

Effect of Low Dose X-Irradiation on Alloantigen Sensitized and Unsensitized Lymphocytes*

Kiyohiko DOHI, Hiroshi YAHATA, Yasuhiko FUKUDA,
Toshimasa ASAHARA, Eiji ONO and Haruo EZAKI

The Second Department of Surgery, Hiroshima University School of Medicine,
1-2-3, Kasumi, Minami-ku, Hiroshima 734, Japan

(Received September 25, 1984)

Key words: Rejection, Local graft irradiation, MLR, CML

ABSTRACT

The effect of local graft irradiation on immune response in allograft in which acute rejection occurs was studied using an *in vitro* model. Unidirectional mixed lymphocyte culture (MLC) was used as the *in vitro* model of acute rejection. 150 and 300 rad x-irradiation suppressed mixed lymphocyte reaction (MLR) but did not cell-mediated-lympholysis (CML) of unsensitized lymphocytes. X-irradiated alloantigen sensitized cells (ASC) generated in 6-day MLC suppressed MLR and CML of unsensitized lymphocytes. Suppressive effects of x-irradiated ASC were of the same degree by x-irradiation doses of 150-500 rad. Suppressive effect of x-irradiation was maintained for only a short period after x-irradiation. Potential function of suppressor precursor cells among unsensitized lymphocytes was abolished by x-irradiation of 300 rad.

INTRODUCTION

Local graft irradiation (LGI) is usually administered as treatment for acute rejection in human renal transplantation. In animal experiments, many reports^{2,7,10,16} have been published on the efficacy of immunosuppressive effects of LGI. But, in human renal transplantation, its efficacy has not yet been established. For example, Fidler et al.⁶ reported treating acute rejection in life-threatening infection cases with LGI alone which resulted in achieving favourable graft survival. Abramson et al.¹¹ reported that LGI alone was useful in reversing acute rejection. Godfrey et al.⁸ reported that LGI was effective in controlling acute rejection but was not effective in improving graft survival rate one year after transplantation. Nakajima et al.¹³ reported that they treated acute rejection using LGI and high-dose-steroids at the same time, with no improvement of graft survival rate. Pilepich et al.¹⁵, on the other hand, reported that treat-

ment with LGI lowered the graft survival rate. Perhaps, one of the reasons why LGI does not improve graft survival rate is that acute rejection readily recurs in recipients treated by LGI^{8,15}, although it is effective in reversing acute rejection. Despite host lymphocytes newly enter into the graft after LGI, why is there reversal of acute rejection by LGI? Why does acute rejection readily recur after LGI?

In this paper we will report on immune response in graft after LGI, based on the results of our study on effects of low dose irradiation using unidirectional mixed lymphocyte culture as an *in vitro* model of acute rejection.

MATERIALS AND METHODS

Isolation of lymphocytes. Human peripheral lymphocytes were obtained from healthy non-transfused adults by Ficoll-Conray density gradient⁹. After washing 3 times, the lymphocytes were suspended in RPMI 1640 (Gibco) supplemented with 20% pooled heat inactivated human male serum, 100 u/ml of penicillin and

*) 土肥雪彦, 八幡 浩, 福田康彦, 浅原利正, 小野栄治, 江崎治夫: アロ抗原感作および未感作リンパ球に対する低線量X線照射の効果

100 µg/ml of streptomycin, and used for culture. All cultures were done at 37°C in a humidified incubator with 5% CO₂.

Generation of alloantigen sensitized cells (ASC). To generate ASC, unidirectional bulk mixed lymphocyte culture (bulk MLC) was performed. That is, 1 × 10⁷/10 ml of responding cells were mixed with 1 × 10⁷/10 ml of mitomycin C (33 µg/ml) treated allogeneic stimulating cells in Falcon 3013 culture flask and cultured. After 6 days of culture, ASC were harvested from bulk MLC. Dead cells and cell debris of ASC were removed by Ficoll-Conray (1090) density gradient⁹, after which viability of ASC was measured by trypan blue exclusion test. ASC viability was in excess of 95%. Although different donors were used in different experiments, to simplify the description, responding cells in all experiments were described as A, and all donors used as stimulating cells were described as B. Thus, the generated ASC were called ABm.

Mixed lymphocyte reaction (MLR) assay. Micro MLC was established by adding 1 × 10⁵/0.1 ml of responding cells (A) and 1 × 10⁵/0.1 ml of stimulating cells (Bm) to wells in a flat bottom microculture plate (Falcon 3042). At initiation of culture, 0.05 ml of medium, or 5 × 10⁴/0.05 ml of x-irradiated ASC (X-ASC), or 5 × 10⁴/0.05 ml of x-irradiated responding cells A (X-A) were added to micro MLC, which were performed in triplicates. Determination of MLR was carried out by taking counts, with a liquid scintillation counter (Packard Model 3255), of the incorporated ³H-thymidine (3H-TdR, Amersham) which had been added in amounts of 0.5 µCi/well 18 hr prior to harvesting. The MLR values were obtained using the following equation.

$$\text{MLR} = \frac{\text{cpm of test MLC}}{\text{cpm of control MLC}} \times 100$$

$$\frac{(A+Bm+X-ASC)}{(A+Bm+X-A) \text{ on day 6}} \times 100$$

Cell-mediated-lympholysis (CML) assay. Determination of CML was made in accordance with the ⁵¹Cr releasing assay method of Lightbody et al.¹¹. That is, 5 × 10⁶/5 ml of responding cells A were cultured with 5 × 10⁶/5 ml of stimulating cells Bm in a Falcon 3013 culture flask to generate effector cells (macro MLC). At the initiation of culture, 2.5 ml of medium, or 2.5 × 10⁶/2.5 ml of X-ASC, or 2.5 × 10⁶/

2.5 ml of X-A was added to each flask. After 6 days of culture, the cells were harvested and, after removing the dead cells, the remaining cells were used as effector cells. Stimulators B, which were cultured with PHA (Difco) for 3 days, were labelled with Na₂⁵¹CrO₄ (New England Nuclear), and then used as target cells. Aliquots of 5 × 10⁵/0.1 ml of effector cells and 1 × 10⁴/0.1 ml of target cells were cultured in wells of round bottom micro-culture plates (Nunc, 163-320). After 4 hr of culture, 0.1 ml of the supernatant was collected from each well and the released ⁵¹Cr was counted by a gamma counter (Shimazu RAW-600). The results were described as percent cytotoxicity using the following equation.

$$\text{CML} = \frac{\text{Experimental } ^{51}\text{Cr release}}{\text{Maximal } ^{51}\text{Cr release}} \times 100 (\%)$$

$$\frac{\text{— Spontaneous } ^{51}\text{Cr release}}{\text{— Spontaneous } ^{51}\text{Cr release}}$$

X-irradiation was administered by Shimazu x-ray machine (50 rad/min) at room temperature.

RESULTS

First, the effect of ASC on MLR was studied (Fig. 1). Micro MLC (A+Bm) was established by adding x-irradiated ASC or A at its initiation, and determination of MLR was made with the elapse of time. Similar determination was made using as control, MLC to which only medium was added. MLR (A+Bm+X-A) showed the same response as MLR (A+Bm+medium). MLC (A+Bm+X-ASC) to which 150 rad x-irradiated ASC had been added showed reduced MLR with no kinetic change (p < 0.001). The degree of the suppressive effect of X-ASC on MLR did not change with x-irradiation dose in the range of 150–500 rad. The peak of MLR was observed to be produced earlier, in 4-day culture, with the addition of non-x-irradiated ASC. Secondary MLR of ASC themselves peaked in 4-day culture. Therefore, ASC x-irradiated with doses of 150 to 500 rad were considered to have MLR suppressive effect.

Next, study was made of the effect of x-irradiated ASC on CML (Table 1). Macro MLC (A+Bm), to which X-ASC or X-A were added, were cultured for 6 days. As control, similar culture was done adding 2.5 ml of medium instead of X-ASC. Macro MLC (A+Bm+X-

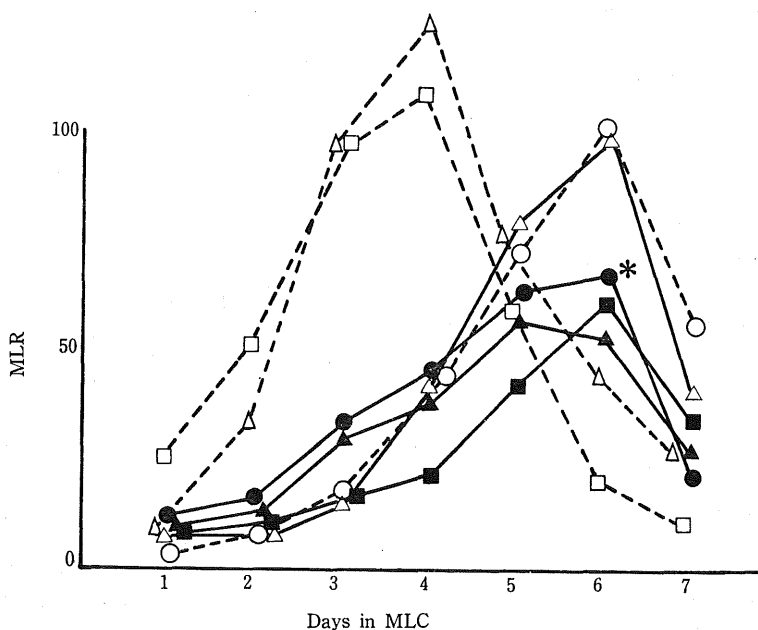


Fig. 1. Effect of X-irradiated ASC on MLR

- △-△ A+Bm+X-A
- A+Bm+Medium
- A+Bm+X-ASC (150 rad)
- ▲-▲ A+Bm+X-ASC (300 rad)
- A+Bm+X-ASC (500 rad)
- △-△ A+Bm+ASC
- ASC+Bm

* $p < 0.001$ Peak of $^3\text{H-TdR}$ incorporation of MLC with addition of X-A versus peak of $^3\text{H-TdR}$ incorporation of MLC with addition of X-ASC (150, 300, 500 rad)
 Results are expressed as the mean of 6 different experiments.

Table 1. Effect of X-irradiated ASC on CML

Macro MLC	CML (%)
A+Bm+Medium	52.5±17.9
A+Bm+X-A (150 rad)	51.7±18.7
A+Bm+X-ASC (150 rad)	26.1±11.9*
A+Bm+X-ASC (300 rad)	24.5±11.1*
A+Bm+X-ASC (500 rad)	24.2± 9.6*

* $p < 0.02$ CML with addition of X-A versus CML with addition of X-ASC (150, 300, 500 rad)
 Results are expressed as the mean±S.D. of six different experiments.

Table 2. Cytotoxic Activity of ASC Generated After X-irradiation

MLC	MLR	CML (%)
A+Bm	100	56.6±14.1
Ax+Bm ^a	41.6±24.7	55.3±14.5
Ax+Bm ^b	17.1±10.6	42.9±8.6

a : responding cells were x-irradiated with 150 rad prior to initiation of bulk MLC

b : responding cells were x-irradiated with 300 rad prior to initiation of MLC

Results are expressed as the mean± S.D. of four experiments.

ASC) to which 150 rad x-irradiated ASC had been added showed reduced CML ($p < 0.02$). The suppressive effect of X-ASC on CML did not change with x-irradiation dose in the range of 150–500 rad.

Next, the effect of x-irradiation on suppressor and cytotoxic precursor cells among unsensitized lymphocytes was studied. Bulk MLC (A + Bm) was initiated after responding cells A were x-irradiated with doses of 150 or 300 rad. As control, non-x-irradiated responding cells A were similarly cultured (A + Bm). The cytotoxic and suppressive effect of ASC was determined After 6 days of culture. ASC (AxBm 150) generated after x-irradiation of 150 rad and ASC (AxBm 300) generated after x-irradiation of 300 rad showed CML of almost the same level (Table 2). ASC (AxBm 150) showed suppression of MLR and CML (Table 3). However, no suppression was demonstrated by ASC (AxBm 300). Therefore, the interpretation was made that the suppressor precursor cells among the responding cells were either destroyed or devitalized by x-irradiation of 300 rad, but cytotoxic precursor cells were not abolished by 300 rad. This indicates that x-irradiation of 300 rad is over dose for LGI.

Next, study was made of the suppressive effects of suppressed bulk MLC cells (A + Bm + X-ASC) which had been cultered for 6 days with the addition of X-ASC to elucidate the effect of LGI 6 days after irradiation. Bulk MLC cells (A + Bm + X-ASC) which was cultured for 6 days is called ASC'. The sup-

Table 3. Suppressive Effect on MLR and CML of ASC Generated After X-irradiation

MLC	MLR	CML(%)
A + Bm + X-ASC (ABm)	48.5 ± 12.1	6.2 ± 5.6
A + Bm + X-ASC (AxBm 150) ^a	50.0 ± 10.9	10.3 ± 5.4
A + Bm + X-ASC (AxBm 300) ^b	99.3 ± 16.3	48.1 ± 14.3
A + Bm + X-A	100	44.3 ± 15.6

a : ASC generated after responding cells were x-irradiated with 150 rad prior to initiation of bulk MLC

b : ASC generated after responding cells were x-irradiated with 300 rad prior to initiation of bulk MLC

Results are expressed as the mean ± S.D. of four experiments.

Table 4. Suppressive Effect on MLR and CML of cells (ASC') Cultured for Six Days in MLC with Addition of X-irradiated ASC

MLC	MLR	CML (%)
A + Bm + X-A	100	60.6 ± 24.5
A + Bm + X-ASC	35.6 ± 29.5*	28.4 ± 18.2**
A + Bm + X-ASC' ^a	85.2 ± 18.4	58.9 ± 18.5

a : MLC cells cultured for six days with addition of X-ASC

* $p < 0.005$ MLR with addition of X-ASC versus MLR with addition of X-ASC'

** $p < 0.01$ CML with addition of X-ASC versus CML with addition of X-ASC'

Results are expressed as the mean ± S.D. of six different experiments.

pressive effect of ASC' on MLR and CML was determined (Table 4). MLC added X-ASC showed reduced MLR and CML. However MLC added X-ASC' showed the same MLR and CML as the control. Therefore, it was interpreted that the suppressive activity of LGI was maintained for only a short period after x-irradiation.

DISCUSSION

Makinodan et al.¹²⁾ reported that irradiation of 100 rad caused reduction of unsensitized or sensitized immunocompetent cells, that this effect was proportional to irradiation dose, and that irradiation of over 300 rad suppressed antibody response. Nichols et al.¹⁴⁾ reported that gamma-irradiation of 200 rad inhibited MLR of unsensitized lymphocytes and secondary MLR of sensitized lymphocytes. We, also, found that fresh MLR and secondary MLR were inhibited by x-irradiation of 150 rad (unpublished data).

Although, effect of x-irradiation to immunocompetent cells was different *in vivo* and *in vitro*, the results presented in this paper strongly suggest that immunosuppressive mechanism by LGI has not only the direct immunosuppressive effects described above but also the indirect immunosuppressive mechanism of irradiated alloantigen sensitized lymphocytes in the graft to suppress the attack of unsensitized lymphocytes newly entering graft after LGI. By this indirect immunosuppressive effect, acute rejection is considered to be mitigated despite the entry of host lymphocytes into the graft after LGI.

Because suppressor precursor cells are abolished by 300 rad x-irradiation, cytotoxic precursor cells are not abolished by 300 rad x-irradiation and the degree of immunosuppressive effect of x-irradiated alloantigen sensitized lymphocytes do not change with doses in the range of 150-500 rad, 150 rad which is usually used is believed to suffice as x-irradiation dose per LGI. However, the disappearance of the immunosuppressive effect of x-irradiation 6 days after irradiation suggests, as reported by Godfrey et al.⁸⁾, that acute rejection readily recurs though it is temporarily reversed by LGI.

LGI is known to have little effect on the systemic immune system⁴⁾. In fact, Fidler et al.⁶⁾ reported that they treated acute rejection crisis in recipients with complication of life-threatening infection by LGI, with favourable results in reversing acute rejection. Therefore, LGI is believed to be ideal as a non-specific immunosuppressive therapy if recurrence of acute rejection can be suppressed by some method. One possible method to prevent the recurrence of acute rejection is to increase the frequency of LGI and administer it for a longer period as reported by Abramson et al.¹⁾ instead of irradiating 150 rad every other day, three days. Also, as it is reported that steroid induces suppressor T cells⁹⁾, another possible method is to administer steroid pulse therapy one week after LGI when the immunosuppressive effect of LGI disappears instead of administering the two at the same time.

If suppression of recurrence of acute rejection after LGI is possible, LGI will contribute largely to the graft survival rate and the survival rate of patients.

REFERENCES

1. **Abramson, N., Ornitz, R. and Cavanaugh, P. J.** 1974. The effect of local irradiation on rejection of the transplanted kidney. *Radiology* 112 : 413-416.
2. **Ascher, N.L., Hoffman, R. A. and Simmons, R. K.** 1979. Local graft irradiation: Effect on cytotoxic cell infiltration. *Surg. Forum.* 30 : 272-274.
3. **Böyum, A.** 1968. Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* 21 (Suppl 97) : 77-91.
4. **Chatterjee, S.N.** 1979. Immunosuppressive drugs, p. 101-118. *In* S.N. Chatterjee (ed.), *Manual of renal transplantation*, Spinger-Verlag, New York.
5. **Davidson, W.F. and Parish, C.R.** 1975. A procedure for removing red cells and dead cells from lymphoid cell suspensions. *J. Immunol. Methods* 7 : 291-300.
6. **Fidler, J.P., Alexander, J.W., Smith, E.J. and Muthiah, V.** 1973. Radiation reversal of acute rejection in patients with life-threatening infections. *Arch. Surg.* 107 : 256-260.
7. **Foster, R. G.** 1974. Local graft irradiation as an adjunct to pharmacologic immunosuppression. *J. Surg. Research* 16 : 457-462.
8. **Godfrey, A.M. and Salaman, J.R.** 1977. Is graft irradiation of value in renal transplant rejection? *Transplant. Proc.* 9 : 1005-1006.
9. **Hirschberg, T., Randazzo, B. and Hirschberg, H.** 1980. Effects of methylprednisolone on the in vitro induction and function of suppressor cells in man. *Scand. J. Immunol.* 12 : 33-39.
10. **Kauffman, H.M., Cleveland, R.J., Dwyer, J. J., Lee, H.M. and Hume, D.M.** 1965. Prolongation of renal homograft function by local graft radiation. *Surg. Gynecol. Obstet.* 120 : 49-58.
11. **Lightbody, J. and Frost, P.** 1980. Cell-mediated lympholysis test with ⁵¹Cr or ¹¹¹In in pre- and posttransplant patients, p. 1034-1041. *In* N.R. Rose and H. Friedman (ed.), *Manual of clinical immunology*, 2nd ed. American Society for Microbiology, Washington, D.C.
12. **Makinodan, T. and Price, G.B.** 1972. Radiation, p. 251-271. *In* J.S. Najarian (ed.), *Transplantation*, Lea and Febiger, Philadelphia.
13. **Nakajima, N., Sreepade Rao, T.K., Sakai, A., Butt, K.H. and Kountz, S.L.** 1977. Effect of intravenous bolus dosage methylprednisolone and local radiation on renal allograft rejection and patient mortality. *Surg. Gynecol. Obstet.* 144 : 63-66.
14. **Nichols, W.S., Troup, G.M. and Anderson, R.E.** 1975. Radiosensitivity of sensitized and nonsensitized human lymphocytes evaluated in vitro. *Am. J. Pathol.* 79 : 499-508.
15. **Pilepich, M.V., Sicard, G.A., Breaux, S.R., Etheredge, E.E., Blum, J. and Anderson, C. B.** 1983. Renal graft irradiation in acute rejection. *Transplantation* 35 : 208-211.
16. **Wolf, J.S., McGavie, J.D. and Hume, D.M.** 1969. Inhibition of the effector mechanism of transplant immunity by local graft irradiation. *Surg. Gynecol. Obstet.* 128 : 584-590.