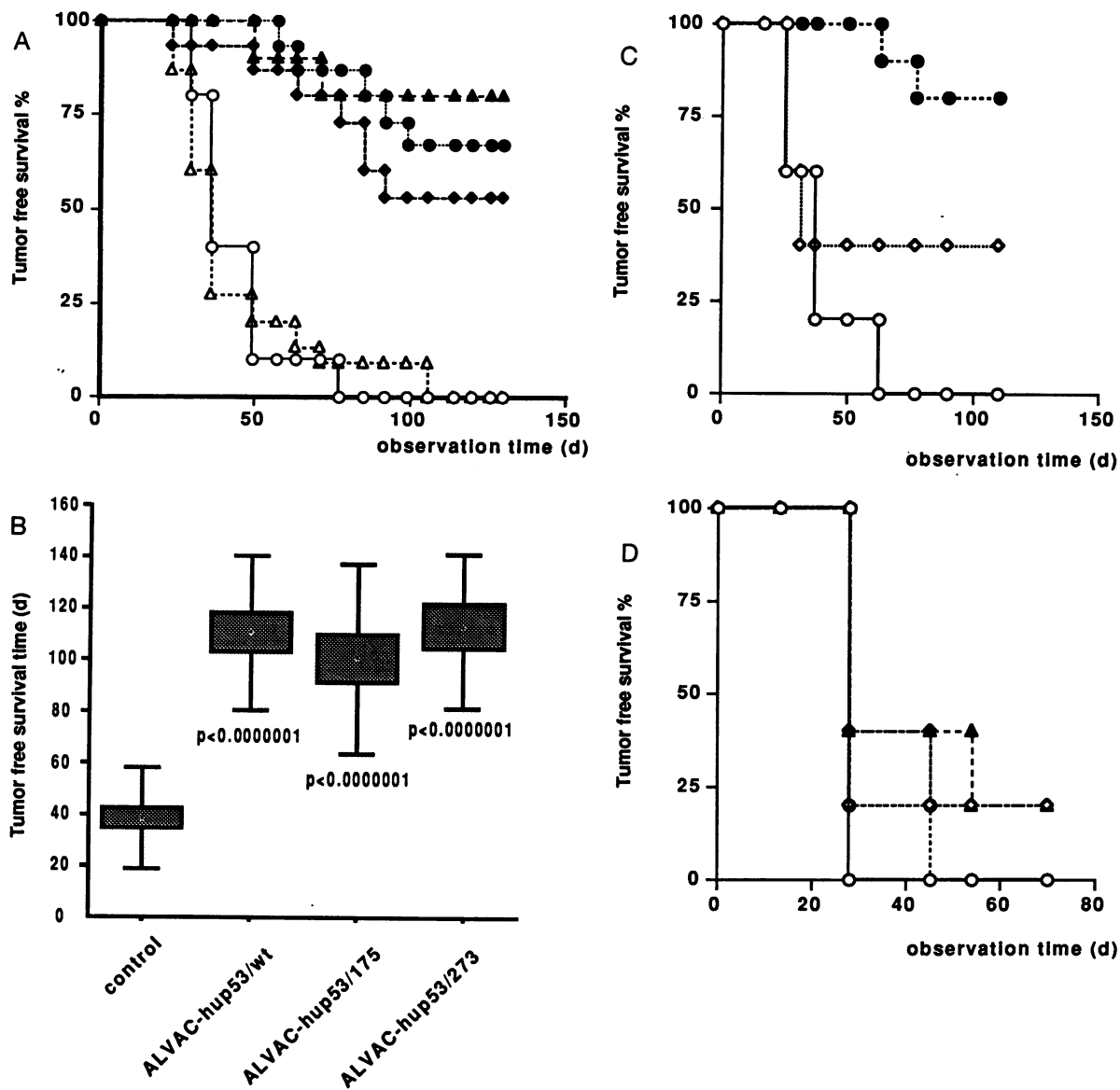


FIG. 1. Protection against tumor cell challenge after vaccination with ALVAC-hu p53. (A and B) Groups of mice were vaccinated twice with ALVAC-hup53/wt ($n = 15$) (●), ALVAC-hup53/175 ($n = 15$) (◆), ALVAC-hup53/273 ($n = 10$) (▲), with ALVAC ($n = 15$) (△), or were not vaccinated (mock, $n = 10$) (○) and then were challenged with 10(3)273.1NT24 cells. (A) Kaplan-Meier plot showing the percentage of tumor-free mice over the observation time of 130 d. B shows the average tumor-free survival time for all control mice (mock and ALVAC) and all vaccinated mice (ALVAC-hup53/wt, ALVAC-hup53/175, ALVAC-hup53/273). Average values are shown as dots; boxes represent the standard error, and stackers show the standard deviation. (C) Mice were challenged with 10(3)273.1NT24 cells, and only 24 h later vaccinations were started with ALVAC-hup53/wt ($n = 10$) (●), ALVAC ($n = 5$) (◇), or were not vaccinated (mock, $n = 10$) (○). (D) Mice were vaccinated twice with ALVAC-hup53/wt ($n = 5$) (●), ALVAC-hup53/175 ($n = 5$) (◆), ALVAC-hup53/273 ($n = 5$) (▲), with ALVAC ($n = 5$) (△), or were not vaccinated (mock, $n = 5$) (○), and all mice were challenged with 10(3)Tx87 cells.

8-week-old animals were injected s.c. with 5×10^7 plaque-forming units of one of the ALVAC viruses. All animals were challenged s.c. on the left flank with 10^5 10(3)273.1NT24 or 10(3)TxBT87 cells in 0.5 ml of PBS. Animals were monitored weekly for the presence of tumors, and tumor size was measured by caliper in two dimensions.

Anti-p53 Antibody Response. Tail blood was obtained before immunization, 8 days after the second immunization, and after mice were sacrificed with tumors of at least 10×20 mm in size. Anti-p53 antibody levels were determined by using 2 μ l of mouse serum and 10 μ l (packed volume) of protein A-Sepharose (Pharmacia Biotech) to immunoprecipitate equal amounts of *in vitro* translated 35 S-labeled human wild-type p53 (24). The immunoprecipitated protein was separated by SDS/PAGE and visualized by quantitative autoradiography using the PhosphorImager system (Molecular Dynamics).

Expression of the p53 Protein in Tumors Escaping Immunologic Rejection. Eight tumors of about 10×5 mm in size from mice immunized with ALVAC-hup53 or ALVAC-mup53 and nine tumors from control mice that were either not immunized or immunized with nonrecombinant virus were excised under sterile conditions. Tumors were cut into small fragments, digested for 30 min in 0.5 mM EDTA, pH 8.0/PBS and subsequently incubated with DMEM plus 10% fetal bovine serum until tumor cells had grown out to about 80% confluence. After two passages, cells were analyzed for expression of the p53 protein. The cells were labeled metabolically with [35 S]methionine for 18 h. Equal amounts of labeled protein were subjected to immunoprecipitation followed by SDS/PAGE and quantitative autoradiography.

RESULTS

Vaccination with ALVAC Recombinant Viruses Expressing Human p53 Protect Against Tumor Cell Challenge. Various ALVAC-based recombinant viruses were engineered to express human or murine p53 in wild type or mutant form. To confirm p53 expression, chicken embryo fibroblasts were infected with the different recombinant viruses, and the p53 protein was detected by immunoprecipitation (not shown). Similar results were obtained after infection of murine 10(3) cells, which are nonpermissive for ALVAC replication (not shown). The ability of ALVAC-hup53 to protect against tumor cell challenge was tested (Fig. 1). BALB/c mice were immunized with either an ALVAC recombinant virus (ALVAC-hup53/wt or ALVAC-hup53/273 or ALVAC-hup53/175) or the ALVAC parental wild-type virus or were not immunized (mock). Four weeks later, each group received a similar booster immunization with subsequent challenge 2 weeks later with the highly tumorigenic cell line 10(3)273.1NT24 cells. As shown in Fig. 1A, all control animals (mock, ALVAC) developed tumors (25/25). In contrast, 8/10 (80%) of the animals vaccinated with ALVAC-hup53/273, 10/15 (67%) of the animals vaccinated with ALVAC-hup53/wt, and 8/15 (53%) of the animals vaccinated with ALVAC-hup53/175 remained tumor-free over an observation period of 130 days. The tumor-free survival time of mice immunized with ALVAC-p53 recombinant viruses as compared with the control mice was increased on a highly significant level ($P < 0.0000001$ using an independent *t* test, Fig. 1B). However, the actual increase in tumor-free survival time is even greater than indicated, since a large proportion of vaccinated mice were still alive at the end of the observation period. No significant difference in tumor protection was conferred by different ALVAC recombinants expressing either wild type or the various p53 mutant protein forms (Fig. 1B), indicating that the protective effect is not specifically directed against these mutated regions of p53. There was a highly significant difference in the tumor-free survival time between mice immunized with nonrecombinant virus (ALVAC) and mice immunized with the recombinant

viruses ($P < 0.0000001$ using an independent *t* test) (cf. Fig. 1A). This demonstrates that the tumor-protective effect is dependent upon p53 expression and cannot be attributed to nonspecific immunostimulation by the parental virus. Significantly, six of the mice remaining tumor-free at day 130 were reboosted with recombinant ALVAC and rechallenged with the same amount of 10(3)273.1NT24 cells as previously. All mice remained tumor-free throughout an additional 120-day observation period (not shown). Next, the efficacy of the vaccination with recombinant ALVAC was tested for the treatment of 24 h established microscopic tumors (Fig. 1C). BALB/c mice were first challenged with 10^5 10(3)273.1NT24 cells. After 24 h, they were vaccinated with either ALVAC-hup53/wt or nonrecombinant ALVAC, or they were not vaccinated. Four booster inoculations were administered every

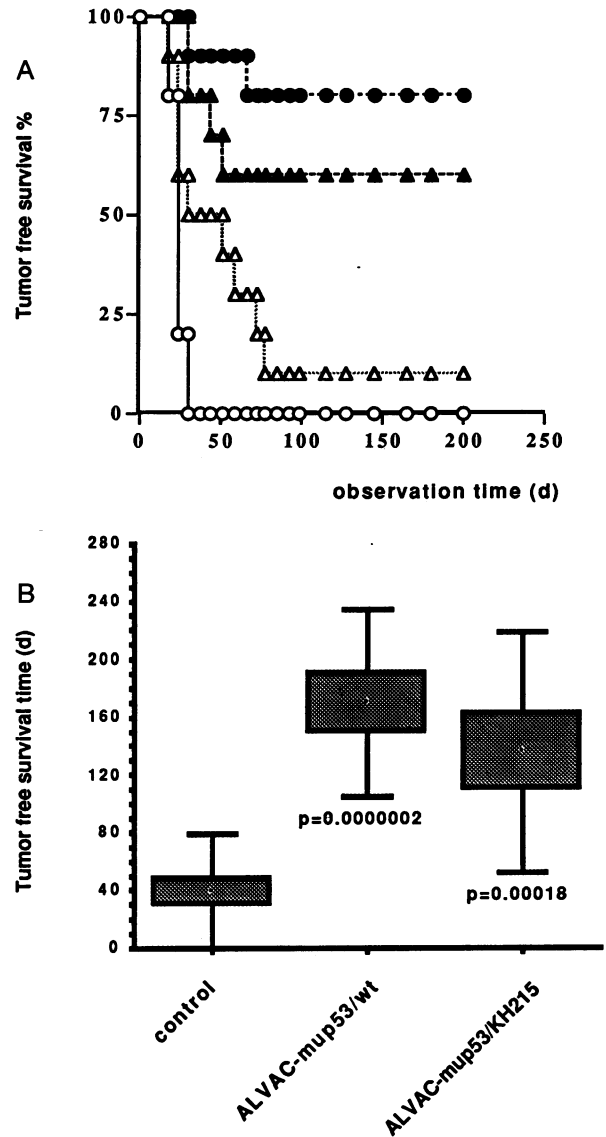


Fig. 2. Protection against tumor cell challenge after vaccination with ALVAC-mup53. Groups of mice were vaccinated twice with ALVAC-mup53/wt (●) ($n = 10$), ALVAC-mup53/KH215 (▲) ($n = 10$), with ALVAC ($n = 10$) (△), or were not vaccinated (mock, $n = 10$) (○) and then were challenged with 10(3)273.1NT24 cells. (A) Kaplan-Meier plot showing the percentage of tumor-free mice over the observation time of 200 days. (B) Average tumor-free survival time for all control mice (mock and ALVAC) and all vaccinated mice (ALVAC-mup53/wt, ALVAC-mup53/KH215). Average values are shown as dots; boxes represent the standard error, and stackers show the standard deviation.

2 weeks. Eight of 10 mice vaccinated with ALVAC-hup53/wt remained tumor-free over a 110-day observation period in contrast to only two of 10 of the control animals (Fig. 1C). However, when the vaccination was started after the formation of 1×1 mm tumors, there was no impact on tumor growth by this vaccination protocol (not shown). To further demonstrate that the tumor-protective effect induced by the vaccination is directed specifically against the p53 protein, BALB/c mice were vaccinated with the different recombinant viruses as described, and 2 weeks after the booster immunization they were challenged with 10^5 10(3)TxB87 tumor cells, which do not express the p53 protein. Significantly, no tumor protection was induced by any of the different viruses (Fig. 1D).

Vaccination with ALVAC Recombinant Viruses Expressing Murine p53 Protect Against Subsequent Tumor Challenge.

We next tested whether tumor protection was also achieved by vaccination with recombinant ALVAC expressing murine p53 (ALVAC-mup53). BALB/c mice were immunized as described above with the ALVAC-p53 recombinants, ALVAC-mup53/wt and ALVAC-mup53/KH215, or nonrecombinant ALVAC virus, or they were not immunized (mock). Two weeks after the second immunization, all mice were challenged with 10^5 10(3)273.1NT24 cells. As shown in Fig. 2A, only 1/20 (5%) of the control mice (mock, ALVAC) remained tumor-free, whereas 8/10 (80%) mice immunized with ALVAC-mup53/wt and 6/10 (60%) mice immunized with ALVAC-mup53/KH215 remained tumor-free over an observation period of 200 days. The increase in tumor-free survival time of mice immunized with ALVAC-p53 recombinant virus compared with the control mice was significant to $P = 0.000002$ and $P = 0.00018$ using an independent *t* test (Fig. 2B). These results show that vaccination with murine p53 also leads to a tumor-protective immune response.

Anti-p53 Antibodies Are Induced by Tumor Formation but Not by Vaccination with ALVAC-p53 Recombinant Virus.

To assess the ability of ALVAC-hup53 to induce an anti-p53 antibody response, we determined the anti-p53 antibody (IgG) response in a subset of mice vaccinated with recombinant ALVAC. Serum was taken prior to immunization, 2 weeks after the last immunization, and after mice were sacrificed with tumors of at least 10×20 mm diameter in size. These sera were first incubated with protein A-Sepharose, which is known to bind predominantly to IgG molecules contained in the sera. These beads were used for immunoprecipitation of *in vitro* translated and radiolabeled human p53, followed by SDS/PAGE and quantitative autoradiography. At least 10 mice were examined in each group: mice vaccinated with recombinant virus (ALVAC-hup53), nonrecombinant virus (AL-

VAC), or not vaccinated (mock). As shown in Fig. 3, anti-p53 antibody levels did not increase significantly after immunization with the ALVAC-p53 recombinant virus. In contrast, mice in all three groups that were sacrificed with tumors, developed high anti-p53 antibody levels. This suggests that the tumor-protective effect did not correlate with the amount of IgG anti-p53 antibodies at the time of tumor cell challenge.

Down-Regulation of the p53 Protein Is a Common Event in Tumors Escaping Immunologic Rejection. A subset of mice developed tumors despite prior administration of ALVAC-p53 recombinant viruses. These tumors were analyzed for down-regulation of their p53 protein expression as a possible escape mechanism. We established eight cell lines from tumors of mice that had been vaccinated with ALVAC-hup53 and nine control cell lines of mice that were not vaccinated or were vaccinated with ALVAC parental virus. For analysis of the p53 protein expression, all 17 cell lines were metabolically labeled with [35 S]methionine, and p53 protein was immunoprecipitated from whole cell lysate using the anti-p53 antibody PAb421. The expression of p53 was markedly reduced in cell lines derived from tumors of immunized mice compared with the control cell lines (Fig. 4). It might be argued that the down-regulation of p53 protein expression is due to prolonged growth of these tumors in mice in the absence of G418, which is used to select for the p53 expression vector. This seems unlikely, because prolonged growth of the parental cell line 10(3)273.1NT24 under G418-free conditions does not lead to down-regulation of p53 (not shown). These results demonstrate, that down-regulation of the tumor-specific antigen p53 is a common event in tumors escaping the immunologic rejection, suggesting that the vaccination is specifically directed against p53.

DISCUSSION

The experiments presented here demonstrate that ALVAC recombinant viruses expressing either murine or human, mutant or wild-type p53 can protect mice against the growth of tumor cells that express high levels of mutant p53. Administration of ALVAC parental virus did not confer protection, suggesting that the observed protection against tumor challenge was due to a p53-specific immune response. This is further supported by the lack of tumor protection after challenge with a cell line that failed to express p53 protein (the 10(3)Tx4BT87 line). Additional evidence for the p53 specificity is provided by the fact that tumors escaping the immunosurveillance were all found to have down-regulated p53 protein expression. There was no significant difference in the efficacy

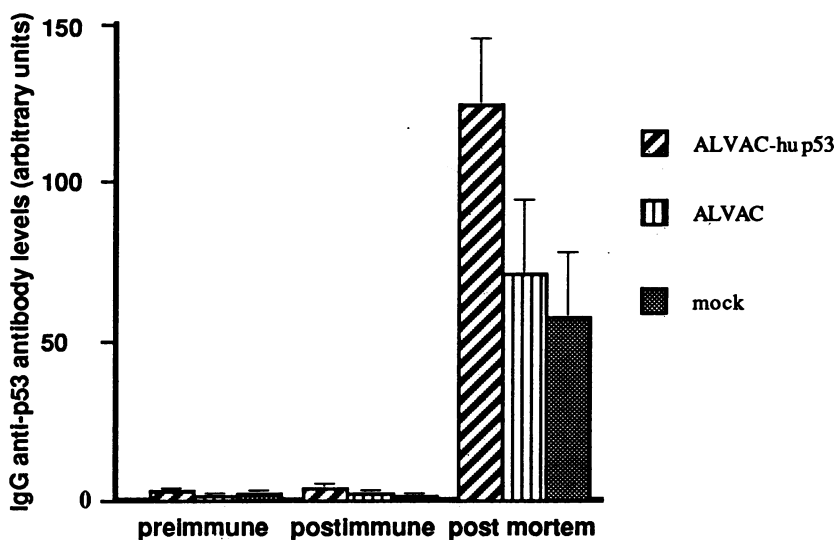


FIG. 3. ALVAC-hup53 does not induce detectable levels of anti-p53 antibodies. p53 antibody levels were measured in serum from mice vaccinated with ALVAC-hup53, from mice vaccinated with nonrecombinant ALVAC, and from nonvaccinated mice. Preimmune sera, post-immune sera, and postmortem sera were analyzed (at least 10 in each group). Anti-p53 antibody levels in the mouse sera were determined by immunoprecipitation of *in vitro* translated [35 S]methionine-radiolabeled p53 followed by SDS/PAGE and quantitative autoradiography. Average quantities of immunoprecipitated p53 are shown along with standard errors.

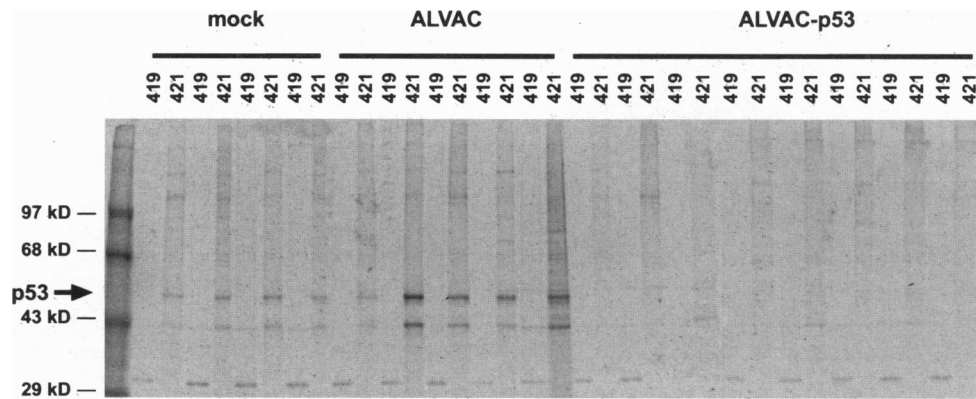


FIG. 4. Down-regulation of the p53 protein expression in tumors of mice vaccinated with ALVAC-p53 recombinant viruses and of control mice. Eight tumors from mice immunized with ALVAC-hup53 or ALVAC-mup53 and nine tumors from control mice (ALVAC, mock) were analyzed for expression of the p53 protein by immunoprecipitation of equal amounts of p53 protein (PAb421) from radiolabeled cell lysate followed by SDS/PAGE and quantitative autoradiography. Immunoprecipitation with PAb419 served as negative control.

between ALVAC viruses expressing human or murine p53. There was also no significant difference in the tumor-protective effect of the various mutants compared with wild-type p53. This indicates that the protective immune response was directed against one or several epitopes of p53, which are shared between the different mutants and between human and murine p53 as opposed to a mutant p53 epitope. Thus, ALVAC recombinant viruses expressing p53 represent a vaccine candidate that might be effective against a wide variety of human tumors harboring different p53 mutants.

The induction of a tumor-protective effect against murine p53 apparently overcomes self-tolerance. What might be responsible for this phenomenon? Likely, the high levels of the p53 protein in the infected cells and in the tumor cells lead to an immune response directed against an antigen that is otherwise present in low amounts. It is conceivable that the density of complexes consisting of MHC and peptides derived from p53 is critical for the activation of an otherwise silent T cell repertoire (cf. ref. 25 for review). Further stimulatory signals may contribute to this phenomenon, including the adjuvant effects of the viral infection. In support of this concept, an immune response against the self-tumor-antigen HER-2/neu has been shown previously in cancer patients (5).

No IgG serum antibodies directed against p53 were detected in vaccinated mice. However, mice with a large tumor burden did produce high levels of p53-specific antibodies. This is in accordance with previous studies reporting that about 10–20% of sera from patients with various carcinomas, mainly breast and lung carcinomas, contain anti-p53 antibodies (26, 27). It can be speculated that tumor necrosis leading to the release of p53 into the extracellular space represents the main source of p53 antigen eliciting a humoral response. As p53 is located inside the vital tumor cell, it is unlikely that such anti-p53 antibodies could be of protective value. This result is in accordance with the concept that T-cell responses, rather than antibody responses, are the primary target of effective antitumor immunotherapeutic strategies.

A crucial requirement for eliciting a cytotoxic T-cell response is the presentation of antigenic peptides on MHC class I molecules, which are of the H-2^d class in BALB/c mice. A computer-assisted analysis of the p53 amino acid sequence based on the predictions of Falk *et al.* (28) and Corr *et al.* (29) revealed that the p53 mutants expressed in the ALVAC-p53 recombinant viruses presented here or in the 10(3)273.1NT24 cells are unlikely to result in the presentation of novel peptides binding with high affinity to the H-2K^d or H-2D^d molecules (not shown). Therefore, at least with respect to these haplotypes, it is not surprising that the vaccination efficiency using

ALVAC-based recombinants expressing wild-type or mutant p53 did not lead to significant differences in tumor protection.

For clinical applications, it would be highly desirable to immunize against wild-type p53 sequences. A vaccination strategy against epitopes created by specific p53 mutations might be possible in a subset of cases (11, 12, 30, 31), but it would require the precise assessment of the p53 mutation in each case and would only be applicable to patients carrying a specific HLA subclass leading to the presentation of a mutant p53-specific peptide. It is therefore encouraging that in these studies wild-type p53 can efficiently be used for vaccination. A cellular immune response against wild-type p53-derived peptides has been described recently. Patients with breast cancer harboring a mutant p53 gene were found to have a proliferative T-cell response to wild-type p53 (32).

Immunization of HLA-A2.1 transgenic mice with suitable peptides derived from human wild-type p53 can elicit a cytotoxic T lymphocyte response capable of lysing tumor cells harboring mutant p53 *in vitro* (33). Interestingly, the strongest cytotoxic T lymphocyte responses were obtained by immunization with four peptides (amino acids 25–35, 65–73, 149–157, 264–272) that are all located outside of the frequently mutated regions in human cancers. These results further support the hypothesis presented here that overexpressed p53, although seen as self, can be used as a target for immunotherapy. Hence, a successful vaccination directed against the p53 protein would represent a widely applicable immunologic treatment of malignant tumors.

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