The Investigation of the Responses to Phytohemagglutinin in the Patients of non-Hodgkin's Lymphoma who were Candidates for High Dose Chemotherapy, Autologous Bone Marrow, or Peripheral Stem Cell Transplantation

İ. Halil Özerol, MD[1], Mustafa Şenol, MD[2], Ana Ageitos, MD[1], James E. Talmadge, PhD[1]

Phytohemagglutinin (PHA) is principally a T lymphocyte mitogen, producing a greater stimulatory effect on $CD4^+$ helper/inducer T lymphocytes than on $CD8^+$ suppressor/cytotoxic T cells. In this study, we investigated if there are differences in the mitogenic response to PHA between autologous bone marrow (BM) and granulocyte-monocyte colony stimulating factor (GM-CSF)-mobilized peripheral stem cell (PSC) products as well as to normal peripheral blood leukocytes (PBL). BM and PSC products from 24 patients with non-Hodgkin's lymphoma (NHL) were collected and assays of cell proliferation in response to PHA were performed both before and following 5 days co-incubation with IL-2. The proliferative response to PHA by cells from both PSC and BM products were significantly lower than normal PBL ($p<0.001$). The PHA response of PSC was significantly higher than that observed with BM ($p<0.01$). The response to PHA of PSC was higher than BM ($p<0.05$). When compared before culture with IL-2, the PHA response of PBL was decreased ($p<0.05$), but PSC and BM was not changed. This study showed that the PSC products prior to IL-2 incubation had higher mitogenic response to PHA. According to these results the depressed mitogenic response of lymphocytes from non-Hodgkin patients was associated with an increase in "baseline" proliferation due to membrane bound IL-2 resulting in continued proliferation. [Journal of Turgut Özal Medical Center 1996;3(4):306-310]

***Key Words**: Non-Hodgkin lymphoma, PHA response, bone marrow, peripheral stem cell, transplantation*

Yüksek doz kemoterapi, otolog kemik iliği veya periferik stem cell transplantasyonu yapılacak non-Hodgkin lenfomalı hastalarda fitohemaglutinin'e cevabın araştırılması

Fitohemaglutinin (PHA), genellikle bir T lenfosit mitojenidir. $CD4^+$ helper/inducer T lenfositlerine, $CD8^+$ suppressor/cytotoxic T lenfositlerine göre daha fazla stimulatör etki yapar. Bu çalışmada, biz normal periferik kan lökositleri (PBL) ile otolog kemik iliği (BM) ve granulosit-monosit koloni stimulan faktör (GM-CSF) ile mobilize edilmiş periferik stem cell (PSC) ürünlerinin PHA'e verdikleri mitojenik cevapların farklı olup olmadığını araştırdık. Non-Hodgkin lenfomalı 24 hastadan kemik iliği ve periferik stem cell ürünleri toplandı ve IL-2 ile 5 gün kültür yapılmadan önce ve yapıldıktan sonra PHA hücre proliferasyon cevabı incelendi. Hem PSC hem de BM hücrelerinin PHA proliferatif cevapları anlamlı şekilde normal PBL'den düşüktü ($p<0.01$). PSC'in PHA cevabı BM'unkinden daha yüksekti ($p<0.05$). IL-2 ile kültür yapıldıktan sonra PBL'nin PHA cevabı azaldı ($p<0.05$) fakat PSC ve BM'unki değişmedi. Bu çalışma sonucunda IL-2 ile inkübasyona PSC ürünleri PHA'e daha yüksek mitojenik cevap verdi. Bu sonuçlara göre non-Hodgkin lenfomalı hastaların lenfositlerindeki deprese mitojenik cevap, membrana bağlı IL-2'nin sürekli stimüle olması nedeniyle yüksek baz proliferasyona sebep olduğunu düşündürmektedir. [Turgut Özal Tıp Merkezi Dergisi 1996;3(4):306-310]

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[1] University of Nebraska, Department of Pathology/Microbiology, Omaha, NE 68198, USA
[2] University of Pittsburgh, Department of Pediatrics Section of Allergy/Immunology, Children`s Hospital of Pittsburgh, Pittsburgh, PA 15213, USA

A mitogenic substance causes DNA synthesis and induces blast transformation and division by mitosis. Mitogens have been widely used in both experimental and clinical immunology to evaluate T and B lymphocyte function in vitro. Phytohemagglutinin (PHA) is principally a human and mouse T lymphocyte mitogen, produces a greater stimulatory effect on $CD4^+$ helper/inducer T lymphocytes than on $CD8^+$ suppressor/cytotoxic T cells. It has a weaker mitogenic effect on B lymphocytes (1). In the lymphocyte culture techniques for mitogen activation, obviously there are a multitude of technical as well as conceptual variables that can affect the results of this sensitive assay system. The degree of lymphocyte activation is also a function of the cellular regulatory influences present in the culture. These include the concentration of cells, the geometry of the culture vessel, contamination of cultures with nonlymphoid cells or microorganisms, the dose of mitogen, the incubation time of cultures, and techniques of harvesting cells. Confusion may result from a nonstandardized format for presentation of data. Many investigators present results of lymphocyte stimulation as a ratio of counts per minute (cpm) in stimulated culture to those in control cultures so-called stimulation index (SI). The stimulation index is a ratio, and marked changes can therefore result from changes in background or control cpm of the denominator. So, it is best to report data in the ways to permit better interpretations (2).

Recent studies have shown that the immune function of PBL following autologous bone marrow transplantation (ABMT) and peripheral stem cell transplantation (PSCT) is significantly suppressed compared to normal PBL. Following stem cell transplantation, patients undergo a prolonged period of T cell immunodeficiency including their immune responses to antigen and mitogen induced T cell proliferation and IL-2 production (3-5). In contrast to these observations, lymphocyte proliferative response to high concentration of IL-2 in the absence of antigen stimulation has been reported to normalize more rapidly following transplantation (6).

IL-2 is an autocrine and paracrine growth factor that is secreted by activated T lymphocytes and is essential for clonal T cell proliferation. It supports continuous growth of normal T cells in culture. So, it is called T cell growth factor. Its essential role in T cell proliferation, together with its effects on cytockine production and on the functional properties of B cells, macrophages and NK cells, places IL-2 among the most critical immunoregulatory cytokines (7,8). Resting T lymphocytes do not synthesize or secrete IL-2 but can be induced to do both appropriate combinations with antigens and costimulatory factors or by exposure to policlonal mitogens. Studies of isolated T cell subtypes indicated that antigen-induced IL-2 production occurs mainly in $CD4^+$ helper T cells. When normal human lymhocytes are exposed to a T cell mitogen, IL-2 mRNA expression becomes detecteable after 4 hours, reaches peak concentration at 12 hours and thereafter decrease rapidly. When exposed to appropriate activating stimuli, resting $CD4^+$ T lymphocytes begin to express both IL-2 and IL-2R and shortly thereafter begin to proliferate (2).

Because IL-2 has a critical role in lymphocyte proliferation, we have examined the ability of IL-2 and PHA to extend the proliferation of lymphocytes from both BM and PSC products of NHL patients compared to that of the PBL from healthy donors using short-term (5 day) cultures with IL-2.

MATERIALS AND METHODS

Patients. Between August 1995 and April 1996, a total of 24 consecutive intermediate grade NHL patients who were candidates for high dose therapy (HDT) and PSC transplantation (n=14) or autologous BM transplantation (n=10) at the University of Nebraska Medical Center (UNMC) were entered into these studies. Written informed consent for stem cell collection and autologous transplantation was obtained from each patient. Peripheral blood progenitor cells were mobilized with granulocyte-monocyte colony stimulating factor (GM-CSF) by intravenous administration of 250 $\mu g/M^2$ and a target dose of $6.5x10^8$ mononuclear cells/kg body weight, and samples were collected and cryopreserved. A minimum of three apheresis sessions were performed beginning at three or more days after the initiation of GM-CSF administration. Following stem cell transplantation, all patients received GM-CSF until their absolute neutrophil count was $\geq 500/mm^3$ on two consecutive days. BM products were collected according to protocol established at the University of Nebraska

Medical Center. All samples were obtained using protocols approved by the Institutional Review Board of UNMC. In addition, PBL were obtained from 20 normal healthy volunteer donors.

Cell isolation. The PSC, BM, or PB was diluted 1:1 (1:2 for PSC products) in Hank's Balanced Salt Solution (HBSS) (Gibco BRL, Grand Island, NY), layered on Ficoll Hypaque (Organon Teknika, Durham, NC) and centrifuged for 20 minutes at 1400 rpm. After centrifugation, the aqueous layer was removed and the mononuclear cell layer transferred to another tube. The cells were then washed twice in HBSS and adjusted to $4x10^6$/ml in RPMI-1640 (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Hyclone, Logan UT), 10 mM HEPES (Research Organics, Cleveland, OH), 40 µg/ml Gentamycin (Gibco BRL) and 2 mM L-Glutamine (Gibco BRL).

Five days IL-2 co-culture. Fresh PSCs, BM cells, and PBLs were cultured in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine and 40 µg/ml gentamycin. For each experiment, a T-25 tissue culture flasks (Costar, Cambridge, MA) was established with $1x10^6$ cells per ml. These flasks were supplemented with recombinant human IL-2 (Chiron Corporation, Emeryville, CA) (specific activity $3x10^6$ units/mg) at a final concentration of 100 IU/ml. Cultures were incubated at 37°C in a 5% CO_2 incubator for 5 days, after which period cells were harvested and tested for PHA proliferation.

Mitogen (PHA) response assay. Cells (100,000/well) were added to 96-well flat bottom microplates (Falcon, Sacramento, CA) and cultured in a humidified 37°C, 5% CO_2 incubator for 72 hours in presence of PHA (Difco Laboratories, Detroit, MI). The PHA was added as 5 µg/ml, 2.5 µg/ml, 0.3125 µg/ml or no PHA. For the final 18 hours of culture, 50 µl of 20 µCi/ml ^{3}H-thymidine (Amersham Life Science, Arlington Heights, IL) was added to each well. At the end of incubation, cells were harvested onto fiberglass strips using an automated multi-well harvester, scintillation cocktail was added, and specific incorporation of ^{3}H-thymidine was determined by counting radioactivity using a Top Count™, Microplate beta Scintillation Counter (Packard, Meridan, CT).

Statistics. Results of experimental data obtained from multiple experiments were reported as mean ± standard error of the mean (SEM). Significance levels were determined by the Student's unpaired t-test analysis using SPSS for Windows®.

RESULTS

Patients. The median age of the PSCT patients was 45 years (range 32 to 68 years) and 49 years (range 34 to 68 years) for the autologous BMT patients. The PSCT patients were 8 males and 6 females while the BMT patients were 7 males and 3 females.

PHA mitogenic response. The mitogenic response to PHA within these same cell populations were also studied (Figure 1). The results shown in Figure 1 demonstrate that the mitogenic response to 0.3 µg/ml of PHA (optimal concentration) of both BM and GM-CSF mobilized PSC products were significantly decreased as compared to that of observed in normal PBL ($p<0.001$ in both instances). In addition, the PHA response of the BM cells was significantly reduced compared to that of observed with the GM-CSF mobilized PSC products ($p<0.01$). The stimulation indexes are consistent with the observed proliferative results showing an stimulation index (SI) of 181, 26, and 2 for the PBL, PSC, and BM leukocyte products, respectively. Following co-culture with IL-2 (100 IU/ml) for five days, the PBLs again had significantly better proliferative capacity in response to PHA than PSC or BM cells ($p<0.01$ and $p<0.001$, respectively). However, in contrast to the PHA mitogenic response with the fresh products, there was no significant difference between the BM and PSC proliferative responses. The SI within the IL-2 cultured cells was much lower than that of observed prior to culture with SI's of 4.8 and 4 for PBL and PSC, respectively, as well as an SI of 1.3 for the BM cells. The depressed SI was associated with an increase in "baseline" proliferation due to membrane bound IL-2 resulting in continued proliferation.

DISCUSSION

PSCT has been used increasingly as an alternative to ABMT after myeloablative therapy in treatment of malignancies. Perhaps the greatest area of apparent improved efficacy with PSCT as

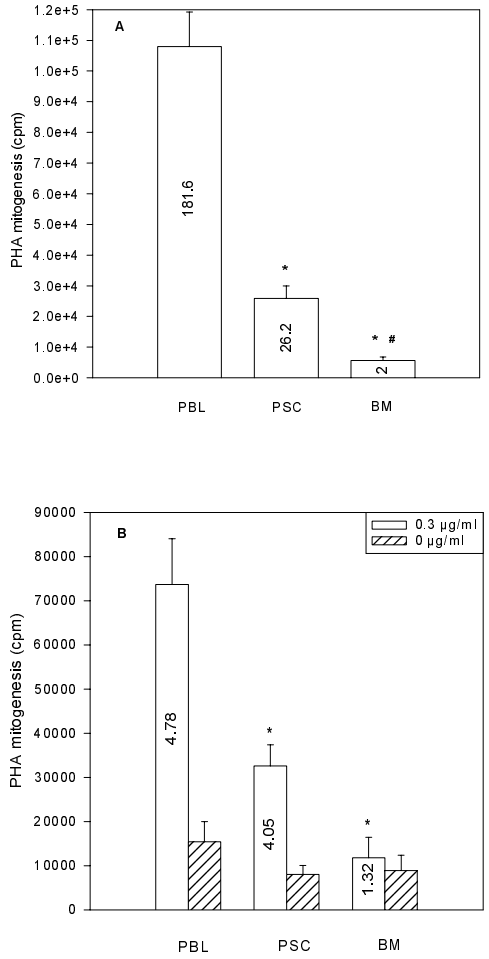

Figure 1. Mitogenic responses to PHA in PBL from normal donors and in PSC and BM from NHL patients (A). Mitogenic responses to PHA in PBL from normal donors and in PSC and BM from NHL patients after in vitro activation with IL-2 (100 U/ml) for 5 days (B). Data represent the mean cpm ± SE. The stimulation index is shown in the bar. *Significant difference compared to normal PBL ($p<0.001$). #Significant difference compared to PSC ($p<0.01$).

compared to ABMT is the more rapid myeloid (9,10) and immunologic reconstitution (10) following PSCT as compared to BMT (11,12). The former advantage is somewhat controversial, as the patients receiving ABMT have not received mobilized BM and this is not felt to be a valid comparison. The apparent more rapid immunologic reconstitution following PSCT has been an interesting observation (11). PSCs harvested from patients during the recovery phase from chemotherapy-induced neutropenia are an alternative source of pluripotent hematopoietic progenitor cells with the possible added advantage of a lower tumor cell contamination frequently (12). Recently, there has been several studies using purged stem cell products which have suggested that the removal of the tumor cells from within the products results in a significant prolongation of failure free and overall survival (13). However, regardless of the use of purged products many of the patients still relapse due to the inability of the conditioning regimen to fully remove all the tumor cells. Therefore, additional adjuvant therapeutic strategies are needed to improve the therapeutic efficacy of these different approaches. One such approach is currently focused upon is the use of IL-2 augmented stem cell products (11) or the use of IL-2 following transplantation (12). The latter is particularly important as recent studies from our laboratory have suggested that there are suppressor cells within PSC products which may significantly impact on the proliferative and T cell responses of PSC products. The aim of this study was to evaluate the proliferative response to IL-2 and PHA in PSC and BM products from NHL patients.

A number of studies have shown a reduced PHA response in the PSC of NHL patients (14-17). The available data suggest that if patients are given IL-2 augmented BM that they may reduce immunosuppression that is normally observed.

Lymphocyte response to PHA is a sensitive test for T lymphocyte function. The loss of immunoreactivity observed in NHL patients following PSCT and BMT may be due to an inhibitor (cellular or secreted) in the stem cell products (18). Our results showed that proliferative responses to PHA of both PSC and BM are lower than that of found in normal PBL ($p<0.001$). PHA response of PSC was statistically higher than that of observed with BM ($p<0.01$). IL-2 stimulated proliferative response of PSC was similar to that of observed with normal PBL, but there was a significantly lower response between PBL and BM

as well as PSC and BM ($p=0.014$ and $p=0.0189$, respectively). After culture with IL-2, the PHA proliferation of PSC and BM products were significantly lower than that of PBL ($p<0.001$). Further, the response to PHA of PSC cells was higher than that of BM cells ($p<0.05$). Additionally, IL-2 stimulated proliferations of PSC and BM were significantly lower than that of PBL. Although IL-2 stimulated proliferation of PBL was significantly increased, PSC and BM responses were not increased.

In this study, we showed that decreased PHA response of NHL patients is associated with an increase in "baseline" proliferation due to membrane bound IL-2 resulting in continued proliferation and it can not be increased in culture of the stem cell product with IL-2.

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Correspondence address:
İ.Halil ÖZEROL, M.D
İnönü University Faculty of Medicine
Department of Microbiology and
Clinical Microbiology
44100 MALATYA
E-mail: ibrahim.halil@ihlas.net.tr