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Potential Clinical Applications of Signal Transduction Measurements in Marrow Transplantation and HIV-1 Infection^a

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INTRODUCTION

The use of flow cytometry to identify and isolate cells by surface antigen expression was a fundamental advance for immunologists and hematologists and now has an increasing role in the clinical practice of medicine. Similarly, the use of the flow cytometer to study lymphocyte activation and cellular metabolism has proved to be a powerful technique. Knowledge of signal transduction mechanisms has progressed to a point where clinical applications of signal transduction measurements can now be anticipated.

The development of a number of fluorescent probes for the study of intracellular ion concentrations in single, viable cells has permitted a substantial advance in the understanding of many areas of immunology.¹ It is well established that changes in intracellular free calcium concentration play a key role in some, but not all forms of B and T cell activation.^{2,3} Calcium was the first ion to be studied in lymphocytes by flow cytometry and the results of early studies involving lymphocytes have been summarized.⁴ Presently, two probes are commonly used for flow cytometric calcium measurement, indo-1 and fluo-3. A limitation of indo-1 is that it requires ultraviolet excitation, which is not available on all flow cytometers. Fluo-3 is a newly available probe¹ that, in part, circumvents this limitation.⁵⁻⁷ This probe can measure cellular

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ionized calcium with 488-nm excitation and is therefore universally applicable to currently available flow cytometers. Fluo-3 does not have intrinsic fluorescence properties that permit ratiometric determinations and is therefore less sensitive to calcium flux in small subpopulations of cells than indo-1. These limitations can be circumvented, in part, by the simultaneous use of another indicator, SNARF-1, which allows the variation in signal due to cell size and brightness to be eliminated.⁷ With proper optics, fluo-3 can be used in cells that are also surface-labeled with phycoerythrin-conjugated antibody.⁶

In this report, abnormal calcium homeostasis in the T lymphocytes from patients with two forms of acquired immunodeficiency is illustrated. Patients with HIV-1 infection have impaired calcium mobilization early in the course of the infection, before the onset of clinical immunodeficiency. This abnormality occurs in asymptomatic patients, before significant CD4 cell depletion. Finally, it is speculated that signal transduction will be clinically useful in other disease states.

BONE MARROW TRANSPLANTATION

Bone marrow transplantation (BMT) results in a severe immunodeficiency of both cellular and humoral immune functions in all patients. In cases of successful engraftment, the immune system returns to normal one to two years postgrafting.⁸ This can be delayed even further in cases where graft versus host disease develops or where the marrow is manipulated, as, for example, with T cell depletion of the donor marrow. Impaired T cell immunity is a major cause of morbidity and mortality after marrow transplantation, as evidenced by the high incidence of fatal pneumonia from cytomegalovirus.

A key question concerning the immunodeficiency is to what extent the immunodeficiency is the result of lymphopenia and to what degree impaired cellular function might contribute to the immunodeficiency. To answer this question, the responses of T cells were tested in a series of patients derived from the Medical College of Wisconsin and Wayne State University.⁹ In this study, the T cells from 23 patients were assessed in the first year after BMT. The calcium signal occurring after anti-CD3 monoclonal antibody stimulation of the peripheral blood lymphocytes from BMT patients was quantitated. The peak mean change in intracellular free calcium concentration ($[Ca^{2+}]_i$) was graded as "normal" (70–100% of control), "blunted" (35–70% of control), and "poor" (0–35% of control). Only 4 of 23 patients studied within the first year after transplantation had normal responses, whereas 5 of 7 patients studied more than one year after transplantation had normal responses.

The mechanism of this defect in calcium signal transduction was further studied in another group of patients at Wayne State University. To date, 29 patients have been studied, with a median age of 32 (range of 10 to 54). The responses of B and T lymphocytes to anti-Ig and anti-CD3 stimulation have been tested using flow cytometry as previously described.¹⁰ The cells were obtained from BMT patients at a median 94 days after grafting (range of 24 to 264 days). Twenty-seven of the patients had a marrow allograft and 2 had an autologous graft. Fourteen of the patients had clinically evident graft versus host disease and 26 of the patients were receiving some form of immunosuppression.

In the present study, a substantial proportion of patients were found to have a marked decrease in the percent of responding T cells after anti-CD3 stimulation (FIGURE 1, lower panel). Under these conditions of anti-CD3 treatment, more than 50% of normal T cells can be observed to respond in the flow cytometric assay, whereas more than half of the BMT patients had <20% of their T cells respond. Most intriguingly, this defect in T cell responsiveness appears to be specific, as the fraction of responding B cells appears to be normal (FIGURE 1, upper panel). These results are preliminary because fewer observations of B cell responses are available, as this part of the assay was added to the protocol more recently.

The mean calcium response of patient T cells was also reduced (TABLE 1). In TABLE 1, the mean calcium response of T and B cells from BMT patients was compared to the response of the companion control sample. Approximately two-thirds of the BMT patients had poor or blunted responses, whereas B cell responses were again relatively preserved. The impaired T cell responses tend to recover with increasing time after BMT (TABLE 2), confirming earlier results.⁹

The mechanism of the impaired calcium signal transduction after BMT remains to be elucidated. Multivariate analysis has failed to show a clear association of any specific form of immunosuppression, such as cyclosporine, or any complications of BMT, such as graft versus host disease. It remains to be determined whether this defect in signal transduction might be related to the risk of developing opportunistic infections after BMT. It is intriguing to speculate that the abnormal thymic microenvironment after BMT may result in abnormal or delayed T cell maturation. Finally, the pace of normal maturation of lymphoid signal transduction remains to be determined in the peripheral human immune system. Ethical constraints have prevented the assessment of B and T cell signal transduction during human prenatal and postnatal development.

HIV-1 INFECTION

Patients with HIV-1 infection often remain asymptomatic for years after the initial infection. Whereas the late and progressive phase of HIV-1 infection is characterized by a decline in circulating CD4⁺ T cells, the latent phase is characterized by normal or nearly normal CD4⁺ T cells that have functional impairments. For example, CD4⁺ T cells from patients with late-stage HIV-1 infection have decreased proliferation to specific recall antigens, whereas proliferation to mitogens remains normal.¹¹ Similarly, IL-2 production and T cell proliferation in patients with AIDS were impaired in the autologous mixed lymphocyte reaction, whereas alloantigen-induced proliferation was intact.¹² In a large study of patients with asymptomatic, early-stage HIV-1 infection, impaired IL-2 production to recall antigens such as tetanus toxoid was commonly observed.¹³

Many studies suggest that defective signal transduction in HIV-1-infected cells might account, in part, for the functional impairments commonly observed in the cells from HIV-infected patients (summarized in reference 14). Baseline inositol trisphosphate levels are increased in an HIV-1-infected cell line.¹⁵ We found that T cell receptor-induced calcium fluxes were impaired in CD4⁺ T cells infected *in vitro* with HIV-1.¹⁶ This signal transduction defect was selective, as anti-CD2 monoclonal antibody-induced calcium flux remained intact.

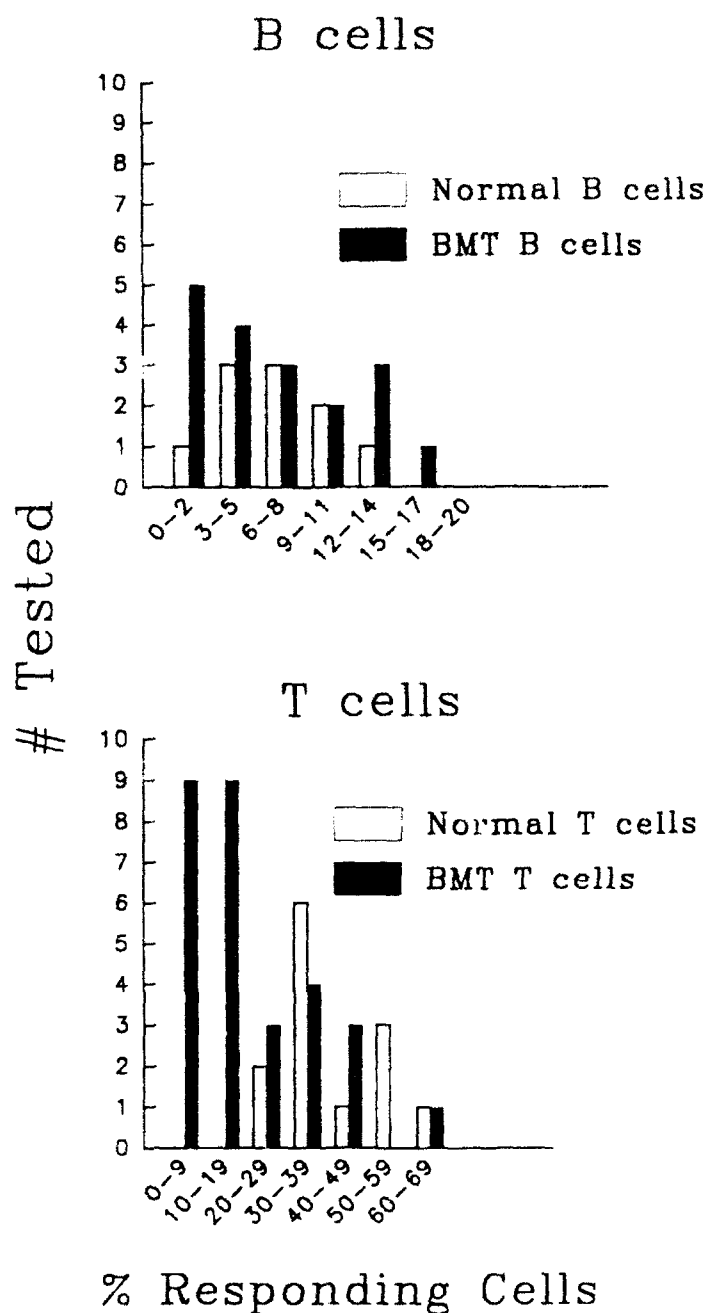


FIGURE 1. Calcium mobilization in B and T cells from marrow graft recipients. Peripheral blood lymphocytes were isolated by gradient centrifugation, loaded with indo-1, and stained with a cocktail of phycoerythrin-labeled monoclonal antibodies so that B or T cells could be identified by negative selection.¹⁰ The cells were equilibrated at 37 °C and anti-CD3 antibody or anti-Ig was added in order to stimulate T and B cells, respectively. The percent of responding cells at 2 SD's above the baseline value was calculated as previously described.¹⁰ Control samples from normal volunteers were analyzed at the same time as the samples from the BMT patients. To date, T cells from 29 BMT patients and 13 controls and B cells from 18 BMT patients and 10 controls have been tested. Histograms of the percent responding cells are depicted.

TABLE 1. Relative Calcium Mobilization in B and T Cells from Marrow Transplant Recipients

Cell Subset ^b	Type of Response ^a [No. of Patients (%)]		
	Poor	Blunted	Normal
T cells	11/29 (38%)	9/29 (31%)	9/29 (31%)
B cells	5/18 (28%)	3/18 (17%)	10/18 (56%)

^aResponses were assessed by comparison to control samples. The responses were classified to an arbitrary scale of normal, blunted, and poor, representing 70–100%, 35–70%, and 0–35% of the control peak mean $[Ca^{2+}]_i$ response.

^bLymphocytes were obtained from marrow graft recipients, loaded with indo-1, and stained with phycoerythrin-conjugated antibodies in order to identify B and T cells by negative selection.¹⁰ The cells were stimulated with anti-CD3 monoclonal antibody or anti-Ig to activate T and B cells, respectively. Responses were quantitated by flow cytometry by measuring the changes in the mean $[Ca^{2+}]_i$ as assessed by changes in the violet and blue indo-1 fluorescence. The response of B and T cells was determined by gating on the red fluorescence using negative selection.

We have now studied a series of patients with early-stage HIV-1 infection to determine whether impaired signal transduction occurs during *in vivo* infections. Twenty-one patients with early-stage HIV-1 infection (Walter Reed stages II–III) were studied. Normal volunteer blood donors served as controls. Peripheral blood lymphocytes were isolated by Ficoll density gradient centrifugation. The cells were loaded with indo-1 and CD4⁺ T cells were identified by staining with anti-CD5 and anti-CD8 monoclonal antibody and gating on the CD5^{bright} CD8⁺ cells. The cells were stimulated with anti-CD3 monoclonal antibody and the changes in the mean $[Ca^{2+}]_i$ and the fraction of responding cells were analyzed by flow cytometry as previously described.¹⁰ For these studies, the flow cytometer was modified to prevent the generation of biohazardous aerosols.

The peak mean $[Ca^{2+}]_i$ response of the cells from HIV-1-infected cells was significantly lower than the response of the control cells (TABLE 3). Comparison of

TABLE 2. Time-dependent Recovery of T Cell Responses after Marrow Transplantation

Days after BMT	No. of Patients	% Control Ca^{2+} Response ^a
30	1	2
60	4	34
90	9	40
120	6	55
150	2	96
180	3	69
240	4	65

^aThe response of indo-1-loaded T cells from marrow graft recipients was assessed as in TABLE 1 and FIGURE 1. The cells were stimulated with anti-CD3 monoclonal antibody and the peak mean change in $[Ca^{2+}]_i$ was determined. Cells were tested from recipients who were between 24 and 264 days after transplantation and the responses were assessed by comparison to control samples.

the responses of individual patients and controls shows little overlap between the data points (not shown). The percent of responding cells was also decreased in the HIV-1-infected patients (TABLE 3). The decrease was specific in that the response to calcium ionophore remained intact. The impaired response of the HIV-1-infected patients was not a shift in the kinetics of the response, as the time to peak response was the same in patients and controls. Some patients have severely impaired responses, whereas some are only moderately impaired. When the response to anti-CD3 stimulation is analyzed at the single cell level, the decrease in the peak mean response is a result of two components. First, there is a population of nonresponding CD4 T cells in HIV-1-infected patients and, second, some patients have a population of responding cells that exhibit a blunted response when compared to control patients.

The mechanism of the decreased calcium mobilization in HIV-1-infected patients remains undetermined. In uninfected T cells, there is evidence from fluorescence resonance energy transfer experiments indicating that CD4 is physically associated with the T cell receptor.^{17,18} It is possible that gp120 disrupts this association. However, many other mechanisms are possible.¹⁴

TABLE 3. T Cell Receptor-mediated Calcium Signals in Patients with Early-Stage HIV-1 Infection

	Anti-CD3 Stimulation	
	Peak Mean [Ca ²⁺] _i (nM) ^a	% Responding Cells ^a
Patients (n = 21)	443 ± 180	52 ± 14
Controls (n = 17)	1036 ± 381	81 ± 6

^aMean ± 1 SD.

Further studies will be required to determine if the defects in signal transduction correlate with disease outcome or are prognostic of the clinical course. If this proves to be the case, then signal transduction assays will be useful clinical tests to monitor the efficacy of many new and often expensive therapeutic interventions. At this time, the best validated marker is the CD4 count. The long natural history of HIV-1 infection and the increasing use of interventional therapies dictate a requirement for a sensitive test of immune function early in the course of infection, when the CD4 cell count remains normal. It is possible that an assay of cellular signal transduction might subserve this function.

Blackman and colleagues¹⁹ have recently shown that some forms of peripheral (nondeletional) T cell tolerance are accompanied with impaired signal transduction. When, in these studies, transgenic mice with T cell receptors specific for the Mls superantigen were bred to a strain that expresses self-superantigen, the T cells from the mice were hyporesponsive when compared to transgenic mice that did not express self-superantigen. Thus, it is possible that some forms of self-tolerance are the result of T cell receptor desensitization. If so, then many common autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis that are thought to represent a loss of self-tolerance might be assessed with clinical studies of signal transduction. Finally, it is likely that signal transduction will be useful for a

variety of clinical situations involving cells other than T cells. For example, platelet calcium homeostasis appears to be abnormal in women with preeclampsia.²⁰

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