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Immunologic monitoring

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Summary: The development of reliable *in vitro* assays that could allow the quantitation and characterization of anti-donor alloimmune responses has always been a goal in clinical transplantation, both to predict presensitization to the transplanted tissue and to be able to identify rejection without resorting to more invasive tests. With recent development in our understanding of transplantation biology and therapeutics, there is a real expectation that these tests may be used to identify tolerance as much as to predict rejection. The traditional limiting dilution assays still have a contribution to make and are being complemented by an array of tools, such as ELISpot, flow cytometry-based techniques, and microarray analysis. The assays that have been informative, to date, are discussed in this review. This information will lead, at least, to a better understanding of how and when the rejection process occurs. More interestingly, the objective is to apply this information to evaluate tolerance-inducing strategies or to identify patients that have become tolerant to their graft and can be weaned of immunosuppression. Of course sensitive, accurate and specific immunologic monitoring has applications well beyond the field of transplantation.

Introduction

The development of assays that allow us to monitor the current state of an immune response is of interest for several reasons. These assays have the potential to identify rejection without resorting to invasive tests. Possibly more importantly, a reliable index of immune status could allow the customizing of the prescription of immunosuppressive drugs. In some cases, the identification of immunological tolerance would allow the partial or complete cessation of immunosuppressants, a highly desirable goal, given the morbidity and mortality associated with long-term administration of such therapy. It is also clear that such assays will bring with them a more complete understanding of the mechanisms underlying the generation of tolerance and rejection, which will open the door to new and better targeted therapeutic interventions aiming the immune response in the desired direction.

Improving HLA matching

One of the major obstacles that has to be overcome in achieving organ and hematopoietic stem cell (HSC) transplantation is

the immune response to the highly polymorphic human leukocyte antigens (HLA) expressed by almost all nucleated cells of the body. The application of faster and more precise techniques for HLA typing has led to improved donor : recipient matching with better clinical results. The effect of HLA matching on clinical outcome varies greatly with the organ being transplanted. The HLA effect is most pronounced in allogeneic HSC transplantation; whereas in the case of liver transplantation for autoimmune disease, donor matching might actually be detrimental (1). In renal transplantation, the benefits of HLA-A, -B, and -DR matching are evident both for long-term and in the early post-transplant period (2). Recently, interest has focused on strategies to define acceptable mismatches, particularly in highly sensitized patients (3, 4). These approaches have met with variable success (5).

Even with modern high resolution typing, the effect of a set of HLA mismatches cannot be completely quantitated. Like the beasts in *Animal Farm*, not all mismatches are equally undesirable. Accordingly, much interest has focused on quantitating the immune response, in particular donor–recipient mismatches, analogous to the well-defined Ir gene effects identified in inbred mice. Such work is dependent on the availability of reliable functional tests of the immune response. Moreover, it has the potential not only to allow the avoidance of particularly immunogenic combinations but also to permit the use of certain less immunogenic mismatches, which might otherwise deny access of a particular individual to a significant number of organs that become available.

Managing the transplant recipient

Monitoring the effector responses to allogeneic stimulation may help us understand the mechanisms that result in graft rejection. If assays become available that are able to identify key steps in the process, they might ultimately be used as predictors of potentially detrimental events, prior to their clinical manifestation. This ability would allow intervention at a much earlier stage in the rejection process. Increased anti-donor responses have been measured in association with rejection in solid organ transplantation (6–8), but these results have not been consistent (9, 10). No large prospective studies have been conducted that evaluate clinically useful anti-donor responses, probably due to the lack of definition of an assay that can be conducted easily in a large number of patients, that requires an acceptably small volume of blood, and that can be repeated on several occasions.

Advances in the efficacy of immunosuppression over the past two decades have led to a considerable improvement in the short-term survival of organ transplants. Not withstanding this, almost all transplanted patients have to endure immunosuppression for the rest of their lives. Long-term immunosuppressive drug treatment is associated with significant morbidity and mortality, mainly due to cardiovascular disease, opportunistic infections, and an increased incidence of malignancy. The ultimate goal in the management of transplanted patients is the induction of donor-specific tolerance-antigen-specific immunological unresponsiveness that is sustained in the absence of chronic immunosuppression. There is an increasing body of knowledge focused on how to specifically control the immune responses that transplanted tissues initiate, much of which is discussed in this issue. Immunological monitoring could contribute by quantitating pro-inflammatory and anti-inflammatory components of the anti-donor response. If reliable assays were available, then it would be possible to monitor the evolution of anti-donor responses in individual patients and to determine the effectiveness of potentially tolerogenic therapeutic strategies. As new drugs and biological agents are introduced, such assays are vitally important in determining whether they are 'tolerance-promoting' or whether they impede the development of immune tolerance. In patients who have already received a transplant, the assays would be used to identify those in whom tolerance had developed and, therefore, whose immunosuppression could be weaned, avoiding much of its detrimental effects.

We can define immunological monitoring as the *ex vivo* measurement of pro-inflammatory and anti-inflammatory responses with clinical utility. Assays that conform to this definition are the subject of this review.

To monitor responses in recipients of transplants, we are mainly constrained to use lymphocytes from peripheral blood. It could be argued that the responses in peripheral blood do not necessarily mirror what will happen in the tissue, as this area is regulated by infiltrating lymphocytes. The nearest attempt to mirror a transplant situation in a mouse model was provided by Orosz *et al*. (11), in an elegant model of allograft using polyurethane sponges bearing allogeneic splenocytes. Donor-reactive cytotoxic T cells represented, at most, 0.2% of the cells recovered from these allografts, which is similar to the frequencies found in limiting dilution assays (LDAs) on peripheral blood from completely mismatched human samples (12). Whilst acknowledging that peripheral blood is not the ideal source of information, these data argue that there are reasons to believe it is good enough.

While individual kinetic assessment of alloreactivity by different methods is feasible in human peripheral blood, in

murine systems peripheral blood is a poor source of cells, and, thus, individual kinetic assessment of alloreactivity in the same individual can be challenging. The advantage in murine systems is that transplantation groups can be bigger and the variation from one individual to the next in littermates is so little that the kinetic studies can be performed on different individuals. Moreover, graft-infiltrating lymphocytes are available for study, arguably providing the most interesting source of information. Most of the assays described below can be applied in rodents using the appropriate reagents. However, most reports on animal models of transplantation assess alloreactivity by graft survival, and accurate quantitation of responses to donor antigens is surprisingly scarce in the literature. Where *in vitro* assays have been informative in rodent systems, we have included these data.

It is important to comment on the practicalities of performing these assays before going further. Almost certainly, no single assay will provide all the answers; rather, each will analyze the immune response in a subtly different fashion. Combining the results of several assays, it should be possible to determine the fingerprint of the immune response at any given time in a given individual.

Assays being developed for the immunological monitoring of the alloimmune response can be broadly divided into the antigen specific and the antigen non-specific. In the following sections, we summarize the impact such assays have had in clinical transplantation.

Antigen-specific assays to monitor responses to grafts

Several key concepts underlie the design of assays to monitor anti-donor T-cell immunity. The first concerns the two pathways of the major histocompatibility complex (MHC) alloantigen recognition (*Fig. 1*). The 'direct' pathway requires the recognition of intact donor MHC alloantigens on the surface of donor cells. This pathway is responsible for the vigor of the mixed lymphocyte reaction (MLR) and could be an important driver of early acute transplant rejection. Direct pathway T-cell activation is most efficiently achieved by donor bone marrow-derived antigen-presenting cells (APCs) and, most importantly, tissue dendritic cells that migrate to draining lymphoid tissue shortly after transplantation.

The second pathway of MHC allorecognition is referred to as the 'indirect' pathway and involves the internalization, processing, and presentation of alloantigens as peptides bound to recipient MHC molecules. The involvement of this pathway in transplant rejection was first proposed on the basis of observations in a rat kidney transplant model (13, 14). Since those early observations, others and we have provided evidence that indirect allorecognition is an important driver of transplant rejection (12, 15–18) and that the induction of tolerance in this pathway is a requirement for long-term transplant survival (19, 20).

The second key idea follows the emergence of regulatory T cells that hold the anti-donor immune response in check. The evidence for such cells is long-standing and comes from adoptive transfer studies in which tolerance can be transferred to a naïve recipient by $CD4^+$ T cells. Although the mechanisms of this regulation remain incompletely understood, some progress has been made in defining the phenotype of this regulatory population. These cells have the same phenotype, $CD4^+CD25^+$, as the spontaneously arising population that plays a vital role in the prevention of autoimmune disease. Depletion of these $CD4^+CD25^+$ cells prevents the transfer of tolerance by $CD4^+$ T cells from a transplant-bearing animal (21). Over the past decade, an ever-increasing body of data both in human and animal models has established the role of these and other naturally occurring regulatory cells (such as natural killer T cells) in transplantation. We and others have recently reviewed this phenomenon elsewhere (22, 23). The picture of the mechanisms underlying the regulatory function of these cells is far from completely defined, but it does appear that this population of T cells plays an important role in the maintenance of experimental (23) and possibly clinical (24) transplantation tolerance.

A set of assays has been developed to quantify lymphocytes recognizing donor antigen (Summarized at the end on *Table 1*). The literature is dominated by the presentation of antigens in the direct pathway, but modifications in the culture conditions can be set in place for almost all the assays to measure indirect pathway responses. The primary *in vitro* response to the direct recognition of allogeneic molecules emerges in the MLR. This reaction was first described in the 1960s and has been extensively used to study anti-donor responses. However, in its conventional form, proliferative MLR bulk cultures have very little predictive value in the context of transplantation (25). For this reason, different assays have been developed to obtain information of immunological responses that are of clinical utility. The special challenge that measuring indirect pathway responses poses is due to the low frequency of T cells with this specificity. In many occasions, these responses are at or below the limit of detection of the assays currently available. Therefore, all steps taken to increase the sensitivity of the assays will help in our ability to measure such responses.

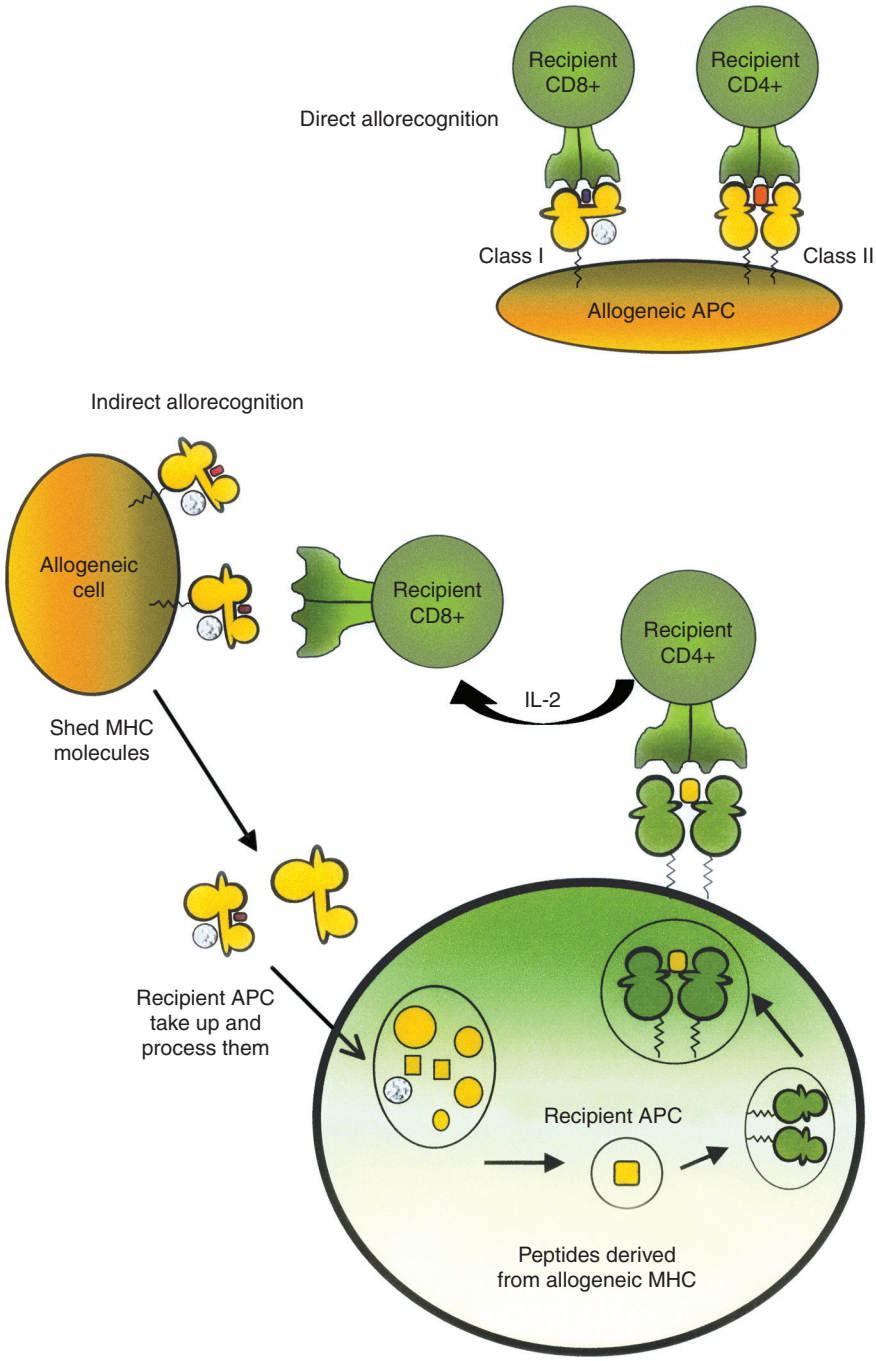

***Fig. 1.* Direct and indirect pathways of allorecognition.** Direct pathway: intact allogeneic MHC molecules are being recognized by recipient's T cells. $CD8^+$ T cells are activated by recognition of class I molecules of the MHC complex, whereas $CD4^+$ cells are activated by the recognition of class II molecules. Indirect pathway: allogeneic MHC molecules are shed from the graft, and these molecules are taken up and processed by the recipient APCs. Peptides derived from the allogeneic molecules are presented in the context of the appropriate restriction elements. Indirect pathway $CD4^+$-specific T cells are able to provide help to direct pathway specific $CD8^+$ T cells.

Limiting dilution assays

These assays, first described by Lefkovits (26), provide more precise quantitation of immunity to a given stimulus and allow the estimation of frequencies of antigen-specific cells participating in an immune response (27). They have become a standard experimental tool for estimating frequencies of defined function in a population of cells.

The technique consists of setting up multiple replicates of graded dilutions of responder cells (usually patients' unselected peripheral blood lymphocytes or purified populations of $CD4^+$ or $CD8^+$ cells) in wells containing a non-limiting stimulus (e.g. donor stimulator cells). The readout from a particular well is only considered positive if the measure chosen exceeds the mean of controls (cultures lacking responder cells) by a factor of three or more. The number of 'negative' wells at each dilution of responder cells is determined. As the concentration of the responder cells increases, the proportion of 'negative' wells will tend to be less; the relation between the number of negative cultures and the mean number of precursors can be plotted and a frequency obtained (27, 28).

The ability of an LDA assay to predict the frequency of precursors depends on the number of replicates and the number of responder cells added per dilution (29). An important issue concerns the statistical method used to estimate the unknown frequency. A number of methods are available to estimate the effector frequency from the experimental data: least squares, weighted mean, minimum chi-square, and maximum-likelihood. Extensive evaluation of the methods using artificial data concluded that the last three were useful (30). We have favored a maximum-likelihood-based method that introduces bias reduction (31).

Different effector functions can be measured at different time points. These include proliferation, cytokine secretion (allowing determination of helper T-lymphocyte precursors 'HTLp'), and cytotoxicity (allowing determination of cytotoxic T-lymphocyte precursors 'CTLp'). In each case, each well is 'scored' as positive or negative, and the frequency of precursor cells able to mount such response can be, thus, calculated.

LDA assays have been shown to be specific and reproducible as a measurement of alloreactivity (32). Several refinements have been described to increase the specificity and sensitivity in the measurement of interleukin (IL)-2-secreting HTLp (33–35) and CTLp frequencies (36). The production of different cytokines can be measured in the presence of these cultures such as interferon-γ (IFN-γ), IL-5, IL-4, IL-10, IL-13, or tumor necrosis factor-α (TNF-α) (37, 38). A comprehensive study of the kinetics of T helper (Th) 1 and Th2 cytokines produced in alloresponses has recently been published (39). The clinical utility of anti-donor cytokine HTLp frequency measurement has been extensively demonstrated for IL-2 (40, 41) and less extensively for IFN-γ (42, 43). Our laboratory has data to support the assertion that the frequency of IFN-γ-secreting $CD8^+$ T cells is of value in measuring the alloresponses to HLA class I disparities in unrelated donor hematopoietic cell transplantation (Brookes and Lechler, unpublished observations). There have also been claims that raised frequencies of T alloreactive T cells secreting the Th2 cytokine, IL-4, are associated with a better outcome in heart (44) and bone marrow transplantation (45). Further development in the detection of cytokines may help in dissecting mechanisms of tolerance and rejection.

In the context of bone marrow transplantation, there is vast experience in the utility of CTLp frequency measurement to predict graft-versus-host disease (GVHD) and survival (46–50). In solid organ transplants, the data is less abundant, and conflicting data have been reported in the ability of CTLp measurement to predict rejection (51–53).

With our increasing appreciation of the importance of regulatory cells in the control of effector responses in the immune system, the estimate of the frequency of T lymphocytes in unfractionated cell populations poses a particular challenge. This difficulty reflects the fact that there are no specific immunophenotypic markers to identify regulatory cells, and, therefore, they cannot be clearly isolated. Although coexpression of CD4 and CD25 does characterize a population of dedicated regulatory cells, activated $CD4^+$ T cells have the same phenotype, leading to difficulties particularly in the context of an active immune response, such as transplant rejection. A unique advantage of LDAs is that they allow the study of complex responses at the population level, in that they can reveal the presence of different populations of cells (such as regulatory cells) that affect the response. These complex responses usually manifest themselves as deviations from the single-hit kinetics and graphically give 'zig-zag' curves when cell-dose is plotted against fraction of negative cultures (54) (see *Fig.* 2 for further explanation of this concept). Indeed, in LDA experiments if $CD4^+CD25^+$ cells are added back to the $CD4^+CD25^-$ fraction, there is a dose-dependent effect between the percentage of $CD4^+CD25^+$ cells and the deviation of the data from single-hit kinetics (55). Mathematical models for the accurate estimation of the frequencies of interacting cell types and of the parameters for their multi-hit interaction were developed by Dozmorov *et al.* (56). Recently, a novel theoretical approach for quantifying the frequency of suppressor cells in a responding population has been developed. This method is based also on limiting dilution data modeling, and it allows the simultaneous estimation of the frequencies of both proliferating and suppressor cells (57).

Albeit labor intensive and complex in data analysis, LDAs are still a valuable tool to monitor donor-specific responses, particularly in the era of computerized calculations. Their specificity and relationship to clinical outcome has not been surpassed by any other assay to date. Their ability to unmask regulatory cell effects and the range of readouts that can be measured will ensure its ongoing usefulness.

ELISpot

This assay is based on the detection of a cytokine produced by single cells after stimulation with mitogens or antigens (58, 59). The secreted cytokine is detected by specific monoclonal antibodies and revealed by the generation of discrete spots, reflecting the number of cytokine-secreting cells (60) (*Fig.* 3). Automated video image analysis has developed the potential use of this assay (61). Presently, it is widely used in monitoring

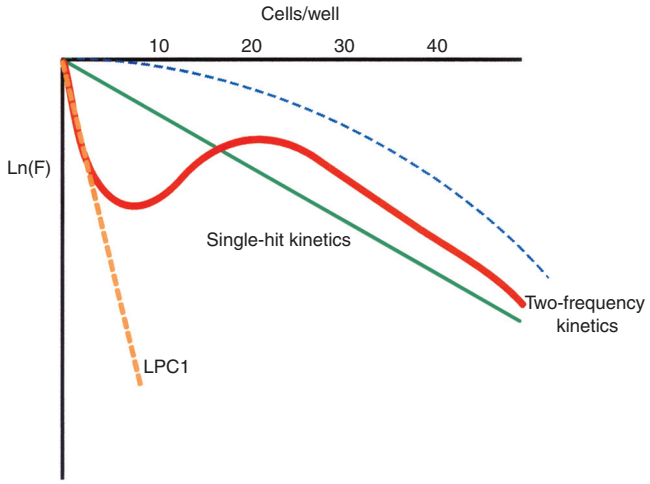

***Fig. 2.* Single-hit and two-frequency kinetics in limiting dilution assay.** Single-hit and multiple-hit LDA curves are represented. An experiment with only one population of cells responding gives a straight line (single-hit kinetics, green line). An experiment where the population of responding cells is complex gives the classic 'humped' curve (red line). In this model, several assumptions need to be made to derive a curve. First, at a given ratio of regulators-to-responder cells, there will be suppression, but below this ratio suppression will not occur. Second, if enough regulators are present, they too will be able to proliferate. If studied in isolation, the responder population is represented by LPC1 (orange line), a single-hit kinetic where there are a small number of cells per well will sharply decrease the number of wells 'scoring negative'. In isolation the regulatory population is represented by LPC2 (blue line); at a low number of cells per well, the well still scores negative. As the number increases, some cells proliferate under the influence of the other regulators thus producing a curve. If the populations are mixed (red line), at a low frequency the responders predominate, but as the number of cells per well increases the regulatory cells can exert their suppressive effect and more wells will start to score negative, hence the hump. The wells will not score positive again until there are both enough regulators to start proliferating and an excess of responders to prevent suppression. Once this happens, the hump is overcome and the line tends towards a straight line. The actual frequency of the two populations can be derived from the gradients of LPC1 and LPC2.

antigen-specific responses in the context of vaccine development for infectious diseases (62), cancer (63), and autoimmunity (64). In the context of transplantation, it has been used to identify the presence of donor-specific T cells in patients prior to surgery (65).

Several studies have found that ELISpot frequencies correlate with LDA precursor frequencies of varied effector functions (66), although in one it correlated only when a modification was included in the LDA (67). The advantage of this assay over the LDA is that results are not dependent on clonal expansion, thereby excluding one potential variable. The assays are less labor intensive and are shorter. The main disadvantage is that, even with the aid of specialized imaging equipment, there is a certain degree of subjectivity in the interpretation of results, as a threshold for the size, intensity, and gradient of the spots are user-defined. Comparison of results in different ELISpot readers has not yet been performed. Standardization of parameters that define a positive spot will be needed for reproducibility across laboratories and readers. ELISpot has also been found to be informative to assess direct and indirect allogeneic responses in murine systems (16).

CFSE labeling and cell division

In the area of quantitative flow cytometry, fluorescent dyes are being exploited to track lymphocyte migration and proliferation. Most notably, carboxyfluorescein succinimidyl ester (CFSE), an intracellular fluorescent label that divides equally between daughter cells, has been used to study cell division (68, 69) (*Fig.* 4). Recently, a method was developed using this dye to quantify alloreactive T-cell responses (70). A combination of LDA and CFSE labeling has also been described to measure alloactivation in CD8 cells (71). Accurate quantitation of dividing cells can be achieved by the use of internal standards such as microspheres that allow enumeration of absolute cells as opposed to percentages (72). Using this enumeration method, antigen-specific frequencies have been measured with high sensitivity and reproducibility (Hernandez-Fuentes MP, manuscript in preparation). The advantage of this method, as with other flow cytometric methods, is that different phenotypically defined subsets of cells can be studied simultaneously (73). The limit of detection of this method is established by background proliferation and the number of cells acquired; a higher sensitivity can be achieved if the dividing cells can be identified by a surface marker.

The use of CFSE labeling to assess allogeneic responses has been widely used in murine systems. A fundamental advantage is that by adoptive transfer of CFSE-labeled specific responding cells, enumeration of responses (70) and mechanisms of rejection and tolerance *in vivo* can be readily assessed (74). Not only is enumeration available, but also issues of migration, localization of lymphocyte activation, and antigen presentation now can be addressed. Moreover, this technique also can be used to determine kinetics of immune responses, to track proliferation in minor subsets of cells, and to follow the acquisition of differentiation markers or internal proteins linked to cell division (75).

We have compared different methods to measure CD4 antigen-specific frequencies in healthy controls, and we have found them to be stable over a short period of time. In a mouse model specifically designed to compare the accuracy of these alternative approaches to frequency estimation, ELISpot was found to be marginally superior to LDA or CFSE

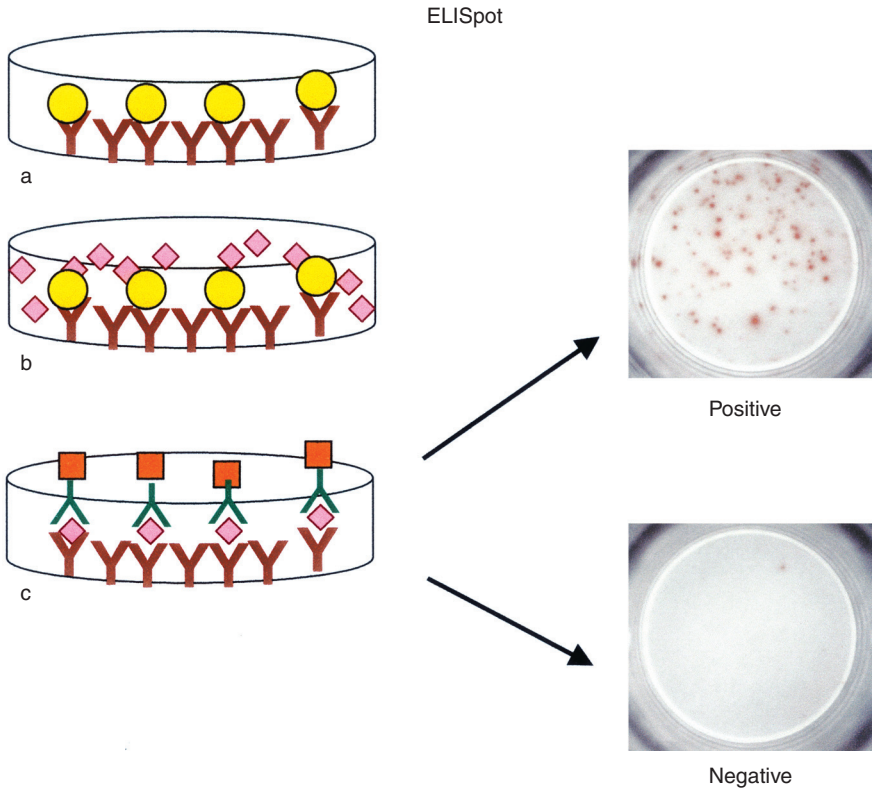

Fig. 3. ELISpot procedure. (A) Culture wells are coated with the capture antibody and cells are then added. (B) Cultures are incubated for 24–48 h, and the cytokine is produced by the cells. Cells are then removed from the plate, and the cytokine remains bound to the antibody. (C) A detection biotinylated antibody is then added, followed by a conjugate of an enzyme with streptavidin. In the final step, the substrate precipitates where the secondary antibody was bound, forming spots that correspond to cells producing the cytokine.

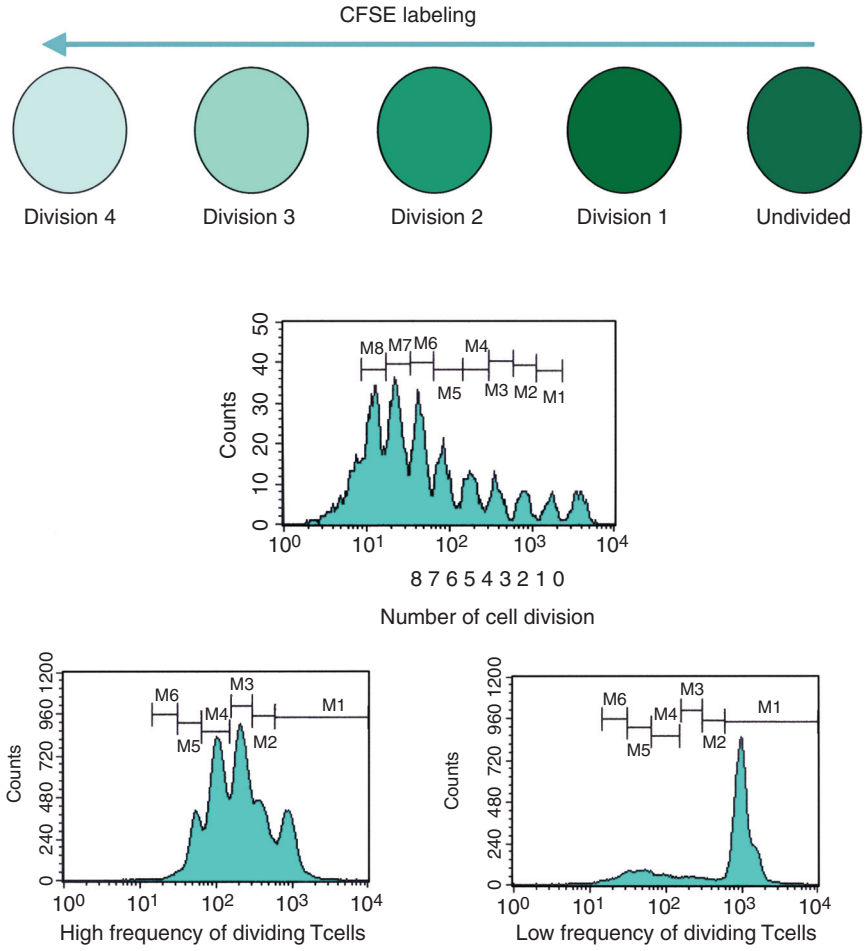

Fig. 4. Flow cytometry analysis of cell division: carboxyfluorescein succinimidyl ester (CFSE) labeling. Cells are labeled with CFSE prior to culture, and with each division cell fluorescence intensity halves. In T lymphocytes, up to eight cell divisions can accurately be distinguished. Applying methods to calculate exact number of cells, we can find out the number of precursors. By relating precursors to the number of cells seeded a frequency can be obtained. The advantage of this method is that two populations of cells can be studied simultaneously, as long as they can be identified with different surface markers.

labeling and flow cytometry (Hernandez-Fuentes MP, manuscript in preparation). Given the speed and relative ease of ELISpot assays, it is clearly an attractive option.

Flow cytometric detection of cytokines

Flow cytometry methods to detect and measure cytokine production by lymphocytes are emerging. The cytokine secretion assay is defined by the creation of an artificial affinity matrix on the cell surface that is specific for the secreted product of interest. Once the matrix is added, the cells are allowed to secrete for a defined time period and the secreted product is 'captured' in the matrix. The affinity matrix is generated by attaching a bispecific antibody to the cell surface, i.e. an antibody that binds CD45 on lymphocytes will cover the surface of the cell, and the second specificity will detect a cytokine, such as IFN-γ or IL-4. The secreted molecules bind to the affinity matrix on the secreting cell and are subsequently labeled with specific fluorescent or magnetic staining reagents (76) (*Fig.* 5). This method has been shown to correlate with tetramer-binding numbers in $CD8^+$ T cells, binding a melanoma-associated peptide, Melan-A (77). However, in a study of healthy volunteers measuring responses to influenza peptides, this method did not correlate with results obtained with ELISpot or intracellular staining of cytokines (78).

Our experience and that of Asemissen (78) is that this assay shows non-specific binding of the secondary anti-IFN-γ antibody. Hence, background staining is often a problem, and the 'noise-to-signal' ratio leads to a lack of sensitivity. It was designed to isolate functional cytokine-producing T cells specific for the stimulating antigen, and it has shown to be successful, even when the frequency of starting antigen-specific cells are very low (76).

An alternative flow cytometry-based cytokine detection method involves the intracellular staining of cytokine in cells (79). Specific activation procedures are always needed to be able to detect cytokines, usually involving the addition of inhibitors of intracellular transport (such as brefeldin or monensin) (80), which can limit the viability of the cells. This method allows the individual characterization of large numbers of cells. With multiparameter staining, it can demonstrate exclusive or mutual coexpression of different cytokines in individual cells. It, therefore, allows the categorization of T-cell subsets, such as Th1 or Th2, rather than just surface markers. Frequencies calculated by this method have been shown to correlate with the number of tetramer-binding cells in human immunodeficiency virus (HIV) patients (67), although in another study on patients with metastatic melanoma, such a correlation was not found (81). It has also been demonstrated to correlate with the frequency obtained by

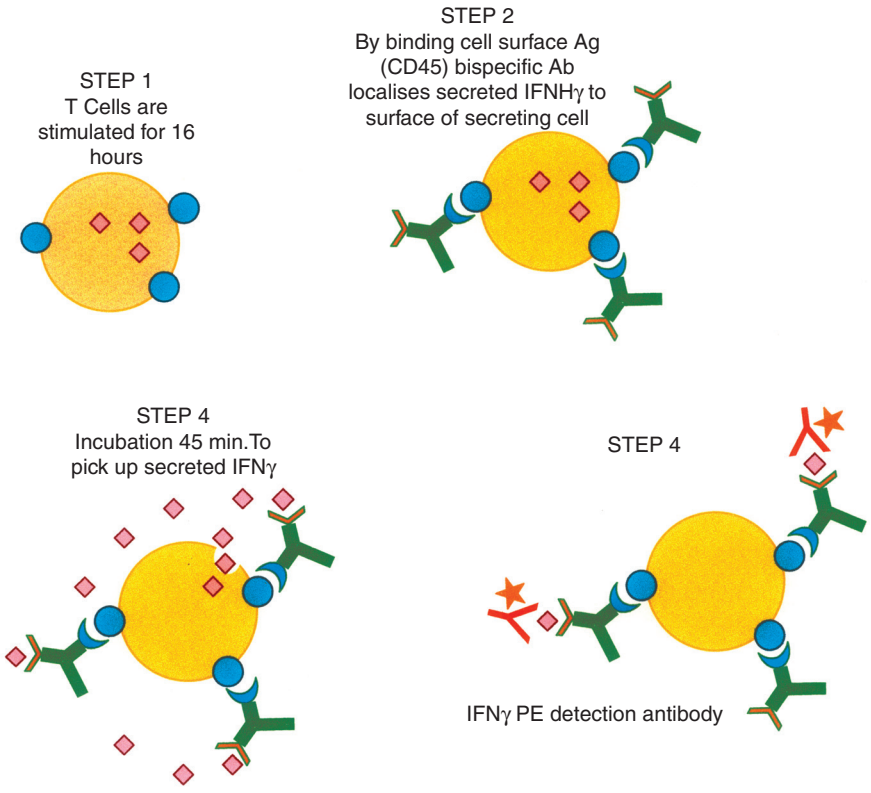

***Fig.* 5. Flow cytometric analysis of antigen-specific T cells: cytokine secretion assay (cell surface affinity matrix technology).** Incubation steps are shown and explained.

ELISpot in responses to influenza peptides in healthy volunteers (78) and to exceed several fold the frequency obtained by LDA using cytotoxicity as a readout in Epstein-Barr virus and allogeneic responses (82).

Tetramer staining

First described by Altman and colleagues (83), these reagents have revolutionized the visualization and quantitation of antigen-specific T cells. It consists of four MHC–peptide complexes linked covalently by a fluorochrome (83). The increase in avidity caused by tetramerization allows consistent binding to T cells (*Fig.* 6). Since their first description, the range of available peptides and restriction elements has increased rapidly and now includes HLA-class II molecules (84, 85). They have proved to be clinically useful in the monitoring of immunity to infectious diseases caused by different viruses, such as HIV (86) or hepatitis-B virus (87). In tumor immunology, they have been used both to study tumor-specific $CD8^+$ cells (88) and to monitor responses to vaccination (81).

In several instances, it was noted that $CD8^+$ T-cell frequencies estimated by tetramer staining were substantially higher than those calculated by conventional LDA using cytotoxicity as the readout (67, 89). The likely explanation for this discrepancy is that tetramer staining can detect naïve, memory and effector cells, which may be substantially expanded during an active infection. In contrast, a conventional assay of CTLp frequency requires *in vitro* clonal expansion, and effector cells are not only incapable of such expansion but also tend to die by apoptosis in prolonged culture (90).

The potential of MHC tetramers lies in the possibility of monitoring peptide-specific T cells over time with very small volumes of blood. In fact, recently, they have been used to monitor minor histocompatibility (mH) antigen-specific T cells in bone marrow recipients and have demonstrated an association between the presence of functional cytotoxic T-lymphocyte-specific for mH antigens and the occurrence of GVHD (91). With the advent of class II tetramers, it should now be possible to measure and detect anti-donor T cells with 'indirect' specificity.

Trans vivo delayed-type hypersensitivity (DTH)

A recent addition to the repertoire of immune reactivity that are currently in use is the '*trans vivo*' delayed-type hypersensitivity (DTH) assay. In this assay, human peripheral blood mononuclear cells (PBMCs) are injected with specific antigens into either the footpad or the pinna of a mouse, and the magnitude of the resultant swelling after 24 h is taken as an index of the reactivity of these cells to that antigen. Carrodeguas *et al.* (92) validated this technique for detecting responses to

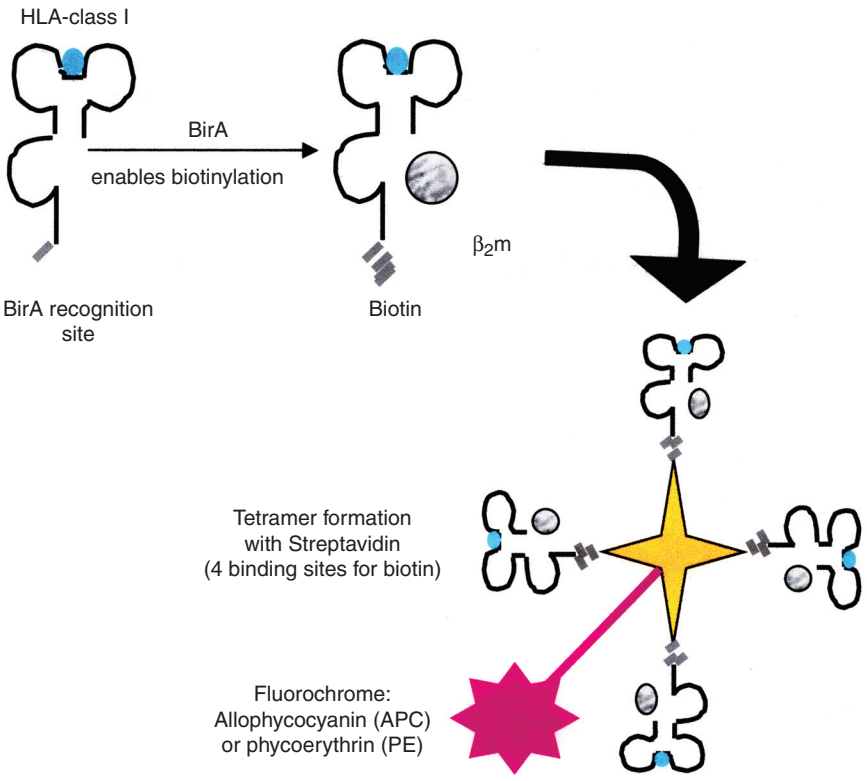

***Fig.* 6. Flow cytometry analysis of determinant-specific T cells: tetramer-staining.** Purified class I MHC molecule-peptide complexes are enzymatically biotinylated by incubation with purified BirA. The heavy-chain fusion protein is folded *in vitro* in the presence of β_2-microglobulin. The complex is then multimerized by mixing the biotinylated protein with phycoerythrin- or allophycocyanin -labeled streptavidin at specific molar ratios. Multimeric peptide-MHC complexes are able to bind more than one T-cell receptor on a specific T cell and, thus, have slower dissociation rates, which allows the identification by flow cytometry of T cells specific for the peptide-MHC molecule complex.

tetanus toxoid and cytomegalovirus. They determined that this response was antigen-specific, that it depended on the presence of injected $CD4^+$ T cells as well as human antigen-presenting cells, and that it was not influenced by the presence of an intact murine immune system. With respect to monitoring the alloresponse, it has, hitherto, been of value only in addressing indirect alloreactivity, because intact 'stimulator' PBMCs can also provoke swelling in the mouse, giving an unacceptable noise-to-signal ratio. Using sonicated allogeneic (but not autologous) PMBCs as stimulating antigens, recipients' PBMCs could be demonstrated to generate an alloantigen-specific response in the *trans vivo* DTH assay. It did not correlate with the parallel humoral alloreactivity, suggesting that it was measuring some other aspect of immune responsiveness.

This same group went on to apply this assay to transplant patients, this time, using immunologically incompetent severe combined immunodeficiency disease (SCID) mice (93). They described four transplant recipients in whom all immunosuppression had been discontinued. Three of these patients, who had prolonged drug-free graft survival, were shown to have alloantigen-specific hyporesponsiveness in the *trans vivo* DTH assay. By contrast, the fourth patient, who underwent delayed graft rejection, had a strong alloantigen-specific *trans vivo* DTH response. This assay proved sensitive to suppression in that recall antigen responses could be inhibited in those patients with alloantigen-specific hyporesponsiveness if recall and alloantigen were coinjected. This group further characterized the nature of the MHC alloantigen that was mediating this process of 'linked suppression' as being an indirectly recognized class I molecule (94). The precise nature of the cellular interaction being measured in this assay remains unclear, but it is certainly different from what is being assessed in a standard *in vitro* MLR, because it was not possible to demonstrate linked suppression in an *in vitro* assay using the same reagents with which it had been demonstrated in the *trans vivo* assay (95). It is also clear that donor-specific *trans vivo* DTH reactivity occurs more commonly in renal transplant recipients than allosensitization, as detected by the development of donor-specific alloantibodies (96). Indeed, there is rather poor functional correlation between these two indices of allosensitization in transplant patients. It could be argued that an *in vivo* assay, such as the *trans vivo* assay, might more closely represent what is going on within the patient is a wholly *in vitro* assay. However, this assertion remains to be validated in larger numbers of patients in various clinical contexts.

Whilst this assay is attractive in that it offers something that actually occurs *in vivo*, it is limited by a need to sacrifice animals, with all the attendant problems. In addition, the readout is a few millimeters of swelling and, as such, presents a problem with quantitation. Nonetheless, it may provide a useful measure, particularly of indirect pathway sensitization and suppression.

Measurement of donor-specific alloantibodies

In the early days of transplantation, the presence of preformed anti-HLA antibodies in recipient serum was recognized as a prominent risk factor for episodes of acute allograft kidney rejection (97), and their presence is still associated with rejection or graft loss (98). Screening patients on transplant waiting lists for such alloreactive antibodies is performed to provide an estimate of the degree to which she or he is sensitized to the graft. Historically, screening to identify antibodies to HLA antigens has been performed with a panel of HLA-typed lymphocytes in an assay that detects complement-dependent cytotoxicity (CDC) against certain HLA specificities. To increase sensitivity an anti-human globulin step was introduced. This assay has served the transplant community well for more than 30 years; however, the need to maintain a panel of viable, HLA-typed lymphocytes and questions of sensitivity and specificity have prompted the development of newer assays.

Kao *et al.* (99) introduced the enzyme-linked immunosorbent assay (ELISA) methodology using soluble HLA molecules adsorbed onto plates. A patient's serum can be tested for the presence of immunoglobulin (Ig) G or IgM against the adsorbed molecules. Flow cytometry-based techniques were introduced in the early 1990s. These methods use either donor T and B lymphocytes (FCXM) (100) or beads coated with purified class I or class II molecules (Flow-PRA) (101). Flow cytometry methods are 10–250-fold more sensitive than CDC (102) and have proved to be reliable and reproducible (103). When ELISA and flow cytometry using Flow-PRA beads were compared, the latter appeared to be the more sensitive and specific. However, ELISA offers the advantage of being more suitable for testing large numbers of samples in a more time- and cost-effective manner (104). The advent of these new techniques generated controversy; not all of the studies found an association between a pretransplant-positive FCXM and graft survival (105). In contrast, other studies confirm the usefulness of these new techniques to predict rejection or transplant failure (106, 107). A sensible approach to the testing for the presence of pretransplant antibodies could be to use a routine test and apply others to confirm the presence or the specificity of the antibody to evaluate risks (107).

Table 1. Comparison of assays for immunologic monitoring

Assay	Technique/detection	Advantages	Disadvantages
LDA	Measures number of responding cells in a given population	Measures function and frequency The function of regulatory cells can be assessed	Requires *in vitro* expansion and is labor intensive and mathematically complex
IL-2 by bioassay	IL-2 production	High sensitivity and high reproducibility	Maintenance of CTL line
Proliferation	Tritiated (3H) thymidine incorporation	Measures complex effector function	Low sensitivity
IFN-γ by ELISA	IFN-γ production	Sensitive and reproducible enough	Longer incubation period
Other cytokines by ELISA	Cytokine production	Measure Th1/Th2 pattern	Lower sensitivity
Cytotoxic frequency CTLp	Cytotoxicity	Reproducibility and large experience in the clinical setting	Longer incubation period and needs use of radioactivity
Bulk MLR	Tritiated (3H) thymidine incorporation Or cytokine production by ELISA	Measures complex function, Th1/Th2 pattern, and kinetics of effector function	Low sensitivity and little reproducibility
CFSE labeling	Flow cytometry-based measurement of proliferation	Measures function and frequency High intra-assay reproducibility Two subsets can be studied simultaneously	Requires stringent controls and careful optimization
ELISpot	Cytokine production at single-cell level	Measures function and frequency Does not require *in vitro* expansion High sensitivity	Data interpretation can be ambiguous Requires optimization
Peptide : MHC multimers	Flow cytometry-based	Does not require *in vitro* expansion If used with intracellular staining, function can be assessed Uses little cells useful in prospective studies	Requires specialized reagents for each MHC/peptide complex of interest Better established for class I than class II
Intracellular staining	Flow cytometry-based	Measures function and assesses Th1/Th2 pattern in immunophenotipically defined cells	Does not allow isolation of specific T cells, because the cells are destroyed
Cell-surface affinity matrix	Flow cytometry-based (cytokine capture technique)	Measures function Does not require *in vitro* expansion Specific T cells can be isolated	Prone to false positives
RT-PCR	Measures production of cytokine mRNA	Highly reproducible High sensitivity Ag specificity is not completely established	Does not allow isolation of specific T cells because the cells are destroyed

LDA, limiting dilution assay; IL-2, interleukin-2; CTLp, cytotoxic T-lymphocyte precursors; IFN-γ, interferon-γ; ELISA, enzyme-linked immunosorbent assay; MLR, mixed lymphocyte reaction, CFSE, carboxyfluorescein succinimidyl ester; MHC, major histocompatiblity complex; RT-PCR, reverse transcriptase-polymerase chain reaction.

The relevance of the *de novo* appearance of post-transplant antibodies has been thoroughly reviewed (98). Numerous reports have been published that find an association between the presence of anti-HLA antibodies and increased acute and chronic rejection and, with them, decreased graft survival. Hence, there are reasonable grounds to test for the presence of anti-HLA in the post-transplant period. However, these results should be interpreted with caution, as in all these studies there is a percentage of patients with positive anti-HLA antibodies whose graft continues to function (98). Additionally, the presence of anti-donor antibodies prior to transplantation might not always be an absolute contraindication to transplantation, because aggressive therapy has proven to be successful, mainly in heart transplants (108). Moreover, 'accommodation' (the development of insensitivity of antibody-mediated damage) of the graft has been shown to develop and be beneficial (109).

In contrast with other assays, the detection of post-transplant alloantibodies has frequently been performed to assess mechanisms of rejection in rodent models, and it has been particularly informative in models of chronic rejection in rats (110–112) and mice (113).

Non-antigen-specific approaches to immunological monitoring

All the assays described in the earlier part of this review have studied the response of T cells to donor antigens. An alternative approach to determining the response of cells to antigen is to determine their phenotype of surface markers or functional state and tries to identify a pattern associated with a particular clinical status. For example, much effort is currently focused on identifying distinctive features of regulatory T cells. At the level of cell-surface markers, such an approach has been used for many years. Following immunosuppression with anti-lymphocyte therapy, both polyclonal and monoclonal, the absolute number of T cells, phenotyped by their ability to rosette sheep erythrocytes or by the expression of CD3, has been used as an index of the degree of immunosuppression. More sophisticated approaches have been suggested in the light of technological advances. In this section, we highlight three of these.

Patterns of T-cell receptor usage

T-cell receptors are heterodimers of α and β chains, each of which has a membrane-distal portion containing the antigen-binding domain. This portion is coded by one of the series of variable region (Vα and Vβ) gene segments. Within these antigen-binding domains, each chain has three complementarity-determining regions (CDRs) of which the third (CDR3) is the most variable. Much attention has been given over recent years to the pattern of T-cell receptor usage in different clinical scenarios. Little clinical applicability has emerged from these studies. However, as has been discussed above, our renewed understanding of the development of regulatory cells has given an additional impetus to considering whether the presence of certain patterns of T-cell receptors could inform us about the nature of the immune response taking place.

Soullilou's group (114) has addressed patterns of T-cell receptors in the context of transplantation. They have shown that the pattern of Vβ-gene usage in an alloimmune response is very different from what is seen in the unperturbed immune system. They looked at the T cells infiltrating 18 human grafts that had been lost to chronic rejection and found a strong bias in Vβ-gene usage (114). In the same study, they found that 55% of Vβ families shared common and oligoclonal Vβ–Cβ rearrangements, if the patients had acute rejection superimposed on the chronic rejection. Two Vβ genes showed a common Vβ–Jβ rearrangement. In contrast, T-cells infiltrating grafts that showed only chronic lesions showed an unaltered Gaussian-type CDR3 length distribution. Similarly, in a xenograft model in which long-term survival of a hamster-to-rat cardiac graft was generated using cyclosporine and cobra venom factor; they found that, when immunosuppression was discontinued, a Gaussian pattern of CDR3 lengths rapidly changed to a much more restricted pattern when the grafts began to be rejected (115).

Using a system that combines analysis of the CDR3 region with a quantification of each Vβ family mRNA within a T-cell pool, known as T-cell landscape (TcLand), this group has analyzed the T-cell receptor repertoire used, following direct alloantigen presentation (116). They have shown that T-cell interaction with MHC-mismatched APCs triggers the activation of T cells with specific Vβ families, regardless of their CDR3 regions (as seen in indirect recognition) but specific to the combination being studied.

It may be that these patterns of Vβ-gene usage and CDR3 length may contribute to the development of a 'fingerprint' of tolerance. Regulatory T-cell clones characterized by the presence of an altered T-cell receptor repertoire have been implicated in the tolerance that is induced by pregraft donor blood transfusion (117, 118). In a model of infectious tolerance in a rat cardiac allograft system, a unique pattern of CDR3 spectratyping was identified (119).

This approach is still some way away from clinical applicability. However, direct correlations with functional states of

interest have reawakened interest in this method, and it may yet prove to be of significant prognostic value in transplant recipients.

Microarray analysis

We are now able to use microchips to analyze the pattern of expression of tens of thousands of genes in tissues, in a given scenario. It is hoped that it will be possible to characterize the genes whose expression contributes to a protective phenotype and those which contribute to an aggressive one. This technology, although highly sophisticated, remains in its infancy with respect to clinical application. However, with our understanding that the process of tolerance is an active one, it seems likely that it will be associated with a pattern of activation of certain genes.

Work has already begun in the transplant sphere. Sarwal *et al.* (120) used a DNA microarray chip to try to establish a pattern of gene expression within renal biopsy specimens that was associated with acute rejection. They identified three patterns that correlated with differences in cellular proliferation and patterns of cellular infiltrate on immunohistochemical staining. Specifically, there was a striking association between an infiltrate that included large numbers of $CD20^+$ B cells and both steroid resistance and graft loss. The power of this technique is such that it will be surprising, if it does not provide many more insights into the underlying biology of transplantation and tolerance and, thereafter, the care of patients.

Cytokine mRNA quantification by real-time polymerase chain reaction

The above approaches will be particularly sensitive to all-or-none phenomena; either a gene is or is not transcribed. Clearly, to some extent, the phenomenon is the aggregate effect of a number of different phenomena, and the population of transplant recipients is more likely to represent a spectrum of immunological responses rather than two discrete populations. One implication of this range is that some apparently tolerant individuals will be living on a knife-edge and find it easy to have their tolerance broken. Alternatively, the immune response of some people to their grafts may be easily converted into tolerance. A sensitive approach to quantitative assessment of the immune response using cytokines, the molecular messengers of the immune response, is quantitative real-time polymerase chain reaction (PCR).

Using fluorogenic molecules, it is possible to measure the accumulation of product during PCR in a quantitative fashion. Its use on cytokine mRNA has been well established. It remains to be determined whether this measurement can be correlated with functional status in the transplant recipient (121). These methods have also been used in murine systems to measure cellular immune responses (122).

The experience of immune monitoring in the transplant setting

Different attempts at monitoring lymphocyte responses to assess donor-specific immunity or tolerance have been published using LDAs with different readouts (proliferation, cytokine production, or cytotoxicity) in the transplantation context. The largest clinically useful experience in immune monitoring has been carried out in the context of hematopoietic cell transplantation from an unrelated donor for the prediction of GVHD and also for donor selection. The need was driven here by the lack of high-resolution tissue typing techniques, and functional assays were critical up until 10 years ago. LDAs were initially applied to the measurement of human alloreactive CTLp (32). The estimation of host-reactive CTLp in peripheral blood of donors has been shown to be predictive of acute GVHD and survival in a series of studies (46–48, 50), but this finding has not been universal (123). Host reactive IL-2 HTLp frequencies have been shown to correlate with outcome in bone marrow transplant in identical siblings (40, 124) and unrelated donors (47, 50, 125). In all these studies, where the donor and the recipient share the majority of HLA-molecules, it is assumed that both direct and indirect responses are being detected simultaneously. Functional assays will have a lasting role in bone marrow transplantation, even in the era of high-resolution typing. These assays can define permissible mismatches, and they can be used to discriminate the less immunogenic donor in the absence of a completely matched donor.

The experience in solid organ transplantation is less extensive, though a renewed effort is underway to dissect mechanisms of tolerance and rejection. Direct pathway responses have been the subject of a number of studies. Donor-specific cytotoxic T-cell hyporesponsiveness that develops sometime after kidney (52), heart (44, 45, 126), or lung (10) transplantation has been described. However, as before, this finding has not been universal (53,127–129). Hyporesponsiveness of helper T lymphocytes has also been described (126, 130, 131), although conflicting data have been reported (132). In several studies the strength of direct pathway anti-donor responses correlated with rejection episodes and clinical status (7–9, 44, 131, 133) but not in others (52, 126, 130). Three groups

have attempted to measure responses via the direct and the indirect pathways in the same group of patients (12, 134, 135). The tentative conclusions from these studies were that the direct pathway anti-donor response diminishes with time after transplantation and that raised frequencies of T cells with indirect anti-donor reactivity correlate with the presence of chronic transplant rejection. If the indirect pathway is critical in mediating transplant rejection, it follows that abolition of the direct pathway alone will not achieve allograft tolerance. Indeed, strategies that promote tolerance in the indirect pathway should increase allograft survival, as has been demonstrated in animal models (136).

What lies ahead

This area of research clearly has potential applications outside the transplantation setting. Several other therapeutic strategies will benefit from developments in this field. Vaccine effectiveness both to infectious agents and malignancies are already using some of the techniques described here.

In this era of increased opportunities to translate developments in basic immunology for the benefit of the transplant patients, where tolerance-inducing strategies are being transferred from the bench to the bedside, it is of central importance that the *in vitro* assays described here are improved to allow the identification and perhaps even quantitation of tolerance. It will be crucial to be able to differentiate this outcome from a lack of response or assay insensitivity. With the variation in values that is so commonly found with different assays, further clinical data need to be gathered to identify which method or combination of methods gives the most clinically informative frequencies. No gold standard can be established as yet. For this reason, a collaborative group in Europe and USA has been formed with a view of finding the fingerprint of tolerance. Results from these and other similar initiatives will provide information that will be invaluable in the context of designing clinical trials of putatively tolerance-promoting protocols and in guiding decisions about drug weaning.

For too long, the clinical practice of immunology has not had the power to quantitate its interventions in a straightforward way that has now become second nature to the cardiologist or the pulmonary physician. There is now reason to be optimistic that this era of ignorance and paralysis may soon come to an end.

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