A MODIFIED METHOD OF MIXED LYMPHOCYTE REACTION: ESTABLISHMENT OF THE ASSAY SYSTEM AND ITS APPLICATION TO EXTRACTS OF FUNGAL CULTURES

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A modified mixed lymphocyte reaction (MLR) assay was developed to screen immunosuppressive agents. In the MLR, irradiation of splenocytes with UV light was employed for the preparation of stimulator cells, and the highest response was observed with the combination of splenocytes of C3H/He mice as a stimulator and those of C57BL/6 as a responder. The blastogenesis of the responder cells was quantified by a colorimetric method using MTT in 96-well microculture plates. For screening immunosuppressive substances, the MLR in combination with a cytotoxicity test can distinguish immunosuppressors from cytotoxic agents. The applications of this assay system to extracts of fungal culture was also described.

Immunosuppressive drugs are used for supression of allograft rejection in transplantations, and therapy of autoimmune diseases and hypersensitivity. Recent introduction of effective immunosuppressors such as cyclosporin A (CsA)[1~3] and FK506[4~6] isolated from a fungus and a strain of actinomycetes increased the survival rate of patients after transplantation. For the evaluation of immunosuppressive chemicals, several *in vivo* model systems with organ transplantation technics are proposed. However, these *in vivo* systems are time-consuming and not effective for mass screening of suspected chemicals.

To evaluate *in vitro* effects of chemicals on the cellular immune system, mitogen-induced blastogenesis of lymphocytes[7] and mixed lymphocyte reaction (MLR) are used. In the former system, mitogens such as concanavalin A and phytohemaggulutinin stimulate T lymphocyte non-specifically, whereas antigen-specific stimulation is induced in the latter system. In these assay systems, an incorporation of [^{3}H]-thymidine into the cellular DNA has been generally employed for measuring the proliferation of cells. This measurement using isotope-labeled compounds is rather troublesome for handling. A colorimetric assay method to measure the growth of cells using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was established.[8,9] The procedure of the assay is simple compared with the

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isotope method, namely several washing steps and collection of cells by filtration can be omitted.

In this paper, we describe studies on the method for preparation of the stimulator cells and the combination of mice strains for the stimulator and responder cells to develop a simplified system of the MLR using mice splenocytes and the MTT assay. For assaying immunosuppressive agents, cytotoxicities of samples were also determined with myeloma cells, to avoid false positive which exhibit non-specific cytotoxicity to mammalian cells. The MLR in combination with the cytotoxicity test was applied to extracts from fungal cultures for screening immunosuppressive metabolite(s).

Materials and Methods

Chemicals

Cyclosporin A (CsA) and prednisolone were obtained from Sandoz Pharmaceutical Co. Ltd. (Basel, Switzerland) and SIGMA Chemical Co. (M.O, U.S.A.), respectively. FK506 was kindly supplied by Dr. K. OKUMURA (Juntendou University Medical School). Mitomycin C was a product of Wako Pure Chemicals Co. Ltd. (Osaka, Japan). RPMI 1640 medium and Hanks solution were obtained from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). HEPES buffer was purchased from Gibco Laboratories (N.Y. U.S.A.). Fetal calf serum was a product of Whittaker M. A. Bioproducts (M.D. U.S.A.).

Splenocytes

Syngenic 6-week old male mice of C57BL/6, C3H/He and BALB/C strains obtained from Sankyo Labo Service Co. Ltd. (Tokyo, Japan), were sacrificed by cervical dislocation and spleen was taken off aseptically. Splenocytes were obtained by squeezing the tissue using frosted glass. The cells were washed three times with GUOLB's phosphate buffered saline (GPBS: 4.38 g of NaCl, 20.41 g of $Na_2HPO_4 \cdot 12H_2O$, 2.44 g of KH_2PO_4 in 1,000 ml), suspended in RPMI 1640 medium containing 10% fetal calf serum, 10 mM HEPES buffer, 6 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol and 60 μg/ml of kanamycin (CCM: complete conditioned medium), and filtered through a stainless steel mesh (120 meshes).

Preparation of Stimulator Cells

For preparation of the stimulator cells, the splenocytes obtained from one mouse were suspended in 10 ml of CCM in a polystylene culture dish (diameter 10 cm, No. 25010, CORNING Laboratory Science Co., N.Y., U.S.A.) and irradiated with UV light (254 nm, 7 mW/cm^2) transilluminator (TS-36 Ultra Violet Products Inc., C.A., U.S.A.) through bottom of the culture dish for 8 minutes. The irradiated cells were harvested by centrifugation at $300 \times g$ for 10 minutes and resuspended in the fresh medium. In the preparation using mitomycin C, suspension of the splenocytes was treated with 50 μg/ml of the drug for 30 minutes and the cells were washed three times with Hanks solution.

Mixed Lymphocyte Reaction (MLR) and Cytotoxicity Assay

Responder and stimulator cells (each 5×10^5 cells/well) were mixed in a well of 96-well flat bottom culture plate (CORNING No. 25860MP) in a total volume of 100 μl, and cultured at 37°C with 5% CO_2 fumigated air for four days. The growth of the cells was measured by the MTT method.[8] The MTT-reducing activity was expressed as an optical density of the solution in each well which was determined on a plate reader, (type MTP-22, Corona Electric Co. Ltd., Tokyo, Japan) with a wavelength of 570 nm and a reference wavelength of 630 nm.

For cytotoxicity assay, mouse myeloma X63.Ag8. 6.5.3 cells (6.5.3 cell, 1×10^4 cells) were cultured in 100 μl of the CCM in a well of 96-well flat bottom culture plate in the presence of chemicals for two days and the cell growth was measured by the MTT method.

Preparation of Fungal Extracts

Fungi isolated from soil in Japan were inoculated to a test tube (16×180 mm) containing 2 g of polished rice grains and 0.6 ml of water, and cultured at 25°C for 14 days. The molded rice grains were

collapsed with a rod, 8 ml of methanol was added, and the test tube was kept in a sonicator (type UC-0310, Tokyo Cho-onpa Co. Ltd., Tokyo Japan) for several minutes. The mixture was filtered through a paper and the methanol solution was evaporated to dryness. The extracts were dissolved in ethanol and diluted stepwise with CCM to give the final concentrations of 1, 10 and 100 μg/ml of the extracts and 1% of ethanol.

Results

Preparation of Stimulator Cells

In the assay of MLR, preparation of stimulator cells is an important step. Generally treatment of lymphocytes with mitomycin C or irradiation with γ-ray are employed for the preparation. In the present study, we have examined UV irradiation for preparation of the stimulator cells. To know the relationship between the duration of irradiation and the viability of cells, myeloma 6.5.3 cells (1×10^5/ml) were irradiated for the desired periods in a culture dish and cultured for 2 days. The viability of the cells were determined by trypan-blue exclusion method. The numbers of viable cells irradiated for 1 minute were similar to that of non-irradiated cells (7.04×10^5/ml), while the irradiation for 4 minutes decreased the number to 3.06×10^5/ml. Irradiation for 8 minutes brought remarkable damage for the cells to give the viability of about 1.6% (1.12×10^4/ml) of the non-irradiated cells. Thus, it was elucidated that the UV irradiation could suppress the growth of the cells.

The nature of stimulator cells prepared by the UV irradiation method was compared with those prepared by mitomycin C treatment in the MLR. Splenocytes obtained from C57BL/6 mice as a responder and those from C3H/He mice as a stimulator were used. Immunosuppressive drug CsA was used as a positive control of suppressor in the MLR. As shown in Table 1, the response in the MLR using the UV-irradiated stimulator cells was similar to that using the mitomycin C-treated cells and the both responses were similarly inhibited by CsA, suggesting that the UV irradiation is useful for preparation of the stimulator cells.

Strain Difference in Combination of Mouse Splenocytes for MLR

To obtain high response in the MLR, the combination of responder and stimulator cells were examined using splenocytes of C57BL/6, C3H/He and BALB/C mice. As is seen in Fig. 1, the combination of C57BL/6 splenocytes as a responder and UV-irradiated C3H/He splenocytes as a stimulator gave the highest response (OD 0.74) and this response was suppressed by 0.2 and 1 μg/ml of CsA. On the other hand, the response in C3H/He responder cells co-cultured with UV-irradiated C57BL/6 stimulator cells was low. The combinations including BALB/C splenocytes as a responder or a stimulator gave low responses. The OD of the mixed culture of non-treated splenocytes of C57BL/6 and C3H/He was 0.61, but the suppression by cyclosporin A was low. Thus we adopted the combination of C57BL/6 splenocytes as a responder and UV-irradiated splenocytes of C3H/He as a stimulator for the MLR.

Table 1. MLR induced by stimulator cells prepared by UV irradiation or mitomycin C treatment and its inhibition by cyclosporin A.

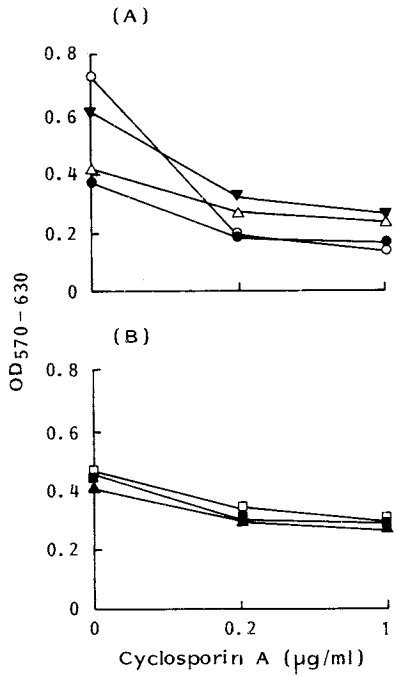
Cyclosporin A (μg/ml)	MLR ($OD_{570-630}$)[a]	
	UV irradiation	Mitomycin C treatment
0	0.736±0.041[b]	0.793±0.058
0.2	0.200±0.005	0.270±0.012
1.0	0.142±0.002	0.149±0.006

[a] The MLR was performed with splenocytes of male C57BL/6 mice as a responder and those of C3H/He treated either by irradiation of UV light at 254 nm for 8 minute or with 50 μg/ml of mitomycin C for 30 minute as a stimulator.

[b] The values are the means of four experiments and standard deviations.

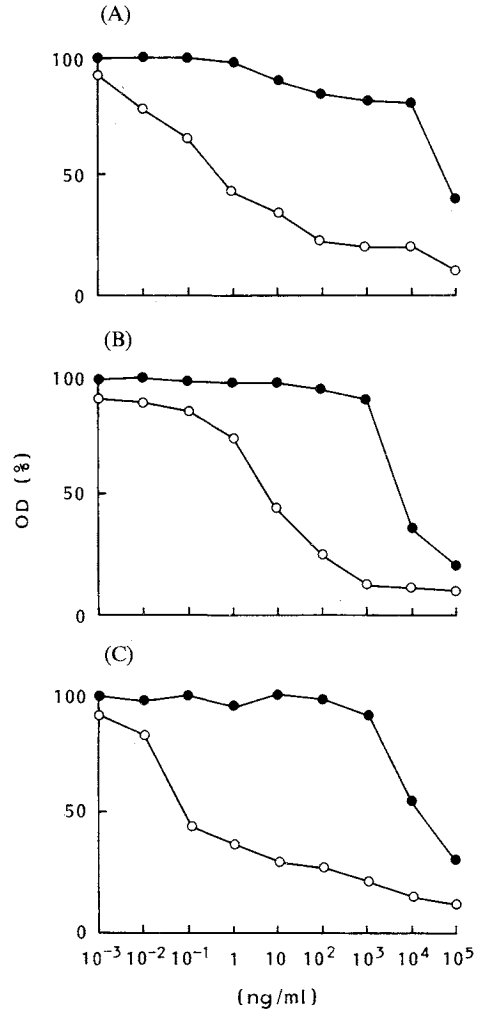
Fig. 1. MLR with different combinations of stimulator and responder mice splenocytes, and its suppression by cyclosporin A.

(A): C57BL/6 and UV-irradiated C3H/He (○), C3H/He and UV-irradiated C57BL/6 (●), C57BL/6 and non-treated C3H/He (▼), C57BL/6 and UV-irradiated BALB/C (△); (B): BALB/C and UV-irradiated C57BL/6 (▲), C3H/He and UV-irradiated BALB/C (□), BALB/C and UV-irradiated C3H/He (■).



The data are presented as the optical density determined by the MTT method. Each point is the mean of the triplicate assays.

Fig. 2. Effects of (A): prednisolone, (B): cyclosporin A and (C): FK506 on the MLR (●) and myeloma X63.Ag8. 6.5.3. cells (○).



The data are expressed as % of the control experiment and each values are means of the triplicate assays.

Effects of Immunosuppressive Agents on the MLR

To evaluate the assay system, effects of immunosuppressive agents on the MLR and on the growth of 6.5.3 cells were examined (Fig. 2). Prednisolone markedly inhibited the MLR with the IC_{50} of 1 ng/ml, while it did not affect the growth of 6.5.3 cells below 10 μg/ml. CsA and FK506 also inhibited the MLR with the IC_{50} of 10 ng/ml and 0.06 ng/ml, respectively, but not inhibited the 6.5.3 cells below 1 μg/ml. These results indicated that immunosuppressive potential of chemicals can be evaluated by comparison of the suppressive effect on the MLR and the non-specific cytotoxicity to the myeloma cells in this assay system.

Application of the Screening Method to Fungal Cultures

The assay was applied to extracts of fungal cultures, to know whether this assay system can be used for screening immunosuppressor(s)-producing fungi. The extracts of 32 isolates of fungi were added to

Table 2. MLR-supressing activity and cytotoxicity of extract of fungal culture.

Fungi		Inhibition (%)					
		MLR			Cytotoxicity		
		1[a]	10	100	1	10	100
Aspergillus versicolor	R1048	65[b]	87	91	8	57	71
Chaetomium quandrangulatum	R9043	4	18	29	3	6	9
Emericella acristata	R1712	12	26	68	8	13	39
E. nidulans	R1713	7	32	86	9	21	50
E. rugulosa	R1714	87	92	92	41	57	73
Fusarium oxysporum	R2336	0	6	9	1	11	15
F. oxysporum	R2337	3	0	2	0	0	2
Fusarium species	R2338	5	0	58	3	3	60
Fusarium species	R2339	0	0	49	4	9	90
Fusarium species	R2340	0	0	0	4	2	10
Fusarium species	R2341	0	0	6	0	0	13
Fusarium species	R2342	0	0	64	0	0	42
Fusarium species	R2343	4	6	41	1	5	17
Fusarium species	R2344	0	3	9	0	3	4
Fusarium species	R2345	2	7	0	0	2	6
Fusarium species	R2346	0	0	9	1	4	10
Fusarium species	R2347	25	55	83	0	5	24
Fusarium species	R2348	7	4	18	0	0	2
Fusarium species	R2349	6	9	30	0	4	44
Fusarium species	R2350	4	3	90	3	4	42
Fusarium species	R2351	0	0	35	3	3	0
Fusarium species	R2352	3	1	10	3	4	42
Hamigera striata	R3501	16	29	73	0	7	35
Talaromyces bacillosporus	R3502	50	90	91	15	48	83
T. flavus	R3503	0	1	22	5	4	17
T. helicus	R3504	3	0	2	9	3	9
T. luteus	R3505	19	32	87	3	5	46
T. panasenkoi	R3506	14	11	90	17	11	67
T. rutundus	R3507	6	4	12	4	6	15
T. stipitatus	R3508	15	3	23	5	7	12
T. trachyspermus	R3509	9	9	74	11	17	28
T. ucrainicus	R3510	12	11	90	11	10	70

[a] Concentrations (μg/ml) of methanol extract of fungal culture.
[b] Means of triplicate experiments.

each well of the culture plate at concentrations of 1, 10 and 100 μg/ml. As shown in Table 2, *Aspergillus versicolor* R1048, *Emericella rugulosa* R1714, *Fusarium* species R2347 and *Talaromyces bacillosporus* R3502 exhibited inhibition higher than 50% in the MLR at 10 μg/ml, while only *Fusarium* species R2347 was not cytotoxic to the 6.5.3 cells at the same concentration.

Discussion

In this study, a simplified MLR method which can evaluate immunosuppressive activities of enormous samples has been developed. Generally in the MLR, mitomycin C or γ-ray irradiation have been employed for the preparation of stimulator cells and incorporation of [^{3}H]-thymidine is measured for determination of cell growth. For example, KINO *et al.*[10] examined the immunosuppressive activity of FK506 in mouse MLR of splenocytes employing the method using mitomycin C and measurement of incorporated

[^{3}H]-thymidine. However, in the present study, we have prepared stimulator cells by irradiation with UV light. The growth of splenocytes could be stopped by the irradiation with UV light at 254 nm for 8 minutes and the irradiated cells were useful as an allo-antigen (Table 1). In the method using mitomycin C, it is necessary to wash the cells several times for removing the residual drug in the culture medium. Irradiation with γ-ray is also extensively employed, but UV irradiation is much easier and safer for the purpose. Thus, we propose the UV irradiation as a simple and safe method for the preparation of stimulator.

For measurement of the cell growth, the MTT method has been introduced. This method is also much easier than the measurement of incorporation of [^{3}H]-thymidine into the cells, which requires cell harvesting. The MTT method reflects a mitochondrial enzyme activity of viable cells, while the incorporation of [^{3}H]-thymidine indicates the DNA synthesis in the cells. Therefore, it is suggested that the MTT method is less sensitive than the measurement of [^{3}H]-thymidine incorporation when applied to early stage of cell cycle in which only DNA synthesis is taken place. On the other hand, inhibitors of mitochondrial dehydrogenase might result in the decrease in the formation of the formazan from MTT. Therefore, these chemicals might be judged as inhibitors of the MLR or cytotoxic agents.

It was shown that the immunosuppressive drugs, prednisolone, CsA and FK506, inhibited the MLR in a dose-dependent manner at the concentration which did not inhibit the growth of 6.5.3 cells (Figs. 2 A, B and C). Chemicals inhibiting the MLR at a low concentration but not the 6.5.3 cells are supposed to be specific immunosuppressive agents and such compounds can be detected by comparison of effects on the MLR and on the 6.5.3 cells. For screening specific immunosuppressors for clinical use, the ratio of the IC_{50} in 6.5.3 cells to that in the MLR should be larger at least one hundred. In the case of an inhibitor of mitochondrial dehydrogenase, it would be judged as a cytotoxic agent because such chemical inhibits both the MLR and the 6.5.3 cells in this assay system. The IC_{50} of prednisolone, CsA and FK506 in the present MLR were 1, 10 and 0.06 ng/ml, respectively, while KINO *et al.*[10] reported the IC_{50} of these compounds to be 6, 32 and 0.25 ng/ml, respectively, indicating that the MLR developed in the present study is more sensitive. This may be due to the combination of the mouse strains. We employed the combination of C57BL/6 responder cells and C3H/He stimulator cells, while their system was a combination of C57BL/6 responder and BALB/C stimulator cells. The MLR response of the latter combination was about a half (OD 0.42) of that induced in our system and the suppression by CsA was also low (Fig. 1).

For application of this screening system, 32 extracts of fungal culture were examined (Table 2). Among the extracts of fungal culture, that of *Fusarium* species R2347 inhibited the MLR without strong cytotoxicity. The ratio of inhibition of the MLR to that of the cytotoxicity seemed to be about one hundred, suggesting that this isolate produced an immunosuppressive agent(s). In addition, extracts of three isolates strongly inhibited both the MLR and the 6.5.3 cells although the inhibition of the latter was weaker. In these cases, it seemed that the active metabolites in the three extracts were cytotoxic rather than immunosuppressive. However, it is also possible that both immunosuppressive and cytotoxic metabolites are produced. Isolation of the active substances in the extracts is now undertaken in our laboratory.

For the culture of fungi, we have employed rice grains as a substrate. In the case of liquid culture, it is necessary to assay the activity of extract from both medium and fungal mass. Thus, the screening of fungi producing active metabolite(s) can be assayed with extract from rice culture, and then their presence in the medium or the fungal mass and the productivity can be examined with liquid culture.

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