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Expression of interleukin-7 in the model of orthotopic liver transplantation in rats

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ABSTRACT

The purpose of this experiment was to determine whether interleukin-7 (IL-7) is involved in the development of acute liver rejection in the model of orthotopic liver transplantation in rats. We evaluated IL-7 expression using samples serially obtained from the various organs, on 1, 3, 5, 7, 10 post-operative day (POD, n=5 at each POD) in two groups: Group I --BN ($RT1^n$) to BN rats; Group II --DA ($RT1^a$) to BN rats. IL-7 gene sequence and IL-7 mRNA were detected with PCR and Northern blotting. IL-7 levels were determined with enzyme-linked immunosorbent assay (ELISA), and data were expressed as mean±standard error. Group I animals (n=5) survived indefinitely. Whereas group II animals (n=5) died within 12.8±2.7 days due to the acute rejection proved histologically. In both groups, IL-7 gene sequence could be detected in every organ examined by PCR. In Northern blotting, IL-7 mRNA could be detected in 100% of thymic and bone marrow samples, and one third of the kidney in both groups. In ELISA, IL-7 levels of group I remained low throughout the experiment in each organ, whereas in group II, IL-7 levels increased until POD 10, with statisticaly significant differences against those in group I on POD 10, in the serum, liver and thymus ($p<0.05$) (serum--3.60±0.39 vs. 0.63±0.09, liver--7.29±1.4 vs. 2.66 ±0.38, thymus--13.3±5.8 vs. 0.91±0.32). In conclusion, IL-7 was increasingly produced in the thymus during the acute rejection of the graft liver. The thymus and its IL-7 are thought to be playing an important role in the acute rejection of the graft liver. *Ryukyu Med. J., 17(2)79~84, 1997*

Key words: interleukin-7, liver transplantation, acute rejection, thymus

INTRODUCTION

In 1988, IL-7 was purified as a proliferative factor of pre-B cell from mouse myeloid stroma cell line[1], followed by isolation of it's cDNA[2]. It was a natural speculation that IL-7 might be involved in proliferation of the thymocyte and T cell lineage because of its mRNA expression in the thymus[2]. In fact, in vitro studies revealed that IL-7 was playing an important role in maintaining or proliferating several T cell subsets in the thymus, either with or without other cytokines, like IL-2, IL-4 or IL-6[3-6]. IL-7 was also proved to induce proliferation of CD4+or CD8+peripheral T cells[7-9] and LAK cells[9,10].

Although these cells are known to participate in graft rejection, in vivo role of IL-7 has not been clarified in transplant immunology. In this experiment, we hypothesized that IL-7 might be involved in the development of acute rejection of the liver graft. Our specific aim in this experiment is to serially evaluate IL-7 levels in various organs of the rat recipients, with or without acute rejection of the graft liver.

MATERIALS AND METHODS

Animals:

Adult male DA ($RT1^a$) and adult Brouwn Norway (BN: $RT1^n$) rats, 10 to 16 weeks of age, were purchased from Ryukyu-Biotec (Okinawa, Japan). All animals were housed in wire-bottomed cages with controlled light/dark cycles and adequately air-conditioned environment, fed on a standard laboratory diet, and given free access to water.

Orthotopic Liver transplantation:

Orthotopic rat liver transplantation was performed using previously described techniques[11] under sterilized conditions. All recipients were isolated in a private

cage and were administered antibiotics subcutaneously (tobramycin, 1 mg/day) for 2 consecutive days post transplant.

Experimental Group:

Two groups were used in this study: adult male BN ($RT1^n$) rats served as recipients of donor liver grafts, that were obtained from adult BN ($RT1^n$, Group I, syngeneic) or DA ($RT1^a$, Group II, allogeneic) rats. Nine animals in each group were allowed to survive until death for survival analysis. No animal received immunosuppressive drugs in either of the two groups. An autopsy was performed immediately after death to confirm patency of the vascular anastomosis and to rule out biliary obstruction or infection. Five rats were sacrificed from both groups on post-operative day (POD) 1, 3, 5, 7, 10 to obtain samples from various organs (liver, kidney, thymus, bone marrow, spleen, bile and serum) of the liver grafted recipients. Bile was collected by cannulating a polyethylene tube (PE-10, Imamura Inc., Japan) into the graft bile duct, starting one hour prior to sacrifice. Samples obtained were either snap frozen or fixed in 10% formalin. Frozen samples were stored in liquid nitrogen until use.

Polymerase chain reaction:

Sampled organs were diced coarsely, rinsed in iced RNase-free saline, and frozen immediately in liquid nitrogen. Genomic DNA was purified using phenol-chloroform extraction, followed by suspension in TE buffer and storage at -70℃. For the bone marrow, 50 μl of the sample was suspended in 500 μl TE buffer, and washed twice. The sample was then incubated with 50 μl (200 μg/ml) proteinase K and 0.5% NP-20 solution (50 μl) for 90 min at 55℃ and 10 sec at 95℃.

One mg of extracted DNA was amplified by polymerase chain reaction (PCR) in a total volume of 100 μl, which contained 2 mM deoxynucleoside triphosphatase, 50 pmol 5' and 3' oligonucleotide primers, 1 mM KCl, 100 mM Tris-HCl (pH 8.9), 15 mM $MgCl_2$, and 2U Taq polymerase (Boehringer, Manheim). The amplification profile consisted of 50 cycles of denaturing at 95℃ for 1 min, annealing of primers at 50℃ for 2 min, and extension at 72℃ for 2 min. Primer sequences were chosen from separate eons of the genes such as described in the literature[12].

Reverse transcription polymerase chain reaction (RT-PCR).

Total cytoplasmic RNA was extracted from the tissue by the Acid Guanidinium-Phenol-Chloroform (AGPC) method[13]. Complementary DNA was synthesized using total RNA sample as a template. Total RNA (1 μg) was incubated at 42℃ for 30 min with a mixture of 200 units of reverse transcriptase (Super Script: Bethesda Research Laboratories, Galthersburg, USA), 50 mM Tris-HCl (pH8.3), 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 20 mM each dNTP, 20 units of RNase inhibitor (RNA guard: Pharmacia, USA) and 0.2 μg of DNA random hexamers (Takara Shuzo Co., Kyoto, Japan) in a volume of 30 μl. An aliquot (5 μl) of reverse transcribed products was mixed with 2 units of Taq DNA polymerase (Ampli Taq DNA pol, Hoffmann-La Rache), and 20 p mol each of sense and antisense primers in a buffer containing 20 mM Tris-HCl (pH 8.3), 40 mM KCl, 2.5 mM $MgCl_2$, 100 mg/ml BSA and 20 μM each dNTP in 50 μl. The mixture was overlaid with 30 μl mineral oil to prevent evaporation and then amplified by 50 cycles of PCR (Perkin-Elmer, Cetus). The thermal cycle profile used in this study is; a) denaturing for 60 sec at 94℃, b) annealing primers for 90 sec at 40℃, and c) extending the primers for 60 sec at 72℃. A portion (10 μl) of PCR mixture was electrophoresed in a 3% agarose gel in 0.04M Tris-acetate and 0.001M EDTA buffer. The gel was stained with ethidium bromide and photographed. Primer sequences used in this RT-PCR were identical with the one used in PCR analysis[12].

Southern blotting of PCR product.

Ten micro liter of RT-PCR products were electrophoresed in 3% agarose gel for 3 hr and transferred for 3 hr to a nitrocellulose filter using capillary method. Filters were then UV cross linked and hybridized at 42℃ in a solution containing a IL-7 cDNA probe labeled with ^{32}P, which was made from the murine IL-7 plasmid DNA (Sumitomo Bio-Science Inc.). Filters were then exposed to film.

Protein assay:

Tissue homogenates were prepared using a Brinkmann polytron (Brinkmann Instruments, Inc., Westbury, NY) in 1.5ml phosphate-buffered saline containing 2 mM phenylmethylsulfonyl fluoride protease inhibitor (Sigma, USA). Tissue homogenates were briefly centrifuged in a microcentrifuge to remove excess particulate matter. Supernatants were then transferred to microtubes and stored at -70℃ until use. Total protein levels were quantitated using BCA protein assay reagent (Pierce Inc., USA). Briefly, 20 μl of sample was transferred to the microplate and thereafter incubated with 200 μl of BCA protein assay reagent for 30 min at 37℃. Results were analyzed spectrophotometrically with a microplate reader.

IL-7 mRNA studies by Northern blotting:

Total cellular RNA was prepared by AGPC method. RNA was quantitated spectrophotometrically by using O.D.260 nm determinations. After electrophoretic separation in a 1.0% formaldehyde agarose gel, RNA was transferred for 3 hr to a nitrocellulose filter using capillary method. Filters were then hybridized overnight at 42℃ in a solution containing a IL-7 cDNA probe labeled with ^{32}P. Filters were then exposed to film.

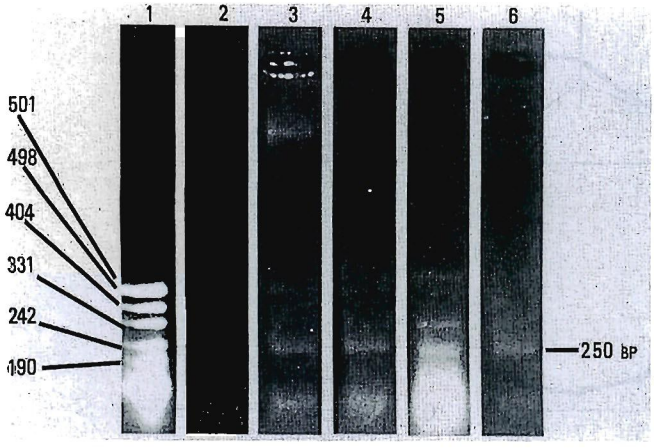

Fig. 1 PCR analysis for the detection of the IL-7 gene in various tissue. Lane 1: pUC19. digested with Hap II as a size marker [sizes in base pairs (bp) at left], lane 2: bone marrow (group I, POD5), lane 3: bone marrow (group II, POD5), lane 4: thymus (group II, POD5), lane 5: liver (group I, POD5), lane 6: kidney (group I, POD5). The band located at 250 bp was uniformly detected in various tissue except liver. In lane 5, multiple bands at 240, 330, 380, >700 bp were detected, and was impossible to decide the positivity for IL-7.

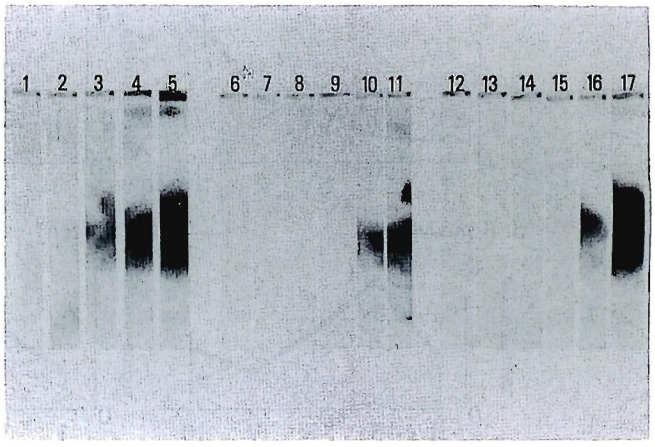

Fig. 2 Northern blot analysis of IL-7 mRNA in the samples obtained from various tissues of the recipients. Lanel-5: group II, POD5 (1: liver, 2: spleen, 3: kidney, 4: thymus, 5: bone marrow), lane 6-11: group I, POD5 (6: liver, 7: spleen, 8: serum, 9: kidney, 10: thymus, 11: bone marrow), lane 12-17: group II, POD7 (12: liver, 13: spleen, 14: serum, 15: kidney, 16: bone marrow, 17: thymus). IL-7 mRNA could not be detected in the samples from the liver, spleen, or serum (Lane 1-3, 6-8, 12-14). In the bone marrow and thymus IL-7 mRNA was uniformly detected in both groups (Lane 4, 5, 10, 11, 16, 17).

ELISA assay:

The expression of IL-7 in the tissue homogenate and the serum was assayed using ELISA assay. Each 0.1 μg of goat anti-murine IL-7 neutralizing antibody (R & D System INC., USA) in 50 μM carbonate-buffer was dispensed into 96-well microtiter plates. The plates were incubated overnight at 4℃ and blocked with 1% BSA-PBS. Duplicate samples of 100 ml were plated and incubated for 2 hr at room temperature. After adding 0.2 μg of biotinylated (NHS-LC-Biotin: Pierce Inc., USA) anti IL-7 antibody, the plates were incubated for 2 hr at room temperature. The plates were further incubated with 100 μl of HRP streptavidine conjugate (diluted at 1:5,000) (Zymed Inc., USA) and 100 μl of OPD solution (13mg / 12ml solution; Wako-Junyaku Inc., Japan). Finally, optical density (OD) was measured at 492/650 nm using the plate reader. In this ELISA, >0.1 ng/ml of IL-7 can be detected.

Statistical analysis:

Data are expressed as mean±standard error (SE) of the mean value. Group comparisons were performed with t-test, when appropriate. Differences were considered to be statistically significant at $p<0.05$.

RESULTS

Syngeneic control (group I) survived indefinitely. Allogeneic liver transplanted recipients (group II) died within 12.8±2.7 days post transplant due to the rejection proved histologically. Liver histology in group II revealed rapid development of acute rejection with mononuclear cell infiltration to the portal tract area as early as POD 3, and severe rejection with extensive mononuclear cell infiltration on POD 10.

Bile was collected for 1 hr until sacrificing animals, by cannulating a polyethylene tube into the graft's bile duct. Until POD 7, bile flow of the recipients ranged from 0.2 to 0.4 ml/h showing no statistical differences between the two groups. But in the allogeneic group II, bile flow was remarkably reduced (<0.1 ml/hr) on POD 10.

PCR analysis detected the IL-7 gene sequence in every organ examined both in group I and II. Detected sequence of the IL-7 gene corresponded to 251 bases as anticipated from the nucleotide sequence of the murine IL-7 cDNA[12] (Fig. 1).

In Northern blotting, IL-7 mRNA could be detected in the thymus, bone marrow and one third of samples from the kidney. In the liver, we could not detect positive band for IL-7 mRNA in either group I or II. The bands expressed in the thymus and bone marrow were detected in both the syngeneic and allogeneic groups, and transcripts were present at the level of 18S (Fig. 2).

To confirm the result obtained in Northern blotting, the RT-PCR and the Southern blotting for the product of RT-PCR were performed. In both of these assays, bands located at 251 bp were detected in the thymus, bone marrow and some of the kidney samples from both groups. None of the samples from the liver and spleen showed detectable bands in both groups.

The levels and kinetics of IL-7 expression were

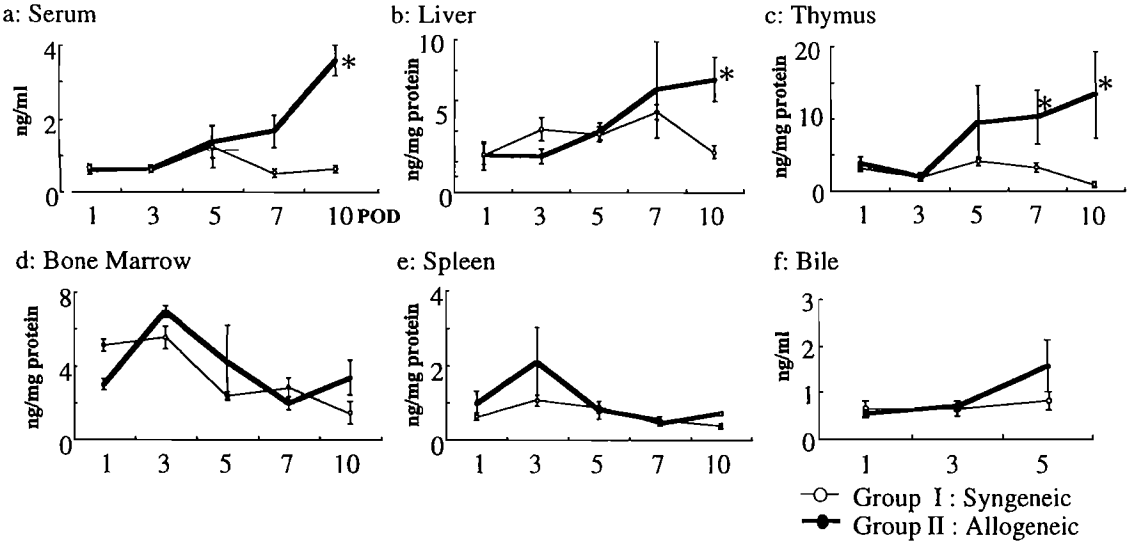

Fig. 3 Protein levels of the IL-7 assayed by ELISA (mean±SE). Thick lines with closed circles indicate the allogeneic group (group II) and thin lines with open circles indicate the syngeneic group (group I).

evaluated using the sandwich ELISA method. Serum IL-7 levels after liver transplantation are shown in Fig. 3-a. In the syngeneic group I, 28% of the serum samples were undetectable for IL-7 (<0.5 ng/ml) and 99% of the samples had a value less than 1 ng/ml. On the other hand, every sample from the allogeneic group showed detectable IL-7 level after POD 5. IL-7 levels in group II reached its maximum level on POD 10 (3.6±0.8ng/ml) and significant differences were observed between these two groups ($p<0.005$).

IL-7 levels in the liver are shown in Fig. 3-b. Allogeneic group II showed time-dependent increase of IL-7 levels after POD 5. IL-7levels were statistically higher in group II (7.3±1.4ng/mg protein) than in group I (2.7±0.4ng/mg protein) on POD 10 ($p<0.05$).

IL-7 levels in the thymus showed more prominent differences between these two groups compared to the serum or liver. IL-7 levels started to elevate on POD 5 (9.5±4.9ng/mg protein) in group II reaching the maximum level on POD 10 (13.2±5.8ng/mg protein), whereas those levels in group I remained below 4ng/mg protein throughout the experiment (Fig. 3-c). There were statisticaly significant differences in IL-7 levels between group I and II on POD 10 ($p<0.05$).

In the bone marrow (Fig. 3-d) and the spleen (Fig. 3-e), IL-7 levels peaked on POD 3 in both groups (spleen; 1.07±0.14 ng/ml in group and 2.05±0.95 in group II, bone marrow; 5.5±0.18 ng/ml in group I and 6.97±0.29 ng/ml in group II) and showed no further activity after POD 5. There was no statistical difference at any point between these two groups.

In the bile, 45% of the samples from both groups showed undetectable levels for IL-7 (<0.5 ng/ml) and 75% of the samples showed values below 1 ng/ml throughout the experiment (Fig. 3-f).

DISCUSSION

Interleukin 7, originally described as a pre-B cell growth factor[1, 2], was also found to have a potent costimulus for the growth of both murine and human CD4+and CD8+T cells[7, 8, 14, 15]. IL-7 was also found to induce generation of both the CTL and LAK cells in bulk cultures[16]. Since these cells are also known to participate in allograft rejection, we hypothesized that IL-7 might be playing an active role in allograft rejection. In the experiments reported here, we investigated the expression of IL-7 in the recipient of orthotopic liver transplantation in rats.

In PCR analysis, primers which were originally designed to detect the murine IL-7 cDNA[12] detected the IL-7 DNA sequences in every organ in both groups. However, in Northern blotting, IL-7 mRNA expression could be detected only in the thymus, bone marrow and some of the kidney samples, but not in the liver. Negative detection of IL-7 mRNA in the graft liver, which has ongoing acute rejection, indicated that detected IL-7 in the liver was mainly produced at the extra-hepatic organ (s) and delivered into the graft liver. In the thymus, increased production of IL-7 could be detected with ELISA as well as the positive detection of mRNA with Northern blotting, in response to acute rejection. These data directly indicated that IL-7 was increasingly produced in the thymus, along with the development of acute rejection and resulted in increased levels of IL-7 in the serum and liver. Watanabe et al. reported that IL-7 may be produced locally in the thymic and bone-marrow microenvironments and that IL-7 plays an important role in the proliferation and, potentially, in the differentiation of immature T cells[17]. These IL-7 mediated proliferation and differentiation of T cells might also play an

important role in our model.

In our experiment, serum IL-7 level reflected the thymic level of IL-7 with a high-fidelity, both in the syngeneic and allogeneic groups. Therefore, elevated serum IL-7 might be derived from the thymus, in which T-cell development would be activated in response to acute rejection of the liver. Serum IL-7 could be used as an indicator of T cell activation in the acute rejection of the liver.

In the spleen and bone marrow, both groups showed monophasic elevation at POD 3, decreasing its level after POD 5. There was no difference between the two groups in these organs. Although we do not have a definite explanation for the monophasic pattern of IL-7 elevation at POD 3, it might be related to surgical stress, because of an identical pattern of elevation in both syngeneic and allogeneic groups at an early post operative period.

To obtain further understanding of the results obtained in this experiment, we performed immunohistochemical staining with the same anti-IL-7 antibody used in ELISA. However, we could not detect any difference between the two groups in any organ (data not shown). Further efforts would be needed to elucidate the in vivo role of IL-7, including immunohistochemical staining.

In conclusion, IL-7 was increasingly produced in the thymus along with the development of acute rejection of the liver. Serum IL-7 level accurately reflected the thymic IL-7 level and it might be used as an indicator of T-cell activation in the thymus. The thymus and its IL-7 were thought to be actively participating in the immunology of acute rejection of the liver.

We followed "Standards Relating to the Care and Management of Experimental Animals" (Notification No. 6, March 27, 1980, Prime Minister's Office, Tokyo, Japan) for care and use of animals. The animals used in our studies were handled humanely in accordance with animal experimental protocols approved by the Animal Care and Use Committee of the University of the Ryukyus.

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