A NOVEL METHOD OF SCREENING FOR IMMUNOMODULATING SUBSTANCES, ESTABLISHMENT OF AN ASSAY SYSTEM AND ITS APPLICATION TO CULTURE BROTHS OF MICROORGANISMS

AKITO NAKAMURA, KAZUO NAGAI, SEIKICHI SUZUKI†, KUNIO ANDO and GAKUZO TAMURA

Laboratory of Microbiology, Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo, Japan
†Research Laboratories, Chugai Pharmaceutical Co., Ltd., Takada, Toshima-ku, Tokyo, Japan

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A novel method of screening for immunomodulating substances is developed employing lymphocytes and three mitogens. Concanavalin A (Con A) and phytohemagglutinin (PHA) are applied as T cell specific stimulants and lipopolysaccharide (LPS) as a B cell specific stimulant respectively. The lymphocytes obtained from mouse spleen are cultured with an antibiotic or a sample extract in the presence or absence of mitogen for three days and pulsed with [^{3}H]thymidine for five hours before harvest. Differential effects of a sample compound on [^{3}H]thymidine incorporation by the activated and quiescent lymphocytes are scored. In this procedure most of the tested antibiotics or chemical compounds with different mode of actions show non-specific effects. Cyclosporin A, a potent immunosuppresive substance, suppresses both Con A and PHA responses more extensively than LPS response and quiescent cell growth, and two cytochrome bc_1 complex inhibitors, funiculosin and antimycin A_3, are less suppressive to PHA response than to the others. The present system was also applied to the methanol extracts of the culture broths prepared from the type strains of Actinomycetes and *Penicillium*.

The immunomodulating substances which attack specific target sites of cells are expected to be potent tools for the study of cellular and biochemical events of the immune responses and also provide a useful prototype of drugs for immunotherapy. Several groups have tried to search for immunomodulating substances among microbial metabolites using immunological assay system, and some new immunoactive low molecular weight substances have been discovered.[1~3] It would be of value if an assay system for the immunomodulating substances with limited target spectrum could be established, because the immune system is composed of interactions between the lymphocytes which belong to different groups by direct contact as well as *via* soluble factors like cytokines.

Polyclonal mitogens induce lymphocytes to proliferate *in vitro*. Since many of them are virtually exclusive activators of T or B cells,[4] they are used for developing model systems to study the roles of T and B cells in the immune system.[5~7] The typical mitogens specific for T lymphocytes are two plant lectins, concanavalin A (Con A) and phytohemagglutinin (PHA). Both of them activate T lymphocytes and make them secrete various lymphokines but the population of their target cells[8] and their dependency on the accessory cells[9] for expression of mitogenic activity are different from each other. One of the most popular murine B lymphocyte mitogens is bacterial lipopolysaccharide (LPS). LPS activates B lymphocytes directly and differentiates them to immunogloblin-secreting cells.[6]

Employing the above three different stimulants, Con A, PHA and LPS, we tried to establish a novel method of screening for immunomodulators. The activity of samples in the assay system was evaluated by the differential effects on [^{3}H]thymidine incorporation among the quiescent spleen cells and those stimulated by mitogens.

The screening system was applied to several compounds including antibiotics and chemical agents and the extracts of culture broths of 235 type strains of Actinomycetes and *Penicillium*. The results are presented in this report.

Materials and Methods

Mice

Specific pathogen free BALB/c mice (male 6~8 weeks old) were obtained from Charles River Japan Co., Ltd., Tokyo.

Mitogens

Concanavalin A (Con A, C2010 highly purified, Sigma Chemical Co., St. Louis, MO, U.S.A.), phytohemagglutinin (PHA, HA16 purified, Wellcome Research Laboratories, Beckenham, England) and lipopolysaccharide (LPS, *Salmonella enteritidis*, Difco Laboratories, Detroit, MI, U.S.A.) were used throughout this work.

Isolation of Murine Splenocytes

Normal mice were sacrificed and the spleen was teased with a plastic syringe in a Petri dish containing 5 ml of Eagle's minimum essential medium (MEM, Nissui Seiyaku, Tokyo). The product was gently strained through a 100-mesh screen to remove clumps and produce a single cell suspension. These cells were washed three times with MEM and resuspended in RPMI-1640 medium (Nissui Seiyaku) supplemented with 10% fetal calf serum (FCS, Flow Laboratories, Rockville, U.S.A.).

Determination of Lymphocytes Proliferation *In Vitro*

Murine splenocytes were cultured in RPMI-1640 medium supplemented with 10% FCS, at a concentration of 5×10^{5} cells/ml (1×10^{5} cells/well) with or without Con A, PHA or LPS and a test sample in the flat bottom plastic microtrays (A/S NUNC, Rockilde, Denmark). Most of the test samples were applied as methanol solution. Since 0.5% methanol in the culture medium showed no effect on the mitogenic responses, the final concentration of methanol was adjusted to 0.5% in the present assay system. Cells were cultured for 72 hours at 37°C in a humidified atmosphere of 5% CO_2 and air. At the end of the culture period, they were pulsed with [*methyl*-1′,2′-^{3}H]thymidine (113 Ci/mmol, NEN, Boston, MA, U.S.A.) (0.2 μCi/well) for 5 hours and harvested by a cell harvester (Labo Mash, Labo Science Co., Tokyo). The amount of [^{3}H]thymidine incorporated into cells was determined by a liquid scintillation counter or a gas flow counter.

Cultivation of Microorganisms

Type cultures of Actinomycetes were cultivated at 30°C in test tubes for 4 days. The culture medium contained glucose 2%, soybean meal 2%, L-methionine 0.1%, KH_2PO_4 0.1%, $MgSO_4\cdot7H_2O$ 0.05% and $CaCO_3$ 1% and the pH was adjusted to 6.5. Strains of *Penicillium* were cultivated at 27°C in test tubes for 3 days. The culture medium contained lactose 2%, glucose 2%, corn steep liquor 3%, $NaNO_3$ 0.1%, KH_2PO_4 0.06%, KCl 0.05%, $MgSO_4\cdot7H_2O$ 0.02% and $CaCO_3$ 0.5% and the pH was adjusted to 6~6.5. Culture broths of microorganisms were lyophilized and extracted with MeOH. The extracts were applied to the assay system.

Antibiotics

Tunicamycin, brefeldin A and funiculosin were from the stock of our laboratory. Cyclosporin A was a gift from Dr. K. KASAHARA, Jichi Medical School, Tochigi. Monensin, cytochalasin B and antimycin A_3 were from Sigma. Bleomycin, mitomycin C and vincristine were the commercial pro-

ducts of Nippon Kayaku Co., Ltd., Tokyo, Kyowa Hakko Kogyo Co., Ltd., Tokyo, and Shionogi & Co., Ltd., Osaka, respectively.

Results

Proliferation of Splenocytes Induced by Mitogens

The rate of proliferation of splenocytes was originally estimated by morphological assessment.[10] However the method is seldom employed owing to the extreme variability and subjectivity of the results. Currently, DNA synthesis which is estimated by the incorporation of tritiated thymidine is generally accepted as an indicator of cell proliferation and we employed this in the present system.

All of the three mitogens, Con A, PHA and LPS, afforded their maximal effect on DNA synthesis of lymphocytes three days after initiating the culture. The rate of DNA synthesis at day 3 was dependent on the concentration of mitogen in the culture medium as shown in Fig. 1. The optimal concentration for induction of mitogenic response was $2\sim3$ μg/ml with Con A or PHA. In the case of LPS the stimulation was shown to be nearly constant at the concentrations above 4 μg/ml. In the present screening system we adopted sub-optimal doses of the mitogen so that both augmentation and inhibition of mitogenic response by the added sample could be detected. Cytotoxicity as well as mitogenic activity of the samples were also assayed by the culture in the absence of mitogen.

Effects of Antibiotics on Proliferation of Splenocytes

Among the 25 substances including antibiotics and synthetic compounds which were assayed in this system no significant enhancement of [^{3}H]thymidine incorporation into cells was detected. All of them had inhibitory effects on mitogenic responses as well as on quiescent cell growth at proper concentrations. Table 1 shows 80% inhibitory concentration (IC_{80}) of typical antibiotics on [^{3}H]-thymidine incorporation. With the exceptions of funiculosin and cyclosporin A all compounds tested failed to exhibit significant selective inhibition among the three mitogenic responses. Funiculosin inhibited Con A and LPS responses more significantly than PHA response. This antibiotic is an anti-viral substance[11,12] and supposed to be an attacker of cytochrome bc_1 complex like antimycin A_3[13]. The similar differential effect on mitogenic responses was also observed with antimycin A_3.

Cyclosporin A is a potent immunosuppressive agent and has been applied to prevent organ allograft rejection.[14] Fig. 2 shows the effects of cyclosporin A on mitogenic responses. The antibiotic

Table 1. Inhibitory effects on [^{3}H]thymidine incorporation.

Agents	Target	IC_{80} (μg/ml)		
		Con A	PHA	LPS
Bleomycin	DNA	1.2	1.2	1.2
Mitomycin C		0.03	0.03	0.03
Tunicamycin	Membrane	0.008	0.008	0.004
Brefeldin A		0.2	0.2	0.2
Monensin		0.003	0.003	0.005
Cytochalasin B	Microfilament	0.005	0.005	0.003
Vincristine	Microtuble	0.006	0.006	0.003
Funiculosin	Cytochrome	0.025	0.1	0.013

The mouse spleen cells were cultured and labeled as described in the legend of Fig. 2. The concentrations of each compound which showed 80% inhibition on [^{3}H]thymidine incorporation are indicated.

Fig. 1. Proliferative responses of spleen cells to mitogens.

The mouse spleen cells were cultured with indicated amount of Con A (A), PHA (B) or LPS (C) for 3 days. The cells were allowed to incorporate [^{3}H]thymidine for 5 hours before harvest.

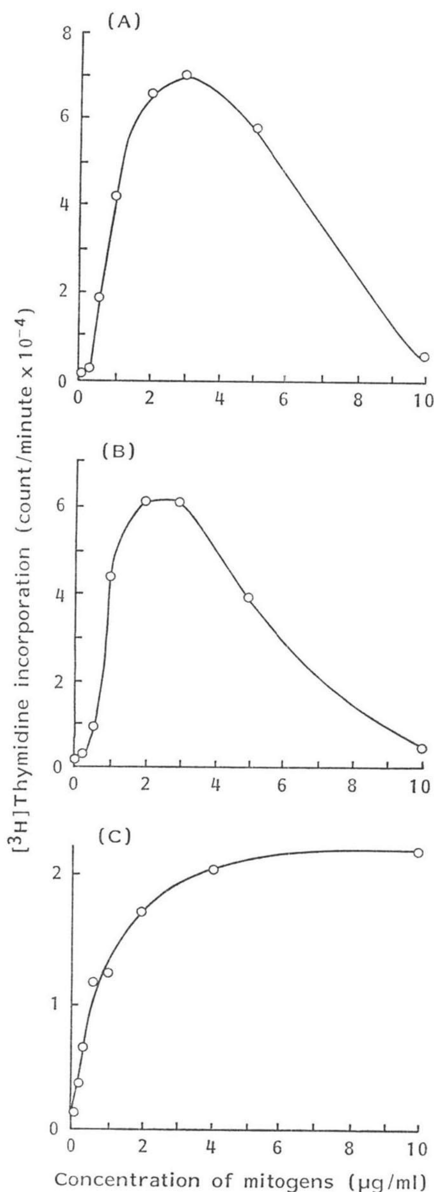
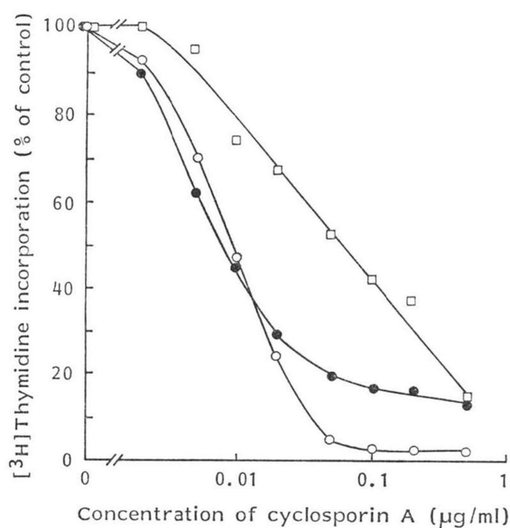


Fig. 2. Effects of cyclosporin A on mitogenic responses of murine splenocyte.

The mouse spleen cells were cultured with 1 μg/ml Con A (○) or PHA (●) or 4 μg/ml LPS (□) in the presence of indicated amount of cyclosporin A for 3 days. The cells were allowed to incorporate [^{3}H]thymidine for 5 hours before harvest.



suppressed Con A and PHA response more extensively than LPS response. The results are in good agreement with the previous notion that cyclosporin A affects T cell function considerably leaving B cell functions relatively intact.[15]

Application of the Screening Method to the Extracts of Culture Broth

The present assay system for detecting immunomodulating substances was applied to the methanol extracts of 235 culture broths of microorganisms including Actinomycetes and *Penicillium*. As shown in Table 2, 43 samples were seen to be associated with immunomodulators. Fourteen samples had mitogenic activity showing two-fold or more stimulation of [^{3}H]thymidine incorporation in the absence of mitogen. Those were derived from culture broths of *Streptomyces flaveolus*, *S. flavovirens* (two strains), *S. griseolus* (two strains), *S. griseus*, *S. microflavus*, *S. olivaceus*, *S. parvullus*, *S. tanashiensis*, *Streptomyces* sp. (two unidentified strains), *S. zaomyceticus* and *Actinoplanes philippinensis*. In a preliminary results, none of them could be extracted with ethyl acetate. Seventeen were specific inhibitors of LPS response. These included *Penicillium claviforme* (three strains), *P. lilacinum*, *P. nalgiovensis*, *P. violacea*, *Strepto-*

Table 2. Classification of the culture broths by selective effects on mitogenic response.

Mitogens				Number of active strains
None	Con A	PHA	LPS	
↑	→	→	→	14
→	↓	↓	→	2
→	↓	→	↓	6
→	→	↓	↓	0
→	↓	→	→	2
→	→	↓	→	2
→	→	→	↓	17
↓	↓	↓	↓	177
→	→	→	→	15
Total number of strains				235

→: No effect, ↑ : stimulation, ↓ : inhibition.

myces bikiniensis, *S. griseolus*, *S. griseoluteus*, *S. griseoruber*, *S. griseus*, *S. olivaceus*, *S. roseochromogenes*, *Streptomyces* sp. (unidentified), *S. sulphureus*, *S. tanashiensis* and *S. thioluteus*. Selective inhibition of Con A response was shown by the extracts of *S. albus* and *S. viridochromogenes*. The samples from *S. hygroscopicus* and *S. noboritoensis* suppressed PHA response specifically. Six of the samples, *P. chrysogenum*, *P. citrinum*, *P. crustosum*, *P. cyclopium*, *P. funiculosum* and *P. jenseni*, suppressed Con A and LPS responses more extensively than PHA response. Two of the samples, *S. hiroshimensis* and *S. roseochromogenes* inhibited Con A and PHA responses more extensively than LPS response. No preferential effect on either PHA or LPS responses could be detected among the samples tested in the present work. The rest of the cultures showed non-specific inhibition on mitogenic responses and quiescent cell growth or no effects at the assayed concentrations.

Discussion

Research in immunopharmacology has made remarkable progress in the last decade and several low molecular weight immunomodulators have been isolated from microbial sources.[1,2,16,17] Some of these compounds have been examined for their efficacy in the treatment of cancer[18] and infectious diseases.[19] These agents could be valuable in therapeutic use but they might not be useful tools in research of the cellular and biochemical events of immune response since they have multi-functional effects on the immune system.[18~20] Therefore, we elaborated a screening system for immunomodulating substances which should attack specific targets on the limited immune cell population.

We are interested in lymphocyte proliferation induced by Con A, PHA and LPS because of their specificity and tried to apply it to a screening system for immunomodifiers among the microbial metabolites. Both Con A and PHA activate T lymphocytes specifically, but their functions are different in several aspects such as target cell population and adherent cell requirement *etc.* As to target cell population, it is generally accepted that helper T lymphocytes are preferentially stimulated by PHA and suppressors are by Con A.[8] In the human system, the OKT4$^-$ T cell subset (corresponding to Lyt1$^-$2,3$^+$ T cells in the murine system) is less responsive to PHA than the OKT4$^+$ population which is the analog of Lyt1$^+$2,3$^-$ T cells. The mitogenic responses of the spleen cells to Con A and PHA were affected by depletion of adherent cells. Dependency on the function of adherent cells is more significant in the case of Con A response than PHA response.[9] Recently, genetic control of Con A and PHA stimulation was studied and it has been reported that Con A and PHA responses are mediated by the different gene systems.[21] LPS is a B lymphocytes specific mitogen. Addition of anti-Ia serum into a culture of splenocytes results in a significant inhibition of LPS response.[22] This suggests that the Ia$^+$ cells are responsive to LPS. Thus, the cell populations stimulated by the three mitogens are

different from each other in several aspects. Therefore, differential effects of some compounds on those responses could be correlated to the specific immunomodulating activity.

For the assay of mitogenic responses, it was possible to adopt morphological changes of the spleen cells which were detected by microscopic observation. However, in the present system, [^{3}H]thymidine incorporation by the cells was scored and used as an indicator of mitogenic responses. By this evaluation method, we estimated the effect of the added compound numerically and avoided variability depending on the investigators.

All of the tested antibiotics and synthetic drugs inhibited lymphocyte proliferation induced by mitogens at proper concentrations. With the exception of a few compounds, the similar rate of inhibition among the responses induced by three mitogens has been observed at those concentrations. Cyclosporin A suppressed only T cell proliferation at the concentration which left B cells unaffected. The results is in good agreement with the effects of cyclosporin A on lymphocytes which have been reported by several investigators.[15,23] This indicates that the present screening system is rational to detect immunosuppressors of the cyclosporin A type. Antimycin A_3 as well as funiculosin suppressed cell proliferation induced by Con A and LPS more significantly than that induced by PHA. At present, it remains to be elucidated if the specificity is correlated to inhibitory activity of both antibiotics on cytochrome bc_1 complex.

The rate of detection of active samples among the extracts of culture broths was higher than we expected and most of the activities belong to mitogens or selective inhibitors of LPS response. Thus, these two groups appeared in high frequency, and most of the possible combination of inhibition were observed. This suggest that the present assay system could be applied as a primary screening method for immunomodulating substances with different specificity.

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