B and T Lymphocytes: Quantitation, Function, and Clinical Applicability

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ABSTRACT

Methodologies for T and B lymphocyte quantitation, lymphocyte blast transformation (LBT) and carbohydrate (CHO) metabolism are important for assessing host lymphocyte response in the clinical laboratory. Modifications of methods for each of these techniques are presented. Results from studies of normal ambulatory adults, patients with diabetes mellitus, sickle cell disease and hyperlipidemia are reported. LBT of normal lymphocytes before and after ethanol exposure are examined. LBT during pregnancy is evaluated. T cell populations are abnormally high in black diabetics and decreased in patients with sickle cell anemia. B cell subpopulations are increased in patients with sickle cell anemia. LBT responses are decreased in maturity onset diabetes, during pregnancy and in patients with sickle cell disease. Ethanol in amounts attainable during human consumption results in significantly decreased LBT response. CHO metabolism (especially hexose monophosphate shunt [HMPS] and HMPS by pentose sugar recycling) is abnormal in diabetic lymphocytes. The low HMPS activity is partially reversible by treatment with prostaglandin synthetase inhibitors. Information related to lymphocytes in normal states remains to be collected by further clinical application of these techniques of quantitation and in vitro function.

Introduction

Clinical relevance of T and B cell typing is most clear in the analysis of immunodeficiency diseases. T lymphocytes form rosettes with sheep erythrocytes (E). This E rosette test is the basic assay for the quantitation of T cells. Thymocytes and a subset of peripheral blood T cells will rosette at low sheep cell to mononuclear cell ratios at 37°C. These experimental conditions define the so-called “active” E-rosette test.4,8,9 This “active” test is more useful in lymphomas (e.g., childhood acute lymphoblastic leukemia) than the “total” E-rosette test. Both active and total T cell quantitation are used to elucidate immunopathological mechanisms for a variety of diseases.18,22

To study immunodeficiency states, it is necessary to know the percent and absolute number of T lymphocytes. Care must
be taken in calculating absolute numbers of subpopulations of lymphocytes from the raw percentage data. The absolute number is calculated by multiplying the percentage of T cells by the concentration of lymphocytes in a Wright stained peripheral blood smear. The Ficoll-Hypaque interface can contain other cells such as eosinophils or early myeloid cells. The presence of these cells must be determined so that they do not complicate the calculation of the total number of T cells.\textsuperscript{5,22}

B cells are quantitated by assaying a number of cell membrane markers. Despite considerable overlap of the various markers (e.g., slg, Fc) within the B cell lineage, the existence of subsets of B cells bearing only one or two markers means that use of any one marker alone may not be adequate for typing B lymphocytes.\textsuperscript{3-6} B cell markers (slg, Fc and C' receptors) have been detected on T cells under a variety of conditions.\textsuperscript{13} Therefore, the interpretation of the presence or absence of a given marker is defined within the context of the test used to detect the marker.\textsuperscript{13,21,23} Staining of surface immunoglobulins with fluorescein conjugated anti-immunoglobulin is a popular method for identification of B cells. Cells other than lymphocytes may have complement receptors and can cause confusion in the identification of macrophages versus lymphocytes. Macrophages or monocytes present no problem if marked by latex ingestion before testing.\textsuperscript{23}

Lymphocyte transformation can be used to measure lymphocyte function in genetic or acquired immunodeficiency states and to follow responses to therapy for these conditions. Since 1960, when Nowell discovered that normal lymphocytes will transform into large blast-like cells when cultured with phytohemagglutinin (PHA), this phenomenon has been used as the basis for the most widely used \textit{in vitro} test of lymphocyte function.\textsuperscript{19} Lymphocyte blast transformation (LBT) in response to lectins or specific antigens can be quantitated by measuring the associated increase in deoxyribonucleic acid (DNA) or protein synthesis by use of either tritiated thymidine (\textsuperscript{3}H TdR) or leucine, respectively. Transformation to nonspecific mitogens, such as PHA, Concanavalin (CON-A) or pokeweed mitogen (PWM), occurs in many different lymphocytes. Responses to specific antigens occur only in lymphocytes previously sensitized to that antigen and are actually secondary responses \textit{in vitro}. In general, LBT correlates well with other tests of delayed hypersensitivity such as skin tests.\textsuperscript{10}

The whole blood LBT assay, which will be described, was chosen for its simplicity, sensitivity and small amount of blood required. It is useful as a screening test. Abnormal responses can be further evaluated by using separated mononuclear cells and testing for serum inhibitory factors.

Carbohydrate (CHO) metabolism can be assessed as an index of lymphocyte function. Hexose monophosphate shunt (HMPS) activity increases as lymphocytes undergo blast transformation. Decreased HMPS activity is found during \textit{in vitro} lymphocyte blast transformation in patients with leukemia and diabetes.\textsuperscript{5} Glucose-6-phosphate dehydrogenase (G-6-PD) and 6-phosphogluconate dehydrogenase (6-PGD) levels are decreased in leukemic patients.\textsuperscript{1} These two enzymes are important rate-limiting factors in HMPS activity.

Quantitation, functional response to mitogens and assessment of lymphocyte populations are of increasing importance. This paper describes the methods and clinical applications of: (1) T and B lymphocytes subpopulation counts (2) LBT as measured by \textsuperscript{3}H TdR uptake and (3) CHO metabolism of diabetic and nondiabetic lymphocytes.
Materials and Methods

T AND B CELL QUANTITATION

T Lymphocytes are quantitated by total E rosette method described previously in detail.\(^9\) \(^{22}\) B lymphocytes are quantitated by assaying integral surface immunoglobulin. The identification is made by labelling an anti-human immunoglobulin with fluorescein. The positive cells complex with the fluorescein-conjugated antibody can be identified with the aid of a fluorescent microscope. Lymphocytes with demonstrable surface immunoglobulins (sIg) are quantitated by expressing the percent of positive cells among the total number of lymphocytes counted.\(^9\) \(^{22}\)

LYMPHOCYTE BLAST TRANSFORMATION

A modified LBT assay using heparinized whole blood is utilized. PHA-P\(^*\) is used at a dilution of 1:2,000; CON-A\(^†\) at a concentration of 2.5 \(\mu\)g per ml and PWM\(^†\) at a 1:50 dilution. The media for lymphocyte suspension is RPMI\(^§\) supplemented with 20 percent heat inactivated fetal bovine serum, 150 units of penicillin per ml and 150 \(\mu\)g streptomycin per ml. PHA-P and CON-A responses peak on day 4 and PWM on day 6; as a compromise, cultures are processed on day 5 after incubating at 37\(^{\circ}\)C in a 5 percent \(\text{CO}_2\) incubator with 1 \(\mu\)Ci \(^3\)H TdR per ml for 16 to 18 hours. The mononuclear cell count of the specimen has little effect on the results as long as it remains less than 3.5 \(\times\) 10\(^6\) per ml. When counts are higher, the specimen is diluted 1:2 in media.\(^10\) \(^{22}\)

LBT is performed in triplicate or quadruplicate. Results are expressed as the arithmetic mean and the standard devia-

\(^*\) Difco.
\(^†\) Pharmacia.
\(^†\) Gibco.
\(^§\) RPMI = Roswell Park Memorial Institute.

Carbohydrate Metabolism

Ten ml of heparinized whole blood are obtained by venipuncture. Lymphocytes are separated on a Ficoll-Hypaque gradient and concentrated to 1 \(\times\) 10\(^6\)/ml; 0.1 ml of the suspension is used for each assay in 0.9 ml of media. Controls contain 0.9 ml of media, 0.1 ml of cells and 0.1 ml of isotope. Mitogen treated cultures contain 0.8 ml of media, 0.1 ml of mitogen, 0.1 ml of isotope and 0.1 ml of cells. Two \(\mu\)Ci per ml of \(^{14}\)C labelled glucose in either the C-1 (for HMPS), C-2 (for HMPS-Pentose sugar recycling) or C-6 (Krebs cycle determination) position is added in parallel, separate and distinct cultures to evaluate the various cycles. All assays are performed in triplicate.

The mixture of lymphocytes, \(^{14}\)C-glucose and mitogen is incubated with a small piece of filter paper onto which 50 \(\mu\)l of hyamine hydroxide is placed. The filter is stuffed into the top of a micropipet tip. The micropipet tip is dropped into the tube with media, mitogen and isotope. The tubes are capped TIGHTLY and incubated for four days in a 37\(^{\circ}\)C incubator. At the end of the incubation, the filters are
removed from the pipet tip. $^{14}$C labelled CO$_2$ is trapped on the filter. Filter papers are placed into scintillation vials with 10 ml of scintillation fluid and counted in a beta scintillation counter (figure 1).$^{14}$

**Results**

**Quantitation of T and B Peripheral Lymphocytes**

The normal range for lymphocyte subpopulations on 65 white healthy ambulatory adults (40 females and 25 males, 32.1 ± 9.7 years old) was T cells = 71.3 percent ± 6.8; B cells = 7.8 percent ± 2.2 (mean ± 1 s.d.). Normal black volunteers ($n = 40$) had 63.8 percent ± 2.3 T cells and 8.3 percent ± 2.1 B cells, respectively.$^{12}$

Patients from the diabetic clinic ($n = 50$) at the Medical University of South Carolina overall had increased T cells, 78.2 percent ± 10.1 compared to controls ($p < 0.01$). These are predominately black, adult onset non-insulin dependent patients with diabetes mellitus. No significant difference was found in B cell percentages. No statistically significant correlation was found with duration of the disease, type of therapy, age, sex, race or glucose level. No factor examined in this study could account for the increased T subpopulations.$^{12,16}$

Patients with sickle cell disease ($n = 20$) also show abnormal ($p < 0.01$) T and B percentages; 50.2 percent ± 6.2 T cells and 17.0 percent ± 3.4 B cells.$^{14}$

Determinations on nine members of a family with high incidence of carcinoma revealed unusual data. There is a bimodal distribution of the B and T cell populations within five of the nine members of this family; 57.8 percent ± 9.9 T cells, 20.6 percent ± 2.7 B cells. Of inter-
est in this family is that two members found to have elevated B lymphocytes are known to have neoplasms. One individual has carcinoma of the thyroid; another has renal cell carcinoma.9

**LYMPHOCYTE BLAST TRANSFORMATION RESULTS**

Lymphocyte blast transformation has been evaluated in 100 normal ambulatory adults (67 females and 33 males, 35.7 ± 10.2 years old). Stimulation indices (mitogen treated / untreated) were 98.3 ± 23.7 for PHA-P; 93.4 ± 22.8 for CON-A and 45.7 ± 15.9 for PWM.

Lymphocytes from patients with adult onset diabetes mellitus show a decreased response to mitogen challenge (table I). There is a significantly (p<0.01) decreased response to each of the three mitogens. As part of our ongoing study of diabetics, lymphocytes were pretreated with a prostaglandin synthetase inhibitor (PGSI) 24 hours prior to mitogen incubation. Sodium meclofenamate (SMF), a PGSI, was used at 1 µg per ml. SMF brings levels of diabetic lymphocyte transformation to that of the unmanipulated nondiabetic lymphocytes. SMF treatment of nondiabetic lymphocytes increases the LBT of these cells to levels that the diabetic lymphocytes never attain (table II).

Alterations in the immune system during pregnancy have been reported.7 Thirty patients from the OB/GYN clinic at the Medical University of South Carolina in various trimesters of pregnancy show marked decreased LBT compared to non-pregnant controls, 20.6 ± 11.7 for PHA-P, 18.5 ± 10.6 for CON-A and 25.2 ± 14.6 for PWM.

Sickle cell patients also have abnormalities (p<0.01) in response to mitogenic challenge. The stimulation indices are 36.5 ± 57.6 for PHA-P; 34.3 ± 39.0 for CON-A; and 19.7 ± 21.2 for PWM.14

The effect of ethanol on lymphocyte responsiveness has also been studied. Dosages of 100, 250 and 300 mg ethanol per dl have suppressive effects on LBT of normal lymphocytes (table III). Viability of the lymphocytes remains at 90 percent as determined by trypan blue dye exclusion. These are doses compatible with levels reached in the peripheral blood after human ethanol consumption.

Five patients with hyperlipidemia are reported with regard to lymphocyte response to mitogenic challenge. These pa-

<table>
<thead>
<tr>
<th>Table I</th>
<th>Stimulation Indices for Diabetics Compared to Nondiabetics</th>
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<tbody>
<tr>
<td></td>
<td>PHA-P / CON-A / PWM</td>
</tr>
<tr>
<td>Diabetics*</td>
<td>35.5 ± 21.0 / 41.3 ± 16.0 / 18.9 ± 12.7 (n=75)</td>
</tr>
<tr>
<td>Nondiabetes</td>
<td>98.3 ± 23.7 / 93.4 ± 22.8 / 45.7 ± 15.9 (n=100)</td>
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<tr>
<td>*p&lt;0.01 for each mitogen compared to nondiabetic</td>
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<tr>
<th>Table II</th>
<th>Stimulation Indices of Diabetics Before and After Treatment with Sodium Meclofenamate*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>PHA-P / CON-A / PWM</td>
</tr>
<tr>
<td>Diabetic†</td>
<td>48.0 ± 23.3 / 51.3 ± 28.5 / 23.8 ± 16.4 (n=10)</td>
</tr>
<tr>
<td>Nondiabetic</td>
<td>116.7 ± 28.1 / 125.5 ± 15.8 / 48.7 ± 12.8 (n=10)</td>
</tr>
<tr>
<td>Diabetic§ + SMF (n=10)</td>
<td>106.0 ± 33.7 / 114.8 ± 26.0 / 26.7 ± 17.4</td>
</tr>
<tr>
<td>Nondiabetic + SMF (n=10)</td>
<td>166.3 ± 59.0 / 150.8 ± 32.5 / 36.0 ± 16.7</td>
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<tr>
<td>*After sodium meclofenamate (SMF) treatment, diabetics reach a normal level of response (p = n.s.) to mitogens (except PWM) no longer significantly different from untreated non-diabetics.</td>
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<tr>
<td>†p&lt;0.01 diabetic compared to nondiabetic.</td>
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<tr>
<td>§p&lt;0.01 diabetic plus SMF compared to nondiabetic plus SMF.</td>
<td>PHA-P = Phytohemagglutinin, CON-A = Concanavalin, PWM = Pokeweed Mitogen</td>
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TABLE III
Stimulation Indices (mean ± 1 s.d.) of 10 Normal Individuals With and Without In Vitro Ethanol Exposure

<table>
<thead>
<tr>
<th>Ethanol (mg/dl)</th>
<th>PHA-P</th>
<th>CON-A</th>
<th>PWM</th>
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<tbody>
<tr>
<td>None</td>
<td>90.3 ± 24.0</td>
<td>81.7 ± 20.8</td>
<td>48.7 ± 10.0</td>
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<tr>
<td>100*</td>
<td>38.4 ± 7.6</td>
<td>57.9 ± 18.5</td>
<td>35.9 ± 7.8</td>
</tr>
<tr>
<td>250*</td>
<td>17.3 ± 6.8</td>
<td>20.1 ± 5.9</td>
<td>17.8 ± 7.9</td>
</tr>
<tr>
<td>300*</td>
<td>13.6 ± 10.9</td>
<td>19.9 ± 14.6</td>
<td>17.3 ± 9.8</td>
</tr>
</tbody>
</table>

*p < 0.001 compared to control without ethanol exposure.

PHA-P = Phytohemagglutinin
CON-A = Concanavalin
PWM = Pokeweed Mitogen

Patients were suppressed compared to controls. Their indices were 26.9 ± 9.1 for PHA-P; 36.6 ± 11.1 for CON-A; and 11.8 ± 3.0 for PWM.

CARBOHYDRATE METABOLISM RESULTS

When treated with PHA-P, diabetic lymphocytes exhibited less HMPS activity increase than nondiabetics. No significant difference between the Krebs cycle activity of nondiabetics and diabetic lymphocytes with PHA-P stimulation was observed. With the addition of the mitogens CON-A and PWM, response of diabetic lymphocytes was less than that of nondiabetic lymphocytes. Statistically significant differences (p < 0.01) were observed after stimulation with PHA-P, CON-A and PWM between diabetic and nondiabetic lymphocytes in the HMPS activity and HMPS by pentose recycling activity, but no significant difference was seen in the Krebs cycle activity (table IV).

Discussion

Means and standard deviations of the results of the E-rosette, sIg, LBT and CHO assays from normal donors should be generated in each laboratory performing the tests. Results can only be interpreted by comparison with the information generated from the control group.

High values or abnormal cells rosetting with SRBC may be seen in a number of lymphoproliferative states. These include the T cell lymphomas (mycosis fungoides and Sezary syndrome) or T cell chronic lymphocytic leukemia. Elevated T cell percentages and absolute numbers are seen in B cell deficient states, such as Bruton’s sex-linked agammaglobulinemia. Increased percentages of T cells found in black diabetics may represent an attempt to overcome an immunological deficit by increasing the circulating T pool.

Marked depression of T cell percent and absolute numbers is primarily seen in immunodeficiency states involving the thymus dependent arm of the immunologic system. Examples include severe combined immunodeficiency and the DiGeorge syndrome (thymic parathyroid hypoplasia). Slight depression of T cell percentages and numbers as
a manifestation of disease is less well characterized. End stage malignancy may be associated with T cell depression. The presence of autoantibodies on T cells in some collagen vascular diseases (e.g., systemic lupus erythematosus and Sjögren's syndrome) may lead to an apparent depression in T cell percent numbers. Very high and very low levels of T cells are usually interpretable in the light of the patient's clinical status. Minimally abnormal values are enigmatic.3,6,22

Chronic lymphocytic leukemia, usually B lymphocytes, may not always have detectable surface immunoglobulin. This could be because the immunoglobulin is present in amounts below the threshold of detection by standard immunofluorescence or is not present.22

Factors present in the patient's sera may give artifactual results in B and T cell quantitation. For example, the presence of immune complexes in sera or anti-T lymphocyte antibodies can bind to the cells, enhancing or blocking the detection of one or more of the markers. Separation procedures, washing and latex ingestion incubation are usually sufficient to allow elution of complexes or cytophilic antibodies before testing.22,23

LBT by ³H TdR uptake is a useful assay of lymphocyte response. Although semiquantitative, the whole blood assay is a useful screening test for abnormalities of lymphocyte function. LBT responses of an individual vary over time because of a variety of factors. Results obtained at different times can not be compared except for gross changes. LBT is useful in following immune reconstitution, especially in patients with severe combined immunodeficiency treated with bone marrow transplants.

Serum factors can inhibit mitogenic response. Inhibitors have been found in active tuberculosis, carcinoma, secondary syphilis, multiple sclerosis and sarcoidosis. Immunosuppressive drugs can inhibit responses.5,9,22

The plant mitogens (PHA-P and CON-A) transform predominately T-lymphocytes; PWM stimulates B and T lymphocytes. LBT is useful in diagnosis of severe combined immunodeficiency and is a sensitive assay to measure response to specific allergens, pathogens or auto-antigens.

Abnormalities in the HMPS activity of diabetic lymphocytes have been previously documented.5,20 This study enhances the understanding of CHO metabolic pathways and suggests a relationship of these pathways to immune functions. Normal recognition of antigens and satisfactory initiation of the immune response as mediated by lymphocytes may have a metabolic basis. Carbohydrate metabolism is abnormal in patients with diabetes mellitus. Hexose monophosphate shunt activity in lymphocytes of diabetics is impaired to nondiabetics. The defect in the HMPS levels correlated with decreased LBT.19 Proper application of clinical tests of altered carbohydrate response in the lymphocytes may add to the evaluation of medical control of diabetes mellitus.

Summary

B and T lymphocyte subpopulation quantitation is useful in describing normal populations and assessing immunodeficiency states. Quantitation of cell types aids in characterization of lymphoproliferative disorders. Diabetics have alterations in T cell populations. The reason for this remains obscure.

LBT is an effective method of assessing functional lymphocyte response in vitro. Diabetics and pregnant patients have decreased LBT responses to PHA-P, CON-A and PWM. Experiments adding ethanol to normal cells reveals significant decrease in LBT.

Carbohydrate metabolism in diabetic lymphocytes reveals decreased HMPS and HMPS pentose recycle activity. Krebs
cycle activity in normal and diabetic lymphocytes is not significantly different. A PGSI partially corrects HMPS activity of diabetic lymphocytes. Further exploration of the role of the prostaglandins on CHO intermediate metabolism is warranted.

References