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

Pisana Rawson, Ian F. Hermans, Stephanie P. Huck, Joanna M. Roberts, Hanspeter Pircher, and Franca Ronchese

Malaghan Institute of Medical Research, Wellington School of Medicine, Wellington South, New Zealand [P. R., I. F. H., S. P. H., J. M. R., F. R.], and Department of Immunology, Institute of Medical Microbiology and Hygiene, University of Freiburg, Freiburg, Germany [H. P.]

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 immortalizing 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

Received 12/6/99; accepted 6/16/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by research grants from the University of Otago, the Health Research Council of New Zealand, and the Wellington Division of the Cancer Society.

2 To whom requests for reprints should be addressed, at Malaghan Institute of Medical Research, P. O. Box 7060, Wellington, New Zealand. Phone: 64-4-385-5096; Fax: 64-4-389-5095; E-mail: fronchese@malaghan.org.nz.

The abbreviations used are: BMDC, bone marrow-derived dendritic cell; CM, complete medium; DC, dendritic cell; LCMV, lymphocytic choriomeningitis virus; IL, interleukin.

4 The abbreviations used are: BMDC, bone marrow-derived dendritic cell; CM, complete medium; DC, dendritic cell; LCMV, lymphocytic choriomeningitis virus; IL, interleukin.
Activated SepPack C18 cartridges (Waters, Milford, MA) were used to purify cell lines. The cells were pelleted, and the supernatants were clarified by centrifugation. 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).

LCMV 33–41-specific Proliferation Assays. The proliferative response of LCMV 33–41-specific T cells to DCs pulsed with tumor peptide extracts or with synthetic LCMV 33–41 peptide was determined by incubating 2 × 10^6 spleen cells from “318” mice with serially diluted DCs that had been pulsed with antigen for 3 h at 37°C. Proliferative responses to tumor cell lines were determined by incubating 5 × 10^5 “318” spleen cells with serially diluted mitomycin C-treated tumor cells in the presence of 10 units/ml of IL-2 as described (13).

Cytotoxicity Assay. Spleen cells from “318” mice were cultured for 5 days in 24-well plates (Falcon) in CM in the presence of 0.01 μM LCMV 33–41 peptide and 20 units/ml of IL-2 and fed every second day with CM and IL-2. At day 5, viable cells were washed, counted, and assayed for CTL activity by the JAM test (17) as described (13). In some experiments, a ^51Cr-release assay was carried out as described (12).

In Vivo Tumor Protection. DCs (10^6 cells/ml) cultured for 6 days were incubated overnight at 37°C in CM in the presence of 20 ng/ml IL-4 and granulocyte/macrophage-colony stimulating factor and either 10 μM LCMV 33–41 peptide or tumor peptide extracts. DCs were washed three times, resuspended at 10^6 cells/ml in Iscove’s modified Dulbecco’s medium, and used for immunization. Groups of 57BL/6 mice (n = 5) were immunized s.c. into the right flank with 10^6 peptide-loaded or control DCs on days 0 and 7. After 7 days from the last immunization, all animals were challenged with 2 × 10^5 LL-LCMV or B16.F10 GP33 tumor cells, injected s.c. in the opposite flank. Mice were monitored every 3–4 days, and tumor size for each group was calculated as the mean of the products of bisecting tumor diameters as described. Measurements were terminated when the tumor size of one mouse in each group reached 200 mm^2 or whenever animals were visibly unwell. All experiments were approved by the Wellington School of Medicine Animal Ethics Committee and performed in accordance with the University of Otago guidelines.

RESULTS

Experimental Model. We used as a source of antigen three cell lines stably transfected to express the LCMV glycoprotein peptide LCMV 33–41, presented in association with H-2D^d. The expression of H-2D^d 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-2D^d on EL-LCMV and LL-LCMV cells is similar, whereas B16.F10GP33 express very low levels of H-2D^d. This is consistent with what was reported previously for the parental B16.F10 cell line (18). An LCMV 33–41-specific proliferation assay (Fig. 2A) showed that EL-LCMV and B16.F10GP33 cells stimulated similar proliferation of LCMV 33–41-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 LCMV 33–41, 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.F10GP33 cells were lysed by LCMV 33–41-specific CTLs much more efficiently than LL-LCMV tumor cells, suggesting a higher presentation of specific antigen on B16.F10GP33 cells. EL-LCMV cells were not tested in this assay.

Therefore, we had available a cell line, LL-LCMV, expressing good levels of H-2D^d but presenting small amounts of LCMV 33–41 peptide; a cell line, EL-LCMV, expressing similar levels of H-2D^d compared with LL-LCMV cells but presenting higher levels of LCMV 33–41; and a cell line, B16.F10GP33, with very low expression of HMC class I molecules but with a very high relative density of LCMV 33–41 peptide, even when expression of class I is low (B16.F10GP33) 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

![Fig. 1. Expression of MHC class I on parental and transfected tumor cell lines. EL4 (A), EL-LCMV (B), LLTC (C), LL-LCMV (D), B16.F10 (E), and B16.F10GP33 (F) cells were stained with FITC-labeled anti-H-2D^d antibody and analyzed by flow cytometry. Filled curves, nonstained cells; open curves, anti-H-2D^d stained cells. Mean fluorescence intensity values for each staining are shown. All stainings were carried out in the same experiment and analyzed using the same instrument settings and thus are comparable with each other.](image)
levels of H-2D$^b$ and a good ability to stimulate LCMV$_{33-41}$-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 cytolytic 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 mutant cell 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 ($10^9$) 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 effect cells, 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 $\mu$M, Fig. 5A and 1 $\mu$M, 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. 5). Peptides derived from the two unrelated tumor cell lines EL-LCMV and B16.F10 GP33, which presumably only share the model LCMV 33–41 tumor peptide with the LL-LCMV cell line, were less effective at inducing anti-LL-LCMV immune responses. Peptides from EL-LCMV were essentially inactive (Fig. 5, A and B). Peptides from B16.F10 GP33 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 33–41 because peptides from the nontransfected B16.F10 line were completely ineffective.

A similar pattern was observed when the B16.F10 GP33 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 GP33 as compared with LL-LCMV (100 nM versus 1–10 μM). Mice receiving BMDCs and peptides derived from B16.F10 GP33 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 33–41, in different amounts and proportions. The B16.F10 GP33 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 33–41-specific, TCR transgenic T cells (Fig. 2 A) and are efficiently lysed by specific CTLs (Fig. 2 B). These data suggest that the LCMV 33–41 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 GP33 cells (Fig. 1), but the relative density of LCMV 33–41 on their surface is probably much lower, because EL-LCMV and LL-LCMV have respectively similar or weaker abilities to stimulate LCMV 33–41-specific T cells as compared with B16.F10 GP33 (Fig. 2 A).

Cytotoxicity data showing that the LL-LCMV cell line is lysed by LCMV 33–41-specific CTLs much less effectively than the B16.F10 GP33 cell line (Fig. 2 B) also supports this.

Regardless of the different expression of the LCMV 33–41 model...
tumor antigen on these tumor cell lines, we were unable to demonstrate the presence of LCMV33–41 in the material eluted from each of the transected 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 LCMV33–41 peptide in mixtures from the EL-LCMV tumor cell line. The inability to demonstrate the presence of LCMV33–41 contrasts with results reported by another group, where unfractionated peptides derived from OVA-expressing tumor cells were as effective as the purified OVA peptide in 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 LCMV33–41 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 LCMV33–41 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 LCMV33–41, 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.F10GP33 cell line delayed the onset of LL-LCMV tumors, whereas vaccination with EL-LCMV peptides did not have any effect on tumor growth (Fig. 5). The protective effect was attributable to LCMV33–41 in the peptide mixture and not to other unrelated peptides, because peptide preparations from the nontransfected B16.F10 cell line were completely ineffective. B16.F10GP33 and EL-LCMV appear to present approximately similar levels of LCMV33–41, 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 LCMV33–41 peptide, which is likely to be proportionally less represented in the mixtures from EL-LCMV cells because the expression of H-2D^d 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 LCMV33–41 peptide needed to pulse DCs to achieve tumor protection. An optimal antitumor immune response against the LL-LCMV tumor, which presents low levels of the transfected tumor antigen LCMV33–41, 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.F10GP33 melanoma, which presents a much higher density of the LCMV33–41 peptide (Fig. 6). Protection from challenge with the two tumor cell lines involves similar effector cell mechanisms, suggesting that the different peptide concentrations do 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
tumor cells are first the source of antigen and later the targets of the \textit{in vivo} immune response.

**ACKNOWLEDGMENTS**

We acknowledge the personnel of the Biomedical Research Unit of the Wellington School of Medicine for animal husbandry and care.

**REFERENCES**


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

Pisana Rawson, Ian F. Hermans, Stephanie P. Huck, et al.

*Cancer Res* 2000;60:4493-4498.

Updated version

Access the most recent version of this article at:

http://cancerres.aacrjournals.org/content/60/16/4493

Cited articles

This article cites 20 articles, 9 of which you can access for free at:

http://cancerres.aacrjournals.org/content/60/16/4493.full.html#ref-list-1

Citing articles

This article has been cited by 2 HighWire-hosted articles. Access the articles at:

/content/60/16/4493.full.html#related-urls

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.