Interleukin-7-enhanced cytotoxic T lymphocyte activity after viral infection in marrow transplanted mice

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Summary:

Lethally irradiated BALB/c mice were reconstituted by syngeneic bone marrow transplantation (BMT), and injected with recombinant interleukin 7 (rIL-7), recombinant interleukin 2 (rIL-2), or saline 10 days post-transplantation. Intranasal infection with A/PR8/34 influenza virus 2 weeks after BMT was associated with the highest survival rate in the rIL-7-treated group. The protective mechanism elicited by rIL-7, as manifested by very low virus titers in the lung, involves T and B cell functions. High hemagglutinin inhibition antibody levels were observed on days 7 and 12 post-challenge in the rIL-7 mice. Moreover, the anti-influenza cytotoxic T lymphocyte activity was induced primarily by rIL-7, leaving the effect of rIL-2 on the same level as that of the control. Thus, rIL-7 promotes both T cell-mediated function and B cell production during the immunodeficient state after BMT. This cytokine may prove a potential immunotherapeutic modality in BMT recipients.

Keywords: bone marrow transplantation; interleukin 7; T lymphocytes

Autologous bone marrow transplantation (BMT) is being used with increasing frequency to permit administration of otherwise lethal doses of irradiation, chemical agents or various combinations of both, in an attempt to eradicate tumor cells in patients with malignancies.^{1,2} Numerous studies have shown the addition of myeloid growth factors to the treatment regimen after autologous BMT to hasten the time to durable engraftment of hematopoietic cells.³ In contrast to the recovery of the myeloid cells, the reconstitution of lymphocyte function is delayed, making it the causative mechanism for the prolonged immune deficiency state post-BMT, both with regard to protection from infection and immune-mediated anti-tumor activity.^{4–6}

The research addressing the mechanism(s) of immune deficiency and its correction spans a wide variety of immunomodulators, including interleukins (IL), without tangible success. Investigations in BALB/c mice dealing with the

induction of IL-2 mRNA and IL-2 production after syngeneic BMT (sBMT) showed early restoration of this cytokine, implying a relative minor role of endogenous IL-2 in the pathogenesis of post-BMT immune deficient state.⁷

Interleukin 7 (IL-7), a 25 kDa glycoprotein isolated from the murine bone marrow stromal cell line, has been described as a growth promoting, and possibly differentiating, factor of pre-B cells and immature thymocytes.^{8–10} Endogenous IL-7 has also been found to be involved in the development of cytotoxic T lymphocytes (CTL), as evidenced by complete blocking of its activity by anti IL-7 serum.¹¹ The cytokine has further been shown to promote V(D)J gene rearrangement in the precursor T cells.¹² In an earlier investigation, we probed the ability of IL-7 to induce rearrangement of the V β 8 T cell receptor gene after sBMT in mice;¹³ it appeared that rIL-7 is directly involved in signal transduction of both V(D)J and RAG-1 gene expression. Bolotin *et al*¹⁴ very recently found, in line with our results, that administration of IL-7 after BMT enhanced thymopoiesis, even when donor bone marrow was depleted of mature T lymphocytes.

The present murine study investigates the effect of rIL-7-induced gene rearrangement on immune function in the context of viral infection after sBMT in mice. The experiments were drafted so as to follow the kinetics of the specific immune response against influenza virus at the level of antibody production, as well as to detect generation of cytotoxic T lymphocytes.

Materials and methods

All animal procedures utilized in this study were approved by the Institutional Committee for Animal Experimentation.

Mice

Female BALB/c mice, aged 10–16 weeks, were obtained from the Hebrew University-Hadassah Medical School mouse colony. The animals were fed a regular diet and acidified water without antibiotics, and were maintained under standard conditions with no isolation.

Total body irradiation

The mice were positioned in radiation chambers and exposed to a total lethal dose of 800 cGy delivered from

a linear accelerator (Varian clinac 6X) at a dose rate of 170 cGy/min, at a source-to-skin distance of 80 cm.

Bone marrow transplantation

The donor mice were killed by cervical dislocation; the long bones (femora, tibiae and humeri) were removed and the marrow cavities were flushed with RPMI 1640 medium (Biolab, Jerusalem, Israel) using a 25-gauge needle. Cell suspensions were filtered through a fine nylon mesh and washed twice in RPMI medium. Aliquots (0.3 ml medium containing 10^7 nucleated cells) were injected intravenously into the lateral tail vein of recipient mice 24 h post-irradiation.

Administration of rIL-2 and rIL-7 in vivo

All mice were subjected to total body irradiation and subsequent sBMT, after which they were divided into three groups (15 animals/group). One group was injected intraperitoneally with rIL-7 (100 ng $\times$ 2/day for 10 days), the second group received intraperitoneal rIL-2 (1000 units $\times$ 2/day for 10 days), and the third group was given saline (control). A fourth group of non-transplanted mice served as untreated (normal) animals. Each experiment was repeated three times. Human rIL-2 was kindly provided by Eurocetus/Chiron BV (Amsterdam, The Netherlands) as 1 mg proleukin (18×10^6 international units is equivalent to 3×10^6 cetus units) and human rIL-7 was a gift from Peprotech (Princeton, NJ, USA) in the form of lyophilized powder soluble in water. The cytokine was reconstituted in phosphate buffered saline (PBS) to a concentration of 100 ng/ml.

Challenge with influenza virus

Susceptibility of the sBMT recipients and non-transplanted (normal) mice to influenza virus infection was tested by intranasal administration of the live influenza A/PR/8/34 H1N1 virus 2 weeks after BMT. Each mouse received 50 μ l PBS containing 10^6 50%/EID₅₀ under light anesthesia. Survival was monitored daily during the experimental period.

Cytotoxic T lymphocyte (CTL) cultures in vitro

Single-cell preparations from pooled spleens from mice (three mice/group) immunized 2 weeks previously with the influenza virus or recombinant vaccinia virus were cultured.¹⁵ Briefly, 15×10^6 spleen cells were mixed with 5×10^6 syngeneic, irradiated (2000 rad) A/PR/8 influenza virus-infected feeder spleen cells in 10 ml complete medium (RPMI 1640, 10% FCS, 5×10^{-5} M 2-mercaptoethanol, penicillin and streptomycin). The cultures were harvested after 7 days incubation at 37°C and assayed for ⁵¹Cr release. The responding CTL were harvested and tested for their activity.

⁵¹Cr release cytotoxicity assay

For this assay P₈₁₅ cells were used as targets. The cells were washed twice with serum-free medium (RPMI 1640),

infected with 50 μ l of A/PR/8 influenza virus-infected allantoic fluid, and labeled with 150 μ Ci ⁵¹Cr (Amersham, Arlington Heights, IL, USA) for 90 min at 37°C as previously described.¹⁶ Four concentrations of CTL (effector-to-target ratios of 30:1, 10:1, 3:1 and 1:1) were added in triplicate to 10^4 influenza-infected target cells in 96-well U-shaped bottom plates in RPMI 1640 with 10% FCS. The plates were centrifuged and incubated for 6 h at 37°C. An aliquot (100 μ l) of supernatant was then removed from each well, and ⁵¹Cr release was determined in a gamma counter. Spontaneous and maximal release were determined by incubating ⁵¹Cr-labeled A/PR/8 influenza virus infected P₈₁₅ cells with either 100 μ l media or 100 μ l of Triton X-100 (Fisher Scientific, Pittsburgh, PA, USA). The percentage of specific chromium release was calculated by subtracting spontaneous released counts per minute from release in the presence of CTL divided by the maximal released counts per minute minus the spontaneous release. This fraction was multiplied by 100.

Serum hemagglutination inhibition antibody titration

Individual blood samples were collected by bleeding from the retro-orbital sinuses and sera were treated with vibrio cholera receptor destroying enzyme (Sigma, St Louis, MO, USA) for 18 h at 37°C, followed by heat inactivation for 30 min at 56°C. HI antibody titers were determined by microtitration using four hemagglutination units.

Protection

The susceptibility of each experimental group to influenza infection was tested by an intranasal challenge with 50 μ l PBS containing the lethal dose of 10^6 50%/EID₅₀ units, administered under light anesthesia 2 weeks after immunization. Seven and 12 days following challenge, mice were killed and their lungs removed, frozen in PBS containing 0.1% gelatin and stored at -70°C. To detect viable virus in lungs, 2×10^4 MDCK cells were seeded into each well of a 96-well tissue culture microplate and incubated overnight at 37°C. A/PR/8 influenza virus-infected allantoic fluid and the lung sample suspensions were serially diluted in 10-fold steps from 10^{-1} to 10^{-9} in DMEM containing 1% bovine serum albumin, 0.01% sodium bicarbonate, and 0.1% gentamicin. A 25- μ l aliquot of an appropriate dilution of each dilution was added to MDCK cells in triplicate wells of tissue culture plates. After 1 h incubation at 37°C, 175 μ l DMEM with bovine serum albumin, sodium bicarbonate, gentamicin and 2 μ g/ml trypsin (Sigma type IX from porcine pancreas, crystallized, dialyzed, lyophilized) was added to each well. Forty-eight hours later, the virus titers were established by determining the hemagglutination titers of supernatants from each individual well. The TCID₅₀ was calculated using the Reed and Muench method¹⁷ and given in log₁₀ units.

Statistical evaluation

The independent *t* test was used for comparison between the groups. Differences at the *P* < 0.05 level were considered significant.

Results

Effect of rIL-2 and rIL-7 on influenza viral replication in the lung of post-sBMT mice

Lungs were harvested 7 and 12 days post-viral inoculation, homogenized, serially diluted and seeded on MDCK cells. The $\log_{10}$ tissue culture infectious dose of the influenza titers was used to express the rate of viral replication in the lungs in mice that underwent BMT and treated by either saline or each of the cytokines. As seen in Table 1, 7 days after inoculation, the rate of viral replication was highest among the controls, followed by IL-2-treated mice, the IL-7-treated group showing the lowest viral titers. Complete clearance of the influenza virus from the lungs of the IL-7 treated mice was observed by day 12, while the control and IL-2 group still showed a heavy load of the virus (differences between IL-7 vs IL-2 and saline groups were statistically significant, $P < 0.05$ for both days tested). In parallel, survival was monitored within each of the experimental group. Within 2 weeks of influenza challenge, $>90\%$ of the rIL2 and saline-treated mice succumbed; in the rIL-7 group $>40\%$ survived for more than 20 days ($P = 0.0035$) (data not shown). The dosages of the two cytokines used in this study did not lead to toxic death in the virus-free controls.

Effect of rIL-2 and rIL-7 on hemagglutination inhibition antibody titers post-sBMT

To elucidate the mechanism(s) underlying the high viral clearance in the IL-7-treated mice, HI antibody serum titers were measured 7 and 12 days post-inoculation. While all three groups demonstrated HI titers considered protective against influenza infection (1:40),¹⁵ the rIL-7-treated mice had the highest titer on day 7, increasing to significantly high values on day 12 ($P < 0.05$; Figure 1a).

Effect of rIL-2 and rIL-7 on CTL activity level post-sBMT

To assay specific cytotoxicity against the influenza virus, CTL activity was measured in splenic cells 12 days after viral inoculation. The IL-7-treated mice showed the highest

Figure 1 (a) Effect of IL-2 and IL-7 treatment on antibody formation following sBMT and intranasal challenge with live A/PR/8 influenza virus. Values represent mean $\pm$ s.d. of three experiments. (b) Activity of anti-influenza cytotoxic T lymphocytes in mice inoculated with the virus. Percentage of ^{51}Cr released from influenza-infected P_{815} cells by spleen cells from mice inoculated 2 weeks after marrow transplantation. CTL-to-target (E:T) ratios of 30:1, 10:1, 3:1 and 1:1 percentage release was calculated by subtracting spontaneous released counts per minute from release in presence of CTL divided by maximal released counts per minute minus spontaneous release. Values represent 1/3 representative experiments.

CTL activity against the influenza virus, especially at an effector-to-target ratio of 30:1 (Figure 1b). The transplanted saline group (control) showed enhanced cytotoxic activity superseding that of the non-transplanted-virus challenged (normal) mice (Figure 1b). This phenomenon was also reflected in the level of viral infection in the lungs (Table 1). This effect is probably due to the known 'upshoot' of cytotoxicity occurring after TBI. The CTL activity in the IL-2-treated group was similar to that seen in the controls (saline).

Discussion

Recipients of marrow-ablative chemotherapy or chemoradiotherapy prior to BMT are at high risk of viral infection until engraftment of lymphoid precursors evolve into mature T and B cells capable of restoring normal protective mechanisms.^{4,6,18} The precise cellular and molecular *in vivo* events that underlie the immunosuppressive state post-BMT are largely obscure. In the present murine study, IL-7 treatment post-sBMT induced a specific immune

Table 1 Pulmonary infection of mice challenged with live A/PR/8 influenza virus after sBMT and subsequent IL-2 and IL-7 treatment

<i>Treatment group</i>	<i>Log₁₀ TCID₅₀ of lung influenza titers</i>						<i>Mean ± s.d.</i>
<i>Day 7</i>							
Normal	3.5	4.25	3.8				3.850 ± 0.377
Control (saline)	5.8	4.25	4.8	6.25	4.8		5.180 ± 0.819
rIL-2	4.25	4.25	4.5	4.5	4.25	3.8	4.258 ± 0.250
rIL-7	2.8	3.5	3.5	3.25	2.8		3.170 ± 0.353
<i>Day 12</i>							
Normal	<1	1.8	1.8				1.533 ± 0.462
Control (saline)	2.5	2.5	2.5	<1			2.125 ± 0.750
rIL-2	3.5	3.25	3.25	2.5			3.125 ± 0.433
rIL-7	<1	<1	<1	<1			<1

The values refer to individual mice.

response, providing protection from influenza virus infection by increasing both antibody production and cytotoxic cellular activity.

IL-7 is a potent stimulus in generating proliferative signals to B and T cell precursors and, to a lesser extent, in activating mature T cells.^{19–21} In fact, IL-7-deficient mice have demonstrated profound inhibition of lymphoid development, in contrast to gene inactivation of other cytokines.²² It has been suggested that the lymphokine acts via mechanism(s) enabling it to support the rearrangement of the T cell receptor β gene in cultured thymocytes.¹² In a previous study rIL-7 immunotherapy was shown to induce early transcription of the RAG-1 gene, and consequent accelerated V β 8 gene rearrangement.¹³ In the present investigation, we used an *in vivo* approach to pinpoint the fundamental role of T cell receptor gene rearrangement within the realm of IL-7 activity inducing protection from viral infection after BMT. The cytokine boosted survival after challenge with influenza virus post-sBMT.

With regard to IL-2, there is accumulating data that immunotherapy with this cytokine may decrease the danger of relapse of hematologic malignancies.^{23,24} It has been postulated that IL-2 might induce, or augment, the graft-versus-leukemia (GVL) reaction via its effect on T and non-T lymphocytes.^{25,26} In animals, IL-2 has been shown to induce a GVL effect; however, it may also either exacerbate or protect against graft-versus-host disease (GVHD) depending on the model, the IL-2 regimen and time of administration.^{27–29} In the post-transplant set up, IL-2 is a potential immunomodulator affecting primarily mature T cells, inducing an anti-tumor effect.

There exists compelling evidence^{12–14} that IL-7 stimulates immature T and B cells, which turns it into a promising agent in the fight against complications occurring after BMT or in other immunodeficiency states, because (1) it enhances the development of the V(D)J reconstitutive apparatus and promotes immune recovery; (2) it likely generates anti-tumor effects by pathways different from those of IL-2; and (3) it appears to be relatively nontoxic in animals.^{12,19,30}

Using sBMT as a basic model, it appeared that IL-7 is capable of rendering protection from viral infection, as opposed to the lesser efficacy of IL-2. The mechanism underlying the reduced viral replication, to nearly zero on day 12 post-infection, in the IL-7-treated animals can be attributed in part to the correlation found to exist between the kinetics of the serum HI antibodies, CTL activity and viral clearance from the lungs. IL-2 was capable of inducing a certain level of, presumably CD4 cell-mediated, protection against influenza virus infection. The phenomenon of the relatively low CTL activity mounted by the normal mice has been reported before.¹⁵ The augmentation of the CTL response following cytokine therapy might be a result of stimulation-susceptible lymphocytes post-BMT.

In the light of these findings it is justifiable to assume that IL-7 activity was targeted at more than one cell type, that is induction of specific anti-influenza T and B cell response. Within the network of cytokine regulation, non-specific immune response, such as natural killer (NK) and lymphokine-activated killer cells, remains a possibility. We postulate that the prompt response to IL-7 administration

reflects a lack of IL-7 production after BMT, a factor that can only be established once accurate appropriate assays become available.

In a mouse model of fibrosarcoma, IL-7 surpassed IL-2 and IL-4 in generating CTL activity against the tumor,^{31,32} findings that inspired us to examine specific cell cytotoxicity against viral infection. The CTL activity against the influenza virus was primarily IL-7-induced, leaving the effect of IL-2 on the same plane as that of the saline in the controls. Considering that, unlike IL-2, IL-7 is a growth factor for the earliest lymphoid cells,²² its mechanism of action in the protection from viral infection might be primarily CD8-mediated. It should be taken into account, however, that in the murine complex each of the cytokines may elicit CTL activity from different cell populations.¹⁹

As far as we could ascertain, the present study is the first to show that *in vivo* administered rIL-7 boosts specific T cell differentiation and improves B cell function post-sBMT. Although clinical trials with rIL-7 in BMT patients are still awaited, and its effects on the basic malignant disease,³³ GVHD, rejection and, especially, induction of GVL via non-specific NK cells remain to be established, the current data suggest a potential role of this cytokine as an immunotherapeutic modality early after BMT.

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