MINIREVIEW

Monitoring of Antigen-Specific Cytolytic T Lymphocytes in Cancer Patients Receiving Immunotherapy

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Recent progress in molecular and immunologic approaches to discovery of tumor-associated antigens (TAA) in humans has resulted in the characterization of a number of new epitopes (3, 23). In most cases, the success of these efforts depended on the availability of tumor-specific T-cell lines or clones, which were used as probes for isolation and biochemical characterization of TAA (10, 55). Two types of methodologies have largely been used for antigen discovery: (i) biochemical fractionation of naturally processed and presented peptides derived from major histocompatibility complex (MHC) class I molecules expressed by tumor cells (16) and (ii) expression cloning of cells transfected with cDNA libraries derived from tumor cells (54). More recent introduction of the SEREX (serological analysis of tumor antigens by recombinant cDNA expression cloning) technology (47) and of computer-based modeling of peptides that best fit the relevant MHC class I molecules expressed on tumor cells (15) further expands the list of technologies available for antigen discovery and for identification of TAA which might be therapeutically useful. SEREX is based on identification of recombinant tumor antigens by immunoglobulin G (IgG) antibodies present in the patient's serum. To qualify for immunotherapy, e.g., as components of antitumor vaccines, TAA or their newly identified epitopes have to be immunogenic, that is, able to induce and sustain an immune response specifically targeted not only to the immunizing epitope but to the tumor itself. With the exception of the products of mutated genes, few if any TAA epitopes meet the criteria for therapeutic utility, largely because they are self-antigens rather than neo-antigens. As such, they are weakly immunogenic, and tolerance for self-antigens is likely to occur by the process mediated by dendritic cells (DC) and referred to as “cross-presentation.” The importance of DC in immune responses to TAA has been emphasized in view of emerging evidence for frequent, if not universal, defective antigen processing in tumor cells (26, 50). This then means that DC can internalize and present TAA for presentation to T cells bearing the appropriate TcRs, by-passing the need for tumor cells to act as antigen-presenting cells (APC). Still, even if DC assume the role of TAA presentation in vivo and cytolytic T lymphocytes (CTL) are generated as a result of effective cross-presentation, these CTL have to be able to access the tumor site and recognize the relevant peptides expressed on the surface of tumor cells in the context of MHC molecules in order to initiate tumor cell lysis. Therefore, expression on the tumor cell surface of the MHC-peptide complexes is a prerequisite for immunologic recognition and immune cell-mediated tumor cell destruction.

TAA-specific T-cell responses following immunotherapy, and particularly after the administration of natural or synthetic antitumor vaccines, have been studied in patients with cancer (28, 34, 46). Early clinical trials evaluating such vaccines showed tumor regression even in patients with advanced disease (28, 34, 46). Quantitation of antigen-reactive T cells prior to, during, and after therapy is crucial for future development of antitumor vaccines. To detect the frequency of peptide-, protein-, or tumor-specific T cells in the peripheral circulation of patients treated with antitumor vaccines, several methods have been developed. The objective aimed for is a measure of effectiveness of therapy, as judged by the increased number of circulating specific T cells responsive to vaccinating antigens and, optimally, to autologous tumor cells as well. The assays available for measuring of TAA-reactive T cells include (i) cytotoxicity assays, which provide the assessment of the ability of T-cell populations to lyse tumor cells, (ii) cytokine expression or production assays, in which TAA-specific responses of T cells are evaluated based on antibody-mediated detection of intracellular cytokines or cytokines released by T cells following stimulation with the relevant antigen, (iii) direct quantitation in peripheral blood mononuclear cells (PBMC) of T cells able to recognize and bind to a labeled peptide-MHC complex, and (iv) enumeration of T cells expressing a specific type of TcR, using PCR-based amplification. The purpose of this review is to briefly consider advantages as well as disadvantages of these methodologies for monitoring of TAA-specific responses in patients with cancer treated with antitumor vaccines and other immunotherapies.

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CYTOTOXICITY ASSAYS

Cytotoxicity assays have been in use for many years (5) for measuring antitumor responses. Typically, they depend on the use of a labeled tumor cell target, which is susceptible to lysis by T cells recognizing an antigen-MHC complex present on the tumor cell surface. There are multiple formats for performance of cytotoxicity assays, but a chromium release microtiter plate method has emerged over the years as the most widely applicable and reliable for detection of tumor-specific CTL (59).

In fact, the chromium release assay has been the “gold standard” for assessment of antigen-reactive T cells based on their cytolytic effector function. The assay is performed in wells of 96-well plates, with each well containing 1,000 tumor cells (or a surrogate target presenting the immunizing peptide) and a defined number of effector T cells. Usually, no more than 10^6 effector T cells are placed in a well, resulting in the effector-to-target ratio of 100:1, to avoid high levels of nonspecific lysis. It is necessary to perform the assay at several (at least four different effector/target ratios to ascertain linear kinetics (58).

In order to observe lysis, 100 to 200 specific effector T cells have to be present in the well, assuming that each effector T cell can eliminate five consecutive targets during the 4-h incubation period. Thus, for a cytotoxicity assay to be positive, the frequency of CTL in the population has to be at least 1 in 1,000 cells, providing the detection limit of 10^3. The available data indicate that the frequencies in PBMC of CTL able to respond to some of the well-defined MHC class I-restricted epitopes are considerably below this limit of detection (11, 45) and imply that cytotoxicity assays are not sufficiently sensitive to be useful for monitoring of tumor-specific CTL in the peripheral blood. However, it is possible to stimulate PBMC in bulk cultures with antigen, using the procedure called “in vitro sensitization” (IVS). To expand specific CTL to the numbers detectable in chromium release assays, three to four rounds of consecutive weekly stimulations with the antigen are required. While IVS facilitates expansion of CTL from their precursors (CTLp), it yields only a qualitative estimate of the presence of specific CTL in PBMC or other lymphocyte populations. In general, methods for evaluation of CTL responses based on ex vivo expansion may greatly underestimate the number of specific T cells, because some T cells have a reduced proliferative potential, particularly in patients with cancer or certain infections (30, 36).

The kinetics of CTL generation in IVS may allow for a distinction to be made between primary and secondary T-cell responses. However, for quantitative assessments of the frequency of CTL in cellular populations, cytotoxicity assays have to be performed following limiting dilution and clonal expansion of CTLp.

Limiting-dilution analysis (LDA) is a microculture technique in which lymphocytes, plated at various cell doses (e.g., 50,000 to 1,000/well) in wells of 96-well plates in the presence of antigen, APC, and interleukin-2, undergo rounds of antigen-driven replication, resulting in the formation of microcultures in a proportion of the plated wells (31). A statistical formula is then used to determine the frequency of proliferating CTLp in the population of plated cells (53). The obtained microcultures or clones (if they are derived from wells containing a single CTLp) of T cells can then be tested in cytotoxicity assays against the relevant target to determine the proportion of wells containing effector CTL. LDA has been extensively used in the past for the quantitation of both virus- and tumor-specific CTL (11, 12, 45, 53), and until recently it has provided the best available estimates of these effector cell numbers in various cellular populations. LDA is, however, very tedious and technically demanding. It is not easily applicable to monitoring of patients undergoing immunotherapy. Furthermore, the assay is notoriously variable and has been shown to grossly underestimate the size of the viral effector CTL population in murine studies (6, 13). For these reasons, the LDA has been largely replaced today by newer and more accurate technologies discussed below.

A multiple-microculture assay, involving stimulation of PBMC in a limited number of microcultures (e.g., 24 wells, each containing 10^5 responding PBMC or 10^4 enriched CD8+ T cells), was introduced to avoid the labor-intensive LDA and to provide a semiquantitative estimate of peptide-specific T-cell frequencies (44). The cells are restimulated twice at weekly intervals with irradiated autologous PBMC pulsed with the peptide in the presence of cytokines, and on day 7 following the third stimulation the cells are tested in chromium release assays against suitable peptide-expressing targets. Cytotoxicity assays are performed following cold-target inhibition with K562 targets to block NK-like activity. Simultaneously, proliferation or cytokine production can be assayed in split wells, provided T-cell expansion yields adequate numbers of responding lymphocytes. Comparing the number of wells with CTL activity in pre-versus postvaccination specimens, it is possible to obtain a semiquantitative assessment of CTLp specific for single CTL epitopes and to use the assay for monitoring of effector cells in clinical trials (unpublished data). More recent reports suggest, however, that the multiple-microculture assay is not sufficiently reproducible and that it may grossly overestimate or underestimate the frequency of tumor-reactive T cells relative to LDA or to enzyme-linked immunospot (ELISPOT) (see below).

Overall, cytotoxicity assays remain firmly established in the repertoire of available CTL measurements. The ability to kill a tumor cell target is, after all, the key functional attribute of antitumor CTL. The specificity of killing, easily confirmed in this assay by the inclusion of anti-MHC and anti-TcR antibodies, may be in many instances more important than the assay sensitivity. Clearly, the assay is not acceptable for screening of CTLp frequencies in PBMC. As a confirmatory method, however, for measuring specific cytokotoxicity, this assay is likely to continue serving as a gold standard for antitumor effector cell function until comparisons validate the equivalent performance for cytokine-based or tetramer-based technologies.

CYTOKINE-BASED CTL ASSAYS

Upon activation, T lymphocytes up-regulate expression of and secrete a number of cytokines (7). Polarization of the cytokine repertoire in Th1 and Th2 lymphocyte subpopulations has been well documented (33, 43). A number of methods have been introduced to measure cytokine expression in T cells responding to specific stimuli at the protein or mRNA level, as reviewed recently (42). Both the population-type and single-cell assays for cytokine expression are available (42). Here, the focus will be on the single-cell assays applicable to CTL frequency estimates, because these assays are increasingly frequently used for monitoring of responses to tumor vaccines in clinical trials.

Staining for intracellular cytokines involves in vitro stimulation of T cells with a relevant antigen in the presence of either monensin or brefeldin A to block secretion of the cytokine and enhance its accumulation in the cells. The cells are stained for surface markers (e.g., CD3, CD4, or CD8), fixed with paraformaldehyde, and then permeabilized in the presence of a detergent to allow for access of labeled anticytokine antibody inside the cell (21, 40). The positively stained cells are quantified by multicolor flow cytometry. This procedure has been
The ELISPOT assay is another antibody-based technique for quantitation of single cells secreting cytokines in response to a challenge with antigen (2, 18, 20, 41, 48, 49). For detection of IFN-γ-secreting cells, nitrocellulose-lined or plastic microtiter plates coated with a capture antibody are used. Graded numbers of PBMC, enriched CD8⁺ or CD4⁺ T cells, or cultured T cells are plated in wells of the microplate together with the appropriate APC plus antigen to stimulate secretion of the cytokine. The number of cells plated is critical, because a uniform lawn of single cells, only some of which (not too few and not too many) secrete the cytokine, is optimal for assay quantitation. After an incubation period of 24 h, a detection antibody labeled with an enzyme such as horseradish peroxidase is added, followed by a suitable substrate for color development. The cells secreting IFN-γ are detected as discrete colored spots, which are microscopically evaluated and counted. Using a computer-assisted video image analysis system developed especially for this purpose (18). Under optimal assay conditions, each spot corresponds to a single cytokine-producing cell (18). In addition to objective enumeration of spots in this system, the spot area can be determined to obtain an indication of the level of produced cytokine and thus the strength of the response to an antigen. The assay has been found to be highly reproducible, convenient to use with cryopreserved PBMC, and sufficiently sensitive to detect 1 IFN-γ-secreting T cell among 100,000 (2). When used with autologous DC pulsed with lysates of tumor cells, for example, the assay can detect not only CD8⁺ but also CD4⁺ responses (19). This is important in view of accumulating evidence that CD4⁺ T cells play a critical role in the induction and maintenance of antitumor responses (37). Responses to MHC-restricted peptides presented on correctly matched APC or to non-MHC-restricted antigens processed and presented by autologous DC can be measured in ELISPOT assays. Because of these attributes and its versatility, the ELISPOT assay has been widely used for monitoring of the frequency of antigen-reactive T cells in patients treated with cancer vaccines and especially of T cells responsive to MHC class I-restricted melanoma antigens, including MAGE, tyrosinase, Melan-A/MART-1, and gp100 (2, 20, 38). In addition, it has been successfully used for identification of a novel DR4-restricted Melan-A/MART-1-derived peptide (Melan-A/MART-1₃₈₋₄₇) recognized by CD4⁺ T cells obtained from HLA-DR4-positive patients with melanoma or normal donors (60). The ELISPOT assay, which measures cytokine secretion (a relatively late event following antigen stimulation), does not discriminate between primary or memory responses, unless it is performed with previously separated precursor or memory T cells (Table 1).

Assays based on detection of cytokine production, as opposed to cytokine expression, have been steadily gaining ground, largely due to the perception that they are functionally more relevant. Since these assays depend on the use of two antibodies recognizing distinct epitopes on the cytokine which is being measured, they are highly specific. They are also highly sensitive, because of the amplification step that is generally associated with the application of antibody-based techniques. Limited comparisons of ELISPOT with cytotoxicity assays performed in my laboratory indicated good agreement between the two (unpublished data). In comparison to cytotoxicity, ELISPOT assays are less labor-intensive, more reproducible, and more cost-effective. The choice of ELISPOT versus single-cell flow cytometry-based cytokine production assays, such as MACS IFN-γ secretion assay, depends on the availability of a flow cytometer for serial monitoring. The requirement for a dedicated flow cytometer may discourage some users from implementing the MACS IFN-γ secretion assays. On the other
hand, ELISPOT, whose general format resembles that of the enzyme-linked immunosorbent assay, lends itself remarkably well to monitoring of clinical protocols and offers an opportunity for quantitative assessments of CD8$^+$ as well as CD4$^+$ T-cell frequencies in freshly isolated or cultured populations. It is, therefore, highly likely that ELISPOT will emerge as the assay of choice for the frequency analysis of tumor- or virus-specific effector T cells after comparisons with other assays are completed.

**MHC-PEPTIDE COMPLEXES FOR DIRECT ASSESSMENT OF LIGAND-BINDING T CELLS**

An attractive approach to isolation and quantitation of peptide-specific T cells in mixed lymphocyte populations was recently introduced based on the use of the fluorescent oligomeric complexes containing the peptide itself linked to MHC class I molecules (1). Commonly referred to as “tetramer binding,” this technology involves formation of oligomeric complexes of MHC molecules with the relevant peptide. Because a monomeric peptide-MHC has a very weak affinity for TcR, a strategy was devised by M. Davis and colleagues of labeling the MHC molecules in the complex with biotin and assembling such biotinylated complexes to form tetrameric arrays on a scaffold of avidin (1, 25). These oligomeric peptide-MHC reagents have increased avidity for T cells expressing specific TcR, and when they bind, a strong fluorescent signal detectable by flow cytometry is generated, thus marking the T cell which recognizes the peptide. Another approach uses genetic linking of MHC molecules to IgG1 to produce a dimer in which IgG1 serves as a scaffold (24). The specificity of peptide-MHC reagents is their greatest asset, and as long as binding properties of the peptide to TcR are preserved or improved by oligomerization, they lend themselves readily to monitoring at this time, it appears to be a promising tool for confirmatory studies of antigen-specific T cell subsets and for “fishing out” small numbers of antigen-specific T cells from mixed populations of lymphocytes for their phenotypic and functional characterization. Furthermore, these cells can be cloned in vitro for further characterization (14). It is likely that future improvements of this promising technology will eliminate some of these limitations and facilitate its broader use in clinical laboratories.

### TABLE 1. Assays for monitoring CTL

<table>
<thead>
<tr>
<th>Function</th>
<th>Assay</th>
<th>Sensitivity (no. of cells)</th>
<th>Specificity for the following CTL type:</th>
<th>Effector</th>
<th>Precursor</th>
<th>Memory</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{51}$Cr release (cytotoxicity)</td>
<td>ELISPOT (cytokine production)</td>
<td>$1/10^3$</td>
<td>+ (after LDA frequency estimates)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>ELISPOT (cytokine production)</td>
<td>$1/10^6$</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Expression</td>
<td>Single-cell flow cytometry (intracellular cytokine)</td>
<td>$1/10^3$</td>
<td>+ (CD45$^+$ RA$^+$)$^a$</td>
<td>+ (CD45$^+$ RO$^+$)$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tetratetramer (binding to unique TcR$^+$)</td>
<td>$1/10^3$</td>
<td>+ (CD45$^+$ RA$^+$)</td>
<td>+ (CD45$^+$ RO$^+$)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Time to response, 6 h.

$^b$ Time to response, ≥24 h.
IMMUNOSCOPE ANALYSIS OF CDR3 DOMAINS IN T CELLS

The complementarity-determining regions (CDR) of TcR are the most variable parts of the receptor protein, endowing it with diversity. The CDR are found on six loops at the distal end of variable domains, with three loops protruding from each of the two variable domains of TcR. The CDR3 are the most variable of the three. The CDR are in direct contact with the binding ligand and determine the receptor specificity. Molecular cloning of TcR genes and sequencing of hypervariable CDR3 indicated that a broad range of specificities exist in the TcR repertoire of an individual (35). However, for certain antigens, the TcR repertoire is quite restricted, in the sense that a few closely related TcR are recognized by responding antigen-specific clones of T cells (8, 52). In principle, clonotypic Vβ-specific primers can be used to detect the presence of antigen-specific T cells (i.e., T cells with a restricted Vβ repertoire) among mixed lymphocyte populations by reverse transcriptase-PCR-based methodology. One quantitative approach involves an initial PCR with unlabelled Vβ- and Cβ-specific primers to determine the length of the CDR3 region. The PCRs are set up to amplify the cDNA of interest, using primers to the regions on either side of CDR3 (a Cβ-specific primer and 1 of 24 Vβ-specific primers). The product of each amplification is then visualized by performing a runoff reaction, which includes an additional fluorescently labeled probe. The runoff products are sequenced on an automated sequencer in the presence of fluorescence size markers. The size and fluorescence intensity of the fragments are then analyzed using Immunoscope software (35). The Immunoscope analysis provides results in the form of a bell-shaped curve with an average of eight peaks for PBMC of a normal donor. The emergence of one prominent peak signifies the presence of one or a few cDNAs with identical or similar CDR3 regions. This means that the T cells utilize a restricted repertoire of Vβ genes and may be clonal or oligoclonal. While this technique allows for the detection of restricted TcR repertoires of T cells, it does not identify the ligands recognized by these T-cell clones. It now appears that antigen-specific T cells can utilize quite diverse TcR repertoires (8, 52, 22). Thus, this technology cannot be applied to monitoring of antigen-specific CTL responses, simply because it is impossible to predict a priori whether a TcR repertoire for a given antigen will be diverse or restricted. However, the method is applicable to following changes in the TcR repertoire in individual patients during therapy (57).

CONCLUSIONS

Several new methods have been identified for monitoring of antigen-specific CTL. A better understanding of the processes of antigen processing, presentation, and recognition by T cells has significantly contributed to the development of these technologies. The availability of these technologies has focused attention on monitoring activities and frequencies not only of antigen-specific effector T cells but also of memory and precursor T cells. Table 1 lists the assays that are currently available for monitoring of these populations in humans and provides estimates of the limits of detection for each assay. The advantages and disadvantages of these assays most relevant to their application in patient monitoring are discussed above. The possibility for quantitation as well functional characterization of antigen-specific T cells in populations of lymphocytes has provided new opportunities for monitoring immune responses to individual antigens in vitro and in vivo. Application of these methods to monitoring of patients with cancer treated with biologic therapies is likely to result in a definition of new immunologic end points. However, to meet criteria for monitoring, the current available methods have yet to be validated. Work is currently in progress to compare the performances of various assays in the clinical setting, and before long it should be possible to recommend those that are biologically and clinically most relevant and economically acceptable.

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