Comparison of ATP Production in Whole Blood and Lymphocyte Proliferation in Response to Phytohemagglutinin

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Lymphocyte proliferation in response to mitogens, phytohemagglutinin (PHA), concanavalin A, pokeweed, and/or specific antigens has been the method of choice for in vitro diagnosis of cell-mediated immune dysfunction. Recently, an assay to measure intracellular adenosine triphosphate (ATP) production in response to PHA has been developed that requires a shorter, overnight incubation. We compared a standard 5- to 7-day lymphocyte mitogen stimulation assay utilizing tritiated thymidine (³H-thy) incorporation to one in which ATP production in response to PHA by CD4-positive cells is measured in a luminometer that requires only 18–24 hr. A total of 20 patient samples suspected of having decreased cell-mediated immunity

submitted for mitogen induced lymphocyte proliferation and 21 normal controls were tested in both assays. A comparison of these two methods has demonstrated that the screening ATP assay has a sensitivity at 24 hr of 100% in detecting decreased PHA induced lymphocyte proliferation at 5 days and a specificity of 85% in the samples obtained from normal controls. The data indicate that the ATP assay may be a useful screening tool for more rapid detection of blood samples with decreased cell-mediated immune responses. However, a positive screen should always be confirmed by ³H-thy uptake using mitogens and recall antigens like candida and tetanus. J. Clin. Lab. Anal. 21:265–270, 2007. © 2007 Wiley-Liss, Inc.

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INTRODUCTION

Patients with T-cell immunodeficiency diseases, including severe combined immunodeficiency disease (SCID), DiGeorge syndrome, Wiskott-Aldrich syndrome, ataxia telangiectasia, and chronic mucocutaneous candidiasis may have defective lymphocyte responses to nonspecific mitogens and/or to specific antigens, such as candida or tetanus. Moreover, patients with malignancies, following bone marrow transplantation, with severe viral infections, or undergoing immunosuppressive therapy for other reasons, may have severe suppression of cell mediated immune responses (1–4). The traditional method for cell-mediated immune function testing has been the lymphocyte proliferation assay. This assay involves a fairly large amount of blood (10 mL), isolation of lymphocytes

from whole blood by density gradient centrifugation, incubations varying from 4 to 6 days and the use of radioactive material, tritiated thymidine (³H-thy). Recently an assay for production of adenosine triphosphate (ATP) in whole blood has been approved by the U.S. Food and Drug Administration (FDA) for use in measuring immune cell function in solid organ transplant patients. The assay, developed by Cylex[®] Inc., known as the ImmuKnow[™] Cell Function Assay

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(Cylex[®] Inc., Columbia, MD), has been used extensively as an assessment tool for immune function in patients who have had solid organ transplants, including liver, kidney, and pancreas (5,6). This method utilizes small amounts (less than 0.5 mL) of whole blood, an overnight incubation, and does not require the use of radioactivity for detection of lymphocyte activation. We sought to determine if this phytohemagglutinin (PHA)-induced ATP production assay could be used as a rapid screen capable of detecting, in 24 hr, those samples that would require further analysis by standard lymphocyte antigen and mitogen induced ³H-thy uptake.

MATERIALS AND METHODS

Clinical Samples

Whole blood from 20 individual patients suspected of having defects in cell-mediated immunity and 21 normal controls were included in this study. All patient information was eliminated from the study in accordance with our Institutional Review Board (IRB #7275) to meet the requirements of the Health Insurance Portability and Accountability Act (HIPAA).

All samples were stored at room temperature until assayed. The ATP assay was performed within 30 hr as suggested by the manufacturer and proliferation was set up within 24 to 48 hr of blood draw.

Lymphocyte Proliferation

Lymphocyte proliferation was carried out as previously described (7–11).

Whole blood collected from both patients and normal control subjects, in acid citrate dextrose (ACD) Solution A, anticoagulated Vacutainer tubes (B-D, Franklin Lakes, NJ) was diluted, two parts blood to one part Hanks' Balanced Salt Solution (HBSS; Cambrex, Walkersville, MD). In some cases whole blood collected in sodium heparin was used and results were comparable to those collected with ACD. Mixed mononuclear cells (MMCs) were separated by density gradient centrifugation with Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ). MMCs were washed twice with HBSS and cell counts were adjusted to 1.0×10^6 /mL culture media made up of RPMI-1640 supplemented with 100 mM HEPES (Cambrex), 200 mM l-glutamine (Cambrex), 10 mg/mL gentamicin (Cambrex) and 10% heat-inactivated fetal bovine serum (Δ FBS) (ARUP Reagent Laboratory, Salt Lake City, UT). The MMCs were plated in sterile 96-well tissue culture plates, and media alone or phytohemagglutinin-P (PHA-P) (Sigma Chemical, St. Louis, MO) at final concentrations of 0.5, 2.5, and 5 μ g/mL, was added in triplicate wells. Incubations were carried out at 37.5°C with 5% CO₂ in

a humidified incubator. Cultures were incubated for 3 days, then pulsed with 1 μ Ci H³-thy (MP Biomedicals, Irvine, CA) and incubated an additional 6 hr. The cultures were then harvested onto glass fiber filtermats (Wallac, Wellesley, MA) using distilled water and a Brandel CH-055 Harvester (Brandel, Gaithersburg, MD) and the filtermats were counted in a Wallac 1450 MicroBeta Trilux (Wallac) scintillation counter. For each triplicate culture, the mean counts per minute (CPM) were calculated and a stimulation index (SI) (mean CPM of stimulated cells/mean CPM of media alone) was also calculated. An average of greater than 84,040 CPM was considered a normal response. Since there is disagreement in how different laboratories interpret and report data from lymphocyte proliferation assays, we have chosen to report both the CPM as well as the SI. SIs as well as mean CPM were compared to both the date-matched client-submitted control and a freshly drawn laboratory control obtained when the patient samples arrived in the laboratory.

ATP Assay

The Cylex[®] ImmuKnow[™] Cell Function Assay (Cylex[®] Inc.) was used to measure ATP production by CD4 cells after overnight incubation with PHA (12). All reagents and materials were included in the kit. Whole blood from both patients and normal control subjects, collected in sodium heparin anticoagulated Vacutainer tubes (B-D, Franklin Lakes, NJ) was assayed within 30 hr of collection. A 100- μ L volume of a 1:4 dilution was incubated in quadruplicate with or without PHA-L (2.5 μ g/mL) in an eight-well assay strip provided by Cylex[®]. The incubation was carried out overnight (15–18 hr) in a humidified 5% CO₂ incubator at 37.5°C. Magnetic beads coated with mouse monoclonal anti-human CD4 (Dynabeads[®] Dynal Biotech A.S.A., Oslo, Norway) were added, and CD4-positive cells were selected for both the stimulated and non-stimulated wells. After intermittent shaking during a 30 min incubation at room temperature, the cells were washed three times using a wash buffer containing buffered saline with bovine serum albumin provided by Cylex[®]. The cells were then lysed, utilizing the Cylex[®] Lysis Reagent, to release intracellular ATP. (The lysing reagent contained a hypotonic basic solution with detergent.) A 50- μ L volume of each stimulated and nonstimulated sample was transferred to an opaque measurement plate and 150 μ L of Luminescence Reagent (Cylex, Inc., Columbia, MD) containing luciferin and luciferase was then added to the cell lysate. Within 10 min, the bioluminescent product was measured in a luminometer. (GloRunner[™], Turner Bio-Systems, Sunnyvale, CA) The amount of light emitted

was compared to a standard curve of ATP calibrators (0, 1, 10, 100, and 1,000 ng/mL). The concentration of ATP in each sample was calculated based on the ATP standards, using an Excel-based software program provided by Cylex[®]. Values of ≤ 225 ng/mL ATP were considered a low response. A moderate response was reflected in the range of 226–524 ng/mL ATP and a strong response was considered anything above 525 ng/mL of ATP produced in response to PHA. For pediatric patients less than 12 years of age, values of less than 175 ng/mL of ATP were considered a low response. A response of 176–395 ng/mL ATP was considered a moderate response in pediatric patients and anything greater than 395 ng/mL ATP was considered a strong response (13).

RESULTS

Clinical specimens from 20 patients submitted to the ARUP Cellular Immunology Laboratory for lymphocyte proliferation were tested in both the traditional assay (isolated MMCs) and the ATP assay (whole blood). In addition, nine samples submitted along with patient samples as client controls, and 12 normal control subjects drawn in our laboratory, for a total of 21 normal controls, were tested by both methods.

Of the 20 patient samples tested, nine (45%) exhibited normal responses in both assays (Table 1). Seven samples (35%) had results considered as very low

responses in both assays. Four (20%) had low ATP production but normal proliferation in response to PHA. There was agreement between both assays in 16 out of 20 (80%) of the patients tested. Exact diagnoses were not available on most patients because of HIPAA regulations.

Whole blood samples from 21 normal controls were assayed in the ATP assay and isolated lymphocytes were tested in the traditional proliferation assay. A total of 20 (95%) of the 21 controls had results that agreed in both assays. A total of 19 (90%) had both normal proliferation and moderate to strong responses to PHA in the ATP assay. Two controls (10%) had low ATP levels but normal proliferation responses to PHA. These two laboratory controls were from normal individuals without known disease but one was on a nonsteroidal antiinflammatory agent for back pain. Laboratory controls ranged in age from 22 to 61 years, with an even distribution of males and females.

One patient had a diagnosis of SCIDs.

This patient had a SI of four with 320 CPM (Fig. 1) of ³H-thy uptake detected (normal > 84,040 CPM) in response to PHA, when tested in the traditional proliferation assay and a significantly low ATP level of 79 ng/mL (adult range: low response: ≤ 225 ng/mL ATP; pediatric range: low response < 175 ng/mL ATP). Thus, this patient's cells exhibited very low responses in both the traditional method of determining lymphocyte proliferation as well as the ATP assay.

TABLE 1. Comparison of ATP levels and lymphocyte proliferation in response to PHA in 20 patient samples*

Patient #	Age (years)	Sex	Proliferation (CPM)	ATP (ng/mL)	Agreement	Dx
1	3 mo	M	320 L	79 L	Yes	SCIDS
2	13	M	219,659 N	420 N	Yes	N/A
3	10	M	9,284 L	38 L	Yes	N/A
4	1	M	7,500 L	30 L	Yes	N/A
5	1 mo	M	7,200 L	149 L	Yes	N/A
6	9 mo	F	170,337 N	110 L	No	N/A
7	9	M	721,904 N	202 N	Yes	N/A
8	2	M	509,814 N	187 N	Yes	N/A
9	7 mo	M	657,421 N	210 N	Yes	N/A
10	11	M	364,950 N	258 N	Yes	N/A
11	50	F	388,619 N	80 L	No	N/A
12	1	F	47,297 L	27 L	Yes	SCIDS
13	1 mo	F	186,359 N	268 N	Yes	N/A
14	1	F	970 L	122 L	Yes	N/A
15	1	M	458,260 N	394 N	Yes	N/A
16	4	F	356,329 N	93 L	No	N/A
17	4	F	478,578 N	153 L	No	N/A
18	29	F	171,691 N	230 N	Yes	N/A
19	1	F	209,907 N	337 N	Yes	N/A
20	6 mo	F	2,325 L	130 L	Yes	N/A

*Proliferation normal value: >84,040 CPM. ATP normal value: pediatric (<12 year: >175 ng/mL); adult >225 ng/mL. Dx, diagnosis; N/A, Dx not available due to HIPAA regulations; N, normal; L, low; mo, month(s); M, male; F, female.

Fig. 1. A comparison of two lymphocyte function assays in a patient with severe combined immunodeficiency (SCID). Proliferation data is expressed as CPM of ³H-thy incorporation. The dashed line represents the normal value cutoff of 84,040 CPM for LMP as indicated on the right y-axis. The solid line depicts the low cutoff (<225 ng/mL), for ATP responses on the left y-axis. The black bars represent the ATP levels and the superimposed white bars, the proliferation data.

Fig. 2. A comparison of two assays of lymphocyte function in a patient with an undefined combined immunodeficiency. Proliferation data is expressed as CPM of ³H-thy incorporation. The dashed line represents the normal value cutoff of 84,040 CPM for LMP as indicated on the right y-axis. The solid line depicts the low cutoff (<225 ng/mL), for ATP responses on the left y-axis. The black bars represent the ATP levels and the superimposed white bars, the proliferation data.

Another patient, though not diagnosed with classic SCID, had a combined immunodeficiency with lymphopenia suggestive of SCID, and went on to receive a marrow transplant (Fig. 2). When tested in the traditional lymphocyte proliferation assay the patient had a SI of 135 but with only 47,297 CPM (normal > 84,040 CPM) in response to PHA and ATP produc-

tion of only 27 ng/mL (low response < 175 ng/mL). Both are low responses, suggestive of significantly depressed cell mediated immunity.

When compared to the “gold standard” of lymphocyte proliferation, the ATP assay demonstrated 100% sensitivity in detecting samples with low ³H-thy uptake, and 85% specificity in normal controls.

DISCUSSION

The Cylex[®] ImmuKnow[™] assay for measuring ATP in whole blood in response to PHA has potential as a screening method for rapidly screening for decreased cell-mediated immunity in blood samples. We do not propose that it be utilized to replace traditional antigen- and mitogen-induced ³H-thy uptake but rather it could serve as a rapid 18–24 hr screen prior to the traditional 5- to 7 day proliferation assay.

The data indicate that an abnormally low value in proliferation was always accompanied by a low ATP result.

The production of intracellular ATP is one of the first steps in cellular activation upon stimulation with mitogens such as PHA. The incorporation of ³H-thy in the process of proliferation occurs much later. Other events include the rearrangement of cell surface receptors and cytokine production resulting from stimulation by mitogens and antigens. ATP production is, thus, utilized as an early indicator of a response to immune stimuli, while proliferation and thymidine incorporation is obviously a much later response.

The use of whole blood rather than isolated lymphocytes in the ATP assay may, in fact, provide a better measurement of actual immune function than proliferation as the cells remain in the same milieu as that in vivo. In the two cases in which the ATP generation was low and the traditional proliferation assay was normal, one might question whether the prolonged incubation time in media in the proliferation assay allowed the isolated cells to recover from immunosuppression. Possible bacterial or endotoxin contamination of samples submitted for the proliferation assay may also contribute to the higher values observed since there could be as long as a 48-hr time lapse between the blood draw and assay set up and 3 to 5 more days of incubation. The ATP assay must be performed within 30 hr of the blood draw, however, a fact that was confirmed by studies in our laboratory (data not shown).

Although there is no adjustment for cell number in the ATP assay as in the proliferation assay, the anti-CD4 monoclonal antibody-coated magnetic beads immunoselect the CD4-positive cells, which may include helper-inducer T cells as well as a small percentage of monocytes. The abbreviated format of the ATP screening assay, which again has been FDA-approved for assessing cell-mediated function in transplants, does not require specific enumeration of cell numbers but employs a small volume of whole blood. We ran the two assays as described and did not do cell counts on the whole blood used in the ATP screen but only enumerated the mononuclear cells isolated from whole blood in the proliferation assay. Still we were able to

detect 100% of the samples which had decreased proliferative responses with whole blood. An excellent review (14) on measuring human T-lymphocyte function points out how difficult it is to enumerate T cells before and after stimulation, “which is not possible in most cases because the responding cells represent only a small percentage of the total cell population at the start of the assay” (14). In our present comparative studies, we did not adjust to a standard cell population but we likely measured the responding cells in both assays and our data suggests the ATP assay can be used as a sensitive screen to detect 100% of the samples with low proliferative responses. In future studies we plan to investigate other potential markers such as enumeration of CD3, CD4, and CD8 T cell counts, and determine T-cell excision circle numbers, as well as CD69 expression, in patients suspected of SCIDs and other T-cell abnormalities (15–17).

Clinicians who suspect that a patient has depressed cell-mediated immunity may be able to obtain a preliminary answer about the status of a patient’s cell-mediated immune response using the whole blood, ATP assay, after only 24 hr. The screening assay could then lead to the more expensive, time-consuming proliferation assay, or a reflexive assay panel could be developed, employing the ATP assay first, followed by lymphocyte proliferation in response to both mitogens and antigens. Early notification of the results of the ATP assay may allow clinicians to institute *Pneumocystis carinii* and fungal prophylaxis in suspected SCIDS patients as well as instituting partial isolation in order to prevent severe and potentially fatal infections prior to receiving the results of the classic proliferation assay. The results of a low ATP screening assay might, therefore, significantly affect patient care and improve survival in these often fatal disorders.

The Cylex[®] ImmuKnow[™] assay has been FDA-approved for use in monitoring immune function in solid organ transplant patients. We suggest that this same method may provide an efficient screening assay, providing more rapid results prior to the availability of the more lengthy, lymphocyte proliferation procedure now utilized as the gold standard for diagnosing cell-mediated immune dysfunction. Obviously, the cost of the FDA-approved assay as well as the need for a luminometer designed to measure ATP production must also be included in evaluating the cost effectiveness of the test as a screen for cell-mediated immune function.

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