PROTECTIVE ANTITUMOR IMMUNITY INDUCED BY IMMUNIZATION WITH COMPLETELY ALLOGENEIC TUMOR CELLS

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Abstract

We have shown previously that immunization of B6 mice (H-2b) with tumor cells of B6 origin transformed by the human adenovirus type 5 early region 1 (Ad5El) induces an H-2D8-restricted CTL response against an EL-encoded CTL epitope. We now report that immunization of B6 mice with Ad5El-transformed tumor cells of BALB/c origin (H-2d), apart from inducing a B6 anti-BALB/c allo-response, also induces a strong CTL response against the BALB/c H-2D8-presented CTL epitope. BALB/c Ad5El-transformed tumor cells are not recognized by EL-specific CTLs, indicating that nontumor cells have processed the EL-encoded CTL antigen and have presented the EL epitope to EL-specific CTLs. These data also show that the B6 anti-BALB/c allo-response does not overweigh the anti-EL response induced by the allogeneic tumor cell vaccination. Moreover, B6 mice immunized with allogeneic BALB/c Ad5El cells are, in contrast to mice vaccinated with untransformed BALB/c cells, protected against a subsequent challenge with B6 Ad5El-expressing tumor cells. These data show that immunization with completely allogeneic tumor cells can lead to protective syngeneic antitumor immunity, indicating that completely allogeneic tumor cell vaccines can be used for the induction of antitumor immunity.

Introduction

An effective T cell-mediated antitumor immune response requires activation of tumor-specific T cells (1, 2). It is generally accepted that MHC class II-restricted tumor-specific CD4+ T cells directed against MHC class II+ tumors are primed by APCs3 that have picked up tumor antigens (1, 3). The defined cellular pathways leading to MHC class I-antigen presentation are different from the pathways leading to MHC class II-peptide presentation (4). MHC class I-antigen presentation requires that the antigenic peptide becomes available in the endoplasmatic reticulum of the APC to be transported to the cell membrane in the context of MHC class I. Therefore, it seems likely that virus-infected cells or tumor cells prime and activate CTL responses directly by presenting peptides derived from virus or tumor antigens on their own cell surface. However, the mechanisms by which MHC class I-restricted tumor-specific CD8+ T cells are primed are still a matter of debate. Allogeneic cells expressing a given antigen may effectively prime an antigen-specific CTL response restricted by MHC class I molecules of the immunized mouse, regardless of the haplotype of the immunizing cells (5, 6). A role for bone marrow-derived APCs in presenting MHC class I-restricted tumor antigens has been implicated by studies in which tumor-specific CTLs were induced by melanoma cells that, due to the absence of MHC class I, were not capable of presenting MHC class I-restricted tumor antigens (7). Although the antigenic peptides recognized by the CTLs were not identified in these studies, the outcome suggests that tumor-specific CTLs can be induced by a phenomenon called cross-priming (8). In contrast, fibroblasts transfected with viral proteins can directly induce CTL responses in vivo, without the involvement of host APC (9). This observation suggests that CTL responses in this case are not induced by cross-priming but by direct interaction between CTLs and target cells.

These observations might have implications for the development of, for example, tumor cell-based anticancer vaccines. If CTL responses are induced directly by tumor cells, then MHC matching between the tumor cells used in the vaccine and the patient is obligatory. However, in the case of cross-priming, MHC matching between the patient and tumor cell vaccine becomes redundant. The use of tumor cell-based vaccines as a tool to augment antitumor responses has received great attention in the last decade (10-13). Both autologous tumor cells derived from a patient’s own tumor and allogeneic tumor cells, sharing at least one HLA class I-restricting element, have been used for these purposes. The rationale for the use of allogeneic tumor cells sharing at least one HLA class I molecule with the recipient comes from the difficulty in obtaining sufficient amounts of autologous tumor cells, the requirement to make the tumor more immunogenic by genetic engineering, and the identification of common tumor antigens (14-16). Nonetheless, MHC matching (often for HLA-A2) of the tumor cell vaccine with the MHC of the recipient might form an obstacle to the large-scale application of tumor cell-based anticancer vaccines.

To study whether completely allogeneic tumor cells are able to induce syngeneic, protective, T cell-mediated antitumor responses, we generated tumor cells transformed by the human Ad5El of C57BL/6 (H-2b) and BALB/c (H-2d) origin. Ad5El-transformed tumor cells of C57BL/6 origin present at least two Ad5El-encoded H-2D8-restricted CTL epitopes to the immune system (17, 18). Immunization of C57BL/6 mice with H-2b-expressing Ad5El-transformed tumor cells induces a protective CTL response that is predominantly directed against the El-encoded peptide VNIRNCCYII (19). We also show that allogeneic Ad5El-transformed tumor cells of BALB/c origin induce a protective H-2D8-restricted ElB-directed CTL response, indicating that ElB-specific CTLs are induced by cross-priming and that allogeneic tumor cells not matched for any MHC molecule can be successfully used for vaccination purposes.

Materials and Methods

Mice. C57BL/6 (B6 Kh, H-2b) mice were obtained from the Netherlands Cancer Institute (Amsterdam, the Netherlands). C57BL/6 mice (B6, H-2b) were obtained from the University Hospital Leiden, Department of Immunohematology and Blood Bank.

Cell Lines and Culture Conditions. Untransformed MECs of C57BL/6 and BALB/c origin were generated as described previously (17). Transformed MEC-derived cell lines were obtained as described previously (17, 18). All cell lines were tested for expression of the transfected Ad5ElA and Ad5ElB genes by Northern blotting (data not shown). All MECs and Ad5El-transformed MECs were maintained in Iscove’s modified Dulbecco’s medium (Biokom KG, Seromed, Berlin, Germany) supplemented with 4% FCS (HyClone Laboratories, Logan, Utah), penicillin (100 IU/ml; Brocades Pharma, Leiderdorp, the Netherlands), and 2-mercaptoethanol (20 μM) at 37°C in a 5% CO2 atmosphere. CTL clones were cultured as described previously (17, 18).

Generation of CTL Bulk Cultures. Ad5El-specific or B6 anti-BALB/c-specific CTLs in bulk culture were generated as follows: 5 × 10⁶ spleen
cells/well derived from B6 mice taken 3 weeks or more after the second s.c.
immunization with 1 × 10^7 irradiated (25 Gy) MECs or AdSE1 MECs were
cocultured for 5–6 days with 10% irradiated (25 Gy) IFN-γ (10 units/ml)-
treated stimulator cells in 24-well plates. Next, effector cells were harvested,
and dead cells were removed by density centrifugation on lymphocyte M
(Cedarlane, Hornby, Canada). These cells were used in a cell-mediated lym-
phocyte cytotoxicity assay.

Cell-mediated Lymphocyte Cytotoxicity. Experimental procedures to
measure cell-mediated cytotoxicity were performed in a Europium (Eu^{3+})-
release assay as described elsewhere (18, 20). In short, varying numbers of
effector cells were added to a 1 × 10^3 Eu^{3+}-labeled target cells in 0.15 ml of
culture medium in 96-well U-bottom plates. After a 4-h incubation at 37°C,
supernatants were collected and mixed with Enhancer solution® (Wallac,
Turku, Finland). Measurement of the samples took place in a 1234 Delfia®
fluorometer (Wallac). The mean percentage specific lysis of triplicate wells
was calculated as follows: % specific lysis = [(cpm experimental release −
cpm spontaneous release)/(cpm maximum release − cpm spontaneous
release)] × 100.

Peptides. Peptides were generated by solid-phase strategies on a multiple
peptide synthesizer (Abimed AMS 422) as described previously (21).

FACS Analysis. The haplotype of B6 and BALB/c cells was verified by
FACS analysis (data not shown). FACS analysis using a FACSscan flow
cytometer (Becton Dickinson, Mountain View, CA) was performed as de-
scribed elsewhere (17). Monoclonal antibodies recognizing H-2D<sup>+</sup> (28.14.8S;
Ref. 22), H-2K<sup>d</sup> (8B.2.43; Ref. 23), H-2K<sup>d</sup> (24), and I-A<sup>40</sup> (17.227R7; Ref.
25) were used to stain MHC class I and class II gene products. All MECs and
AdSE1 MEC cell lines were MHC class II-negative.

TNF Secretion Assay. The TNF secretion assay was performed as de-
scribed previously (26). In short, 1.5 × 10<sup>5</sup> CTLs and 2 × 10<sup>3</sup> stimulator cells
were incubated in 10 Cetus Units of recombinant interleukin 2 (Cetus Corp.,
Emeryville, CA) in a 96-well U-bottom plate. After 24 h, 60 μl of supernatant
were collected, and the TNF content of the supernatant was determined by measuring its cytotoxic effect on WEHI-164
cells/well derived from B6 mice taken 3 weeks or more after the second s.c.
immunization with 1 × 10<sup>7</sup> irradiated (25 Gy) MECs or AdSE1 MECs were
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cells/well derived from B6 mice taken 3 weeks or more after the second s.c.
immunization with 1 × 10^7 irradiated (25 Gy) cells in 0.25 ml PBS. Three weeks later the mice
were injected s.c. with 0.5 × 10<sup>6</sup> AdSE1 + ras-transformed cells of C57BL/6
origin, administered to each animal’s other flank in 0.25 ml PBS.

Tumor Cell Vaccination. C57BL/6 mice were immunized s.c. with
1 × 10<sup>7</sup> irradiated (25 Gy) cells in 0.25 ml PBS. Three weeks later the mice
were injected s.c. with 0.5 × 10<sup>6</sup> AdSE1 + ras-transformed cells of C57BL/6
origin, administered to each animal’s other flank in 0.25 ml PBS.

AdSE1 + ras-transformed cells (tumor cell clone 5R5) form tumors in naive
immunocompetent mice (19). Tumor volumes were measured with a caliper.
Animals were sacrificed when their tumors grew larger than 1000 mm<sup>3</sup> to
avoid unnecessary suffering.

RESULTS

BALB/c AdSE1 MECs Are Not Recognized by B6 AdSE1-
specific CTL Clones. Previously, we reported that B6 mice immun-
ized with B6 MECs (H-2<sup>b</sup>) transformed by the AdSE1 region (B6
AdSE1 MECs) mount a strong AdSE1-specific H-2D<sup>b</sup>-restricted CTL
response (17, 18). In the context of H-2D<sup>b</sup> molecules, B6 AdSE1
MECs present at least two AdSE1-encoded CTL epitopes to the
immune system. One of them is encoded by the EIA region (se-
quency: SGPSNTPPEI; Ref. 17), whereas the other is encoded by the
EIB region (sequence: VNIINCCYI; Ref. 18). No additional genes
are present in AdSE1 MECs. Therefore, no immunity will be raised
against other viral gene products, nor will these cells be able to produce infectious adenosivirus particles. AdSE1 MECs of BALB/c
origin (BALB/c AdSE1 MECs) are of the H-2<sup>d</sup> haplotype and do not
express the restricting H-2D<sup>b</sup> molecule. To study whether BALB/c
AdSE1 MECs are able to present the EIA and EIB peptide to H-2D<sup>b</sup>-restricted EIA- and EIB-specific CTLs, respectively, AdSE1
MECs of BALB/c origin were tested for their ability to present the
AdSE1 peptides to B6 AdSE1-specific CTL clones. BALB/c AdSE1
MECs were not lysed in a cytotoxicity assay by B6 EIA- or EIB-specific
CTLs (Fig. 1). Nor were BALB/c MECs recognized by EIA- or
EIB-specific CTLs when incubated with the synthetic peptides
representing the EIA- or EIB-encoded CTL epitope, respectively
(Fig. 1). Likewise, the EIA- or EIB-specific CTL clones were not
triggered to produce TNF when incubated with BALB/c AdSE1
MECs in a TNF secretion assay (Fig. 2). Taken together, these data
show that BALB/c AdSE1 MECs are not able to present the EIA-encoded CTL epitope SGPSNTPPEI or the EIB-encoded
CTL epitope VNIINCCYI to AdSE1-specific CTLs of B6 origin.

B6 Mice Immunized with Allogeneic BALB/c AdSE1 MECs
Mount a Strong Syngeneic EIA-specific CTL Response. It has been suggested that protective T cell-mediated immune responses
can be generated by immunization with completely allogeneic tumor
 MECS not recognized by host T cells, through a mechanism in
which nontumor cells process and present tumor-derived tumor
 antigens (7). To test whether this is indeed feasible in our model,
we immunized B6 mice with syngeneic B6 AdSE1 MECs or
allogeneic BALB/c AdSE1 MECs. Three weeks later, the spleen
cells of these animals were restimulated with syngeneic B6 AdSE1

Fig. 1. BALB/c cells are not able to present the H-2D<sup>b</sup>-restricted EIA-encoded CTL epitope SGPSNTPPEI or the EIB-encoded CTL epitope VNIINCCYI to EIA- or EIB-restricted
CTL clones, respectively. The percentage specific lysis as determined in a Eu^{3+}-release cell-mediated lymphocyte cytotoxicity assay of untransformed MECs of B6 or BALB/c origin
(unloaded or peptide-loaded) and AdSE1-transformed MECs of B6 or BALB/c origin by EIA-specific CTL clone 5 (A) or EIB-specific CTL clone 01 (B) at different E:T ratios is shown.

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MECs. Bulk CTL cultures derived from mice immunized with B6 Ad5E1 MECs were able to recognize B6 Ad5E1-transformed tumor cells and B6 MECs incubated with the E1B-encoded CTL epitope VNIRNCCYI (Fig. 3). These bulk cultures did not recognize the E1A-encoded CTL epitope. This is due to the fact that the anti-Ad5E1-specific CTL response is dominated by E1B-specific CTL, whereas anti-E1A-specific CTL responses are hardly detectable after tumor cell vaccination (19). Interestingly, B6 mice immunized with allogeneic BALB/c Ad5E1 MECs were also able to mount a strong CTL response directed against Ad5E1-transformed tumor cells and the E1B-encoded CTL epitope (Fig. 3). This CTL response did not result from the in vitro restimulation protocol because spleen cells from naive mice, stimulated in vitro with B6 Ad5E1 MECs, did not recognize Ad5E1-transformed tumor cells or the E1B-encoded CTL epitope VNIRNCCYI. Thus, these results indicate that allogeneic tumor cell vaccination can induce syngeneic tumor-specific CTL responses.

To exclude the possibility that B6 anti-BALB/c allo-specific CTLs induced by immunization of B6 mice with BALB/c cells cross-react on the E1B-encoded CTL epitope explaining the observation that syngeneic tumor-specific CTL activity can be induced by allogeneic tumor cell vaccination, we vaccinated B6 mice with untransformed BALB/c MECs or BALB/c Ad5E1 MECs. Three weeks later, the spleen cells of these animals were restimulated with syngeneic B6 Ad5E1 MECs to propagate Ad5E1-specific CTLs, or allogeneic BALB/c Ad5E1 MECs to expand allo-reactive T cells. Bulk CTL cultures derived from B6 mice immunized with untransformed allogeneic BALB/c MECs were not able to kill syngeneic B6 Ad5E1 MECs or B6 MECs incubated with one of the Ad5E1-encoded CTL epitopes (Fig. 4). This CTL activity was induced when the mice were vaccinated with BALB/c MECs expressing the Ad5E1 region (Figs. 3 and 4). This indicates that the observed anti-E1B response cannot be explained by a cross-reaction of B6 anti-BALB/c allo-specific CTLs on B6 target cells because Ad5E1-specific CTL activity was only induced when mice were immunized with Ad5E1-expressing tumor cells. Moreover, the Ad5E1-specific bulk CTL cultures did not lyse BALB/c Ad5E1 MECs, confirming the observation that Ad5E1-transformed cells of BALB/c origin are not able to present Ad5E1-derived peptides to B6 Ad5E1-specific CTLs. When the same spleen cells derived from mice immunized with allogeneic BALB/c MECs or BALB/c Ad5E1 MECs were restimulated with allogeneic BALB/c Ad5E1 MECs, a B6 anti-BALB/c response was revealed (Fig. 4). This response was also induced in vitro when B6 spleen cells derived from naive mice were grown in the presence of BALB/c cells (data not shown), indicating that the B6 anti-BALB/c allo-specific response is a relatively strong response because it does not require in vivo induction. These results show that immunization of B6 mice with allogeneic BALB/c Ad5E1 MECs not only induces an anti-E1B-specific response that lyases syngeneic tumor cells but also induces a strong B6 anti-BALB/c allo-specific response, indicating that the allo-response does not overwhelm the antitumor response, and that the antitumor response coexists with the B6 anti-BALB/c allo-specific response.
**ANTITUMOR IMMUNITY INDUCED BY ALLOGENEIC TUMOR CELLS**

In vivo: BALB/c MEC

In vitro: B6 Ad5E1 MEC

In vivo: BALB/c Ad5E1 MEC

In vitro: B6 Ad5E1 MEC

Fig. 4. The B6 anti-BALB/c allo-response induced by immunization with allogeneic BALB/c Ad5E1 MECs does not explain the cross-reactivity on syngeneic B6 Ad5E1-transformed tumor cells. B6 mice were immunized with allogeneic untransformed BALB/c MECs or allogeneic Ad5E1-transformed MECs. Spleen cell suspensions derived from mice immunized with BALB/c MECs were split in half and were restimulated in vitro with either syngeneic B6 Ad5E1 MECs or allogeneic BALB/c MECs. The same was done with spleen cells derived from mice immunized with allogeneic BALB/c Ad5E1 MECs. Five days later bulk CTL cultures were tested in vitro with allogeneic B6 Ad5E1 MECs or syngeneic Ad5E1-transformed tumor cells. The percentage of specific lysis at different E:T ratios is shown.

**DISCUSSION**

Immunization with Allogeneic BALB/c Ad5E1 MECs Induces Protection against a Challenge with Ad5E1 + ras Cells. The above data show that immunization with allogeneic BALB/c MECs harboring the Ad5E1 region is able to induce lytic CTL responses against syngeneic Ad5E1-expressing tumor cells. To test whether mice vaccinated with BALB/c Ad5E1 MECs are indeed protected against a lethal challenge with Ad5E1-expressing tumor cells, we challenged these mice with tumor cells transformed by the Ad5E1 region and an activated EJras oncogene. These tumor cells are tumorigenic in immunocompetent mice. B6 mice immunized with syngeneic B6 Ad5E1 MECs or allogeneic BALB/c Ad5E1 MECs were protected against a lethal challenge with Ad5E1 + ras cells (Fig. 5). These results show that immunization with allogeneic tumor cells expressing the Ad5E1 region induces protective immunity against syngeneic Ad5E1-expressing tumor cells. This protection is Ad5E1-specific because it is not induced by allogeneic cells lacking the Ad5E1 region.

Immunization of B6 mice with completely allogeneic tumor cells of BALB/c origin expressing the Ad5E1 region induces a powerful syngeneic E1B-specific CTL activity that is associated with protection against a lethal challenge with syngeneic Ad5E1-expressing tumor cells. Because the allogeneic BALB/c Ad5E1 MECs are not recognized by Ad5E1-specific CTLs of B6 origin at the polyclonal (Fig. 4) or monoclonal (Figs. 1 and 2) level, these results indicate that these antitumor CTL responses are induced by cross-priming by nontumor...
Fig. 5. Vaccination with allogeneic BALB/c AdSE1 MECs induces protective immunity against a lethal challenge with syngeneic AdSE1 + ras-transformed tumor cells. B6 mice were immunized s.c. with irradiated allogeneic untransformed BALB/c MECs, allogeneic AdSE1-transformed BALB/c AdSE1 MECs, and syngeneic B6 AdSE1 MECs or were left untreated (8 animals/group). Two weeks later the mice were injected on the other flank with live B6 AdSE1 + ras cells. B6 AdSE1 + ras cells form tumors when injected into B6 mice. Mice immunized with AdSE1-expressing tumor cells are protected against the outgrowth of B6 AdSE1 + ras cells. The percentage of surviving animals is shown (P = 0.001; log-rank test).

cells that have processed and presented the E1B-encoded tumor antigen.

Although the target antigens were not known, indications that cross-priming is an important mechanism for the induction of CTL responses came from studies in the late 1970s that show that an antigen-specific T-cell response can be induced regardless of the haplotype of the immunizing cell (5, 6). Similarly, it has been shown that class I-negative tumors are able to evoke CTL-mediated antitumor immunity (27), and that such tumors are able to induce CD8-dependent protective immunity (7). A role of bone marrow-derived cells has been postulated to be important in the induction of tumor-specific CTL responses by cross-priming (7). The way these cells pick up tumor antigens is not known, but several different mechanisms have been suggested (reviewed in Ref. 8). These potential mechanisms include an ill-defined pathway of phagocytes to shunt proteins from the phagosome to the cytosol or the phagocyte uptake of peptide-carrying heat shock proteins released from dying tumor cells (8, 28). Nonetheless, other studies show that the involvement of host APCs in the induction of CTL responses is not necessarily required (9). CTL responses to the glycoprotein or nucleoprotein of lymphocytic choromeningitis virus were only obtained in vivo when the antigen and the restricting MHC class I molecule were present on the same cell (9). The presence of the immunizing cell in a lymphoid environment seemed necessary for the induction of CTL responses. Whether direct presentation by syngeneic AdSE1-transformed tumor cells is involved in the induction of H-2D\(^p\)-restricted E1B-specific CTL responses is not known, but cross-priming is most likely sufficient for the instigation of protective E1B-specific CTL responses because allogeneic AdSE1-transformed tumor cells lacking the restricting H-2D\(^p\) molecule are able to induce strong H-2D\(^p\)-restricted E1B-specific CTL responses.

The observation that allogeneic AdSE1-transformed tumor cells are able to induce syngeneic immune responses that can protect against the outgrowth of syngeneic AdSE1-expressing tumor cells is important for the development of active immunotherapy based on allogeneic tumor cells. Active immunotherapy using irradiated (partially) allogeneic tumor cells or tumor cells products administered with or without adjuvants as Bacillus Calmette-Guérin have been used in a clinical setting (10, 11, 13, 29). Although in these cases some complete and partial responses were observed, these approaches, in general, have met with limited success. The success of these anticancer protocols can potentially be increased by making the tumor cell vaccine more immunogenic. Currently, great effort is directed toward the genetic engineering of tumors to enhance their immunogenicity by the introduction of cytokine genes or genes encoding costimulation molecules (reviewed in Refs. 14 and 30). The notion that completely allogeneic tumor cells can be successfully used for the induction of protective antitumor immunity indicates that one tumor cell vaccine can be used for many different patients without MHC matching between the tumor cell vaccine and patient. On the other hand, matching between tumor antigens expressed by the vaccine and the patient’s tumor might be more appropriate. Moreover, our results point to an alternative approach for the development of more immunogenic allogeneic tumor cell vaccines. Especially in melanoma, more and more tumor antigens shared by many different patients that are recognized by CTLs are being defined (31). By transfection of several known melanoma-associated antigens that have a wide distribution in melanoma patients (e.g., melanocytic differentiation antigens, genes belonging to the MAGE family) into a single highly transflectable cell line, it might be possible to generate a cell-based vaccine that can be used for many melanoma patients. MHC matching between the genetically engineered immunizing cell and the patient would not be necessary, and such tumor cell vaccines could be used on a large scale in a clinical setting.

As a result of the use of allogeneic vaccines, most patients will also develop an allo-specific T-cell response. Our results show that the coinduced B6 anti-BALB/c allo-response does not overwhelm the tumor-specific anti-E1B-specific CTL response, indicating that such allo-specific T-cell responses do not necessarily hamper the antitumor response. These allo-specific T-cell responses might even be beneficial for the development of the antitumor response because introduction of an allo-molecule into a weakly immunogenic tumor has been shown to be an effective way to induce protective immunity against a subsequent challenge with an otherwise lethal dose of parental nontransfected cells (32–34). Indeed, in the AdSE1 tumor system, the magnitude of the anti-E1B response seems to be stronger after vaccination with BALB/c AdSE1 MECs compared to immunization with syngeneic B6 AdSE1 MECs (Fig. 3). Taken together, the observation that completely allogeneic tumor cells can be successfully used for the induction of protective antitumor immunity holds promise for the implementation of allogeneic tumor cell-based vaccines and might open new ways to develop new allogeneic tumor cell vaccines.
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