Surrogate Markers of Antitumor Responses: *In Vitro* Activation of T Cells by Autologous Tumor Peptides

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Abstract

The increasing ability to augment antitumor immunity in model systems has led to increased numbers of clinical trials. However, progress in detecting immune responses by patients against autologous tumors has been slow. Although a considerable number of tumor antigens, as well as peptides derived from them, and the MHC determinants together with which they are presented have been identified for melanoma, this is not so for the majority of solid tumors. Furthermore, tumor cells themselves are poor stimulators of immunity. Thus, approaches that do not depend upon defined antigens or using tumor cells as stimulators would be desirable. To attempt to measure immune responses in these situations, we tested whether total peptides, prepared from autologous tumor tissue, stimulated cytokine release by T cells. Peripheral blood mononuclear cells (PBMCs) were mixed with antigen-presenting cells (APCs), pulsed with tumor peptides, and tested in the ELISPOT assay for IFN-γ secretion. Few spots were obtained when PBMCs were cultured with unpulsed APCs or in wells with peptide-pulsed APC alone. In contrast, a strong response was seen when PBMCs were cultured with APCs that had been pulsed with autologous total tumor peptides. This system should help to identify those immunotherapeutic approaches that induce responses against tumor cells *in vivo*. Because different cytokine profiles are associated with distinct arms of the immune response, testing in the ELISPOT assay may also help us understand the mechanisms responsible.

Introduction

Given the increased rapidity with which findings from the research laboratory are translated into clinical trials, the necessity of determining which have efficacy in patients has grown in importance. Nowhere is this situation more compelling than in the field of cancer immunotherapy, because cancer is a disease that often does not respond to conventional treatment. On the basis of theoretical considerations of which type of antitumor immunity would be most useful for tumor cell killing, results in preclinical models and a large number of protocols are directed at eliciting or augmenting immunity mediated by T cells. Two characteristics of T cells that explain this are their antigenic specificity and their memory. However, a specific antigen is required to activate naive and memory T cells and to monitor their functional ability. This presents a problem with most solid tumor cells, because, with the exception of melanoma (1), few tumor-associated antigens have been identified. Other impediments to examining antitumor responses in patients are limitations in the source and amount of lymphocytes that can be obtained for testing, the poor antigen-presenting ability of most solid tumor cells, and the lack of *in vitro* assays that reflect events occurring *in vivo*.

As part of a developing immunotherapy program, we considered a number of approaches to circumvent these problems. The ELISPOT assay was chosen as the readout for several reasons (2, 3). It detects cytokine secretion on the single-cell level, can measure tumor-specific T-cell responses in peripheral blood, can be carried out with small numbers of cells, can be carried out with antigen in the form of synthetic peptides or cell lysates, requires only overnight culture of responder and stimulator cells, and allows the estimation of the frequency of tumor-specific T cells before and after immunotherapy (4–10).

As a source of tumor antigen, we were committed to using the patient's autologous tumor. Because the chances of establishing continuous cell lines from fresh tumor cells were low, we decided to test whether other forms of autologous antigen could serve as antigen. Our first choice was autologous total tumor peptides, because they could be obtained from a large number of patients and would maximize the chance of using tumor antigens that were actually expressed by the patient's tumor. Moreover, tumor peptides prepared in this manner are being considered as immunogens *in vivo*, because they have been shown able to cause regression of murine tumors *in vivo* (11) and have been shown able to induce CTL responses in a mouse tumor model (12). In this report, we show that peptides isolated from fresh human cells can serve as a source of antigen to measure T cell-mediated immunity.

Materials and Methods

Peptide Isolation. Peptides were isolated as described previously (13). Briefly, fresh colon tumors metastatic to the liver were minced into small fragments and resuspended in a mixture containing NP40 and protease inhibitors. The fragments were homogenized until smooth, stirred for 30 min at 4°C, and brought to a pH of 2–3 with trifluoroacetic acid. The slurry was stirred 1 h and centrifuged at 4°C to bring down large pieces. The supernatant was then spun at 100,000 × g for 30 min at 4°C. After centrifugation, the clear supernatant was concen-

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trated on a Centriprep filter (Amicon) with a molecular weight cutoff of M_r 3000. Small peptides that passed through the filter were collected, dissolved in sterile double-distilled water, and lyophilized three times. Peptide yield was based upon absorbance at A_{230}. In early experiments to establish the system, peptides were isolated from autologous LCLs[2] following the same method.

ELISPOT Assays. Ninety-six-well polyvinylidene difluoride-lined plates (Millipore, Bedford, MA) were coated with 100 μl of anti-IFN-γ monoclonal antibody (15 μg/ml; MabTech, Nacka, Sweden) overnight at 4°C. The plates were washed six times with RPMI 1640 and then blocked for 30 min at 37°C with RPMI 1640 supplemented with glutamine (Life Technologies, Inc.), penicillin, and streptomycin (Life Technologies, Inc.), and 10% pooled human AB serum (Vital Products, St. Louis, MO). All studies were done with PBMC responders that had been cryopreserved previously in 10% DMSO. These cells have been reported to function similarly to fresh cells in the ELISPOT (14, 15). The cells were diluted to 1–2 × 10^6/ml in medium containing 20% human serum, and 100 μl/well of the cell suspension were distributed into triplicate wells. Autologous PBMCs served as APCs. They were diluted to 1-2 × 10^6/ml in serum-free medium containing varying amounts of tumor-derived peptide. After 1 h of incubation at room temperature, the pulsed APCs were centrifuged and resuspended in complete RPMI 1640 containing 10% serum, irradiated (1000 rads), and distributed. Negative controls were responders alone and/or responders and unpulsed APCs. Cultures were incubated for 24–36 h at 37°C in 5% CO_2, after which the cells were washed out with PBS. Biotinylated anti-IFN-γ (1 μg/ml) was added, and the plate was incubated 3 h at room temperature. Wells were washed six times with PBS, and a 1:1000 dilution of streptavidin alkaline phosphatase conjugate (Bio-Rad, Hercules, CA) diluted in PBS was added. The plate was incubated an additional 2 h at room temperature and again washed six times with PBS. After the addition of 100 μl of chromogenic alkaline phosphatase substrate (Bio-Rad), diluted 1:25 with deionized water, and a 30-min incubation at room temperature, the plate was washed with tap water to terminate the reaction. The plate was air dried, and spots were counted under an inverted microscope at ×10–×30.

Statistical Analysis. Groups were compared by the two-sample *t* test for independent samples (unpaired *t* test) with equal or unequal variances. All tests were two sided.

Results

Total Cellular Peptides from Fresh Cells Stimulate T Cells in an Antigen-specific, Dose-dependent Fashion. We were interested in developing an approach that would allow the measurement of T-cell responses by cancer patients to autologous tumor antigens. The ELISPOT assay was selected as the assay for several reasons, among them that it detects antigen-specific responses and can be carried out with small numbers of cells. The source of antigen with which to stimulate T cells was

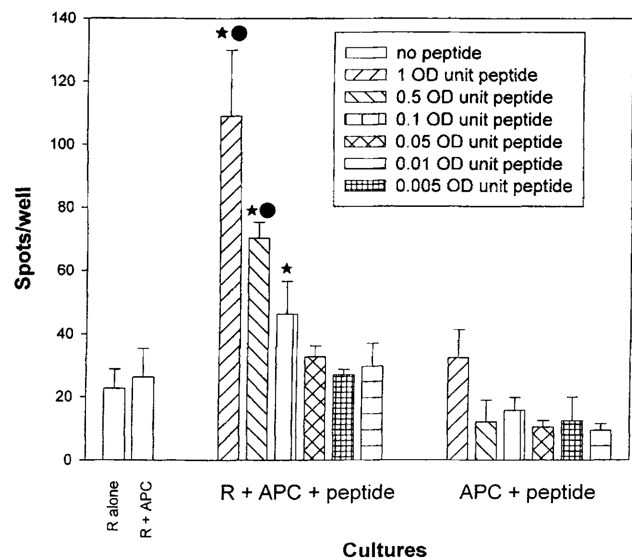

Fig. 1 Secretion of IFN-γ against total cellular peptides is dose dependent. APCs (1 × 10^5) were pulsed with 0.005–1 absorbance unit/10^6 APCs of total cellular peptides and mixed with autologous PBMCs. They were distributed into ELISPOT plates coated with anti-IFN-γ antibodies. Controls included responders alone, responders with unpulsed APCs, and APCs pulsed with peptide, in the absence of responders. After 24 h, the reaction was developed, as described in "Materials and Methods," and spots were counted. Mean values of triplicate wells are shown; *bars,* SD. ★, significantly higher than *R* alone at $P < 0.05$ by the two-tailed unpaired *t* test. ●, significantly higher than *R* + APCs at $P < 0.05$ by the two-tailed unpaired *t* test.

a greater challenge. Tumor cells themselves stimulate T cells poorly. Because the form of antigen recognized by T cells is peptides plus MHC class I or class II, we tested whether professional APCs pulsed with total cellular peptides isolated from fresh cells would stimulate T cells in the ELISPOT assay. Because LCLs express viral antigens as a consequence of their transformation with EBV virus and are potent stimulators of proliferation by autologous T cells, peptides were isolated from LCLs. APCs were pulsed with various amounts of peptides and mixed with autologous PBMCs in ELISPOT plates coated with anti-IFN-γ antibodies. Controls included responders alone, responders plus unpulsed APCs, and APC pulsed with peptides, in the absence of responders. We observed the greatest number of cells that secreted IFN-γ in wells containing responders, APCs, and peptides (Fig. 1). As expected, if the response were dose dependent, as the peptides were diluted out, the response decreased. These results suggested that a heterogeneous mixture of peptides isolated from fresh cells could bind to MHC molecules and activate autologous T cells.

Cytokine Secretion by Patients' T Cells Is Induced by Stimulation with Total Tumor Peptides from Autologous Tumor Tissue. Because for most solid tumors tumor antigens have not been identified, it is difficult to study tumor-specific immunity in patients. On the basis of the results in Fig. 1 showing that total tumor peptides from LCLs stimulated IFN-γ secretion in ELISPOT, we tested whether APCs pulsed with peptides isolated from fresh colon tumor could behave similarly. Autologous APCs, in the form of PBMCs, were pulsed with two

[2] The abbreviations used are: LCL, lymphoblastoid cell line; PBMC, peripheral blood mononuclear cell; APC, antigen-presenting cell.

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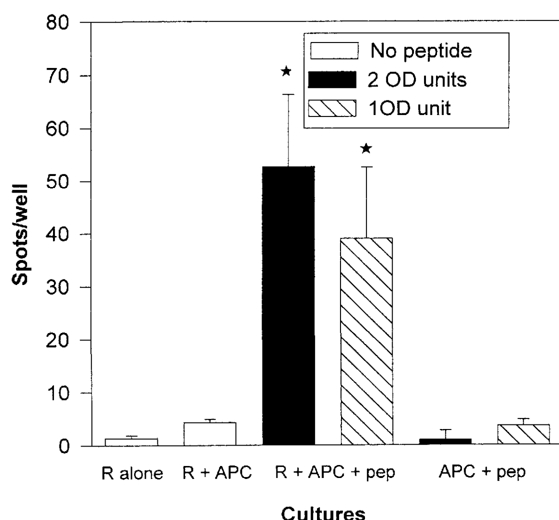

Fig. 2 Autologous total tumor peptides stimulate IFN-γ secretion in ELISPOT. APCs (1×10^5), pulsed with two doses of total tumor peptides isolated from fresh tumor cells, were used to stimulate PBMCs in an autologous ELISPOT system. Controls included responders alone, responders with unpulsed APCs, and APCs pulsed with peptide but in the absence of responders (*R*). The reaction was developed, as described in "Materials and Methods," after 36 h to give the cells additional time to respond to the heterogeneous preparation of peptides prepared from fresh the tumor. Spots were counted, and the mean values obtained from triplicate wells are shown; *bars,* SD. ★, R + APCs + peptide (*R + APC + pep*) significantly higher than *R alone, R + APC,* and APC + peptide (*APC + pep*) by the two-sided unpaired *t* test, $P < 0.05$. *OD,* absorbance.

and one absorbance units of total tumor peptides/10^6 cells from a colon tumor that had metastasized to the liver and distributed in ELISPOT plates coated with anti-IFN-γ, as described in "Materials and Methods." After 36 h, cells were washed out, and the reaction developed. A strong response was seen in wells containing responders cultured with APCs pulsed with total tumor peptides (Fig. 2). As with LCL-derived peptides, the response was dose dependent, because it was stronger when two absorbance units were used compared with one absorbance unit. Similar results have been observed in 6 of 32 patients tested.

Discussion

Considerable evidence supports the hypothesis that tumor-bearing hosts in model systems and a subset of solid tumor patients can respond to their tumors by generating specific T-cell immunity (13, 16, 17). Why this is sometimes insufficient for tumor rejection is unknown, but may relate to how the initial antitumor response evolves and/or whether it can be maintained. In addition to bearing immunogenic tumors, patients' must have immune systems able to mount an immune response and not be immunosuppressed (18–20). Immunotherapy approaches for cancer are designed either to augment insufficient levels of existing antitumor immunity or to induce immunity *de novo.* Although clinical success will await refinements of methods that show initial promise, a large number of approaches have been successful in mouse models, making it important to determine which will be effective in patients. Thus, the development of *in vitro* methods that provide data that correlate with *in vivo* antitumor immunity is very important. One problem in establishing *in vitro* assays is that for most solid tumors, tumor rejection antigens have not been identified and, as such, a source of defined antigen with which to stimulate patients' T cells does not exist. To circumvent this, we tested the ability of total peptides prepared from autologous tumor cells to stimulate T cells. PBMCs pulsed with total peptides from LCLs were effective at stimulating IFN-γ release in the ELISPOT assay in a dose-dependent fashion (Fig. 1). Similar results were reported by Herr *et al.* (21) recently. By fractionating naturally processed EBV-derived peptides isolated from autologous LCLs, the authors were able to identify and then sequence those able to induce memory $CD8^+$ T cells to secrete IFN-γ in the ELISPOT assay.

By extending our results to tumor tissue, it became clear that total peptides isolated from colon carcinoma cells behaved similarly (Fig. 2). The response had specificity, because patients' T cells that responded strongly to autologous peptides responded weakly to some allogeneic preparations of tumor peptides (13). Although this system does not permit identification of the epitopes recognized by the responding lymphocytes, this is not necessary to detect relative changes in patients' immunity over time. In fact, such studies would be prevented by strict limitations on the amount of tumor material that can be obtained from many patients.

Responses similar to those reported here have been seen in ~20% of colon cancer patients tested (13). Because cancer patients are often immunosuppressed at diagnosis (18–20) and the patients were not preselected for immunocompetence, these numbers may be an underrepresentation of the number of patients who make immune responses to autologous tumors. These results suggest that the approach described in this report may be useful to monitor immune responses by patients in immunotherapy trials. Although proliferation and cytotoxicity have more commonly been used, the ELISPOT assay is an especially attractive assay, because it can determine the frequency of T cells secreting cytokines, as well as whether they are T helper 1 or T helper 2 cytokines. Used together, these approaches might provide valuable information about responses to immunotherapy, enabling investigators to select among a variety of promising possibilities.

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