Immunotherapy with Dendritic Cells and Tumor Major Histocompatibility Complex Class I-derived Peptides Requires a High Density of Antigen on Tumor Cells

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ABSTRACT

Immunization with dendritic cells and unfractionated MHC class I-binding peptides derived from autologous tumor cells has been shown to induce effective antitumor immunity. However, the importance of the relative abundance of tumor peptides on the surface of tumor cells is not known. We have addressed this question using peptides isolated from three tumor cell lines transfected with a minigene encoding amino acids 33–41 of the lymphocytic choriomeningitis virus glycoprotein (LCMV33–41). The three cell lines expressed different levels of MHC class I molecules and had different abilities to stimulate proliferation of LCMV33–41-specific T cells in vitro. We found that antitumor immune responses were best elicited by immunizing mice with dendritic cells and synthetic LCMV33–41 peptide. Peptide preparations from a given tumor cell line conferred protection against challenge with the same tumor cell line. However, protective immunity to a different tumor could be induced only if the cell line used for peptide preparation presented a high relative proportion of LCMV33–41 in association with MHC class I. Our results suggest that multiple peptide epitopes are required for the induction of an effective antitumor immune response using MHC class I-binding peptides from tumor cells. Also, the ability to induce an antitumor immune response appears to correlate with the proportion, rather than the absolute amount, of tumor-specific peptide in the mixture used for immunization.

INTRODUCTION

The aim of immunotherapy is to induce or increase the ability of tumor-reactive T cells to mediate antitumor immune responses in vivo. One of the strategies that has been reported recently uses immunization with antigen-presenting cells pulsed with unfractionated MHC class I-binding tumor peptides (1, 2). This approach is simple, does not require the identification of the associated antigens, is not limited by MHC polymorphism, and possibly involves multiple epitopes and thus, is unlikely to result in immune selection of resistant clones of tumor cells.

The potential advantages of using unfractionated tumor peptides as vaccines are counterbalanced by a number of drawbacks that this nonspecific strategy can encounter in its practical application. The level of expression of the tumor peptide on tumor cells is probably the most important issue. Because tumor cells are used first as a source of antigen and later become targets of the antitumor immune response, limited presentation of tumor antigen becomes critical at two separate stages. Other factors that can limit the efficacy of this approach include the immunogenicity of the tumor peptides and the presence of specific T-cell precursors (3). Because tumor-associated peptides are essentially self-antigens, they are possibly weakly immunogenic or not immunogenic at all. Immunodominance of some antigens over others can lead to failure to elicit some T-cell responses (4), although the use of BMDCs as antigen carriers possibly abrogates dominance features among antigens (5). Additionally, other issues that have to be considered are more quantitative and concern the total amount of peptide that can be isolated from tumor cells. Efficient T-cell recognition seems to be critically dependent on adequate tumor antigen concentration (6, 7). However, a frequent feature in tumor biology is down-regulation of MHC class I molecules (8, 9), which leads to a general scarcity of MHC class I-associated peptides and altered recognition of tumor cells by CTLs (10).

In consideration of these restrictions, it is reasonable to hypothesize that the efficacy of using unfractionated tumor peptides as immunogens can be easily compromised by the low concentration of effective tumor peptides on tumor cells (11).

Our aim in this study was to investigate whether the relative abundance of tumor-specific peptides expressed on tumor cells was a limiting factor in the induction of antitumor immune responses. We have adopted an experimental system where different tumor cell lines were transfected to express one MHC class I-associated peptide, LCMV33–41 (12, 13). The same synthetic LCMV33–41 peptide can be used together with DCs to induce antitumor immune responses (13). In this context, we have explored the efficacy of using unfractionated peptides isolated from these tumor cell lines expressing different levels of LCMV33–41.

MATERIALS AND METHODS

In Vitro Culture Media and Reagents. All cultures were in Iscove’s modified Dulbecco’s medium (Sigma Chemical Co., St. Louis, MO) containing 2 mmol/l-glutamine (Sigma), 1% penicillin-streptomycin (Sigma), 5 × 10−3 μg/ml-mercaptoethanol (Sigma), and 5% fetal bovine serum (Life Technologies, Inc., Auckland, New Zealand). The amino acid 33–41 peptide fragment of the LCMV glycoprotein (KAVYNFATM; LCMV33–41) was obtained from Chiron Mimotopes (Clayton, Victoria, Australia).

Mice and Cell Lines. C57BL/6j mice (Jackson Laboratories, Bar Harbor, Maine) and “318” mice (14), transgenic for a T-cell receptor specific for D b 1, and “318” mice (14), transgenic for a T-cell receptor specific for D b 1, were maintained in the Animal Facility of the Wellington School of Medicine by brother × sister mating. The Lewis lung carcinoma (LLC), the murine thymoma EL4, and the murine melanoma B16.F10 were maintained by weekly subculturing in CM. LL-LCMV and EL-LCMV are transfectants of LLC and EL4, respectively, generated by expressing a minigene encoding the LCMV glycoprotein amino acids 33–41 under the control of a cytomegalovirus promoter as described (13). B16.F10 (GP33), derived from parental B16.F10 by transfection of the GP33 LCMV minigene (12), were transfected cell lines LL-LCMV, EL-LCMV, and B16.F10 (GP33) were maintained in CM containing 0.5, 0.4, or 0.2 mg/ml G418, respectively (Life Technologies).

Growth of BM-derived DC. DCs were generated in culture from bone marrow precursors in the presence of IL-4 and granulocyte/macrophage-colony-stimulating factor as described (15). Cultures were fed fresh medium and lymphokines every 2–3 days and incubated at 37°C for 6–7 days.

Flow Cytometry. Fluorescence-activated cell sorter analysis was performed as described previously (15). The anti-H-2D b -biotin monoclonal antibody was purchased from PharMingen (San Diego, CA).

Tumor Peptide Preparation. Peptides were isolated from tumor cell lines as described (16). Briefly, up to 108 cells were washed three times in PBS (Life Technologies).
Technologies, Inc.) and incubated with 10 ml of acid buffer (0.131 m citrate, 0.066 m Na₂HPO₄, pH 3) for 1 min. Adherent cell lines (LL-LCMV and B16.F10(GP33)) were trypsinized and washed prior to the elution. Cell suspensions were then pelleted, and the supernatants were clarified by centrifugation at 2000 rpm for 5 min. The peptides in citrate-phosphate buffer were concentrated on activated SepPack C₁₈ cartridges (Waters, Milford, MA). The cartridges were prewashed with 5 ml of acetonitrile and then with 5 ml of double-distilled H₂O. Acid extracts were then loaded and allowed to gravitate through the cartridges. After washing with 3 ml of double-distilled H₂O, the bound material was eluted in 5 ml of 60% acetonitrile. This material was subsequently lyophilized in a Speed Vac evaporator-concentrator (Savant).

RESULTS

Experimental Model. We used as a source of antigen three cell lines stably transfected to express the LCMV glycoprotein peptide LCMV₃₃–₄₁ presented in association with H-2Dᵇ. The expression of H-2Dᵇ on these transfectants and their parental cell lines was assessed by fluorescence-activated cell sorter analysis. All cell lines were analyzed in the same experiment and using the same instrument settings. Fig. 1 shows that expression of H-2Dᵇ on EL-LCMV and LL-LCMV cells is similar, whereas B16.F10(GP33) express very low levels of H-2Dᵇ. This is consistent with what was reported previously for the parental B16.F10 cell line (18). An LCMV₃₃–₄₁-specific proliferation assay (Fig. 2A) showed that EL-LCMV and B16.F10(GP33) cells stimulated similar proliferation of LCMV₃₃–₄₁-specific T cells, whereas LL-LCMV cells had weaker stimulatory capacity. These differences in stimulatory capacity were most likely attributable to differences in presentation of LCMV₃₃–₄₁, because the assay was carried out in the presence of IL-2 to minimize the effect of costimulatory molecules that might be differentially expressed by the tumor cell lines. A similar conclusion was also suggested by the results of the cytotoxicity test shown in Fig. 2B. B16.F10(GP33) cell were lysed by LCMV₃₃–₄₁-specific CTLs much more efficiently than LL-LCMV tumor cells, suggesting a higher presentation of specific antigen on B16.F10(GP33) cells. EL-LCMV cells were not tested in this assay.

Therefore, we had available a cell line, LL-LCMV, expressing good levels of H-2Dᵇ but presenting small amounts of LCMV₃₃–₄₁ peptide; a cell line, EL-LCMV, expressing similar levels of H-2Dᵇ compared with LL-LCMV cells but presenting higher levels of LCMV₃₃–₄₁; and a cell line, B16.F10(GP33), with very low expression of MHc class I molecules but with a very high relative density of LCMV₃₃–₄₁ presented on H-2Dᵇ.

In Vitro LCMV₃₃–₄₁-specific Bioassays Fail to Detect Eluted LCMV₃₃–₄₁ Peptide. We wished to establish whether the acid elution approach was effective in recovering MHc class I-associated peptide, even when expression of class I is low (B16.F10(GP33)) or when the amount of tumor peptide presented on the tumor cell is relatively small (LL-LCMV). The EL-LCMV cell line provided an optimal model for this type of antigen isolation, having both high
levels of H-2Db and a good ability to stimulate LCMV_{33–41} peptide-specific responses, whereas the B16.F10_{GP33} and the LL-LCMV cell lines would represent more physiological systems.

Peptide mixtures eluted from the LCMV_{33–41}-transfected cell lines were tested for their ability to stimulate proliferation and cytotoxic activity of LCMV_{33–41}-specific T cells in vitro. These assays were chosen as the most sensitive, because T cells can respond even to very few antigen molecules presented on a cell (19). However, in both LCMV_{33–41}-specific proliferation and cytotoxicity assays (Fig. 3), the peptide preparations obtained from B16.F10_{GP33}, EL-LCMV, and LL-LCMV cells were unable to elicit a response. This is despite the presence of considerable amounts of MHC class I-derived peptides in the preparations used, as shown by their ability to induce the renaturation of surface MHC class I on the microcell line RMA-S (data not shown). This observation raised the question of whether LCMV_{33–41}-specific bioassays are sensitive enough to detect the small amounts of LCMV_{33–41} peptide that can be theoretically isolated from the transfected cell lines.

This question was addressed by mixing peptides acid eluted from the nontransfected cell line EL4 with a small amount of LCMV_{33–41} peptide (0.1 ng). This peptide concentration could simulate in excess the quantity of peptide normally bound on MHC class I molecules (10^{-5}–10^{-6} /cell) on the number of cells (10^8) that are normally used for peptide harvesting. In this calculation, we assumed that ~1000 different peptides can be presented by class I molecules on a given cell (20). The resulting peptide mixture was then assayed in a JAM test for its ability to sensitize target cells to killing by LCMV_{33–41}-specific CTLs, but no LCMV_{33–41} specific killing could be demonstrated (data not shown). The same peptide mixture was also tested in an LCMV_{33–41}-specific proliferation assay using BMDCs as stimulator cells, but again no LCMV_{33–41}-specific proliferation was detected (data not shown). We conclude that our in vitro tests are not sensitive enough to detect the very small amount of LCMV_{33–41} peptide that could be eluted from the transfected cell lines.

Irrelevant MHC Class I Binding Peptides Dilute or Inhibit the T-Cell Stimulatory Activity of LCMV_{33–41} Peptide in Vitro. We then addressed the question of whether other MHC class I-binding peptides coisolated in the acid elution process could affect the stimulatory ability of LCMV_{33–41}. Decreasing amounts of LCMV_{33–41} peptide were mixed with irrelevant peptides isolated from the nontransfected EL4 cell line and tested in a LCMV_{33–41}-specific proliferation assay (Fig. 4). At high concentrations of LCMV_{33–41} peptide, the presence of irrelevant peptides did not affect the response to synthetic peptide, whereas at limiting concentrations of LCMV_{33–41} peptide, the proliferative response was inhibited strongly. Thus, the excess of irrelevant peptides present in the mixtures isolated from tumor cells can exert an inhibitory effect on poorly represented tumor epitopes in vitro.

In Vivo Tumor Protection Using Unfractionated Tumor Peptides. We tested the ability of peptides isolated from the LL-LCMV, EL-LCMV, and B16.F10_{GP33} tumor cell lines to elicit an in vivo antitumor immune response when used in combination with BMDCs. We used the LL-LCMV and B16.F10_{GP33} tumor cell lines for the in vivo challenge, as they respectively present low amounts of model tumor antigen and express low levels of MHC class I molecules, and thus may be considered more physiological models of tumor antigenicity.

In the case of LL-LCMV, the most effective protection was observed after immunization with relatively high amounts of LCMV_{33–41} synthetic peptide on BMDCs (10 ^6, Fig. 5A and 1 µM, Fig. 5D). Protective tumor immunity was also observed after immunization with BMDC- and LL-LCMV-derived peptides (Fig. 5, A and B). The effect was reproducible and significant over several experiments (Fig. 5C) but did not induce complete protection because all
treated mice eventually developed tumors (Fig. 5B). Peptides derived from the two unrelated tumor cell lines EL-LCMV and B16.F10<sub>GP33</sub>, which presumably only share the model LCMV<sub>33–41</sub> tumor peptide with the LL-LCMV cell line, were less effective at inducing antilLM-LCMV immune responses. Peptides from EL-LCMV were essentially inactive (Fig. 5, A and B). Peptides from B16.F10<sub>GP33</sub> had a small effect in delaying the onset of LL-LCMV tumor growth (Fig. 5, D and E). The effect appeared to be attributable to the expression of LCMV<sub>33–41</sub> because peptides from the nontransfected B16.F10 line were completely ineffective.

A similar pattern was observed when the B16.F10<sub>GP33</sub> tumor cell line was used for in vivo challenge. Synthetic peptide and BMDCs were the most effective form of immunization, although lower amounts of peptide were necessary for optimal tumor protection against B16.F10<sub>GP33</sub> as compared with LL-LCMV (100 nM versus 1–10 μM). Mice receiving BMDCs and peptides derived from B16.F10<sub>GP33</sub> showed delayed tumor growth as compared with controls (Fig. 6). Again, peptides from the EL-LCMV tumor cell line were completely ineffective.

We conclude that multiple epitopes are required for the induction of antitumor immunity by immunization with BMDCs and peptides eluted from the surface of tumor cells. Individual epitopes are rarely sufficient for the induction of antitumor immune responses.

**DISCUSSION**

The aim of our study was to define how the levels of tumor peptide antigen presented on a tumor cell can influence the induction of an antitumor T-cell response in vivo. We used three transfected tumor cell lines that express the same model tumor antigen, LCMV<sub>33–41</sub>, in different amounts and proportions. The B16.F10<sub>GP33</sub> melanoma cells have a very low expression of surface MHC class I molecules (18) and Fig. 1 but at the same time are potent stimulators of LCMV<sub>33–41</sub>-specific, TCR transgenic T cells (Fig. 2A) and are efficiently lysed by specific CTLs (Fig. 2B). These data suggest that the LCMV<sub>33–41</sub> peptide probably occupies a good proportion of the available MHC class I molecules on these cells. In contrast, the EL-LCMV and LL-LCMV cells express much higher levels of MHC class I than B16.F10<sub>GP33</sub> cells (Fig. 1), but the relative density of LCMV<sub>33–41</sub> on their surface is probably much lower, because EL-LCMV and LL-LCMV have respectively similar or weaker abilities to stimulate LCMV<sub>33–41</sub>-specific T cells as compared with B16.F10<sub>GP33</sub> (Fig. 2A). Cytotoxicity data showing that the LL-LCMV cell line is lysed by LCMV<sub>33–41</sub>-specific CTLs much less effectively than the B16.F10<sub>GP33</sub> cell line (Fig. 2B) also supports this.

Regardless of the different expression of the LCMV<sub>33–41</sub> model
tumor antigen on these tumor cell lines, we were unable to demonstrate the presence of LCMV$_{33-41}$ in the material eluted from each of the transfected tumor cell lines by using either an antigen-specific in vitro T-cell proliferation assay or an in vitro cytotoxicity assay (Fig. 3). Assays using T-cell responses as readouts would be expected to be especially sensitive, because T cells require only a few antigen molecules/cell to express functional activity (19). However, no activity could be demonstrated in those assays. Biochemical analyses such as high-performance liquid chromatography and mass spectrometry also failed to reveal the presence of the LCMV$_{33-41}$ peptide in mixtures from the EL-ELCMV tumor cell line. The inability to demonstrate the presence of LCMV$_{33-41}$ contrasts with results reported by another group, where unfractionated peptides derived from OVA-expressing tumor cells were as effective as the purified OVA peptide at inducing primary CTL responses in vitro (1). This difference may imply that our tumor models more closely simulate naturally occurring tumors, where MHC class I expression is often limited and expression of tumor antigens can be very low (21).

One might assume that the problem of low representation of tumor peptide on the tumor cell could be resolved by using larger numbers of cells as a source of antigen. However, this is not always possible when dealing with ex vivo tumor tissue that may only be available in limited amounts. Furthermore, irrelevant peptides in the peptide mixture may inhibit or dilute the effect of the tumor-specific peptides. Results from in vitro experiments suggest that recognition of synthetic LCMV$_{33-41}$ peptide was inhibited by the presence of other peptides when the concentration of the relevant peptide was limited (Fig. 4). A similar process could be operating in vivo, although we could not test this possibility directly, because the concentration of LCMV$_{33-41}$ at which inhibition by irrelevant peptide has been observed does not induce tumor protection (data not shown). The mechanisms of inhibition may be multiple and may be accounted for by both competition for binding to MHC class I molecules and inhibition of TCR recognition and/or signaling. Using DCs as antigen carriers possibly introduces a further limitation, because the antigen uptake by these cells may be saturable and possibly selective in the uptake of subsets of antigens. On the other hand, the use of BMDCs as carriers possibly abrogates dominance features among antigens (5), and therefore antigens that were subdominant when expressed on tumor cells may become dominant epitopes when presented by DCs.

Our in vivo tumor protection results suggest that the efficacy of vaccination using unfractionated tumor peptides is directly dependent on the relative density of tumor-specific antigen presented on tumor cells. Good tumor protection was observed when mice were immunized with the synthetic tumor antigen LCMV$_{33-41}$, and some degree of antitumor immune response was also achieved using peptides obtained from the same cell line used to challenge mice (Figs. 5 and 6). Therefore, an effective vaccine seems to require either a relatively high amount of a single purified tumor antigen or a mixture of multiple peptide epitopes. Interestingly, vaccination with DCs pulsed with peptides isolated from the B16.F10$\_{GP33}$ cell line delayed the onset of LL-ELCMV tumors, whereas vaccination with EL-ELCMV peptides did not have any effect on tumor growth (Fig. 5). The protective effect was attributable to LCMV$_{33-41}$ in the peptide mixture and not to other unrelated peptides, because peptide preparations from the nontransfected B16.F10 cell line were completely ineffective. B16.F10$\_{GP33}$ and EL-ELCMV appear to present approximately similar levels of LCMV$_{33-41}$, because they are equally able to induce the proliferation of specific T cells in vitro, but their expression of MHC class I molecules differs significantly. The most likely reason for the different effectiveness of the two peptide preparations may be the relative abundance of the LCMV$_{33-41}$ peptide, which is likely to be proportionally less represented in the mixtures from EL-ELCMV cells because the expression of H-2D$^\alpha$ is higher on this cell line. Thus, it is not only the absolute amount of antigen presented on the tumor cell but also its relative concentration that determines the final effect of vaccination.

The amount of antigen presented on the tumor cell was also critical in determining the efficacy of the immune response at protecting against tumor challenge, as reflected by the amount of LCMV$_{33-41}$ peptide needed to pulse DCs to achieve tumor protection. An optimal antitumor immune response against the LL-ELCMV tumor, which presents low levels of the transfected tumor antigen LCMV$_{33-41}$, required DCs to be pulsed with at least 10 nM synthetic peptide (Fig. 5). On the other hand, vaccination with DCs pulsed with 100 nM synthetic peptide conferred maximum protection against tumor challenge with the B16.F10$\_{GP33}$ melanoma, which presents a much higher density of the LCMV$_{33-41}$ peptide (Fig. 6). Protection from challenge with the two tumor cell lines involves similar effector cell mechanisms, suggesting that the peptide concentration does not result in the generation of qualitatively different effector immune responses. Rather, this difference may be attributable to the necessity to prime a more vigorous T-cell response, if the amount of specific antigen on the target tumor cell is limited.

We conclude from these studies that MHC class I-associated tumor peptides can be obtained directly from tumor cells for use in tumor immunotherapy. However, the relative proportion of tumor peptides presented on the tumor cell is an especially critical factor, because

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4. R. Kemp and F. Ronchese, unpublished observations.
tumor cells are first the source of antigen and later the targets of the \textit{in vivo} immune response.

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