Defective Cytokine Production following Autologous Stem Cell Transplantation for Solid Tumors and Hematologic Malignancies Regardless of Bone Marrow or Peripheral Origin and Lack of Evidence for a Role for Interleukin-10 in Delayed Immune Reconstitution

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ABSTRACT

A substantial body of evidence accumulated in recent years indicates a protracted delay in immune reconstitution following autologous stem cell transplantation. In order to investigate the cellular basis of this phenomenon, peripheral blood mononuclear cells were studied from recipients of autologous stem cell transplantation for solid tumors and hematological malignancies. On stimulation with phytohemagglutinin and phorbol 12-myristate 13-acetate, transplant-derived peripheral blood mononuclear cells demonstrate statistically significant decreased production of interleukin 3 (IL-3), IL-4, granulocyte-macrophage colony-stimulating factor, and γ-interferon as compared to normal controls, during the first 6 months following engraftment, which recover to normal levels 6 months or more posttransplant. When the overall group of transplant recipients is compared to the control group, there is a statistically significant lower production of IL-2. In addition, no differences were observed regardless of the source of the engrafted stem cells, whether from bone marrow alone (autologous bone marrow transplantation), from peripheral blood stem cells alone, or from a combination of autologous bone marrow transplantation and peripheral blood stem cells. The defect persisted past 6 months postengraftment. Transplant-derived peripheral blood mononuclear cells were stimulated with combinations of either phytohemagglutinin plus the calcium ionophore A23187, thereby circumventing the requirement for accessory cell function, or with phorbol 12-myristate 13-acetate plus anti-CD28 monoclonal antibody, mimicking the CD28-B7 cell surface-ligand interaction capable of triggering and stabilizing IL-2 gene transcription. In both situations, decreased production of IL-2 as compared to controls was observed in individuals within 6 months of transplantation.

Quantitative polymerase chain reaction indicates that decreased transcription of IL-2 mRNA following transplantation is not due solely to a decrease in the absolute numbers of CD4+ T-cells but is secondary to reduced numbers of transcript copies per cell. Production of IL-10 was found to be decreased regardless of whether the autologous graft was of bone marrow or peripheral blood origin. These findings are consistent with the conclusion that: (a) multiple dysregulations exist in the production of cytokines important in immune homeostasis; (b) a defect occurs at or prior to the level of transcription of IL-2 mRNA; (c) IL-10 does not play a direct role in the pathogenesis of posttransplantation immunosuppression; and (d) there is no evidence that peripheral blood stem cells may be superior to bone marrow-derived stem cells in accelerating immune reconstitution.

INTRODUCTION

Restoration of normal T-cell function in patients receiving high dose chemotherapy followed by ABMT is often delayed for a period of several months to several years, with, characteristically, diminished absolute T-cell numbers, alterations in distribution of T-cell subsets, defective elaboration of cytokines, and decreased cellular proliferation (1–7). Unlike allogeneic bone marrow transplantation, ABMT with or without infusion of autologous peripheral blood stem cells involves no histoincompatibility, no graft-versus-host disease, and no administration of immunosuppressive therapy. The pathogenesis of the defective T-cell physiology therefore likely differs in the two types of grafting, at least in part. Although T-cells in autologous transplant recipients have been observed to have defective production of IL-2, the primary T-cell growth factor, in response to stimulation with mitogen and to interaction with both anti-CD3 and anti-CD2 monoclonal antibody (8), it remains unclear what underlies the defective signal transduction leading to a blunted rise in intracellular calcium concentration ([Ca2+]i) essential for transcription of IL-2 mRNA (9). Defective cytokine production may arise from decreased transcription and/or instability of cytokine mRNA itself, or, alternatively from defective translation and reduced cytoplasmic half-life of the polypeptide product.

Human IL-10, a homodimer with a molecular weight of 39,000 daltons is produced by CD4+ T-cells, by activated CD8+ cells, by Epstein-Barr virus-transformed lymphoblastoid cell lines, and by monocytes (10) and has potent inhibitory effects on antigen-specific T-cell proliferation (11, 12). In addition, human IL-10 depresses the production of a number of cytokines by peripheral blood mononuclear cells activated by either anti-CD3 or PHA, including IFNγ, GM-CSF, IL-3, and IL-2 (13). In vitro, IL-10 inhibits allospecific cytotoxic T-cell responses when allogeneic purified monocytes are used as stimulator cells, while anti-IL-10 antibody enhances the proliferative response, suggesting a role for endogenously produced IL-10 in suppressing proliferation in primary mixed lymphocyte culture (12). Together these observations suggest that IL-10 acts as a strong immunosuppressive factor and that dysregulated overproduction of IL-10 might contribute to the defect in cytokine production and T-cell proliferation seen following autologous transplantation.

Recent evidence has indicated that transplantation of stem cells derived from peripheral blood may lead to a more accelerated hematological and immunological recovery than that provided by ABMT alone, possibly by providing a higher percentage of differentiating progenitor cells for engraftment (14, 15). If that were true, peripheral blood stem cells would be the ideal choice for autologous transplantation not only because of the practical ease of harvest as compared to bone marrow-derived cells but also for functional reasons in promoting immune reconstitution.

In order to address these questions and investigate the nature of impaired cytokine production and defective T-cell immune response postengraftment, we have studied PBMC from recipients of autologous transplantation for solid tumors and hematological malignancies.

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2. The abbreviations used are: ABMT, autologous bone marrow transplantation; IL, interleukin; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; PCR, polymerase chain reaction; GM-CSF, granulocyte-macrophage colony-stimulating factor; BCSN, carbustine; IFNγ, γ-interferon; CDNA, complementary DNA.

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Following *in vitro* stimulation, transplantation-derived PBMC are shown to have significantly defective production of a number of cytokines important in immune homeostasis in the early posttransplantation period. Depressed transcription of IL-2 mRNA is demonstrated in the same early posttransplant group, regardless of the source of engrafted stem cells, whether from bone marrow (ABMT) or from peripheral blood (PBSC), suggesting that there are no qualitative differences between the two types of grafts in promoting immune reconstitution. Furthermore, by quantitative PCR, this deficit is shown not to be related solely to decreased absolute numbers of CD4^+^ cells following transplantation but to decreased number of IL-2 transcript copies per cell. Transplant-derived PBMC were further stimulated with combinations of either PHA plus the calcium ionophore A23187, thereby circumventing the need for accessory cell, or with PMA plus anti-CD28 monoclonal antibody mimicking the CD28-B7 cell surface-ligand interaction capable of triggering and stabilizing IL-2 gene transcription (16). In both of these situations, decreased production of IL-2 as compared to controls was observed in individuals within 6 months of transplantation, suggesting that following engraftment, production of cytokine is dysregulated at more than one step in the early stages of T-cell activation. Lastly, production of IL-10 was found to be decreased regardless of whether the autologous graft was of bone marrow or peripheral blood origin not only indicating a defect in the mononuclear cells producing IL-10 but also suggesting that IL-10 does not play a direct role in posttransplantation immunosuppression. These *in vitro* findings have direct *in vivo* implications in future strategies of stem cell harvesting and possible therapeutic interventions in an effort to accelerate immune reconstitution following autologous transplantation.

**MATERIALS AND METHODS**

**Patients and Grafts.** A total of 66 patients with ages ranging from 14 to 62 years (median, 40 years) were grafted for the following malignancies: non-Hodgkin’s lymphoma (n = 22); small cell carcinoma of the lung (n = 10); breast carcinoma (n = 11); Hodgkin’s disease (n = 6); acute nonlymphocytic leukemia (n = 7); acute lymphoblastic leukemia (n = 2); germ cell tumor (n = 2); rhabdomyosarcoma (n = 1); multiple myeloma (n = 1); adenocarcinoma of unknown origin (n = 2); and undifferentiated carcinoma (n = 1). Thirty-six patients received ABMT alone, 13 received both ABMT and PBSC transplant, while 17 received solely PBSC transplant. Pretransplantation chemotherapy of several regimens consisted primarily of the combinations cyclophosphamide-BNCU-melphalan, cyclophosphamide-BCNU-etoposide, carboplatin-thiotepa-cyclophosphamide, BCNU-etoposide-1-β-arabinofuranosylcytosine-C-melphalan, or 1-β-arabinofuranosylcytosine-C-cyclophosphamide plus total body irradiation) Forty-one patients were studied within 6 months postgrafting (median, 50 days following transplantation), 25 patients 6 months or more postgrafting (median, 654 days). A group of 16 age-matched normals were selected as controls.

Forty to 60 ml of blood were obtained from the transplant subjects or from the normal volunteers with the approval of the Ethics Committee of the Institution. PBMC were isolated from heparinized venous blood by density gradient on Ficoll-Hypaque (Pharmacia, Piscataway, NJ) and washed twice in PBS. The PBMC were counted and analyzed for purity by flow cytometry.

**Reagents.** PHA (Wellcome, Dartford, United Kingdom) was reconstituted in sterile water for a stock of 1 mg/ml and used for *in vitro* stimulation at 1 μg/ml. PMA (Sigma Chemical Co., St. Louis, MO) and A23187 (Calbiochem, La Jolla, CA) were dissolved in dimethyl sulfoxide for stock solutions of 10 ng/μl and 10 mg/ml, respectively, and used for stimulation at 15 ng/ml and 1 μg/ml, respectively. Anti-CD28 monoclonal antibody (CLB-402) was purchased from Janssens Biochemica (Geel, Belgium).

**RNA Extraction and cDNA Synthesis.** Total cytoplasmic RNA was isolated from 5 X 10^6 PBMC by the hot phenol extraction method as described (17). After initial denaturation of the RNA, cDNA was synthesized with reverse transcriptase (Gibco/BRL, Gaithersburg, MD) and random hexanucleotides (Pharmacia) in a reaction containing 50 mM Tris (pH 8.3), 20 mM KCl, 10 mM MgCl_2, 5 mM dithiothreitol, and 1 mM concentrations of each deoxyribonucleotide triphosphate for 1 h at 42°C.

**PCR and Analysis of Amplification Products.** The product of the reverse transcription reaction was diluted to 100 μl, and 5 μl were used for PCR amplification. Quantitative PCR was carried out by comparing the amplification product using as template the cDNA generated from sample PBMC RNA to that obtained using as template a known cDNA probe derived from the human IL-2 gene *pTCGF-11* (American Type Culture Collection, Rockville, MD). In order to clearly differentiate the band sizes of the two PCR products, a 220-base pair *XbaI* site fragment derived from adenovirus type 2 DNA was ligated to a *XbaI* site in exon 3 of the *pTCGF-11* IL-2 gene and the modified gene inserted into the PetB site of the PEGEM-4Z plasmid (Promega, Madison, WI). The plasmid DNA was linearized by digestion with EcoRI, and RNA synthesis was carried out for 1 h at 37°C in 40 mM Tris (pH 7.4), 6 mM MgCl_2, 5 mM dithiothreitol, 4 mM spermidine, 40 units of RNase inhibitor, 1 mM concentrations of nucleotide triphosphates, and T7 RNA polymerase (Boehringer-Mannheim, Mannheim, Germany). cDNA was then synthesized as described above. The upstream 20-mer primer (5’-AGTCGAC-CTACTTCAAGTTC-3’) situated in exon 1 and the 20-mer downstream primer (5’-GCTATTCACATGATGT-3’) situated in exon 4 yielded a PCR product spanning 2 introns and 2 exons. A range of known concentrations of the modified *pTCGF-11* IL-2 template and a constant amount of each reverse transcribed PBMC cDNA sample were coamplified in the same tube in a reaction containing 50 mM KCI, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl_2, 4 mM deoxyribonucleotides triphosphates, 15 pmol of each primer and Taq DNA polymerase (Gibco/BRL). For quantification of the PCR products, 22P-labeled upstream primer was incorporated into each of the amplification reactions. 40 pmol of oligonucleotide was end-labeled with [γ-32P]ATP (>5000 Ci/mmol) and T4 polynucleotide kinase (Gibco/BRL) and unincorporated radiouclide was removed by passage through a NENSORB column (Dupont, New England Nuclear, Boston, MA). Amplification consisted of 30 cycles of 94°C denaturation (1.5 min), 55°C annealing (2 min), and 72°C extension (3 min). PCR products of the correct size were visualized on agarose gels stained with ethidium bromide. The specific bands were excised and radioactivity was measured.

**Assay for Cytokine Production.** PBMC (1 x 10^6 cells/ml) were stimulated with mitogens in 48-h cultures. Supernatants were assayed for production of IL-2, IL-3, IL-4, GM-CSF, IFNγ, and IL-10 in 96-well plates by enzyme-linked immunosorbent assay as recommended by the manufacturer (Amersham International, Amersham, United Kingdom; Biosource International, Camarillo, CA).

**Statistical Analysis.** Values are expressed as the mean ± SEM. The significance of differences between groups was calculated using the Student-Newman-Keuls test.

**RESULTS**

Production of IL-3, GM-CSF, IL-4, and IFNγ. Results of cytokine production in PBMC derived normals versus that seen in recipients of ABMT or autologous peripheral blood stem cell transplantation is presented in Fig. 1. Not unexpectedly, variability in IL-3 production is seen among both the normal controls and the transplant recipients, depending on what may be called the baseline immunological activation of the individual at the time of stimulation with mitogen. Following stimulation with PHA and PMA, mean values for normals were 2,067 ± 441 pg/ml for IL-3 (n = 14), 16,997 ± 2,313 pg/ml for GM-CSF (n = 15), and 8,806 ± 1,417 pg/ml for IFNγ (n = 15). Mean values for IL-4 was 136 ± 33 (n = 15) following stimulation with PHA plus PMA and 406 ± 88 pg/ml (n = 15) with PMA plus A23187.

Mean values for PBMC derived from autologous transplant recipients engrafted less than 6 months is shown in Fig. 1. Because they represent only 2 of the group of 13, recipients of both peripheral blood stem cells either alone or in conjunction with autologous bone marrow are included together with the larger group that received only autologous transplantation.
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Fig. 1. Values of IL-3, GM-CSF, IL-4 and IFNγ as determined by enzyme-linked immunosorbent assay in the supernatant of mitogen-stimulated PBMC (1 × 10⁶ cells/ml) at 48 h: PHA, 1 μg/ml; PMA, 15 ng/ml; A23187, 1 μg/ml. Cells were derived from normal (C) individuals, patients engrafted less than 6 months (<6M) and patients grafted greater than 6 months (>6M). All values are pg/ml.

OGous marrow. Mean values for lectin- and PMA-stimulated lymphocytes showed statistically significant decreases as compared to PBMC derived from normal individuals for IL-3 (676 ± 218 pg/ml; n = 13, P = 0.011), for GM-CSF (4811 ± 1309 pg/ml; n = 13, P < 0.001), and for IFNγ (3259 ± 1199 pg/ml; n = 10, P = 0.011). For IL-4, the mean value after stimulation with lectins and PMA was 23.4 ± 11 pg/ml (n = 19) which represents a difference statistically significant from that of the normal controls (P = 0.001), while that of PMA plus A23187 was 243.7 ± 59.6 pg/ml (n = 19) and was not statistically different from that of the control group (P = 0.125).

The corresponding values for the group of patients longer than 6 months following engraftment are depicted in Fig. 1. As in the previous analysis, the small number of patients who received ABMT plus PBSC or PBSC only are considered together with those who received autologous marrow only.

Mean values for lectin- and PMA-stimulated lymphocytes were 1418 ± 417 pg/ml for IL-3 (n = 18) and 7640 ± 2039 pg/ml for IFNγ (n = 16), which are not statistically different from the values obtained from the normal controls (P = 0.298 and P = 0.646, respectively). However, the mean value of GM-CSF in the supernatants of the PHA plus PMA-stimulated PBMC derived from patients grafted for more than 6 months was significantly higher than the normal stimulated PBMC (36,540 ± 6,186 versus 16,997 ± 2,313 pg/ml; P = 0.002). PHA plus PMA stimulation in this group yielded a mean IL-4 value of 100 ± 27 pg/ml (n = 19) and PMA plus A23187 yielded a mean IL-4 value of 435 ± 87 pg/ml (n = 17), neither statistically different from the normal group (P = 0.399 and P = 0.820).

IL-2 Production in the Presence of Lectin Plus Phorbol Ester. Among the group of normal controls, mean IL-2 production following stimulation of PBMC with PHA plus PMA for 48 h was 48,120 ± 8,346 pg/ml (n = 15) (Fig. 2). When the overall group of transplanted patients is compared to the control group (n = 63), there is a statistically significant lower production of IL-2 (12,004 ± 2,138; P < 0.001) although some patients had normal values. The type of autologous grafting for those engrafted for less than 6 months (ABMT alone, n = 15; PBSC alone, n = 14; or combination of ABMT and PBSC, n = 9) does not substantially influence the production of IL-2, since there were no statistical differences in the mean IL-2 production among the 3 subgroups (8771 ± 3124, 3822 ± 1589, 6668 ± 1943; P > 0.05). When patients are compared according to the time postgrafting, independently of the type of grafting, there is no statistical difference between those engrafted for less than 6 months (n = 38) or longer than 6 months (n = 25).

Following stimulation with PMA plus A23187, the mean IL-2 value was 123,780 ± 44,390 pg/ml (n = 15), while the mean value following stimulation with PMA plus anti-CD28 was 12,813 ± 7,280 (n = 11) for the normal controls. Mean IL-2 values following stimulation with PMA plus A23187 in PBMC derived from transplant recipients less than 6 months postengraftment (ABMT, ABMT plus PBSC, and PBSC) was 20,877 ± 5,762 (n = 14; P = 0.035) and with PMA plus anti-CD28 were 2,672 ± 529 (n = 17; P < 0.001). The corresponding values in patients engrafted for longer than 6 months were 48,973 ± 8,978 pg/ml (n = 17, P = 0.090) and 8,059 ± 2,534 pg/ml (n = 15; P = 0.188), respectively, which were not statistically different from those of normal controls.

IL-10 Production in the Presence of Lectin Plus Phorbol Ester. Production of IL-10 by PBMC was assessed following 48 h of stimulation with PHA plus PMA (Fig. 3). The mean production by control PBMC was 620 ± 161 pg/ml (n = 13). For the transplanted individuals engrafted less than 6 months considered as a whole without subcategorization, the value was 72 ± 15 (n = 63) which is statistically significantly lower than the control group (P < 0.001). This value was not statistically significantly different from the value
obtained in the group engrafted longer than 6 months (101 ± 32 pg/ml; n = 23). It should be noted, however, that several patients had extremely very low values, while some had values in the range of normal controls. Furthermore, among patients engrafted less than 6 months, no differences were seen as a function of the source of engrafted cells. Comparison of IL-10 production between the subgroups of autotransplanted patients grafted for less than 6 months, ABMT alone (50 ± 27 pg/ml; n = 35) versus ABMT plus PBSC (75 ± 43 pg/ml; n = 11) and versus PBSC (48 ± 17 pg/ml; n = 13) showed no statistically significant differences (P = 0.620; P = 0.958).

Transcription of IL-2 mRNA following Transplantation. Consistent with the observed depressed constitutive production of IL-2 in autograft recipients, Northern blot analysis of unamplified mRNA extracted from 5 x 10⁶ PBMC derived from study subjects yielded no detectable IL-2 message, although the presence of intact mRNA was confirmed by hybridization with an actin gene probe (not shown). To overcome this, as well as the unavailability of large numbers of PBMC from transplant recipients, IL-2 mRNA was amplified by PCR. To quantify the amplification product, a concurrent amplification was run in the same tube, using as template a known amount of a size-modified IL-2 gene easily differentiable on fractionating gels from the sample amplification product. Both the sample and indicator products incorporated upstream primers labeled with [γ-32P]ATP. For quantification, a range of known amounts of indicator amplification product was compared to a constant amount of sample amplification product. Characteristic results are shown in Fig. 4. The expected molecular sizes of 546 base pairs for the indicator product and 326 base pairs for the sample product are indicated. By relating the amount of extracted mRNA used for generating PCR template cDNA to the known number of source cells, the number of copies of transcribed IL-2 mRNA per PBMC derived from either normal controls or transplant recipients could be calculated (Table 1). For the 5 normal controls analyzed, the number varied between 100 and 750/PBMC, while for the PBMC derived from autotransplanted recipients the values varied from 5 to 100/PBMC. By flow cytometric determination of CD4⁺ cells, the expression of IL-2 mRNA per CD4⁺ cells in the transplanted patients was calculated to vary between 13 and 714 copies/cell assuming that the CD4⁺ cells are the major producers of IL-2.

DISCUSSION

High-dose chemotherapy and transplantation of autologous bone marrow transplantation and/or peripheral blood progenitor cells have in recent years been investigated as consolidation following standard dose induction therapy in an effort to improve long-term disease-free survival in selected patients with lymphoma (18, 19), with germ cell tumors (20), and to increase the complete response rates in patients with metastatic breast carcinoma (21). However, this approach is frequently associated with an immunodeficiency affecting primarily cellular immunity. Paradoxically, defects have been reported to be more profound in autologous transplantation as compared to allogeneic bone marrow transplantation in in vitro tests for both lymphokine-secreting helper function and IL-2-dependent cytotoxic function (7).

In order to further characterize the nature of this defect, we have analyzed the in vitro production of a number of immunoregulatory cytokines by PBMC derived from autologous transplant recipients. For the purpose of comparison of overall cytokine production in

Fig. 2. IL-2 production by PBMC in the presence of lectin, phorbol ester, calcium ionophore and anti-CD28: PHA 1 µg/ml, PMA 15ng/ml, A23187 1 µg/ml. Values for PBMC stimulated with PHA + PMA are grouped according to the type of autologous grafting (ABMT alone versus ABMT+PBSC versus PBSC alone). Values for PBMC stimulated with PMA + either calcium ionophore or anti-CD28 are presented according to the time post engraftment: less than 6 months (<6M), longer than 6 months (>6M). All values are pg/ml.
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autologous transplantation to previous studies in allogeneic engraftment, the recipients were considered here as a group, regardless of whether they received bone marrow alone, bone marrow plus PBSC, or PBSC alone. Furthermore, transplant recipients were divided into an early group (less than 6 months postengraftment) and a late group (longer than 6 months postengraftment). The rationale for doing so resides in both the protracted and individual heterogeneity in immune reconstitution following transplantation which can require from several months to a full year, as well as a direct means of comparison to the previously analysed group of allogeneic transplant recipients (22). Nevertheless, the two situations are not directly comparable.

While allogeneic transplantation is complicated by the presence of graft versus host reactions and the administration of immunosuppressive agents to prevent it, immune reconstitution following allogeneous transplantation would be a truer reflection of unmanipulated immune ontogeny. In that context, in the present study we report decreased production of IL-3, GM-CSF, IL-4, and IFNγ following PHA and PMA stimulation of PBMC derived from recipients engrafted for less than 6 months. Furthermore, when stimulated with PMA and a calcium ionophore, IL-4 production was only partially corrected.

Our results indicating depressed production of IFNγ in mitogen-stimulated autologous transplant-derived lymphocytes differs somewhat from those reported by Heslop (25, 26) who found a preserved ability of both allogeneic and autologous transplant-derived lymphocytes to augment both IFNγ and tumor necrosis factor secretion in vitro in response to added IL-2 to a degree greater than in lymphocytes derived from individuals treated with chemotherapy alone. This apparent discrepancy is explicable by the fact that mitogen and IL-2 act at different stages of lymphocyte activation. Indeed, in that study the (CD4 CD8) CD3 population was found on addition of IL-2 to be an important source of IFNγ, and it is precisely the IL-2-producing population, principally CD4 T-cells, which we find to be defective in response to mitogen and either calcium ionophore or anti-CD28 (Fig. 2). Bypassing this defect by therapeutic administration of IL-2 to transplant recipients may in fact serve to at least partially overcome the early blocks in T-cell activation which we demonstrate here.

In analysis of IL-2 production, autologous transplant recipients were analyzed in three groups: those receiving bone marrow alone; those receiving bone marrow plus PBSC; and those receiving PBSC alone. The rationale for doing so arises from the suggestion that transplantation of PBSC might provide a more accelerated hematological recovery compared to that observed following ABMT (14, 15), and that this could be due to the grafting of more differentiated progenitor cells. Once engrafted, PBSC-derived transplants might then be expected to achieve immune reconstitution more efficiently than ABMT-derived lymphocytes as reflected in their ability to produce IL-2, the principal T-cell cytokine. In fact, we found PBMC from autologous transplant recipients of less than 6 months to be defective in IL-2 production in response to in vitro stimulation by mitogen and that, in addition, no significant differences existed between those receiving ABMT alone, those receiving PBSC alone, and those receiving both.

Likewise, comparison of production of IL-10 by the three groups shows equally depressed levels (Fig. 3), suggesting that stem cells are qualitatively the same from either source and that, significantly, there may be no biological advantage of accelerated reconstitution in the use of peripheral blood progenitor cells.

Fig. 3. Production of IL-10 by PBMC following stimulation with PHA (1 μg/ml) plus PMA (15 ng/ml). Values shown are those of the normal control group (C), the overall group of patients engrafted less than 6 months (<6M), and the group engrafted greater than 6 months (>6M). All values are pg/ml.

Fig. 4. PCR amplification using as templates cDNA of the size-modified competitor IL-2 gene and reverse-transcribed RNAs of the sample PBMC. The two template cDNAs were amplified simultaneously for 30 cycles in the same tube with identical sets of primers, as outlined in "Materials and Methods." Right, molecular weight. bp, base pairs.
Addition of phorbol ester, bypassing the requirement for accessory cell, fails to stimulate IL-2 production to levels comparable to normal controls in all three groups of recipients following engraftment. When cells were stimulated with PMA and anti-CD28, IL-2 production was likewise found to be decreased in recipients engrafted for less than 6 months, suggesting that either engagement of the CD28-B7 ligand is dysfunctional in transplanted T-cells or that even if engaged, CD28-B7-mediated stabilization of IL-2 transcription and production is quantitatively defective. These findings corroborate those recently reported indicating not only a quantitative decrease in the numbers of CD3+ T-cells expressing CD28 in the first 12 months following autotransplantation but also suppressed CD28 proliferation and IL-2 secretion in response to B7 or anti-CD28 (27). These results and the fact that monocytes obtained from healthy individuals cannot restore normal T-cell function in recipients of ABMT (6) suggest that the accessory cells are not responsible in themselves for the observed impaired immune reconstitution. Although stimulation of T-lymphocytes through their specific receptor by agents that mimic T-cell receptor occupancy in the absence of costimulation may result in a dysfunctional in transplanted T-cells as compared to normal controls. IL-2 mRNA per PBMC suggests that the decreased production of IL-2 following autologous transplantation is not due solely to a fall in absolute numbers of CD4+ T-cells (Table 1), itself a well-described phenomenon implicated in the delayed immune reconstitution following engraftment (6). Single cell in situ hybridization of posttransplant lymphocytes is in progress in an attempt to further clarify the question of functional heterogeneity of engrafted T-cells.

Amplification by PCR was chosen because the low number of peripheral blood mononuclear cells available from patients having undergone ABMT and the low transcript copy number per cell hamper detection of cytokine mRNA by standard Northern or dot blot hybridization. A number of different approaches have been developed to quantify gene expression (30–34). However, because even minute amounts of template mRNA can be artifically amplified and detected, the very sensitivity of quantitative PCR has contributed to the difficulty of the technique. Coamplification of the reverse transcribed mRNA template was carried out in an adaptation of the technique described by Gilliland (31). Amplification by PCR of depressed transcription of IL-2 mRNA per PBMC suggests that the decreased production of IL-2 following autologous transplantation is not due solely to a fall in absolute numbers of CD4+ T-cells (Table 1), itself a well-described phenomenon implicated in the delayed immune reconstitution following engraftment (6). Single cell in situ hybridization of posttransplant lymphocytes is in progress in an attempt to further clarify the question of functional heterogeneity of engrafted T-cells.

A number of recent studies have investigated serum cytokine levels following autologous transplantation. Baiocchi et al. (35) described a sequential coordinated pattern of production of GM-CSF, G-CSF, IL-1α, IL-3, IL-6, and IL-8 in the first 23 days following engraftment and a parallel serum appearance of G-CSF and IL-8 and neutrophil...

### Table 1: Quantitative determination of IL-2 mRNA copies per cell in PBMC derived from control and autotransplanted individuals following 6 hours in vitro stimulation with PHA + PMA

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<td>360</td>
<td>10</td>
<td>27/69</td>
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<td>OH14</td>
<td>NHL</td>
<td>493</td>
<td>100</td>
<td>14/13</td>
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<td>Die</td>
<td>M</td>
<td>510</td>
<td>25</td>
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<td>Sti</td>
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<td>715</td>
<td>5</td>
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<td>Tre</td>
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<td>1095</td>
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<td>19/40</td>
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4 BC, breast carcinoma; NHL, non-Hodgkin's lymphoma; SCLC, small cell lung carcinoma; AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; HD, Hodgkin's disease; GT, germ cell tumor; M, multiple myeloma.

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recovery (35). Similar studies have documented the endogeneous production of growth factors following autologous transplantation at levels commensurate with hematological reconstitution in adults (36–38) and in children (39) However, to date, all studies of in vitro cytokine production, both constitutional and stimulated, have been done in allogeneic transplants, showing defects in production of IL-2 (8, 23), of IL-3 and GM-CSF (24), and of IL-6 and tumor necrosis factor α (40). Immune reconstitution following allogeneic transplantation involves the effects of both histoincompatibility and immunosuppressive drugs administered to combat it. Autologous transplantation, entailing neither of the two, offers a more direct, unmanipulated picture of immune ontogeny. The present study represents the first detailed study of in vitro cytokine production following autologous transplantation.

PBMC used in these studies were obtained from transplant recipients following bone marrow recovery. Therefore, assessments of cytokine production were not designed to be prognostic values in predicting transplantation outcome but rather a mechanistic indicator of defective immune reconstitution following successful autologous engraftment. Furthermore, by design, solely in vitro studies were carried out to avoid variations in in vivo serum cytokine production that may be induced by factors unrelated to the graft itself, such as infection or administered agents. In order to isolate and characterize the posttransplantation T-cell dysfunction in vitro, we used mitogen acting on T-cells and not on other T-cell lineages.

The mechanism of inhibition by IL-10 on antigen-specific T-cell proliferation and on cytokine production is both by a direct negative effect on T-cells (12) and indirectly by inhibiting the antigen-presenting capacity of monocytes, but not Epstein-Barr virus-transformed lymphoblastoid cells, through down-regulation of class II MHC expression (41). The decreased T-cell proliferative response to antigen-presenting monocytes pre-incubated with IL-10 is accompanied by a decreased ability to mobilize intracellular calcium and is only partially restored by high concentrations of exogeneous IL-2 (41). In light of these functional considerations and because of the previously suggested role of increased IL-10 levels in the pathogenesis of human immunodeficiency virus-associated immunosuppression (42) IL-10 production was analyzed in the defecation immune reconstitution of transplantation recipients.

When production of IL-10 was compared in the three groups of autologous transplanted recipients engrafted for less than 6 months (ABMT alone versus ABMT plus PBSC versus PBSC alone) no differences were found. Furthermore, the defect persists in a statistically significant manner in recipients engrafted for longer than 6 months (Fig. 3). This observation suggests that the demonstrated depression in IL-2 production is not related to suppression by IL-10. The absence of quantitative difference between the transplant cells, whether from bone marrow or from peripheral blood, suggests one of two possibilities: either the immune defect following autologous grafting of hematopoietic stem cells is independent of the cell source; or, alternatively, changes induced by prior chemotherapy have effaced such potential differences. In vivo observations have suggested that primitive hematopoietic stem cells and long-term hematopoiesis may be compromised to varying degrees in recipients of cytotoxic agents commonly used in either standard multiagent chemotherapy or in specific bone marrow-conditioning regimens (43), while other evidence has implicated cyclophosphamide-induced changes in bone marrow endothelium and alterations in the traffic of transplanted cells into the hematopoietic compartment (44). Furthermore, in vivo evidence exists that myeloablative therapy is at least partially responsible for posttransplantation immune defects, because such defects are observed following infusion of normal bone marrow in allogeneic transplantation, even after immunosuppression is discontinued. In mice, bone marrow cells injected into unirradiated syngeneic recipients successfully implant into bone marrow, suggesting that bone marrow elements, unhindered by cytotoxic therapy, effectively repopulate the hematopoietic system (45). Nevertheless, the numerical reimplantation is not tantamount to functional immunological reconstitution. Further studies in the murine model and/or in human subjects having received variable rounds of pretransplantation cytotoxic therapy may help to answer these questions and contribute to devising optimal schedules in an attempt to collect maximally functional lymphocytes and accelerate immune reconstitution following transplantation.

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REFERENCES


Defective Cytokine Production following Autologous Stem Cell Transplantation for Solid Tumors and Hematologic Malignancies Regardless of Bone Marrow or Peripheral Origin and Lack of Evidence for a Role for Interleukin-10 in Delayed Immune Reconstitution

Thierry Guillaume, Maryam Sekhavat, Daniel B. Rubinstein, et al.


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