The Effect of Interleukin-2 on Natural Suppressor Activity of Bone Marrow and Peripheral Stem Cell Products from Patients with non-Hodgkin's Lymphoma

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

The activity of natural suppressor (NS) cells of peripheral stem cell (PSC) and bone marrow (BM) products from 24 patients with non-Hodgkin's lymphoma (NHL) were examined both before and following 5 days co-incubation with IL-2. This study showed that in the PSC products prior to IL-2 incubation, NS activity had been higher than both normal peripheral blood leukocytes (PBL) and BM products ($p<0.001$). Following IL-2 activation, NS activity of PSC decreased ($p<0.05$) while BM products' NS activity increased ($p<0.05$) compared to culture in the absence of IL-2. NS activity levels were significantly different between PBL from normal donors and PSC from patients with NHL after culture with IL-2 ($p<0.05$). As these results show, NS activity is higher in PSC from NHL patients than that of controls and in the presence of suppressor cells, lymphocytes from the PSC products of NHL patients did not respond to IL-2 with an increase in proliferative capacity. [Journal of Turgut Özal Medical Center 1997;4(1):1-6]

***Key Words**: Non-Hodgkin lymphoma, IL-2, NS, suppressor cells, bone marrow cell, peripheral stem cell*

Non-Hodgkin lenfomalı hastalardan elde edilen periferik stem cell ve kemik iliği hücrelerinin natural supresör aktivitesi üzerine interleukin-2'nin etkisi

Non-Hodgkin lenfomalı 24 hastadan elde edilen periferik stem cell (PSC) ve kemik iliği ürünlerinin natural supresör (NS) hücre aktivitesi, IL-2 ile kültürden önce ve 5 gün IL-2 ile birlikte kültür yapılarak incelendi. Bu çalışmaya göre PSC ürünleri IL-2 ile stimüle edilmeden önce normal periferik kan lökositleri (PBL) ve kemik iliği (BM) ürünlerinden daha yüksek NS hücre aktivitesi gösterdi ($p<0.001$). İnterleukin-2 aktivasyonundan sonra, aktivasyon öncesine göre, PSC ürünlerinin NS hücre aktivitesi azalırken ($p<0.05$) BM'unki arttı ($p<0.05$). IL-2 ile kültürden sonra, normal donörlerden elde edilen PBL ile NHL'lı hastalardan elde edilen PSC ürünlerinin NS aktivitesi arasında istatistiki bakımdan önemli fark vardı ($p<0.05$). Bu sonuçlar, NHL'lı hastalarda NS aktivitesinin normalden daha yüksek olduğunu ve supresör hücrelerin varlığında NHL'lı hastaların PSC lenfositlerinin IL-2'ye proliferatif cevap vermediğini göstermektedir. [Turgut Özal Tıp Merkezi Dergisi 1997;4(1):1-6]

***Anahtar Kelimeler**: Non-Hodgkin lenfoma, IL-2, NS, supresör hücre, kemik iliği, periferik stem cell*

Lymphocytes that demonstrating nonspesific inhibition of some immune response are called natural suppressor cells. These cells have been found following total irradiation and in neonatal

[1] University of Nebraska Medical Center Department of Pathology and Microbiology, Omaha, NE, 68198
[2] University of Pittsburgh School of Medicine Department of Pediatrics Section of Allergy/Immunology, Pittsburgh, PA 15213, USA

animals. They may share a common lineage and function with natural killer (NK) cells.

The results of some studies indicate that there is an immune suppression after autologous BM transplantation and PSC transplantation as compared to normal PBL (1-4). These patients show a depressed T cell immune function including their immune responses to antigen and mitogen induced T cell proliferation and IL-2 production after stem cell transplantation (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 and to be associated with the presence of normal or increased numbers of circulating NK cells (6). The abnormal immune function may be associated, at least in part, with the immunologic characteristics of the infused PSC product. PSC products after transplantation may contain high levels of suppressor cell activity. The presence of suppressor cells may have an important role in the induction of immunosuppression observed post PSC transplantation and should be considered in any strategy of immune-based adjuvant therapy.

Interleukin-2 (IL-2) plays an important role in the clonal expansion of activated T lymphocytes. In addition to its T cell growth-stimulatory activity, generation of lymphokine-activated killer (LAK) cells, production of cytokines, enhancement of monocyte cytotoxicity, augmentation of NK cell activity, and stimulation of B cells' proliferation (7-11), IL-2 reverse some anergic mechanisms by allowing the T cells to undergo cell division (10,12). The peripheral blood of cancer patients contains B and T cells that recognize antigens expressed by autologous cancer cells, although immune tolerance can reduce the immunologic repertoire against the cancer cells (13). Furthermore, the infusion of T cell populations enriched with cells directed against defined immunodominant epitopes may provide adjuvant therapeutic activity (14).

Natural suppressor cell activity is mediated by cells of BM origin and has the ability to suppress the proliferative response of lymphocytes in a non-major histocompatibility complex (MHC) restricted manner (15). Although, the lineage of suppressor cells are undefined, the activity is detected in tissues undergoing intense hematopoietic regeneration, including neonatal spleen, adult bone marrow, the spleen following total lymphoid irradiation, cyclophosphamide administration, and tumor growth (16,17). It is possible that suppressor cells in the BM migrate to peripheral sites following appropriate stimulation (18,19). Macrophage-like cells, separable by adherence properties, have also been reported to suppress immune responses through the production of soluble factors. Immunosuppressive adherent-macrophage-like cells in rabbit bone marrow (20) have been shown to produce soluble factors capable of blocking both IL-2 induction and activity (21-23). Several studies have also demonstrated that recombinant IL-3 and recombinant granulocyte-monocyte-colony stimulating factor (rGM-CSF), are both efficient at activating bone marrow NS cells, even at very low cytokine concentrations (18,24,25). Activated NS cells produce a cytokine or cytokines which inhibited IL-2 synthesis of spleen cell. Suppressor macrophages appear in some conditions to inhibit the proliferative response of lymphocytes by a nitric oxide (NO)-mechanism dependent upon inducible NO synthetase. Furthermore, IFN-γ is a potent inducer of this enzyme and this cytokine plays a critical role in NS cell activation (26,27).

In a previous study (28), we found a significant depression in T cell proliferation (PHA mitogenesis) in NHL patients as well as significant levels of suppressor cell activity. Because of the potential role of such suppressor cells in delaying the immune reconstitution of PSC transplantation patients (7,26,29), we examined their role in the T cell dysfunction in stem cell products. The aim of this study was to evaluate the response of NS activity to culture using short-term (5 days) cultures with IL-2 in PSC and in BM product from NHL patients.

MATERIALS AND METHODS

Patients. This study was carried on 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 μg/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 ≥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.

Preparation of cells. The PSC, BM, or PB was diluted 1:1 (1:2 for PSC products) in Hanks 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 was 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 PSC's, 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 flask (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 this period cells were harvested and tested for cytotoxicity.

Suppression cell assay. The effect of phagocytic suppressor cells on lymphocyte proliferation in response to IL-2 by the cells in PSC and BM products and in normal PBL was investigated using the following experiment. PSC, BM, and PBL samples were cultured *in vitro* with IL-2 at 100 U/ml doses. The co-culture assay to measure suppressor cell function has been done following routine procedures. Briefly, Ficoll Hypaque purified normal donor PBL (1x10^5/ml) as responder cells were co-cultured with varying numbers of irradiated (500 cGy) putative suppressor cells (PBL or PSC product) starting at a suppressor to responder cell (S:R) ratio of 4:1 and an optimal concentration of PHA. Four, two-fold dilutions of suppressor cells (50 μl) were made in microplate with 96 flat bottom wells. The responder cells were then added (50 μl) together with the PHA (100 μl) to achieve 0.5 μg/ml. Cells were cultured at 37°C (5% CO_2) for three days in RPMI-1640 medium-10% FBS and the mitogenic response by the responder population assayed by pulsing with 1 μCi/well ^{3}H-thymidine over the final 18 hours of culture. Cells were harvested with a multi-well harvester onto fiberglass strips and the radioactivity determined in a Packard beta scintillation counter. The data were presented as the mean ± standart error (SE).

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.

In this study, we found that NS activity of PSC products from patients with NHL in the absence of IL-2 had been higher than normal PBL and BM ($p<0.001$) whereas BM products' NS activity had been lower than normal PBL ($p<0.05$) at S:R ratio equals to 2:1. In the same way, at 1:1 S:R cells ratio, NS activity of PSC products had been higher than normal PBL ($p<0.05$), while BM products' NS activity had been lower than normal PBL ($p<0.05$) again, but its significance degree was different (Figure 1A).

Following co-culture of PSC and BM products from NHL patients and PBL from healthy donors with IL-2 for 5 days, NS activity of PSC was higher than PBL ($p<0.05$), but there was no difference

between PSC and BM products. In the absence of IL-2, PSC products had significantly higher NS activity compared to after culture with IL-2 ($p<0.05$) (Figure 1B).

Significantly increased-NS activity levels in PSC from patients with NHL compared to PBL from healthy person suggested that suppressor cells prevent the proliferative response of lymphocytes to IL-2 in the PSC products from patients with non-Hodgkin lymphoma.

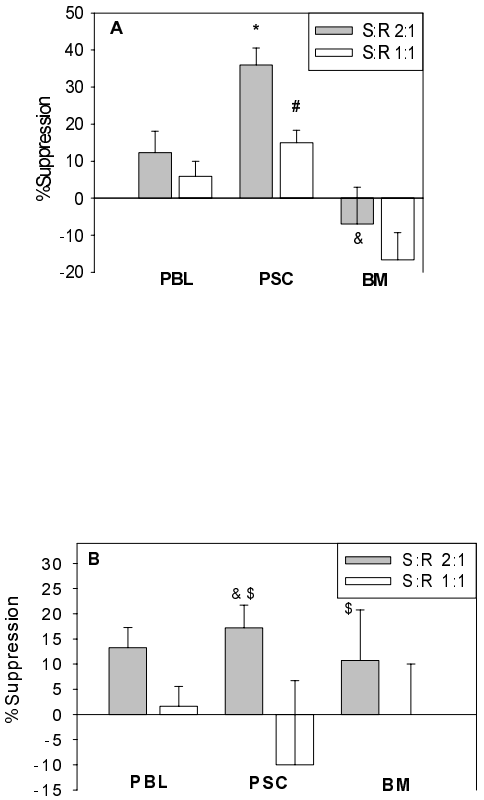

Figure 1. NS activity levels in PBL from normal donors and PSC and BM from NHL patients before in vitro activation with IL-2 (A). PBL, PSC and BM cells cultured 5 days in the presence of 100 U/ml IL-2 (B). Data represent the mean suppressor activity ±SE.

* Significant difference compared to PBL and BM ($p<0.001$)

\# Significant difference compared to PBL and BM ($p<0.05$)

& Significant difference compared to PBL ($p<0.05$)

$ Significant difference compared to before culture with IL-2 ($p<0.05$)

DISCUSSION

Autologous PSC transplantation has been used increasingly as an alternative to autologous BM transplantation after myeloablative therapy in treatment of some malignancies. Perhaps the greatest area of apparent improved efficacy with PSC transplantation as compared to autologous BM transplantation is the more rapid myeloid (30) and immunologic reconstitution (31) following PSC transplantation as compared to BM transplantation (31,32).

Preliminary results have suggested that relapse rate was significantly higher after autologous than allogenic transplantation (33,34). Therefore, the use of consolidative immunotherapy with IL-2 after autologous transplantation is being investigated as a potential means of eradicating residual disease (35,36). A number of studies have evaluated NK cytotoxic activity in bone marrow and mobilized apheresis stem cell products (11,37). NK cells are spontaneously cytotoxic for a variety of fresh and cultured tumor cells and this activity can be augmented by treatment with IL-2 (38,39). Verbik et al. (40) also studied the immunophenotypic characteristics of consecutive PSC collections from cancer patients mobilized with GM-CSF and compared the NK cytotoxic activity of PSC with PBL obtained from normal donors. They found that the cytotoxicity levels of PBL from normal donors were significantly greater, specially after *in vitro* activated with IL-2, compared with PSC obtained from either normal donors or cancer patients.

Monocyte-macrophages play an important role in the immune system and the regulation of lymphocytes, both by direct cellular interactions and by the secretion of soluble factors (41-44). Positive and negative immunomodulatory responses are tightly linked to regulation of IL-2 and its receptors during T cell activation. Proliferation of T lymphocytes is dependent on the interaction of IL-2 with its specific receptor following T lymphocyte activation (20,45). Recent reports have suggested that monocytes can also exert a negative control resulting in an inhibition of IL-2 production and IL-2 stimulation (45,46). Munn et al. have suggested that antigen presenting cells (APC) can induce a selective deletion of mature T cells at a point before their clonal expansion (47). In that study, a form of activation-induced T cell death (apoptosis) was

proposed. They found that proliferation was inhibited by macrophage-derived CSF (M-CSF) in a contact-dependent manner. The macrophage associated apoptosis was affecting resting T cells before blastogenesis and cell division, and preventing functional activation and clonal expansion. Furthermore, lymphocytes cultured with phagocytic suppressor cells lost their capacity to proliferate in the presence of exogenous IL-2 or PHA.

The present study suggests that the suppressor activity (monocyte-like lineage) inhibits the proliferation and expansion of T cells. IL-2 can only stimulate activated T cells, since resting T cells do not express high-affinity IL-2 receptors. In contrast to T cells, IL-2 can stimulate both the proliferation and cytotoxicity of NK cells since these cells normally express IL-2 receptors. Our results suggest that phagocytic suppressor cell primarily exerts its effect on T cell proliferative response to IL-2, by blocking their activation and preventing the expression of IL-2 receptors.

In conclusion, we demonstrate that in the presence of suppressor cells, lymphocytes from the PSC products of NHL patients had not respond to IL-2 with an increase in proliferative capacity. Further studies are needed to identify the mechanism involved in this activity of suppression. The observation of suppressor cell activity within the PSC products suggest that these cells may not only block *in vitro* response of lymphocytes to IL-2 but also immune reconstitution and response to adjuvant immunotherapy.

REFERENCES

1. Kessinger A, Vose JM, Bierman PJ, et al. Peripheral stem cell transplantation in non-Hodgkin's lymphoma patients. J Hematother 1993; 2(3):361-2.
2. Langenmayer I, Weaver C, Buckner CD, et al. Engraftment of patients with lymphoid malignancies transplanted with autologous bone marrow, peripheral blood stem cells or both. Bone Marrow Transplant 1995; 15:241-6.
3. Joshi SS, Kessinger A, Mann SL, et al. Detection of malignant cells in histologically normal bone marrow using culture techniques. Bone Marrow Transplant 1987; 1(3):303-10.
4. Anderson KC, Dzik WH. Transfusion medicine in hematopoietic stem cell and solid organ transplantation. In: Hoffman R, Benz EJ, Shattil SJ, Furie B, Cohen HJ, Silberstein LE, eds. Hematology Basic Principles and Practice. 2nd ed. Churchill Livingstone, USA 1995; 2074-87.
5. Lum LG, Ueda M. Immunodeficiency and the role of suppressor cells after human bone marrow transplantation. Clin Immunol Immunopathol 1992; 63:103-9.
6. Gottschalk LR, Bray RA, Kaizer H, et al. Two populations of CD56 (Leu-19)+ /CD16+ cells in bone marrow transplant recipients. Bone Marrow Transplant 1990; 5:259-64.
7. Talmadge JE, Reed EC, Kessinger A, et al. Immunologic attributes of cytokine mobilized peripheral blood stem cells and recovery following transplantation. Bone Marrow Transplant 1996; 17:101-9.
8. Smith KA. Interleukin-2: Inception, impact, and implications. Science 1988; 240:1169-76.
9. Minami Y, Kono T, Miyazaki T, et al. The IL-2 receptor complex. Its structure, function, and target genes. Annu Rev Immunol 1993; 11:245-67.
10. Mass RA, Dulles HFJ, Den Otter W. Interleukin-2 in cancer treatment: Disappointing or (still) promising? A review. Cancer Immunol Immunother 1993; 36:141-8.
11. Neubauer MA, Benyunes MC, Thompson JA, et al. Lymphokine-activated killer (LAK) precursor cell activity is present in infused peripheral blood stem cells and in the blood after autologous peripheral blood stem cell transplantation. Bone Marrow Transplant 1994; 13:311-6.
12. Beverly B, Kang SM, Leonardo MJ, et al. Reversal of in vitro T cell clonal anergy by IL-2 stimulation. Int J Immunol 1991; 4:661-71.
13. Houghton AN. Cancer antigens: Immune recognition of self and altered self. J Exp Med 1994; 180:1-4.
14. Eberl G, Kessler B, Eberl LP, et al. Immunodominance of cytotoxic T lymphocyte epitopes co-injected in vivo and modulation by interleukin-12. Eur J Immunol 1996;26: 2709-16.
15. Rodriguez R, Angulo I, Vinuela JE, et al. Inhibition of bone marrow-derived natural suppressor activity by glucocorticoids and its reversal by IFN-g or IL-2. Transplantation 1994; 58:511-7.
16. Mortari F, Singhal SK. Production of human bone marrow-derived suppressor factor. Effect on antibody synthesis and lectin-activated cell proliferation. J Immunol 1988; 141:3037-42.
17. Mortari F, Bains MA, Singhal S. Immunoregulatory activity of human bone marrow identification of suppressor cells possesing OKM-1, SSEA-1, and HNK-1 antigens. J Immunol 1986; 137:1133-7.
18. Moore SC, Theus SA, Barnett JB, et al. Cytokine regulation of bone marrow natural suppressor cell activity in the suppression of lymphocyte function. Cell Immunol 1992; 141:398-408.
19. Schreiber KL, Forman J. Effects of bone marrow-derived natural suppressor activity on B cell responses to lipopolysaccharide. Transplantation 1993; 56:700-8.
20. Soderberg LS. Differential activities of rabbit bone marrow suppressor cells. Int Arch Allergy Appl Immunol 1984; 74:341-6.

21. Krakauer T. A macrophage-derived factor that inhibits the production and action of interleukin 2. J Leuko Biol 1985; 38:429-39.
22. Wallis RS, Fujiwara H, Ellner JJ. Direct stimulation of monocyte release of interleukin-1 by mycobacterial protein antigens. J Immunol 1986; 136:193-6.
23. Maes LY, York JL, Soderberg LS. A soluble factor produced by bone marrow natural suppressor cells blacks interleukin-2 production and activity. Cell Immunol 1988; 116:35-43.
24. Sugiura K, Ikehara S, Inaba M, et al. Enrichment of murine bone marrow natural suppressor activity in the fraction of hematopoietic progenitors with interleukin 3 receptor-associated antigen.. Exp Hematol 1992; 20:256-63.
25. Schmidt PA, Wright MA, Matthews JP, et al. Mechanisms of immune suppression in patients with head and neck cancer: presence of CD34+ cells which suppress immune functions within cancers that secrete granulocyte-macrophage colony-stimulating factor. Clin Cancer Res 1995; 1:95-103.
26. Angulo I, Rodriguez R, Garcia B, et al. Involvement of nitric oxide in bone marrow derived natural suppressor activity. Its dependence on IFN-g. J Immunol 1995; 155:15-26.
27. Langrehr JM, Hoffman RA. Nitric oxide: A new endogenous immunomodulator. Transplantation 1993; 55:1205-12.
28. Özerol İH, Şenol M, Ageitos A, et al. 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 J Turgut Özal Medical Center 1996; 3(4): 306-10.
29. Schmidt-Wolf IG, Dejbakhsh-Jones S, Ginzton N, et al. T-cell subsets and suppressor cells in human bone marrow. Blood 1992; 80:3242-50.
30. Gratwohl A, Hermans J, Baldomero H. Hematopoietic precursor cell transplantation in Europa: Activity in 1994. Report from the European group for blood and marrow transplantation (EBMT). Bone Marrow Transplant 1996; 17:137-48.
31. Korbling M, Juttner C, Henon P, et al. Autologous blood stem cell versus bone marrow transplantation. Bone Marrow Transplant 1992; 10 (Suppl 1): 144-8.
32. Goldman JM. Peripheral blood stem cells for allografting. Blood 1995; 85: 1413-6.
33. Appelbaum FR, Buckner CD. Overview of the clinical relevance of autologous bone marrow transplantation. Clin Haematol 1986; 15:1-18.
34. Kessinger A, Armitage JO. The evolving role of autologous peripheral stem cell transplantation following high dose therapy for malignancies. Blood 1991; 77:211-213.
35. Benyunes M, Massumoto C, Higuchi. Interleukin-2 + lymp hokine-activated killer cells as consolidative immunotherapy after autologous bone marrow transplantation for acute myelogenous leukemia: A preliminary report. Bone Marrow Transplant 1993;12:159-63.
36. Klingemann HG. Introducing graft-versus-leukemia into autologous stem cell transplantation. J Hematother 1995; 4:261-7.
37. Arbour S, Toupin S, Belanger R, et al. Phenotypic and functional characterization of peripheral blood and bone marrow natural killer cells prior to autologous transplantation. Bone Marrow Transplant 1996; 17:315-22.
38. Robertson MJ, Ritz J. Biology and clinical relevance of human natural killer cells. Blood 1990; 76:2421-38.
39. Salup RR, Wittrut RH. Treatment of adenocarcinoma in the peritoneum of mice: Chemo-immunotherapy with IL-2-stimulated cytotoxic lymphocytes as a model for treatment of minimal residual disease. Cancer Immunol Immunother 1986; 22:268-71.
40. Verbik DJ, Jackson JD, Pirruccello SJ, et al. Functional and phenotypic characterization of human peripheral blood stem cell harvests: A comparative analysis of cells from consecutive collections. Blood 1995; 85:1964-70.
41. Mills CD. Molecular basis of "suppressor" macrophages. Arginine metabolism via the nitric oxide synthetase pathway. J Immunol 1991; 146:2719-23.
42. Weiss A, Fitch FW. Macrophages suppress CTL generation in rat mixed leukocyte cultures. J Immunol 1977; 119:510-6.
43. Oehler JR, Herberman RB, Campbell DA, et al. Inhibition of rat mixed lymphocyte cultures by suppressor macrophages. Cell Immunol 1977; 29:238-50.
44. Hibbs JB, Vavrin Z, Taintor RR. L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. J Immunol 1987; 138:550-65.
45. Wu MX, Daley JF, Rasmussen RA, et al. Monocytes are required to prime peripheral blood T cells to undergo apoptosis. Proc Natl Acad Sci USA 1995; 92:1525-9.
46. Stevens SR, Shibaki A, Meunier L, et al. Suppressor T cell activating macrophages in ultraviolet irradiated human skin induce a novel, TGF-beta dependent form of T cell activation characterized by deficient IL-2r-alpha expression. J Immunol 1995; 155:5601-7.
47. Munn DH, Pressey J, Beall AC, et al. Selective activation-induced apoptosis of peripheral T cells imposed by macrophages: A potential mechanism of antigen-specific peripheral lymphocyte deletion. J Immunol 1996;156:523-32.

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