Effects of Insulin on Phytohemagglutinin-P, Concanavalin-A, and Pokeweed Mitogen in Diabetic and Nondiabetic Lymphocytes

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ABSTRACT

Blast transformation of lymphocytes from diabetics and nondiabetics was evaluated after adding insulin at various concentrations. Responses to phytohemagglutinin-P (PHA-P), concanavalin-A (CON-A) and pokeweed mitogen (PWM) were measured in the presence of exogenous insulin added in physiologic increments of 0, 10, 20, 30, and 40 microunits of activity per ml of culture medium. A modified method utilizing heparinized whole blood was used. After a four day incubation period, $^{3}$H-TdR uptake was evaluated and used as the index of stimulation.

A standard pooled z test for comparison of $^{3}$H-TdR uptake showed a decrease in blast transformation of diabetic lymphocytes compared to nondiabetic lymphocytes to the mitogens PHA-P ($p < 0.01$), CON-A ($p < 0.01$), and PWM ($p < 0.01$). Added insulin increased the diabetic lymphocyte blast transformation response to each of the mitogens. Blast transformation in diabetics never reached the level of blast transformation in nondiabetics. It is hypothesized that there is a cellular defect in either membrane receptors or intracellular metabolic pathways which accounts for the decrease in diabetic lymphocyte blast transformation.

Introduction

Lymphocyte blast transformation (LBT) is regarded as an in vitro measure of in vivo cell mediated immunological responsiveness. After incubation with the mitogen PHA-P, diabetic lymphocytes have a decrease in blast transformation as compared to nondiabetic lymphocytes. There are differences between the blast transformation levels of well controlled and poorly controlled diabetics and between insulin-dependent and insulin-independent diabetics. The in vitro decrease in cell-mediated and immunological responsiveness of diabetics is clinically substantiated by reports of increased incidences of mycotic and gram negative infections, decreased wound healing, delayed hypersensitivity and graft rejection.

The antigenic properties of insulin in diabetics have been reported. The inactivation of insulin by active processes
such as circulating insulin antibody complexes, passive processes such as decreased insulin binding, or decreased metabolic capabilities of diabetic lymphocytes, may cause decreased LBT responses as demonstrated in diabetes.\textsuperscript{4,12,13,15,16,17}

The purpose of this study was to examine the effects of varying insulin concentrations on the responsiveness of nondiabetic lymphocytes to the mitogens phytohemagglutinin-P (PHA-P), concanavalin-A (CON-A), and pokeweed mitogen (PWM).

Materials and Methods

Subjects

Twenty diabetics were selected from patients treated in the outpatient clinic at the Medical University of South Carolina. The patients were chosen from a group known to have high T lymphocyte percentages (T ≥ 72 percent).\textsuperscript{7} The study group consisted of 20 females (15 blacks and 5 whites) with a mean age of 47.6 ± 11.9 years. Ten patients were treated with insulin and 10 were controlled with oral hypoglycemics. No subjects were experiencing clinical distress or were under treatment for infection at the time of the study.

The controls were ambulatory, healthy, nondiabetic volunteers: 20 females (15 black and 5 white) with a mean age of 46.8 ± 10.1 years. No control subject was under the treatment of a physician at the time of the study.

Sample Collection. Blood is obtained (a.m.) by venipuncture after a 12 hour fast. Ten ml of blood are collected in a heparinized glass tube. Five ml of blood are collected in a flouridated tube. The final concentration of sodium flouride is 7 mg of NaF per ml. This is used for plasma glucose and insulin determinations. Seven ml are collected with ethylene diamine tetra acetic acid (EDTA) at a final concentration of 1.5 mg of EDTA per ml of whole blood. This was used for a complete blood count (CBC) and a differential count.

LBT Materials. The culture media consists of RPMI-1640 plus 20 percent sterile, heat inactivated fetal calf serum, 150 units penicillin per ml, and 150 μg of streptomycin per ml.

Insulin

The stock insulin solution is prepared by adding 49.5 ml of RPMI-1640 to 0.5 ml of U40 regular insulin (Squibb) to form a final dilution of 1:50. Aliquots of this solution are added to the appropriate test tubes to final concentrations of 10, 20, 30, and 40 microunits activity insulin per ml.

Mitogens

Five ml of lyophilized PHA-P are reconstituted with 5 ml of triple distilled, sterile water. This solution is dispensed in 0.1 ml aliquots into sterile tubes and stored at −20°C. A working solution at a final dilution of 1:200 is prepared.

Five ml of lyophilized PWM are reconstituted with 5 ml of triple distilled, sterile water. This solution is dispensed in 1.0 ml aliquots into sterile tubes and stored at −20°C. A working solution at a final dilution of 1:5 prepared.

Thirty mg of CON-A are added to 3.0 ml of RPMI 1640 to make a stock solution containing 10 mg CON-A per ml. This solution is sterilized by filtration and can be stored at 4°C for three months. A working solution at a final concentration of 25 μg per ml is prepared.

Final concentrations of mitogens in culture were PHA-P 1:2,000, CON-A 2.5 μg per ml, and PWM 1:50. Dose curves were performed for each mitogen. These doses represent optimum amounts for maximum lymphocyte stimulation.\textsuperscript{8}

Performance of the LBT

All steps are performed in a sterile environment using a Sterilguard hood. One-tenth ml of heparinized peripheral blood
is added to each tube with the appropriate amounts of insulin, media, and specific mitogen. Each data point represents the mean of an assay performed in triplicate. Three tubes without mitogen serve as controls. The total volume in each tube is 1.0 ml. The tubes are loosely capped and placed in a humidified 5 percent CO₂ incubator at 37°C for four days. At the end of the four day incubation period, 0.1 ml of tritiated thymidine (Schwarz/Mann-specific activity of 6.0 Ci per mM) is added to make a final activity of 1 μCi per culture. The cultures are lightly vortexed to resuspend the cells and are returned to the incubator for 16 to 18 hours.

**Processing the Cells**

The tubes are lightly vortexed and 2 ml of cold (4°C) 3 percent acetic acid are added to lyse the red cells. The tubes are centrifuged at 1800 rpm for five minutes at 4°C. The supernatant is decanted into a radioactive waste container. One ml of cold (4°C) 5 percent trichloroacetic acid is added to the tubes, which are lightly vortexed and centrifuged at 1800 rpm for five minutes at 4°C. The supernatant is decanted, 1.5 ml of 100 percent methanol is added, and the tubes are centrifuged at 1800 rpm for 10 minutes at 4°C. The supernatant is discarded and the pellet is examined. The pellet should be white or cream colored. If the pellet still appears brown, the methanol step is repeated. Once the pellet is the correct color, 0.4 ml of tissue solubilizer is added and the tubes are covered and placed in a 50°C water bath for 30 minutes. After incubation, the tubes are removed from the water bath and cooled to room temperature. Four ml of scintillation fluid are added to each tube then decanted into vials. Again, four ml of scintillation fluid are added to the tubes to wash out any remaining materials. This fluid is decanted into the vials and 6.0 ml of scintillation fluid are added directly to the vial to produce a final volume of 14 ml. The vials are stored in the dark to permit dark adaptation prior to transfer to a scintillation counter. Each vial is counted for two minutes on a full tritium channel.

**Results**

**Glucose Concentration**

The glucose concentration in the culture media was determined before adding blood. The glucose concentration was 198 ± 20 mg per dl (n = 12).

**Mitogen Stimulation**

As shown in table I, the absolute cpm for nondiabetics (139,206 ± 34,540) is greater than that of diabetics (52,277 ± 13,608), when incubated with a 1:2000 dilution of PHA-P. When incubated with a 1:50 dilution of PWM, the normal lymphocytes incorporate significantly more ³H-TdR than do the diabetic lymphocytes. The cpm are 63,191 ± 18,175 and 18,030 ± 7137, respectively. With CON-A (2.5 μg/ml), the cpm of nondiabetics was significantly higher than that of diabetics, 130,217 ± 23,135 as compared to 49,669 ± 18,026. These results confirm previous findings that lymphocytes from diabetics have a lower stimulation index when exposed to PHA-P than do lymphocytes from nondiabetics.² In addition, diabetic lymphocytes are shown to have a decreased cpm when incubated with CON-A and PWM. The pooled z test indicates a statistically significant difference

<table>
<thead>
<tr>
<th>Mitogen</th>
<th>Diabetics (n=20)</th>
<th>Nondiabetics (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA-P</td>
<td>52,277 ± 13,608</td>
<td>139,206 ± 34,504 (p &lt; 0.01)</td>
</tr>
<tr>
<td>CON-A</td>
<td>49,669 ± 18,026</td>
<td>130,217 ± 23,135 (p &lt; 0.01)</td>
</tr>
<tr>
<td>PWM</td>
<td>18,030 ± 7,137</td>
<td>63,191 ± 18,175 (p &lt; 0.01)</td>
</tr>
<tr>
<td>Control</td>
<td>1,134 ± 207</td>
<td>1,322 ± 134 (p = n.s.)</td>
</tr>
</tbody>
</table>

PHA-P = Phytohemagglutinin-P  PWM = Pokeweed mitogen  CON-A = Concanavalin-A  Mean counts per minute (cpm) ± 1 SD of diabetics and nondiabetics in response to various mitogens.
in tritiated thymidine (³H-TdR) uptake between lymphocytes from diabetics and nondiabetics after stimulation by the mitogens PHA-P (p < 0.01), and PWM (p < 0.01).

**INSULIN EFFECT**

Tables II, III, and IV contain mean cpm ± 1 SD using various concentrations of insulin. The data reveal that the cpm for nondiabetic cells is higher at all levels of added insulin. There is a similar pattern of response for both diabetic and nondiabetics. Both types of cells have an increase in response with the addition of insulin, which occurs maximally at 20 μU insulin per ml of media with PHA-P. Nondiabetics and diabetics show a decreased response at insulin additions of greater than 20 μU insulin per ml (table II).

As with the other mitogens studied, the cpm for all levels of added insulin using CON-A is higher for nondiabetics than for diabetics. The greatest stimulation occurs at 20 mU insulin per ml of media for both diabetics and nondiabetics (table III).

Using PWM, the LBT response of the nondiabetics is higher than that of diabetics. Diabetics and nondiabetics show a consistent increase at levels up to 20 mU per ml and a decrease in response at levels greater than 20 mU per ml (table IV).

**Discussion**

This study supports previous findings of decreased diabetic lymphocyte blast transformation in response to the mitogen PHA-P and shows decreased diabetic lymphocyte responses to PWM and CON-A.²,⁹⁻¹⁰ If one accepts the hypothesis that PHA-P predominately stimulates T lymphocytes and PWM predominately stimulates B lymphocytes, the results of this study indicate that the entire population of lymphocytes in diabetics is deficient in their capacity to respond to mitogenic stimuli.

The decreased lymphocyte response may exist prior to the onset of diabetes, although there are no reports of this in the literature. The circulating lymphocytes may be modified at the onset of diabetes.
TABLE IV

Effects of Insulin on Lymphocytes Stimulated by PWM (1:50) Insulin Added (Microunits of Activity)

<table>
<thead>
<tr>
<th></th>
<th>Control (without PWM)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetics*</td>
<td>1,134 ± 207</td>
<td>10,031 ± 7,137</td>
<td>30,731 ± 17,001</td>
<td>66,565 ± 24,843</td>
<td>45,473 ± 19,078</td>
<td>33,793 ± 13,126</td>
</tr>
<tr>
<td>Non-</td>
<td>1,322 ± 134</td>
<td>63,192 ± 18,175</td>
<td>75,486 ± 26,118</td>
<td>104,967 ± 31,054</td>
<td>81,039 ± 23,715</td>
<td>70,198 ± 16,716</td>
</tr>
</tbody>
</table>

*n = 20) PWM = Pokeweed mitogen

Mean cpm ± SD of lymphocytes exposed to varying concentrations of insulin and stimulated by PWM.

and, as a result of this, may express a decreased response to mitogenic stimuli.

Lymphocytes from diabetics and nondiabetics respond differently quantitatively, but similarly qualitatively, to mitogenic stimuli. The response of both diabetic and nondiabetic lymphocytes are increased by the addition of insulin.

Decreased lymphocyte response to PHA-P has been reported as a function of aging and may be due to fewer T cells in the elderly. Subjects used in this study had an increased number of T cells. This implies that decreased response to PHA-P was a cellular defect and not due to a decreased number of T cells. These results indicate that the ability to respond to mitogenic stimuli is present in diabetic lymphocytes, but at a reduced level.

The mechanism responsible for the cellular defect reflected by decreased mitogenic stimulation has not been described. The increased effect of insulin on blast transformation suggests an ineffective utilization of insulin and/or glucose by the diabetic lymphocyte in vivo. Reports of decreased insulin binding owing to a decreased number of insulin receptors in diabetic lymphocytes support this. Absolute fasting serum levels of insulin are similar in maturity onset diabetics and nondiabetics. However, insulin release in the presence of glucose is altered. This delayed insulin release in diabetics, coupled with the time dependent surface changes seen in PHA-P, PWM, and CON-A stimulation, may be responsible for the decreased mitogenic response seen in diabetic lymphocytes. Insulin may not be available at the critical time when transformation should occur. Another explanation may be that the absolute number of insulin receptors are reduced on diabetic lymphocytes.

At this time, there are no data concerning the number of functional insulin receptors present in nondiabetic or diabetic lymphocytes. A threshold number of functional receptors may be necessary to produce an optimum response.

When the antigen or mitogen is bound, time dependent changes precede the irreversible point of transformation. These processes require energy. The efficient intracellular energy production capability of diabetic lymphocytes is questionable. After these surface changes occur, the energy for DNA synthesis necessary for transformation is paralleled by an increase in glucose utilization. Certain enzymes necessary for the efficient operation of the hexose monophoshate shunt (HMPS), the major source of NADPH, and ribosides for RNA and DNA synthesis are decreased in the lymphocytes of patients with diabetes and chronic lymphocytic leukemia (CLL). The reduced levels of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase seen in lymphocytes of CLL patients probably occur in diabetic lymphocytes as well. An increase in intracellular glucose is seen in the lymphocytes of patients with diabetes, CLL, Hodgkin’s, chronic suppuroative processes, and infectious mononucleosis. These findings suggest
an altered intracellular glucose metabolism in diabetic lymphocytes which may contribute to the decreased response to antigenic and mitogenic stimuli.

The mechanism responsible for decreased blast transformation seen in diabetics may be an alteration in membrane surface receptors, metabolic pathways of intracellular energy production, or some combination of these factors.

References