Induction of determinant spreading and of Th1 responses by in vitro stimulation with HER-2 peptides

Received: 10 April 2000 / Accepted: 12 July 2000

Abstract Immunization with tumor antigens induces cellular and humoral immune responses. These responses by T cells are specific for defined epitopes (determinants) in the molecule of the immunizing tumor antigen. Extension of such responses to self-antigens requires induction of autoimmunity to the tumor. As with systems of autoimmune disease, expression of T cell autoimmunity is characterized by diversification of responses from the inducer determinant to other responder (cryptic) determinants. Since similar strategies may be useful for therapy of human cancers, we investigated whether the induction of response to a HER-2 peptide F7 (776–789) induces enhanced reactivity of other HER-2 peptides. We found that stimulation with F7 can expand a response to another epitope F13 (884–899) in both an ovarian cancer patient with progressive disease and a healthy donor who shared HLA-DR11. This response was characterized mainly by increased interferon γ secretion, and proliferation, but was not observed with another donor who shared HLA-DR14 and HLA-DQ5 with the patient. Since repeated vaccination with the same epitope may lead to a decline of primary cell reactivity caused by apoptosis spreading the response to other epitopes, the tumor antigen may provide an approach for maintaining an inflammatory Th1 response during cancer vaccination.

Key words HER-2 · Proliferation · Th1 · Cryptic determinants

Introduction

Studies during recent years have identified tumor antigens that are targets of tumor-reactive cytotoxic T lymphocytes (CTL; reviewed by Boon and van der Bruggen [2]). These antigens are self-proteins that are capable of inducing a cellular response (mediated by CD8+ or CD4+ cells) and/or a humoral response. Recent experimental findings support the concept that CTL and CD4+ cells recognizing tumor antigens can mediate tumor regression and justify the design and development of epitope-directed cancer vaccines [14, 17]. For all tumor antigens the major issue that needs to be resolved is how to generate and optimize an immune response to the tumor. Although both CTL and helper T cells have been identified that recognize peptide epitopes from proteins such as HER-2, gp100, MART-1, p53, etc. [21] and could be easily expanded in vitro [12], the disease progressed in patients, suggesting that either the detected response is too weak to control cancer spread, or the response to tumor is silenced.

Repeated stimulation with the same CTL epitope enhances antitumor CTL expansion with slow kinetics [1, 9]. Since the tumor environment is either tolerogenic or suppressive, CTL induction and expansion depend in many instances on exogenous cytokine help to promote CTL survival and maintain an inflammatory environment. Because of the short half-life of cytokines and side-effects, longer-lasting help can be also provided by
CD4+ cells when they are activated by the appropriate antigen. However, repeated vaccinations with the same CD4+ helper epitope are accompanied by a decline of primary T cell reactivity over time, suggesting that reactive T cells may be eliminated by apoptosis [19]. Thus, an alternative approach to repeated exogenous cytokine administration and repeated CD4+ epitope application is to amplify the Th1 response by spreading [7, 8] it to other epitopes that are endowed with Th1-cytokine-secretory ability. Intramolecular epitope spreading under these circumstances should be more beneficial for an antitumor response than intermolecular spreading because it activates responders to the tumor antigen of interest rather than to irrelevant antigens, which may not be present at that time. This approach requires first the identification of both the inducer and the amplified epitope. To address these questions we reasoned that, if the responses of a cancer patient to a number of HER-2 epitopes are detectable at primary stimulation during the disease-free period, this will indicate an in vivo priming event by an epitope from HER-2 that is presented by antigen-presenting cells (APC). The decline in the responses to some epitopes during disease progression may point to epitopes that are no longer being presented, as well as to a growing tolerance by the existent responders. Analysis of the ability of these epitopes to induce diversification of responses to other epitopes, with concomitant enhancement of type 1 cytokine secretion, may define a stimulation sequence for inducing an inflammatory autoreactive response. We found that priming peripheral blood mononuclear cells (PBMC) from an ovarian cancer patient with the HER-2 peptide F7(776–789) induced diversification of this response to the HER-2 epitope F13(884–899). The response to F7 in this patient was gradually lost during the stable disease period, while the response to F13, which was present during the same period, was lost when the disease progressed. Analysis of specificity and restriction of this response in MHC-I-matched donors indicated that it was associated with HLA-DR11. These results support the hypothesis that intramolecular determinant spreading can be induced by HER-2 peptides and can lead to induction of autoimmunity to cancer antigens. The pattern of spreading identified can provide a basis for epitope selection for cancer vaccine development.

**Materials and methods**

**PBMC**

Peripheral blood was collected from two healthy donors designated as donor 1 (HLA-A2, 23, B7.48, DR7, 11, DQ2.6) and donor 2 (HLA-A11, 68, B51, 67, DR13, 14, DQ5, 6), and from an ovarian cancer patient, designated as patient 1 (HLA-A24, 28, B35, W6, 70, CW3, 4, DR11, 14, DQ5). The HLA typing for the healthy donors was performed in the blood bank of the M.D. Anderson Cancer Center, while the typing of the patient was performed by molecular methods. PBMC were separated by Ficoll-Hypaque and used for stimulation immediately after separation.

**Antigens**

The eight HER-2 peptides, constructed by the Synthetic Antigen Laboratory of the M.D. Anderson Cancer Center, derived from the amino acid sequence of the human HER-2. These peptides have been previously selected on the basis of the computer program ANT.FIND.M., which was used for the prediction of candidate T cell epitopes on HER-2 protein [6]. The sequences of peptides used in this study are as follows: D122 (396–406): QLOVVFETLEET, F12 (449–465) GISWLGLRSLERGSGL; G88 (450–463): ISWGLLRSRLGS; F7 (776–789) GSVYSRLLGICL; G89 (777–790); SPYVSRLGICL; F13 (884–899) VPIKMALESILRRF; G90 (886–898); IKWMALESIRR and F14 (474–487); TVPWDQ LFR NPHQA. The residues that are potential HLA-DR11 anchors are in bold type. Because of the ability of peptides to bind on alternative registers and because of mutations in the MHC-class-II-binding pocket, motifs that predict specific binding to MHC class II are not yet well defined. Most of these HER-2 peptides contain a minimum of two of the three major anchors reported for the major HLA-DR types (i.e. HLA-DR4, -DR1, -DR11). A larger panel of HER-2 peptides was previously tested for the ability to induce proliferation of PBMC from randomly selected healthy donors and ovarian cancer patients [4]. Results of our previous study indicated that F7 and F13 induced proliferative responses in PBMC of healthy donors (57% and 62% respectively) and cancer patients (24% and 21% respectively) with higher frequency than did the other HER-2 peptides tested. In contrast, F12 was less frequently recognized (21% in healthy donors and 4% in cancer patients) [4], F7 and mainly its analog G89(777–790) induced proliferation of PBMC from a large number of HLA-DR4+ breast cancer patients [18]. The HER-2 intracellular and extracellular domains were gifts from Dr. Kenneth Grabstein, Corixa Corporation, and were prepared as described [20].

**T cell proliferation assays**

For characterization of T cell responses to HER-2 peptides, PBMC were plated into 24-well plates at a final concentration of 2 x 10^5/ml in complete RPMI medium supplemented with 5% human AB serum [4, 20]. Each peptide was added at a final concentration of 20 μg/ml. Equal volumes of cells were plated in quadruplicate in a 96-well plate 5 and 6 days later, incubated with 1 μCi tritiated thymidine ([3H]dT) (Amersham), and counted as previously described [4, 20]. Results are expressed as the stimulation index (SI) representing the ratio between the mean radioactivity of the cultured cells stimulated with peptide, and the mean radioactivity of the cultures that had not been stimulated with peptide (NP). For in vitro expansion of T cell cultures, 6 days after the primary stimulation, interleukin-2 (IL-2; Cetus) was added to each culture at a final concentration of 20 Cetus U/ml for 4–5 additional days. Afterwards, over the next 5 days, IL-2 was gradually removed from these cultures. For the last 48 h before restimulation, the lymphocytes were rested by being cultured in the absence of exogenous IL-2. Restimulation experiments were performed in same way as primary stimulation with the difference that APC were X-ray irradiated (100 Gy) autologous PBMC.

**Cytokine assays**

The ability of PBMC to release cytokines in response to antigen stimulation was determined by culturing PBMC either as unstimulated cells (NP) or stimulated with the corresponding peptides. Supernatants were collected after 48 h and stored frozen at −20 °C until assayed for cytokine levels. Interferon γ (IFNγ), IL-4 and IL-10 were measured by double sandwich enzyme-linked immuno- sorbent assay (ELISA) using the corresponding kits provided by Biosource International (Camarillo, Calif.). The assays were calibrated with human recombinant IFNγ, IL-4 and IL-10 to detect each cytokine in the range of 10–10,000 pg/ml.