In Vitro Analysis of Cell-Mediated Immunity: Clinical Relevance
Gene M. Shearer and Mario Clerici

Cellular immunity, a major component of the immune system, contributes to protection against infections and plays a role in the rejection of foreign allografts. T helper cells and the antigen-presenting cells with which they interact provide important functions for cellular immune responses by generating helper signals via soluble molecules known as lymphokines or cytokines. As described here, in vitro analysis of markers of cellular immune function can be used for clinical diagnosis and assessment of therapeutic efficacy and to elucidate the mechanisms of immune dysregulation. Not all helper signals necessarily have the same augmenting effect, because immunoregulatory cytokines that up-regulate the cellular arm of the immune system can conversely down-regulate the humoral arm, and vice versa. The interplay between cross-regulatory cytokines determines which component of the immune system is dominant and can influence the clinical outcome of immunologically controlled diseases. Given the apparent dramatic effects of these cytokines, assays performed in future clinical laboratories should include profiles of immunoregulatory cytokines.

Indexing Terms: immune system/T helper cells/lymphokines/cytokines/HIV/systemic lupus erythematosus/Hodgkin disease

The immune system can be divided into two broad categories based on the effector mechanisms responsible for the prevention or elimination of infection or those for the destruction of foreign tissue transplantation (allograft rejection). These categories are frequently referred to as the cellular (C) and humoral (H) arms of the immune system, and their respective effector mechanisms involve cell-mediated destruction of infected cells or foreign tissue (“C-type” immunity), and antibodies that can bind to free antigen or antigens expressed on cells (“H-type” immunity) (1). Although these two components were initially considered to act in synergy to combat infection or to induce allograft rejection, it is becoming more apparent that C-type and H-type immune responses can mechanistically act in opposition, although both may contribute to an overall effect of eliminating an infection.

Whether a C-type or an H-type response will predominate depends on several factors, only some of which are understood. Of great importance is what type of helper (or regulator) cell is stimulated by antigen. T-helper cells (Th) can be divided into at least two types based on the immunoregulatory cytokines produced, and on whether the Th predominantly activate C-type or H-type responses. Thus, Th1-like or type-1 helpers produce and (or) respond in a positive way to the cytokines interleukin-2 (IL-2), interferon-\(\gamma\) (IFN-\(\gamma\)), and IL-12 and help mainly (but not exclusively) C-type responses. In contrast, Th2-like or type-2 helpers produce and (or) respond in a positive way to IL-4, IL-5, IL-6, and IL-10 and help mainly (but not exclusively) H-type responses. Some of these cytokines are cross-regulatory, in that they not only up-regulate one arm of the immune system but conversely also down-regulate the other arm. Thus, C-type and H-type responses are controlled by type-1 and type-2 regulatory signals, which are mediated by immunoregulatory cytokines.

Our laboratories began to study Th immune-dysregulation in asymptomatic individuals infected with human immunodeficiency virus (HIV\(^+\)) several years ago; we observed, along with other laboratories, that Th responses defined by T-cell proliferation and antigen- or mitogen-stimulated IL-2 production are defective before the more obvious decline in CD4 count occurs (2–4). Our research efforts have continued to focus on this phenomenon, and the descriptive experimental section below summarizes our findings in the HIV work. We have found that empirical immunologic functional tests can be used to stage patients and predict disease states, and have identified three functionally distinct categories of Th dysfunction that are not distinguishable by current clinical markers such as CD4 count or p24 antigen burden. Having used these functional indicators to assess the therapeutic efficacy of anti-HIV reagents, we report these findings and suggest how they can be extended to investigate other conditions of immune dysregulation.

Experimental Studies

Peripheral blood mononuclear cells (PBMC) from HIV\(^+\) individuals were stimulated in vitro with: (a) recall antigens (REC) (influenza virus and tetanus toxoid), (b) irradiated stimulator PBMC from healthy unrelated donors (ALLO), and (c) the T-cell mitogen phytohemagglutinin (PHA). These stimuli were chosen because of their differing requirements for antigen-presenting cells (APC): The response to REC requires autologous APC, the ALLO response can use either autologous APC or APC provided by the allogeneic stim-
ulator leukocytes, and PHA stimulation is less dependent on APC than are the other two stimuli. After 1 week of stimulation, the cultures were assayed for T-cell proliferation and IL-2 production as previously described (4). We generally used 3 x 10^6 responding PBMC in a volume of 0.2 mL plated in 96-well flat-bottom tissue culture plates. For any REC used, we would titrate the antigen for optimal responses by PBMC from healthy control donors. For the ALLO response, we used 1 x 10^6 pooled PBMC (pooled from two or three unrelated healthy donors) irradiated with 5000 rad (50 Gy) to ensure that the response detected is the response of the PBMC from the test donor. Because PHA preparations differ in their mitogenic properties, it is important to titer the PHA for optimal stimulation.

In studying 1000 HIV+ individuals, we observed four different categories of response: (a) individuals who responded to all three stimuli; (b) individuals who responded to ALLO and PHA, but not to REC; (c) individuals who responded only to PHA; and (d) individuals who failed to respond to all three stimuli of stimulation (4). We categorized each response pattern as follows: (a) +++, (b) +++, (c) +++, and (d) ---. The respective frequencies of these four categories were 34%, 40%, 11%, and 15%. We discriminated between the positive and negative responses by determining IL-2 production and proliferation in response to a particular stimulus in a large number of healthy, control donors. The mean response for a particular stimulus was calculated and patients were defined as negative if their response was 2 SD below the mean value for the controls (4).

From our comparisons of IL-2 production with proliferation, we consider the IL-2 assay more accurate, this test being based on a bioassay that used the IL-2-sensitive CTLL cell line and a titration of culture supernates across five twofold dilutions (triplicate samples per dilution). Generating such a titration curve allows calculation of IL-2 units. In contrast, the proliferation assay uses only triplicate wells for measurement of [3H]thymidine uptake. This is not meant to imply that the proliferative assay does not give meaningful results; moreover, it is less expensive and less time-consuming than the IL-2 bioassay.

Perhaps more relevant is the possibility of detecting cytokine production (including, but not limited to, IL-2) by the recent development of sensitive cytokine ELISA assays and kits. ELISAs offer a sensitive and rapid approach that should be readily adaptable to clinical laboratories. Four years ago, we compared the IL-2 bioassay with some of the available IL-2 ELISA kits and estimated that the bioassay was 10- to 100-fold more sensitive than the ELISAs. Current IL-2 ELISA kits (from different suppliers) are now as sensitive as the bioassay. The main advantages of the ELISA kits are convenience and the fact that one can test for multiple cytokines, including IL-2, IFN-γ, IL-4, IL-10, and tumor necrosis factor-α; this will become important for establishing cytokine profiles that may be associated with particular disease states. The main disadvantage of the kits is their cost.

The functional stages (a-d) described above were found to be sequential and progressive such that the loss of response to REC preceeds that to ALLO, which in turn preceeds unresponsiveness to PHA. We also observed that this sequential progression is predictive for three clinically relevant acquired immunodeficiency syndrome (AIDS)-related events: decline in CD4 count; time to AIDS diagnosis; and time to death. Thus, HIV+ individuals who are +++++ do not exhibit an appreciable decline in CD4 count during the subsequent 18-24 months, whereas individuals who are in the categories that have T4 defects show a significant decline in CD4 count (5). Furthermore, ~8% of individuals who are +++ will develop AIDS in the subsequent 36 months; ~23% of individuals who are +++ will be diagnosed with AIDS in the same time interval; and ~48% of those who are +++ or ++++ will develop AIDS within 36 months (6). A similar pattern is seen for time to death (6). These findings indicate that our in vitro T-cell immune functional test detects a predictive course in progression to AIDS that begins before currently identified AIDS symptoms appear.

If an in vitro T-cell assay can be used as a marker for AIDS progression, perhaps the same test could be used as an indicator of therapeutic efficacy. To test this possibility, we categorized the in vitro T-cell immune status of AIDS patients before, during, and after therapy, using three different anti-HIV protocols involving small numbers of patients: zidovudine (ZDV); dideoxynosine (DDI); and soluble recombinant CD4-IgG (rCD4-IgG). We found that 75% (12 of 16) of patients exhibited improved Th function (defined as a threelfold increase in Th function to at least two of the three stimuli) after receiving ZVD (7). Of 22 pediatric AIDS patients on DDI therapy, 11 (50%) showed improved in vitro T-cell responses, and the improved Th function correlated with an absence of recurrent opportunistic infections (8). Finally, 80% (8 of 10) of AIDS patients receiving rCD4-IgG showed improved Th function within 2 weeks of initiating therapy (9).

These findings demonstrate the potential value of an in vitro functional immune test for assessing therapeutic efficacy. Another potential advantage over CD4 count is that changes in Th function can be seen within 1 month of initiating therapy, whereas significant increases in CD4 count often take considerably longer. As Table 1 illustrates, these T-cell functional assays can be more predictive for therapeutic efficacy than CD4 count. Thus, on posttherapy follow-up, 12 pediatric patients who exhibited improved Th function presented with no episodes of recurrent opportunistic infections, whereas 10 of the patients who showed no evidence of improved in vitro Th function presented with 26 episodes of recurrent opportunistic infections (9). This correlation between Th function and recurrent infections was statistically significant, in contrast to the CD4 count, CD4:CD8 ratio, and HIV p24 antigen, none of which correlated with improved Th function.

In vitro Th function can also provide insight into the mechanisms responsible for immune dysregulation,
Table 1. Clinical follow-up of pediatric AIDS patients on dideoxynosine therapy.

<table>
<thead>
<tr>
<th>No. (%) of patients with</th>
<th>Infected</th>
<th>Uninfected</th>
</tr>
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<tbody>
<tr>
<td>Total no. of patients</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Opportunistic infection*</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>Improved T-helper function</td>
<td>9 (75%)</td>
<td>2 (20)</td>
</tr>
<tr>
<td>Increased CD4 count</td>
<td>5 (42)</td>
<td>3 (30)</td>
</tr>
<tr>
<td>Increased CD4/CD8 ratio</td>
<td>6 (50)</td>
<td>6 (60)</td>
</tr>
</tbody>
</table>

* Indicates number of episodes of recurrent opportunistic infections.

thereby providing more options for therapy. As noted above, C-type and H-type responses are regulated by type 1 and type 2 cytokines, which tend to act in opposition. Thus, deficiencies in C-type responses and the accompanying cytokine profile detected in vitro can be up-regulated by exogenous addition of type 1 cytokines such as IL-12 (10) or by antibodies against type 2 cytokines (11, 12). These in vitro demonstrations of changes in immune function can be used to suggest immune-based therapeutic approaches that could be helpful in controlling diseases and conditions that involve immune dysregulation.

In vitro assays of Th function such as antigen-stimulated proliferation or IL-2 production can be used to determine whether individuals have been immunized against infectious agents such as HIV, either as vaccines or by natural exposure or infection. Thus, PBMC from individuals immunized against recombinant gp160 (rgp160) as a candidate AIDS vaccine responded in vitro to restimulation with rgp160, and antibodies specific for HIV were detected in the sera (13). However, low-dose immunization with rgp160 did not elicit serum antibody responses. Surprisingly, the low-dose immunized vaccines generated strong in vitro HIV-specific cell-mediated immune responses, even 1 year after the last boost. We have recently interpreted these results as indicating that very low-dose immunization can result in selective C-type but not H-type responses (14).

Several laboratories have reported that surprisingly high percentages of seronegative individuals exposed to HIV still exhibit in vitro T-cell responses to HIV antigens, despite their failure to seroconvert (15-23). We have suggested that such individuals may be protected against HIV infection (24). The studies raise the possibility that protective immunity does not always occur via antibody-mediated mechanisms. Thus, from a clinical perspective, analyses for correlates of protective immunity should not rely solely on antibody tests (H-type) but should also include cell-mediated immune (C-type) assays. To rely exclusively on serum antibody assays will test for only one arm of the immune system and could miss an important correlate of protection. In retrospect, it may be worth questioning whether some of the failed vaccine trials might have shown more promise had C-type function been included in the assays.

Table 2. Comparison of Th function patterns in HIV+ patients with those with systemic lupus erythematosus (SLE) and Hodgkin disease.

<table>
<thead>
<tr>
<th>% of patients in disease category</th>
<th>HIV+ (n &gt;1000)</th>
<th>SLE (n = 176)</th>
<th>Hodgkin (n = 47)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th functional category</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>REC+/ALLO+/PHA+</td>
<td>34</td>
<td>48</td>
<td>40</td>
</tr>
<tr>
<td>REC+/ALLO+/PHA+</td>
<td>40</td>
<td>42</td>
<td>26</td>
</tr>
<tr>
<td>REC+/ALLO-/PHA+</td>
<td>11</td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td>REC+/ALLO-/PHA-</td>
<td>15</td>
<td>0</td>
<td>15</td>
</tr>
</tbody>
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Discussion

The above summary indicates that analysis of cell-mediated immune function of a syndrome as complex as AIDS can contribute to our understanding of the mechanisms involved in immune dysregulation, provide new strategies for therapy and vaccine design, and open the possibility for applying similar approaches to other immune-dysregulated diseases and conditions. Additional examples of diseases and conditions that appear to involve type 1/type 2 immune dysregulation include leprosy (25), measles (26, 27), systemic lupus erythematosus (28), some parasitic infections (29), insulin-dependent diabetes (30), and certain cancers. In fact, some interesting parallels that suggest type 1/type 2 immune dysregulation have been recently proposed between measles infection and HIV infection (27). We have begun to investigate the cellular immune functional profiles of lupus (28) and Hodgkin disease (31). Table 2 compares the relative frequencies of Th cell responses to REC, ALLO, and PHA in HIV+ individuals with the responses seen in more recent studies of lupus and Hodgkin disease. The profiles of cellular immune unresponsiveness for in the latter diseases were similar to those seen in HIV infection.

It remains to be determined whether immunologic dysregulatory patterns observed in these and other diseases and conditions contribute significantly to the particular disease or condition under investigation, or represent only an epiphenomenon. Irrespective, the types of in vitro tests outlined here should be useful for assessing immunologic markers related to disease state, and in the future may become part of the standard repertoire of immunologic tests used in clinical and diagnostic laboratories. The recent development and availability of sensitive ELISA tests for detecting immunoregulatory cytokines should permit much more rapid and complete assay systems than those that we have used to establish the potential value of in vitro tests of Th-cell function.

References