Hiroshima J. Med. Sci.
Vol.45, No.3, 99～103, September, 1996
HIJM 45–15

A New Method to Detect Response of Lymphocytes to Allo-antigens

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ABSTRACT

The mixed lymphocyte reaction (MLR) is the most commonly used method of investigating the immunological response of lymphocytes. However, this conventional technique requires the use of a radioisotope and is time consuming. Furthermore, there is a possibility that MLR does not reflect the response of each lymphocyte subset individually. The purpose of this study is to determine whether detection of IL-2 mRNA by reverse transcription polymerase chain reaction (RT-PCR) can be used as a new method of studying a precise response of PBL to allo-antigens. The study was conducted using lymphocytes from healthy volunteers, a renal transplant recipient who exhibited donor specific MLR unresponsiveness, the donor and the recipient's father. A kinetics study revealed that the expression of IL-2 mRNA in the MLC reached its maximum at 24–48 h. Lymphocytes from a renal transplant recipient also exhibited the same IL-2 mRNA expression pattern in response to stimulation with PBL from a non-related 3rd party but not to that from the donor. These data suggest that the expression level of IL-2 mRNA in MLC measured by RT-PCR at 24–48 h provides accurate response of PBL to allo-antigens.

Key words: *MLC, IL-2 mRNA, RT-PCR*

Very few methods are currently available for studying immunological response of peripheral blood lymphocytes (PBL) to allo-antigens. The mixed lymphocyte reaction (MLR) and cell-mediated lympholysis (CML) are the most popular methods of estimating the activity of PBL following stimulation with allo-antigens. MLR involves the measurement of the uptake of [^{3}H]-TdR by responder cells in response to stimulation with inactivated PBL by irradiation or mitomycin C, while CML measures ^{51}Cr release from target cells injured by primed responder cells. MLR and CML have both been studied as the methods of detecting HLA antigens[4] and to monitor or predict the rejection during transplantation therapy[10]. However, as a method of predicting the course of transplantation therapy, MLR has been reported to be less reliable than CML[9], since MLR indicates the total response of all subsets of PBL or total DNA synthesis by the whole responder cell population. MLR and CML also require the use of radioisotopes and involve complex procedure. Furthermore, MLR and CML take 6 days and 5 days, respectively, before the results are obtained.

In contrast to these conventional methods, simpler and less time consuming methods of investigating the activity of lymphocytes stimulated by mitogens, such as PHA and Con A, have been reported. These methods measure the expression of cytokine mRNAs and include northern blotting[6] which requires the use of radioisotope and an even simpler method known as the reverse transcription polymerase chain reaction (RT-PCR)[2], which can be performed without a radioisotope. However, the method of studying the reaction of lymphocytes to allo-antigens using RT-PCR has not been reported. The purpose of this study is to determine whether RT-PCR can be used to measure lymphocyte responses following stimulation with inactivated lymphocytes by observing IL-2 mRNA in mixed lymphocyte culture (MLC) in a simple and time-saving manner. An initial MLC was performed using lymphocytes from five healthy adult volunteers, a renal transplant recipient who exhibited donor specific MLR hyporesponsiveness, the donor (the recipient's mother), the recipient's father and a healthy third party. The expression of IL-2 mRNA in responder cells in MLC was then measured by RT-

Abbreviations
CML: cell mediated lympholysis, MLC: mixed lymphocyte culture, MLR: mixed lymphocyte reaction, PBL: peripheral blood lymphocyte, PHA: phytohemagglutinin, RT-PCR: reverse transcription polymerase chain reaction

PCR. IL-2 is known to play the most significant role in lymphocyte proliferation out of 15 cytokines now officially recognized[11]. It can be considered that measuring the expression of IL-2 mRNA is equivalent to measuring the proliferative activity of lymphocytes. The kinetics study presented here shows that the expression of IL-2 mRNA by PBL in response to the stimulation with inactivated allo-PBL at 24–48 h can be used as a method of examining the precise response of PBL to allo-antigens.

MATERIALS AND METHODS

Cells and medium:

Peripheral blood lymphocytes (PBL) were obtained from the venous blood of five healthy adult volunteers, a renal transplant recipient, the kidney donor (the recipient's mother), the recipient's father and a healthy third party by the Ficoll gradient method. The recipient had been transplanted with his mother's kidney 5 years previously and had no history of rejection. PBL were frozen and thawed before each assay. The medium was RPMI1640 (GIBCO BRL) supplemented with 20% human AB serum and 100 U/ml penicillin (GIBCO BRL).

Mixed Lymphocyte Reaction (MLR):

PBL was obtained from healthy volunteers: a renal transplant recipient, the donor (the recipient's mother), the recipient's father and third party. MLR was performed with PBL in a 96 well flat bottomed plate (Falcon 3072) in triplicate. Responder cells ($1\times10^5/100\mu$l) and stimulator cells ($1\times10^5/100\mu$l, irradiated with 2000 cGy) were cultured for 6 days in 5% CO_2 humidified atmosphere. 0.5μCi tritiated thymidine ([^{3}H]-TdR, Amersham) was then added to each well during the last 16 h and the cells were harvested on day 6. Cellular incorporation of [^{3}H]-TdR was assessed using liquid scintillation counter (Aloka).

Reverse transcription polymerase chain reaction after MLC:

Responder cells ($5\times10^5/100\mu$l) and stimulator cells ($5\times10^5/100\mu$l, irradiated with 2000 cGy) were cultured in 5% CO_2 humidified atmosphere. Cells were harvested every 24 h from the beginning of the experiment up to 72 h. Whole mRNA was obtained by the acid guanidinium thiocyanate phenol chloroform extraction method[3]. Briefly, sample PBL were centrifuged in 10000rpm for 5 min after washing with PBS (–). 0.5ml of solution D (4M guanidinium thiocyanate, 25mM sodium citrate; pH7.0, 0.5% sarcosyl, 0.1M 2-mercaptoethanol), 50μl of 2M Na-acetate, 0.5ml of phenol and 100μl chloroform/isoamyl alcohol (49/1) were added successively to the pellet. Samples were vortexed and placed on ice for 15 min. The upper layer was collected after centrifugation at 10000rpm for 20 min. 0.5ml of Isopropanol was added to collected samples. RNA was precipitated by cooling for 1 h in –20°C and centrifugation at 10000rpm for 10 min. Sample RNA was washed with 80% ethanol three times. To prevent DNA contamination, sample RNA was treated with 5U of RQ1 DNAse (Promega). Each mRNA was dissolved in 20μl of DEPEC water. 3μl of each sample was transcribed to cDNA in a THERMAL SEQUENCER TSR-300 (Iwaki Glass Co.). The RT reaction buffer was composed of 50mM of KCl, 20mM of Tris-HCl (pH 8.4 at 25°C), 0.1mg/ml of BSA, 2.5μM of oligo $d(T)_{16}$, 20U of RNasin (Promega), 200U of RTase (GIBCO BRL) and 1mM of dNTP in a reaction volume of 20μl. The $MgCl_2$ concentration was 1.5mM. 2×10^6 copies of pAW 109 RNA[12] were added to RT buffer as an internal standard for IL-2 mRNA. Reverse transcription was performed at 42°C for 16 min and reverse transcriptase was inactivated at 99°C for 6 min. The primer sequences for IL-2 were (GAATGGAATTAATAATTACAAGAATCCC, TGTTTCAGATCCCTTTAGTTCCAG)[11]. The size of PCR products of IL-2 was 222 base pair and that of pAW109 was 305 base pair, respectively. The PCR reaction buffer was composed of 25mM of Tris-HCl (pH 8.5 at 25°C), 0.01% (w/v) of gelatin, 2.5U of Taq DNA polymerase (PERKIN ELMER CETUS) and 50pmol of each primer in a reaction volume of 80μl. PCR buffer was added to the RT products and 100μl of each solution was subjected to 35 cycles of PCR. Each cycle consisted of 85 sec denaturation at 94°C, 2 min annealing at 57°C and 45 sec extension at 72°C. Ten microliters of each product were then analyzed by electrophoresis in a 2% agarose gel run in 1xTBE buffer.

RESULTS

1. MLR measured by thymidine uptake

Among PBL from five volunteers, PBL from K.O yielded 3176±893cpm and 79±18cpm after the stimulation by irradiated PBL from K.S and auto-PBL, respectively. PBL from H.Y yielded 4587±1406cpm and 112±59cpm in response to the stimulation with irradiated PBL from T.O and auto-PBL, respectively. PBL from Y.M yielded 4398±771cpm and 192±17cpm following the stimulation by irradiated PBL from T.O and auto-PBL, respectively (Table 1). These data suggest that the PBL used as responder cells react with irradiated allo-PBL but not with auto-PBL. PBL from the renal transplant recipient yielded 798±753cpm in response to PBL from the donor, 9663±6029cpm in response to PBL from the recipient's father and 19987±3470cpm in response to PBL from the third party, respectively (Table 2). PBL from this renal transplant recipient thus

Table 1. MLR between healthy volunteers

Responder cells	Stimulator cells	
	allo*	auto
K.O	3176± 893	79±18
H.Y	4587±1406	112±59
Y.M	4398± 771	192±17

All results are presented as the mean±SD cpm of thymidine uptake.
* allo: PBL form T.O or K.S.

Table 2. MLR of a renal transplant recipient

Stimulator cells	thymidine uptake (cpm)
donor (mother)	798± 753
father	9663±6029
third party	19987±3470

All results are presented as the mean±SD cpm of thymidine uptake.

showed a low response only to PBL from the donor.

2. MLC measured by RT-PCR

MLC was performed between PBL from three healthy adult volunteers and a renal transplant recipient who showed donor specific hyporesponsiveness during conventional MLR as responder cells and allo- or auto-PBL as stimulator cells. Each set of cells was harvested at 24 h intervals from the beginning of the experiment, up to 72 h, mRNA was extracted and the expression of IL-2 mRNA was evaluated by RT-PCR. The expression of IL-2 mRNA by PBL from K.O in response to the stimulation with PBL from K.S was observed from 24 h to 48 h and almost disappeared at 72 h (Fig. 1-a). Expression of IL-2 mRNA by PBL from H.Y in response to PBL from T.O was seen at 24 h and disappeared after 48 h (Fig. 1-b). PBL from Y.M expressed IL-2 mRNA from 24 h to 48 h but not at 72 h after the stimulation with PBL from T.O (Fig. 1-c). When assessed by RT-PCR, PBL from the renal transplant recipient showed almost the same IL-2 mRNA expression pattern after the stimulation with PBL from non-related third party as was observed in RT-PCR between PBL from healthy volunteers. However, when stimulated by PBL from the donor, PBL from the transplant recipient expressed a little IL-2 mRNA only at 72 h. Furthermore, after the stimulation with the PBL obtained from his father, the PBL from the renal transplant recipient showed a pattern of IL-2 mRNA expression between that observed after the stimulation with PBL from the donor and the third party (Fig. 2).

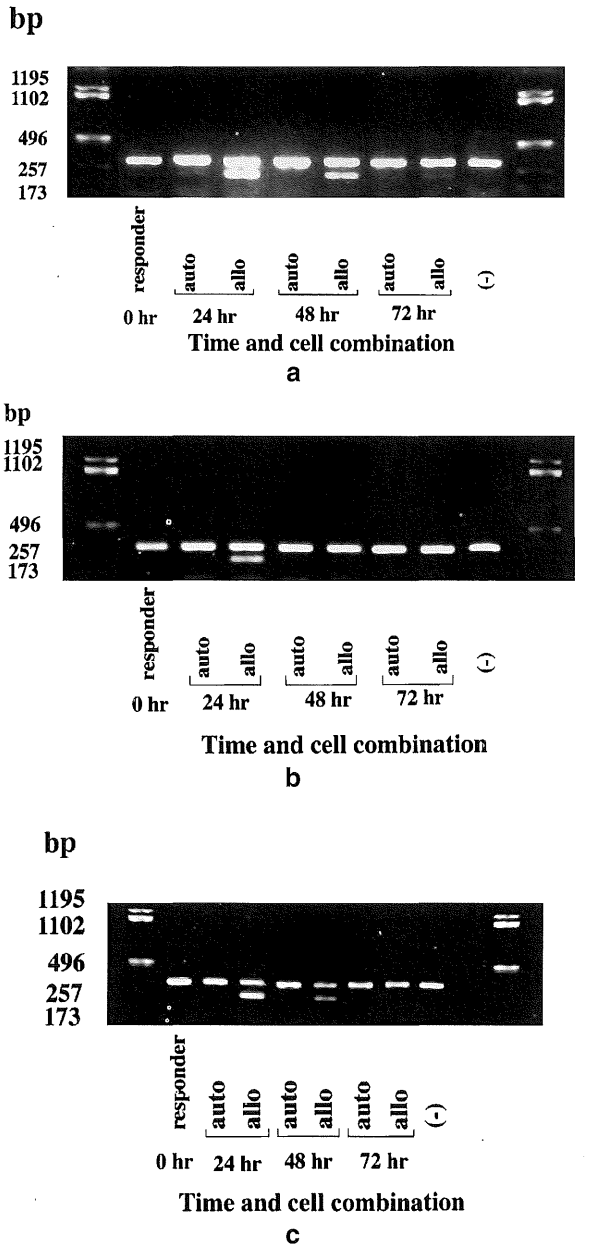

Fig. 1-a, b, c
Kinetic study of IL-2 mRNA after MLC was performed by RT-PCR in three cell combinations using PBL from five healthy volunteers. The upper and lower bands indicate PCR products of pAW 109 and IL-2, respectively.
a: Responder to stimulator cell combination was K.O to K.S. Expression of IL-2 mRNA was observed from 24 h up to 48 h clearly and almost disappeared at 72 h.
b: Responder to stimulator cell combination was H.Y to T.O. Expression of IL-2 mRNA was observed at 24 h and disappeared after 48 h.
c: Responder to stimulator cell combination was Y.M to T.O. Expression of IL-2 mRNA was observed from 24 h to 48 h but not at 72 h.

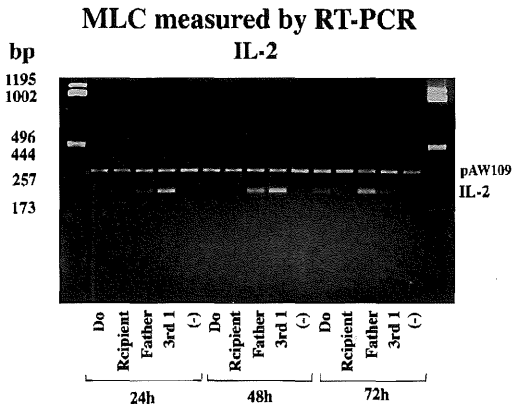

Fig. 2. MLC measured by RT-PCR
Kinetic study of IL-2 mRNA after MLC was performed by RT-PCR using PBL from a renal transplant recipient who exhibited donor specific MLR hyporesponsiveness. The upper and lower bands indicate PCR products of pAW 109 and IL-2, respectively. IL-2 mRNA also reached its maximum before 48 h in response to PBL from non-related third party. In response to PBL from donor, however, little expression of IL-2 mRNA was observed only at 72 h.

DISCUSSION

A conventional 6 days MLR was initially performed to examine the proliferative activity of lymphocytes. Three cell combinations were set using PBL from five healthy volunteers. PBL from these volunteers revealed a normal response to PBL from the other volunteers. PBL from a renal transplant recipient with a well functioning kidney also revealed a normal response to PBL from a non-related third party, but hyporesponsiveness specific to PBL from the donor.

A kinetic study of IL-2 mRNA was then performed using RT-PCR after MLC in the same cell combinations as in MLR.

The expression of IL-2 mRNA in PBL from various volunteers in response to the stimulation with irradiated PBL from other volunteers was observed 24–48 h after the beginning of MLC and disappeared at 72 h. In this experiment, the expression of IL-2 mRNA was not observed in autologous responder-stimulator cell combinations. These data suggest that the expression of IL-2 mRNA after MLC as measured by RT-PCR can be used to estimate the proliferative activity of lymphocytes stimulated by allo-antigens, and that the expression of IL-2 mRNA after stimulation with allo-antigens occurs between 24 and 48 h after the stimulation. Yamamoto et al studied the kinetics of IL-2 mRNA expressed in lymphocytes after the stimulation by PHA using dot hybridization[14]. They concluded that the peak IL-2 mRNA expression occurred 12 h after PHA stimulation and that the high expression level continued up to 48 h. The different kinetics for IL-2 mRNA expression in the current study and that of Yamamoto et al might be explained by the different stimulators, PHA and irradiated allo-lymphocytes used in the experiments.

RT-PCR is generally considered to be a less reliable method for quantitative analysis. In this study, however, the author devised some points to increase the reliability of RT-PCR for quantifying IL-2 mRNA. First, the sample RNA was treated with RQ1 DNAse, which digested DNA in a random manner and removed any contaminating DNA. Second, the PCR machine used in this study was equipped with three oil baths. This system has been reported to provide excellent quantification of PCR products because the temperature of the samples subjected to PCR can be changed instantly by dipping them automatically in each temperature-controlled bath[1]. Third, the peak time of expression of IL-2 mRNA in PBL was easily detectable because it declines rapidly after 48–72 h of stimulation[14].

In clinical renal transplantation therapy, it is well known that some recipients with well functioning kidneys exhibit donor specific immunological unresponsiveness. Clonal deletion[8], the presence of anti-idiotypic antibody[5], donor specific anergy and donor specific suppressor cells[13] have been considered to be responsible for this phenomenon. We have previously reported that specific suppression of IL-2 synthesis in response to donor cells was one of the causes of this phenomenon[7]. In the present study, the process of IL-2 synthesis at the mRNA level in PBL from a renal transplant recipient who exhibited donor specific MLR hyporesponsiveness at the mRNA level was examined using RT-PCR. PBL from the renal transplant recipient showed almost the same pattern of IL-2 mRNA expression in response to the stimulation with PBL from a non-related third party as was observed in the MLC between PBL from healthy volunteers. In these cases, expression of IL-2 mRNA was detectable at 24 h. It reached its peak at 48 h and disappeared at 72 h. However, after stimulation of PBL from the donor, the recipient's mother, no expression of IL-2 mRNA was observed before 48 h and slight expression only was observed at 72 h. When stimulated by PBL from recipient's father, the expression pattern of IL-2 mRNA in PBL from the recipient was between those observed after the stimulation with PBL from the donor and the third party. These data indicate that PBL from the renal transplant recipient failed to express IL-2 mRNA in response to stimulation with PBL from the donor and showed donor specific MLR hyporesponsiveness by a consequent lack of IL-2 synthesis during the initial stage of the MLC.

In conclusion, this study showed, for the first time, that the reactivity of PBL to allo-antigens,

including that from a renal transplant recipient who exhibited donor specific MLR hyporesponsiveness, could be investigated for the detection of the mRNA expression of IL-2 using RT-PCR, which is a key cytokine in the proliferation of lymphocytes. I want to end by emphasizing that this method can be applied to other general examinations that require analysis of proliferative activity of PBL.

ACKNOWLEDGMENTS

The author thanks Dr. H.Yahata and Dr. K. Dohi for critically reviewing this manuscript, Dr. K. Okugawa, Dr. K. Shinozaki and Dr. Y. Miyata for blood donation.

(Received May 13, 1996)
(Accepted August 26, 1996)

REFERENCES

1. **Asakawa, J., Satoh, C., Yamasaki, Y. and Chen, S.H.** 1992. Accurate and rapid detection of heterozygous carriers of a deletion by combined polymerase chain reaction and high-performance liquid chromatography. Proc. Natl. Acad. Sci. U.S.A. **89:** 9126–9130.
2. **Brenner, C.A., Daniel, S. and Adler, R.R.** 1991. Cytokine MAPPing: observation and quantification of cytokine mRNA in small numbers of cells using the polymerase chain reaction, p. 51–59. *In* F. R. Balkwill (ed.), The practical approach series. Oxford University Press, New York.
3. **Chomczynski, P. and Sacchi, N.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:** 156–159.
4. **Keuning, J.J., van den Tweel, J., Gabb, B.W., Termijtelen, A., Goulmy, E., Blokland, E., Elferink, B.G. and van Rood, J.** 1975. An estimation of the recombination fraction between the MLC locus and the FOUR locus. Tissue Antigens **6:** 107–115.
5. **Miyajima, T., Higuchi, R., Kashiwabara, H., Yokoyama, T. and Fujimoto, S.** 1980. Anti-idiotypic antibodies in a patient with a functioning renal graft. Nature **283:** 306–308.
6. **Naylor, M.S. and Balkwill, F.R.** 1991. Northern analysis and in situ hybridization on cytokine messenger RNA, p.31–50. *In* F.R. Balkwill (ed.), A Practical Approach. Oxford University Press, New York.
7. **Okimoto, T., Yahata, H., Fukuda, Y., Hayamizu, K., Shimatani, K. and Dohi, K.** 1994. Donor specific suppressor T cells in a renal transplant recipient with a well functioning kidney. Transplant. Proc. **26:** 858–860.
8. **Pfeffer, P.F., Thorsby, E. and Hirschberg, H.** 1983. Cell-mediated cytotoxicity toward the donor in patients with well functioning kidney grafts. Transplantation **35:** 546–551.
9. **Stiller, C.R., Sinclair, N.R., Abrahams, S., McGirr, D. and Ulan, R.A.** 1976. Anti-donor immune responses in prediction of transplant rejection. New Eng. J. Med. **294:** 978–982.
10. **Thomsen, M., Morling, N., Ladefoged, J., Lokkegard, H., Jakobsen, B., Svejgaard, A. and Thaysen, J. H.** 1980. MLC compatibility, HLA-D and DR typing in cadaver kidney transplantations. Scand. J. Urol. Nephrol. Suppl. **54:** 32–35.
11. **Umlauf, S.W., Beverly, B., Kang, S.M., Brorson, K., Tran, A.C. and Schwartz, R.H.** 1993. Molecular regulation of the IL-2 gene: Rheostatic control of the immune system. Immuno. Rev. **133:** 177–197.
12. **Wang, A.M., Doyle, M.V. and Mark, D.F.** 1989. Quantitation of mRNA by the polymerase chain reaction. Biochemistry **86:** 9717–9721.
13. **Wramner, L., Olausson, M., Söderström, T., Lindholm, L., Rydberg, L. and Brunger, H.** 1987. Evidence of donor specific cellular suppressor activity in donor specific cell mediated lympholysis unresponsiveness in renal transplant patients. Transplantation **44:** 390–395.
14. **Yamamoto, Y., Fujimoto, K., Ohmura, T., Maeda, S., Shimada, K. and Onoue, K.** 1985. Interleukin 2 mRNA induction in human lymphocytes: Analysis of the synergistic effect of a phorbol ester and phytohemagglutinin. J. Biochem. **98:** 49–56.