Immunotoxicological Evaluation of Environmental Chemicals Utilizing Mouse Lymphocyte Mitogenesis Test

Humitoshi Sakazaki,[a] Hitoshi Ueno,[a] Kaori Umetani,[a] Hideo Utsumi,[b] and Katsuhiko Nakamuro*,[a]

[a]*Division of Environmental Health, Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata, Osaka 573–0101, Japan and* [b]*Department of Biophysics, Graduate School of Pharmaceutical Sciences, Kyushu University, Higashi-ku, Fukuoka 812–8582, Japan*

(Received January 5, 2001; Accepted February 1, 2001)

To establish a useful and convenient bioassay for evaluating immunotoxicological effects of environmental chemicals, lymphocyte mitogenesis tests of about 255 chemicals and environmental water were performed. We determined the growth-inhibitory effect of the environmental chemicals on the mouse splenic lymphocyte mitogenesis using lipopolysaccharide and concanavalin A as the specific mitogen for B cells and T cells, and evaluated toxicity on humoral immunity and cell-mediated immunity, respectively. The DNA content of grown cells was determined by the novel ethidium bromide-fluorophotometry method, with 96-well microplates. Of the 255 chemicals tested, 173 chemicals showed inhibitory effects on the mitogenesis. The data were classified into four typical inhibition patterns from the dose-response curves. The chemicals were categorized into six groups in the respect of inhibition selectivity between B cell mitogenesis and T cell mitogenesis. Some chemicals, such as metallic compounds, showed nonspecific effects for B cell and T cell-mitogenesis, which was attributed to the cytotoxicity. The previous reports relating to immunotoxicity found about 123 chemicals; 78 chemicals (63.4%) were positive and one chemical (0.8%) was negative for both experimental results and reported results. In total, the concordance between experimental results and reported results is 64.2%. In the application of river water concentrates for the lymphocyte mitogenesis test, some of the samples inhibited both B cell and T cell mitogenesis at the tested volume of 7.2 ml. These results indicated that the lymphocyte mitogenesis test is applicable in estimating immunotoxicity of pollutants in environmental water as well as authentic environmental chemicals.

Key words —— immunotoxicity, lymphocyte mitogenesis test, environmental chemical, pollutants in environmental water

INTRODUCTION

An increasing number of chemicals have been manufactured every year and continue to be released in the environment. The Environment Agency Government of Japan reported that about 50 thousand chemicals were produced industrially in 1998,[1] and 307 of 775 monitoring chemicals were distributed in the environment during 1974–1998.[2] Among them, many chemicals have been shown to be immunologically toxic.[3–7] Many bioassays for evaluating immunotoxicity have been performed.[8–12] However, no report on application of the lymphocyte mitogenesis method to evaluate immunotoxicity of environmental chemicals is recognized.

The lymphocyte mitogenesis test is one of the representative *in vitro* tests for immunotoxicity used in the safety evaluation of drugs.[13–16] In this test, lymphocytes are stimulated by a polyclonal mitogen specific for either B cells or T cells as an indication of humoral immunity or cell-mediated immunity, respectively, and the effect of drugs on the lymphocyte proliferation is evaluated.[14,15,17]

When lymphocytes are stimulated by a mitogen, the following reactions occur on the lymphocytes: capping of the receptors or immunoglobulins on the surface of lymphocytes, increase of intracellular calcium concentration, activation of intracellular signaling pathways, phosphorylation and activation of each enzyme, synthesis of RNA and proteins, replication of DNA, and finally mitosis of lymphocytes.[13] If lymphocytes have been exposed to an environmental chemical during the period of the mitogen-

*To whom correspondence should be addressed: Division of Environmental Health, Faculty of Pharmaceutical Sciences, Setsunan University, 45–1 Nagaotoge-cho, Hirakata, Osaka 573–0101, Japan. Tel.: +81-72-866-3122; Fax: +81-72-866-3123; E-mail: nakamuro@pharm.setsunan.ac.jp

esis, any inhibition in the sequential reactions can be detected as depression of lymphocyte proliferation. Therefore, the lymphocyte mitogenesis test may be useful for assessing immunotoxicity of environmental chemicals.

To assay the lymphocyte growth, [^{3}H]thymidine incorporation method or 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; triazolyl blue (MTT) assay is widely used. However, the number of grown cells does not always correlate proportionally with [^{3}H]thymidine incorporation,[18,19] and it is too hazardous and troublesome to handle such a radioisotope in bioassays. The measurement of respiratory enzyme activity in mitochondria by MTT is also often modulated by mitogens. Therefore, we tried to evaluate all cell growth by determination of DNA content using the ethidium bromide method.[20] In this method, cells were cultured in 24-well microplates, and after the cultivation, cells were transferred into test tubes to determine cell growth. We improved and modified this method with 96-well microplates throughout the experiment to assay many samples efficiently.

We also evaluated immunotoxicity of the 255 environmental chemicals and environmental water with the lymphocyte mitogenesis test.

MATERIALS AND METHODS

Chemicals and Animals —— Lipopolysaccharide, concanavalin A and ethidium bromide were purchased from Sigma Chemical Co. (St. Louis, Missouri, U.S.A). RPMI-1640 medium was obtained from Nissui Pharmaceuticals Co. (Tokyo, Japan). Sodium dodecyl sulfate (SDS) was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were of the highest commercially available grade. Male C3H/He mice and BALB/c mice, 5 weeks old, were purchased from Japan SLC Co. (Sizuoka, Japan). The animals were kept in a room maintained at 23 ± 1°C with 47–67% relative humidity for 1 week.

Samples Tested —— The 255 chemicals are selected with high priority with respect to possible pollution to the environment, production scale, usage frequency, possible biological effects and exposure level in the environment. These chemicals include 68 pesticides, 17 metal compounds, 41 polycyclic aromatics, 201 mutagens, 160 carcinogens, and 39 endocrine disrupters.

The 255 chemicals listed in Table 1 were dissolved in dimethylsulfoxide (DMSO) or distilled water at the concentration of 0.1–200 mmol/l on the basis of the solubility to the solvent.

To concentrate the water sample, water samples (20 l) from 8 sampling points in Biwa Lake and Yodo River were applied to XAD-2 resin column, and eluted by 500 ml of ethyl acetate. After evaporation to dryness, the residues were dissolved in 2 ml of DMSO.[21] These samples were finally concentrated to 10000 times. All samples were sterilized by filtration and stored at –20°C.

Preparation of Splenic Cells —— Spleen was isolated from C3H/He mouse for the B cell test[22] or BALB/c mouse for the T cell test, and the splenic cells were prepared by the flushing method, injecting 10 ml of RPMI-1640 medium with a syringe into the spleen. The suspended cells were left for 2–3 min on ice to precipitate and be removed of the connective tissue, and thereafter washed twice with the medium and counted with hematocytometer. The cells were dispensed into 96-well microplates at 10^5 cells/well in 200 μl of RPMI-1640 medium containing 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 50 μM 2-mercaptoethanol, 100 IU/ml penicillin, 50 μg/ml streptomycin, 0.18% $NaHCO_3$ and 10% fetal calf serum.

Exposure to Mitogens and the Chemicals —— As the mitogen, 100 μg/ml lipopolysaccharide (LPS) or 2 μg/ml concanavalin A (Con A) was added for B cell mitogenesis or T cell mitogenesis, respectively. The chemicals were prepared by serial dilution and further diluted into 3% by the medium. Twenty-microliters of each prepared chemical solution was applied to the cells and the total volume in each well was 200 μl. The final concentration of DMSO was limited up to 0.3%, in which DMSO exerts less suppression of lymphocyte mitogenesis. The plates were incubated at 37°C in 5% CO_2 for 4 days.

DNA Determination by Ethidium Bromide Fluorescence Method —— After 4-days culture, the total DNA amount in grown cells was determined by the ethidium bromide fluorescence method.[20] The plate was centrifuged at 4°C, 500 × *g* for 10 min and the supernatants were removed. The cells were washed once with 200 μl of PBS, 200 μl of 0.1% SDS was added and left for 30 min to lyse the cells. Then 100 μl of 50 μg/ml ethidium bromide was added to each well, left for 15 min and DNA-ethidium bromide complex was determined fluorometrically at Em 620 nm and Ex 515 nm as indication of cell number. Mitogenesis rate is calcu-

lated as below.

Mitogenesis (%) = $(E - E') / (E_0 - E_0') \times 100$

E: DNA content of the cells exposed to both the sample and the mitogen

E': DNA content of the cells exposed to the sample only

E_0: DNA content of the cells exposed to the mitogen only

E_0': DNA content of the control cells

A cell-growth-curve was obtained by plotting mitogenesis (%) against the sample concentration and subsequently 50% cell growth inhibition-showing concentration (IC_{50}) values for B cells and that for T cells were estimated from the curve to evaluate the potential effect of environmental chemicals on humoral immunity and/or cell-mediated immunity.

Correlation between Reported Data and Experimental Data —— Available literatures were retrieved from MEDLINE database (National Library of Medicine, U.S.A.)[23] using the words "lymphocyte mitogenesis" or "mitogen*" and each chemical name as keywords. The asterisk at the end of a term were used to find all terms that begin with "mitogen" such as mitogens or mitogenic. We retrieved articles describing general immunotoxicity, including lymphocyte mitogenesis, by adding two more keywords "allergy" and "immunotoxic*". The asterisk at the end of a term was used to find all terms that begin with "immunotoxic", such as immunotoxicity or immunotoxicology. Homology between reported data and experimental data was estimated for both experimental results and published results.

RESULTS AND DISCUSSION

Evaluation of Ethidium Bromide Fluorescence Method

To establish a simple and rapid method for immunotoxicological evaluation of many environmental samples, we first investigated applicability of the ethidium bromide fluorescence method (data not shown). The data showed good correlation between the amount of calf thymus DNA and the fluorescence intensity of DNA-ethidium bromide complex. The correlation coefficient between the number of splenic cells and the fluorescence intensity was more than 0.99. It was confirmed that this ethidium bromide fluorescence method is available for estimating cell numbers in this lymphocyte mitogenesis test.

Dose Dependency of Cell Growth Inhibition by Chemicals

The preliminary mitogenesis of environmental chemicals was performed for judgement of the results. The data were classified into four typical inhibition patterns from the cell growth inhibition curves (Fig. 1). In pattern A, the chemicals showed no inhibition within the concentration range assayed. In pattern B, the chemicals showed weak inhibition up to the maximum 50%. In pattern C, the inhibition depended on the sample concentration. In pattern D, the inhibition occurred suddenly at certain concentrations, probably by toxicity. The chemicals that showed pattern A were judged as negative, and the pattern B, C and D were judged as positive. The IC_{50} value for each chemical was obtained when the cell growth inhibition curve showed either pattern C or D.

Specificity for Effect of Test Chemicals on Lymphocyte Mitogenesis

The results of the 255 chemicals were shown in Table 1. Of the 255 tested chemicals, 173 chemicals (about 70%) were positive. The plot of IC_{50} values for B cell versus T cell mitogenesis (Fig. 2) was shown with the chemicals categorized into six groups, as follows: (I) chemicals affected only B cell mitogenesis, (II) chemicals causing stronger effect toward B cells, (III) chemicals affecting B cell and T cell mitogenesis at a similar extent, (IV) chemicals causing stronger effect toward T cells, (V) chemicals affecting only T cell mitogenesis, (VI) chemicals whose IC_{50} values were not obtained for B cell nor T cell mitogenesis (pattern B in Fig. 1). In 173 positive chemicals, many chemicals were distributed to I, III and VI, suggesting that the chemicals which affect lymphocyte mitogenesis tend to have specific inhibition for humoral immunity or nonspecific inhibition for both humoral and cell-mediated immunity. The chemicals that showed sudden inhibition at certain concentrations (pattern D in Fig. 1) were mainly distributed in group III. From this overlapping between pattern D chemicals and group III chemicals, it is thought that nonspecific effects for B cell and T cell mitogenesis is attributed to the cytotoxicity.

Features in Each Category

The chemicals tested were categorized on the basis of the physicochemical properties, chemical structure, usage and toxicity. Physicochemical properties, such as molecular weight, melting point, boiling point and octanol/water distribution coefficient,

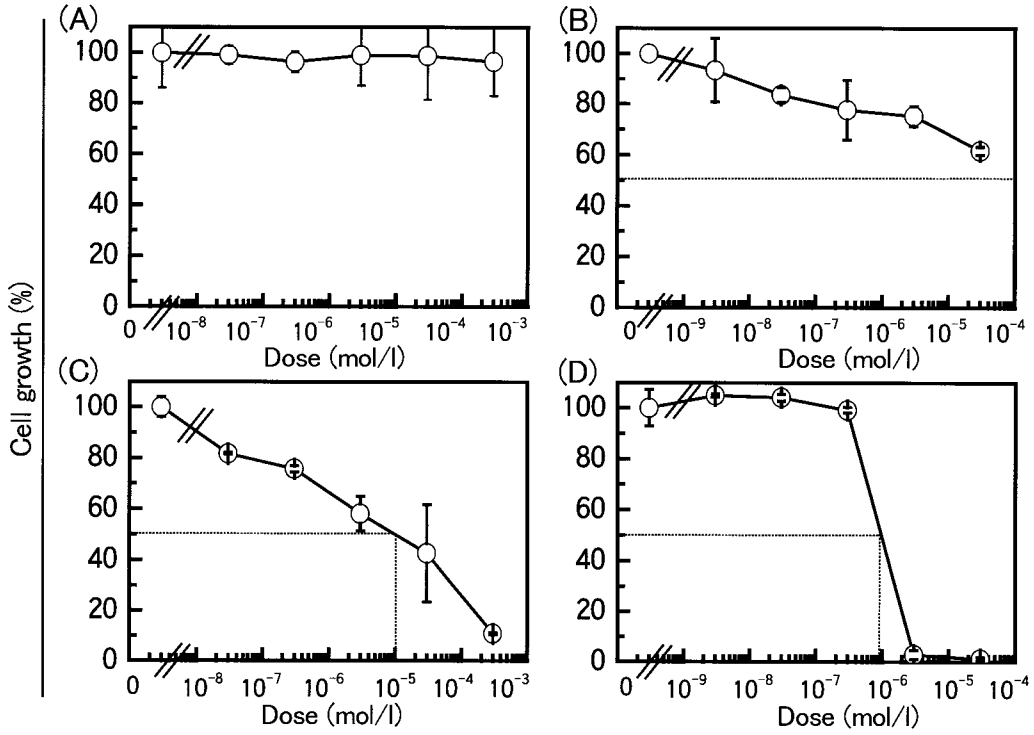

Fig. 1. Four Typical Growth Inhibition Curves of Mouse Lymphocytes Exposed to Environmental Chemicals during Mitogenesis

(A) Effect of benzylalcohol on B cell mitogenesis. No inhibition was shown within the concentration range assayed. (B) Effect of MEP (fenitrothion) on T cell mitogenesis. Weak inhibition was shown up to the maximum 50%. (C) Effect of 2,4-dichloroaniline on B cell mitogenesis. The inhibition depended on the sample concentration. (D) Effect of captan on B cell mitogenesis. The inhibition occurred suddenly at certain concentrations, probably by toxicity. The chemicals that showed pattern A were judged as negative, and the pattern B, C and D as positive. The IC_{50} value for each chemical was obtained when the cell growth inhibition curve showed either pattern C or D. All the values were expressed as the mean ± S.D.

did not show any significant correlation to lymphocyte mitogenesis. Any evidence was not seen in each usage and/or toxicity category, such as agricultural chemicals, mutagens, carcinogens and endocrine disrupters. Agricultural chemicals were further classified on the basis of the groups of organophosphorus agent, organochlorine, carbamate and others, or usage such as insecticides, herbicides, fumigants and others, but no evidence was observed. It is thought that these categories do not have any common characteristics regarding the effect on lymphocyte.

The classification based on the chemical structure did not suggest any evidence. But metallic compounds showed an interesting correlation in this study. Of 19 metallic compounds, 13 compounds were positive and most of those positive compounds inhibited both B cell and T cell mitogenesis at the same extent (group III and VI). Moreover, the cell growth inhibition curve of those metallic compounds indicated pattern D. These pattern D and group III chemicals are thought to show cytotoxicity as described above. It is well known that arsenite inhibits citrate cycle, and it is reported that tributyl tin and triphenyl tin inhibit microtubule assembly in V79 Chinese hamster cells and *in vitro* assembly of bovine brain tubulin.[24] These results suggest that these 13 metallic compounds have nonspecific effects on lymphocytes attributed to the cytotoxicity. Some of these metallic compounds have been reported to have an immunotoxic effect.[25–27] This gives confidence to the immunotoxicological evaluation in this lymphocyte mitogenesis test.

Correlation between Acute Toxicity and Lymphocyte Mitogenesis Inhibition

As some compounds that inhibited lymphocyte mitogenesis at certain concentrations (pattern D in Fig. 1) were suspected to exert toxicity, we estimated the correlation between the IC_{50} value for lymphocyte mitogenesis and acute toxicity as LD_{50} values for mice by i.p. administration retrieved from RTECS (Registry of Toxic Effects of Chemical Substances) database of NIOSH (National Institute for Occupational Safety and Health, U.S.A.). The correlation coefficient between acute toxicity and B cell and T cell mitogenesis inhibition was 0.151 and 0.127, re-

Table 1. IC_{50} Values of 255 Chemicals and their Classification Based on the Lymphocyte Growth Inhibition

No.	Chemicals	B cell		T cell	
		IC_{50} (mol/l)	Inhibition pattern*	IC_{50} (mol/l)	Inhibition pattern*
1	Acephate		B		A
2	Acetaldehyde		A		B
3	Acetamide		A		A
4	Acrylamide		A		A
5	Adipic acid		A		A
6	Aflatoxin B1		B		B
7	Alachlor		A		A
8	Aldicarb		A		A
9	3-Amino-1*H*-1,2,4-triazole		B	2.1×10^{-5}	C
10	2-Aminoanthracene		A	8.4×10^{-6}	D
11	2-Aminoanthraquinone	7.5×10^{-6}	D	8.1×10^{-6}	D
12	2-Aminoethanol		B		B
13	*m*-Aminophenol	7.5×10^{-5}	D	1.4×10^{-5}	D
14	Aniline		B		A
15	Anthracene		A		A
16	Antimony (III) chloride	1.0×10^{-6}	D	3.0×10^{-6}	D
17	Antraquinone		A		A
18	Aplysiaterpenoid A		B		A
19	Barium nitrate		A		A
20	Benzaldehyde		B		A
21	1,2-Benzanthracene	2.1×10^{-6}	C		A
22	Benzo[*a*]pyrene		B	1.0×10^{-7}	C
23	Benzo[*b*]fluoranthene		A		A
24	Benzo[*e*]pyrene	3.0×10^{-5}	C		B
25	Benzo[*ghi*]perylene		A		A
26	Benzo[*k*]fluoranthene		A		A
27	Benzoic acid		A		A
28	Benzophenone		A		A
29	Benzylalcohol		A		A
30	Bifenox		B		B
31	Biphenyl		B		A
32	Bis (2-chloroethyl) ether	3.0×10^{-5}	C		A
33	2,2-Bis (3,5-dibromo-4-hydroxyphenyl) propane	3.3×10^{-5}	C	9.0×10^{-5}	D
34	Bis (2-ethylhexyl) amine		A		A
35	Bis-phenol-A	2.0×10^{-5}	D		A
36	Boric acid	4.0×10^{-6}	D		A
37	BPMC		B		A
38	Bromodichloromethane		A		A
39	Bromoform		A		A
40	*p*-Bromophenol	9.3×10^{-6}	C	6.3×10^{-5}	D
41	1-Butanol	4.5×10^{-6}	C		A
42	*n*-Butyl acrylate	5.1×10^{-6}	C		A
43	*n*-Butylbenzene		A		A
44	*p-t*-Butylbenzoic acid	1.5×10^{-4}	D		A
45	Cadmium chloride	3.0×10^{-6}	C	1.0×10^{-5}	D
46	Captan	9.9×10^{-7}	D		A
47	Catechol	6.0×10^{-8}	C	6.0×10^{-6}	D
48	2-Chloro-1,1,2-trifluoroethyl ethyl ether		A	1.0×10^{-7}	C
49	1-Chloro-2,4-nitrobenzene	6.6×10^{-7}	D	6.0×10^{-7}	C
50	4-Chloro-3-methylphenol	1.4×10^{-6}	C	5.7×10^{-5}	D

Table 1. Continued

No.	Chemicals	B cell		T cell	
		IC_{50} (mol/l)	Inhibition pattern*	IC_{50} (mol/l)	Inhibition pattern*
51	4-Chloroaniline	1.8×10^{-4}	C	1.1×10^{-6}	C
52	Chlorobenzene		A	3.0×10^{-5}	C
53	Chlorodibromomethane	1.1×10^{-5}	B		A
54	4-Chloronitrobenzene		A		B
55	*o*-Chloronitrobenzene		A		A
56	*p*-Chlorophenol	7.9×10^{-5}	D	1.1×10^{-4}	D
57	4-Chlorotoluene	1.0×10^{-5}	C		B
58	CNP	2.0×10^{-7}	C		A
59	Copper (II) sulfate	9.9×10^{-6}	D	6.6×10^{-5}	D
60	Coumestrin		B		A
61	Coumestrol		A		C
62	*p*-Cresol	2.1×10^{-4}	D	1.5×10^{-5}	B
63	Cucumechinoside D		A		A
64	Cumene		A		B
65	Cyclohexanol		A		A
66	Cyclohexanone		B		A
67	Cyclohexyl amine	2.4×10^{-7}	C		A
68	Cyclophosphamide		A		A
69	Cyclosporin A	4.0×10^{-8}	D	4.0×10^{-8}	D
70	DCPA (Propanil)	6.9×10^{-6}	C		A
71	DDVP	1.8×10^{-5}	D	2.4×10^{-5}	D
72	Decabromodiphenyl ether	6.0×10^{-6}	C		C
73	*n*-Decyl alcohol	1.2×10^{-5}	C		A
74	Dexamethasone	3.0×10^{-9}	D	4.0×10^{-9}	D
75	Di-2-ethylhexyl adipate	3.0×10^{-5}	A		B
76	Di-2-ethylhexyl phthalate	3.0×10^{-8}	C	7.0×10^{-6}	B
77	2,4-Diaminotoluene	8.1×10^{-5}	D	5.7×10^{-5}	D
78	Diazinon		A	1.2×10^{-5}	C
79	1,2,5,6-Dibenzanthracene		B		A
80	Dibenzyl ether		A		A
81	1,2-Dibromo-3-chloropropane	1.0×10^{-5}	C		B
82	1,2-Dibromoethane		A		A
83	Dibutyl phthalate	9.0×10^{-5}	D	6.0×10^{-5}	D
84	1,3-Dichloro-2-propanol		A		A
85	2,4-Dichloroaniline	9.6×10^{-6}	B		B
86	2,5-Dichloroaniline	5.1×10^{-5}	D		A
87	3,4-Dichloroaniline	5.0×10^{-7}	D		A
88	4-Dichlorobenzene		A	7.0×10^{-7}	D
89	*o*-Dichlorobenzene		A		A
90	2,4-Dichlorophenol	6.6×10^{-7}	B	6.6×10^{-5}	D
91	2,5-Dichlorophenol	8.0×10^{-7}	C	1.0×10^{-4}	D
92	2,4-Dichlorophenoxy acetic acid	7.0×10^{-5}	D	4.0×10^{-6}	D
93	1,3-Dichloropropene, mixture		A		A
94	Dicyclohexylamine	5.0×10^{-5}	C		A
95	Dicyclopentadiene	3.0×10^{-5}	C		B
96	Diethyl phthalate	9.0×10^{-5}	D		B
97	Diethyl sulfate	7.0×10^{-7}	C		A
98	Diethylbenzene, mixture		A	3.0×10^{-4}	C
99	Diethylene glycol		A		A
100	Diethylstilbestrol	2.0×10^{-6}	D	5.0×10^{-7}	D

Table 1. Continued

No.	Chemicals	B cell		T cell	
		IC_{50} (mol/l)	Inhibition pattern*	IC_{50} (mol/l)	Inhibition pattern*
101	Dimethoate		B		B
102	2,4-Dimethylphenol		A		A
103	Dimethyl phthalate		A		A
104	*N*,*N*-Dimethylaniline		A		A
105	*N*,*N*-Dimethylformamide		A		A
106	2,6-Dimethylnaphthalene	1.2×10^{-5}	C		A
107	1,3-Dimethyl-2-thiourea		B		B
108	2,4-Dinitroaniline	6.0×10^{-5}	D	6.0×10^{-5}	C
109	*o*-Dinitrobenzene	1.8×10^{-8}	C	7.5×10^{-5}	D
110	2,4-Dinitrophenol	1.2×10^{-6}	D	8.4×10^{-5}	D
111	1,8-Dinitropyrene		A		A
112	1,6-Dinitropyrene		A		A
113	1,4-Dioxane		A	1.0×10^{-5}	B
114	Diphenylamine		A		A
115	Diphenylmethane		A		A
116	Diquat dibromide monohydrate	2.0×10^{-6}	C	2.0×10^{-6}	C
117	2,6-Di-*t*-butyl-4-methylphenol	9.0×10^{-5}	C	1.0×10^{-5}	C
118	EDDP (Edifenphos)		A	9.0×10^{-6}	D
119	EDTA 2Na	1.2×10^{-5}	D	8.4×10^{-5}	D
120	Epichlorohydrin		A		A
121	EPN		B	1.4×10^{-5}	D
122	1,2-Epoxyethylbenzene		A		A
123	β-Estradiol-17-acetate	4.0×10^{-7}	C		A
124	Ethyl benzene	1.2×10^{-6}	D		B
125	Ethyl carbamate		A		A
126	*N*-Ethylaniline		B		A
127	Ethylene glycol	2.1×10^{-4}	C		A
128	Ethylene glycol monoethyl ether		A		A
129	Ethylthiomethone		A		A
130	17-α-Ethynylestradiol		A	7.0×10^{-8}	C
131	Formaldehyde	2.0×10^{-5}	C	6.6×10^{-5}	C
132	Fthalide		B		A
133	Genistein		A		A
134	Glyoxal	1.0×10^{-4}	D	1.0×10^{-4}	D
135	Glyphosate		A		A
136	Hexachloro-1,3-butadiene	3.0×10^{-5}	C		B
137	1,2,3,4,5,6-Hexachlorocyclohexane		A	2.0×10^{-6}	C
138	Hexachlorophene	5.2×10^{-6}	C	2.3×10^{-6}	D
139	Hydroquinone	3.6×10^{-6}	D	6.3×10^{-6}	D
140	Hydroxyl ammonium sulfate	7.5×10^{-5}	D	9.0×10^{-5}	D
141	IBP		A		A
142	Isophorone		A		A
143	Isoxathione		B		B
144	Kelthane		B		A
145	Lead nitrate	4.0×10^{-4}	C	2.0×10^{-4}	D
146	Malathion		A		A
147	Maneb		B		B
148	Manzeb		A		A
149	Marthasteroside A1		A		A
150	MeIQx	3.6×10^{-8}	C		A

Table 1. Continued

No.	Chemicals	B cell		T cell	
		IC_{50} (mol/l)	Inhibition pattern*	IC_{50} (mol/l)	Inhibition pattern*
151	Melamine		A		A
152	Menadione	6.0×10^{-7}	D	1.0×10^{-7}	D
153	MEP (Fenitrothion)		B		B
154	2-Mercaptobenzothiazole	1.0×10^{-6}	C		A
155	2-Mercaptoimidazoline		A		A
156	Mercury (II) chloride	3.3×10^{-7}	B	5.7×10^{-7}	D
157	Methomyl		B	1.8×10^{-8}	C
158	Methoxychlor	6.6×10^{-6}	D		B
159	2-Methyl-1-propanol		A		A
160	*N*-Methylaniline		B		B
161	3-Methylcholanthrene	6.0×10^{-7}	C		A
162	Methylglyoxal		A		A
163	Methylmercury Chloride	4.0×10^{-8}	C	9.0×10^{-8}	D
164	1-Methylnaphthalene		A		A
165	2-Methylphenol		A		A
166	2-Methylpyridine	2.7×10^{-4}	C		B
167	a-Methylstyrene		A		A
168	Microcystine RR		A		A
169	Molinate		B		A
170	Monochloroacetic acid		A		A
171	Morpholine	1.8×10^{-5}	C		A
172	MPP (Fenthion)		A		A
173	NAC (Carbaryl)	6.0×10^{-6}	D	1.1×10^{-6}	D
174	Naphthalene		A		A
175	2-Naphthol	7.0×10^{-7}	D		A
176	Nickel (II) chloride		A		A
177	NIP (Nitrofen)	1.7×10^{-5}	C		A
178	Nitrilotriacetic acid	6.0×10^{-7}	C		B
179	2,2′,2″-Nitrilotriethanol		A		A
180	Nitrobenzene		A		A
181	3-Nitrofluoranthene	9.9×10^{-6}	D		A
182	2-Nitrofluorene	1.8×10^{-5}	D	9.3×10^{-5}	D
183	*m*-Nitrophenol	1.1×10^{-4}	D	4.2×10^{-5}	C
184	*o*-Nitrophenol		A		A
185	*p*-Nitrophenol	7.0×10^{-8}	C		A
186	1-Nitropyrene		A	2.0×10^{-9}	C
187	4-Nitroquinoline-*N*-oxide	6.0×10^{-8}	C	3.0×10^{-8}	D
188	*N*-Nitrosodiethylamine		A		A
189	*N*-Nitrosodimethylamine		A		A
190	*N*-Nitrosodiphenylamine		A		A
191	*p*-Nitrotoluene	4.5×10^{-5}	C		B
192	1-Nonanol		B		A
193	*p*-Nonylphenol	5.0×10^{-7}	C	3.0×10^{-6}	C
194	Okadaic acid	3.0×10^{-8}	D	7.5×10^{-9}	D
195	PAP (Fenthoate)		B		A
196	Paraquat		A		A
197	Pentachloronitrobenzene		A		B
198	Pentachlorophenol	1.0×10^{-4}	D	1.0×10^{-7}	D
199	Pentylphenol	5.5×10^{-7}	D		A
200	Permethrin	1.2×10^{-5}	C		A

Table 1. Continued

No.	Chemicals	B cell		T cell	
		IC_{50} (mol/l)	Inhibition pattern*	IC_{50} (mol/l)	Inhibition pattern*
201	Phenol		A		A
202	Propylene glycol	1.5×10^{-7}	C		A
203	*N*-Phenyl-1-naphthylamine	9.0×10^{-5}	D	8.4×10^{-5}	A
204	*N*-Phenyl-2-naphthylamine		A	1.2×10^{-4}	D
205	2-Phenylene diamine	1.0×10^{-5}	D		A
206	Phenylhydrazine	1.7×10^{-4}	D	1.5×10^{-4}	D
207	PhIP		A		B
208	Potassium cyanide		B		A
209	Potassium dichromate (VI)	4.0×10^{-7}	D	3.0×10^{-7}	C
210	Pyrene		A		A
211	Quinoline		A		A
212	Resorcinol		B	2.7×10^{-4}	D
213	Simazine		A	3.0×10^{-5}	D
214	Simetryne		A	2.4×10^{-6}	C
215	Sodium arsenite	6.0×10^{-7}	D	6.0×10^{-6}	D
216	Sodium lauryl sulfate	8.4×10^{-5}	D	4.5×10^{-5}	D
217	Sodium molybdate		A		A
218	Sodium selenate		A		A
219	Styrene monomer		A		B
220	Terephthalic acid		A		B
221	1,1,1,2-Tetrachloroethane		A		B
222	Tetrachloroethylene		A		A
223	Tetraethylenepentamine		A		B
224	Thallium (I) chloride		A		B
225	Thiobencarb	1.0×10^{-5}	C	4.0×10^{-5}	D
226	Thiophanate-methyl	1.8×10^{-5}	C		B
227	Thiourea		A		A
228	Thiuram	5.0×10^{-8}	D	7.0×10^{-8}	D
229	*o*-Tolidine	1.2×10^{-4}	D	5.1×10^{-5}	C
230	Toluene		A		A
231	*p*-Toluenesulfonamide		B		B
232	*o*-Toluidine		B		A
233	TPN	3.3×10^{-8}	C	1.0×10^{-7}	D
234	2,4,6-Tribromophenol	6.6×10^{-5}	D	5.4×10^{-5}	D
235	Tributyl phosphate	5.0×10^{-5}	D	3.0×10^{-6}	C
236	Tributyltin chloride	3.0×10^{-8}	C	7.0×10^{-8}	D
237	1,2,4-Trichlorobenzene		B		A
238	1,2,3-Trichlorobenzene	5.4×10^{-5}	D		A
239	2,4,6-Trichlorophenol	6.0×10^{-5}	D	6.6×10^{-5}	D
240	2,4,5-Trichlorophenol	5.0×10^{-6}	C	8.0×10^{-6}	C
241	2,4,5-Trichlorophenoxyacetic acid		A		A
242	1,2,3-Trichloropropane		B		A
243	Triethylamine		B		A
244	Triethylenetetramine		A		A
245	Trifluralin		B		B
246	2,4,6-Trimethylphenol		A		A
247	Triphenyltin (IV) chloride	7.0×10^{-8}	D	1.0×10^{-7}	D
248	Tris (2-chloroethyl) phosphate	1.0×10^{-5}	C		A
249	Tris (butoxyethyl) phosphate		A		B
250	Tris (2,3-dibromopropyl) phosphate	3.0×10^{-7}	C		A

Table 1. Continued

No.	Chemicals	B cell		T cell	
		IC_{50} (mol/l)	Inhibition pattern*	IC_{50} (mol/l)	Inhibition pattern*
251	Trp-P-2	2.0×10^{-6}	D	2.0×10^{-6}	D
252	Vinclozolin		B		B
253	Vinylacetic acid		A		B
254	Ziram	9.0×10^{-8}	D	9.3×10^{-8}	D
255	Zinc nitrate hexahydrate		A		A

* Effect on lymphocyte mitogenesis. A: No inhibition for lymphocyte mitogenesis. B: Weak inhibition for lymphocyte mitogenesis. C: Dose dependent-inhibition for lymphocyte mitogenesis. D: Cytotoxic response on lymphocyte mitogenesis.

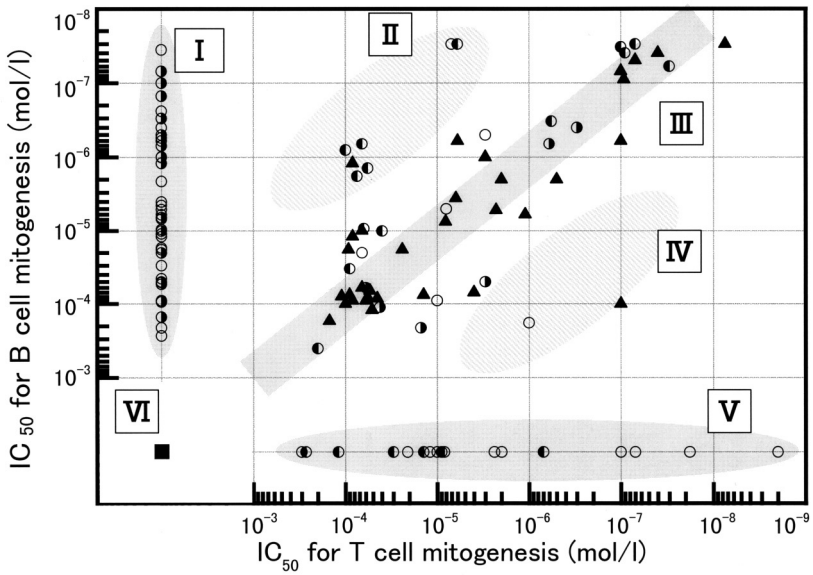

Fig. 2. Distribution of IC_{50} Values of 255 Chemicals for B Cell- and T Cell-Mitogenesis

The IC_{50} values for B cell mitogenesis versus those for T cell mitogenesis were plotted. The chemicals were categorized into six groups. (I) Chemicals affecting only B cell mitogenesis. (II) Chemicals causing a stronger effect toward B cells. (III) Chemicals affecting B cell and T cell mitogenesis at a similar extent. (IV) Chemicals causing a stronger effect toward T cells. (V) Chemicals affecting only T cell mitogenesis. (VI) Chemicals whose IC_{50} values were not obtained for B cell nor T cell mitogenesis (pattern B in Fig. 1). The inhibition pattern of each chemical was indicated as follows; ○: Pattern C for one B cell or T cell, and pattern A, B or C for another. ◑: Pattern D for B cell, and pattern A, B or C for T cell ◐: Pattern D for T cell, and pattern A, B or C for B cell. ▲: Pattern D for both B cell and T cell. ■: Pattern A or B (IC_{50} values were not obtained) for both B cell and T cell mitogenesis.

spectively. Significant correlation was not observed. Therefore, it suggests that although some inhibitions seem to be due to cytotoxicity, the toxic responses on lymphocyte mitogenesis tests differ from results of conventional animal acute toxicity assay. It is thought that some compounds are not lethal but have immunotoxicological effects, and other compounds are lethal but exert little toxicity on the immune system.

Correlation between Reported Data and Experimental Data

Published Data about Lymphocyte Mitogenesis: To give confidence to the immunotoxicological evaluation in this assay, our experimental results about the lymphocyte mitogenesis were compared with the published data (Fig. 3A). Of 255 samples, we found 47 chemicals reported regarding the effects on the lymphocyte mitogenesis. Thirty chemicals (63.8%) were positive in both our experimental results and reported results, and 3 chemicals (6.4%) were negative in both results. Totally, the concordance between experimental results and reported results is 70.2%.

Some compounds showed different results between published data and experimental data. In our experiment, 10 chemicals did not inhibit lymphocyte mitogenesis but they were reported to be posi-

(A)

Experimental results		Found in the database: Positive	Found in the database: Negative	Found in the database: (Sum)	Not found in the database	Total
	Positive	**30 (63.8%)**	4 (8.5%)	34 (72.3%)	139	173
	Negative	10 (21.3%)	**3 (6.4%)**	13 (27.7%)	69	82
	(Sum)	40 (85.1%)	7 (14.9%)	47 (100 %)	208	255

(B)

Experimental results		Found in the database: Positive	Found in the database: Negative	Found in the database: (Sum)	Not found in the database	Total
	Positive	**78 (63.4%)**	9 (7.3%)	87 (70.7%)	86	173
	Negative	35 (28.5%)	**1 (0.8%)**	36 (29.3%)	46	82
	(Sum)	113 (91.9%)	10 (8.1%)	123 (100 %)	132	255

Fig. 3. The Relationship between Reported and Experimental Chemical Data about the Effect on Lymphocyte Mitogenesis and Immunotoxicity

(A) Available literatures were retrieved from MEDLINE database (National Library of Medicine, U.S.A) using the words "lymphocyte mitogenesis" or "mitogen*" and each chemical name as a keyword for lymphocyte mitogenesis. The asterisk at the end of a term was used to find all terms that begin with "mitogen". The hatched cells in the diagram show the concordance between experimental results and published results. (B) We retrieved articles describing general immunotoxicity, including lymphocyte mitogenesis, by adding two more keywords "allergy" and "immunotoxic*" from MEDLINE database. The asterisk at the end of a term was used to find all terms that begin with "immunotoxic". The hatched cells in the diagram show the concordance between experimental results and published results.

tive. These differences are due to animal strain, mitogen, experimental condition such as *in vivo* or *in vitro*, or culture condition. In our experiment a C3H/He male mouse and a BALB/c male mouse were used, splenic cells were stimulated by LPS or Con A, and incubated *in vitro*, while in the reported data, ethyl carbamate was administered to a C57BL/6J female mouse *in vivo*[28] and *N*,*N*-dimethylformamide was added to human peripheral lymphocyte stimulated by phytohemagglutinin.[29] We could not discuss inhibition patterns, IC_{50} values and specificity for B cell and T cell because the results are described in a different manner in each article. The doses of *in vivo* administration could not be compared with the concentrations of *in vitro* exposure. Other mitogens, such as phytohemagglutinin, have different specificity on stimulating B cells and T cells. Therefore, a standardized assay method and uniform indication should be used for assessment of immunological toxicity of environmental chemicals.

In spite of the difference in experimental conditions, there is still the high (70.2%) concordance between experimental data and published data. Our method was confirmed to be effective in evaluating the inhibition of lymphocyte mitogenesis.

Published Data about Immunological Effect: We compared our experimental results about the lymphocyte mitogenesis with the published data about the general immunotoxicity, including lymphocyte mitogenesis (Fig. 3B). The reports relating to

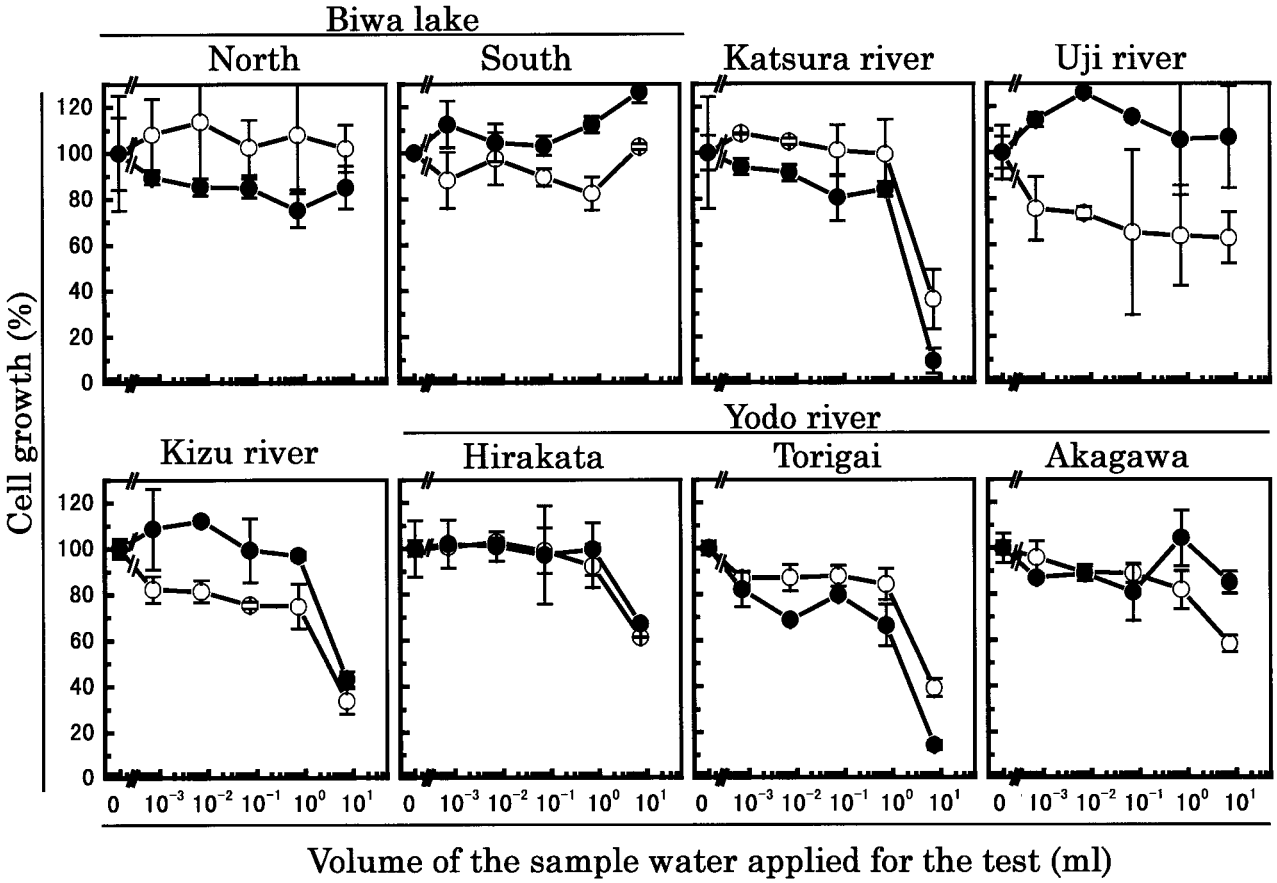

Fig. 4. Effect of Biwa Lake and Yodo River Water Samples Concentrated by XAD-2 Resin on Lymphocyte Mitogenesis

River water was concentrated by the XAD-2 resin method to 1000 fold and each concentrate was prepared by serial dilution and applied to the lymphocyte mitogenesis test as described in MATERIALS AND METHODS. The volume indicated in the x-axis shows the volume of original sample water. ●: B cell. ○: T cell. All the values were expressed as the mean ± S.D.

immunotoxicity found about 123 chemicals; 78 chemicals (63.4%) were positive for both experimental results and reported results, and one chemical (0.8%) was negative for both experimental results and reported results. Totally, the concordance between experimental results and reported results is 64.2%. The experimental data have a high homology with the reported data, including allergy and other immunotoxicity. In our experiment, 36 chemicals did not inhibit lymphocyte mitogenesis in spite of positive results reported in the database. Some of the chemicals may accelerate allergy and suppress macrophage or neutrophils but not lymphocyte. We could not discuss inhibition patterns, IC_{50} values and specificity for B cells and T cells as mentioned above.

These results, therefore, indicate that the results of the lymphocyte mitogenesis test represent broad immunotoxicological effects. The lymphocyte mitogenesis test is a useful bioassay, and available in primary immunotoxicological evaluation for environmental chemicals.

Application to Environmental Water Samples

To confirm the efficacy of the test for complex pollution of multiple chemicals, including unknown substrates,[30] we applied environmental water to the lymphocyte mitogenesis test. Since pollutants in environmental water are diluted, we evaluated the sensitivity of this assay for environmental water. River water was concentrated by the XAD-2 resin method to 1000 fold and tested by the lymphocyte mitogenesis test. The water samples inhibited both B cell and T cell mitogenesis (Fig. 4). These results, therefore, suggest that the lymphocyte mitogenesis test is applicable in estimating immunotoxicity of environmental water using the 1000-fold concentrates.

Acknowledgements This work was supported by funds from the Environment Agency Government of Japan.

REFERENCES

1) Environment Agency Government of Japan (1999) *Quality of the Environment in Japan*, Printing Bureau, Ministry of Finance, Tokyo, pp. 212–215 (in Japanese).
2) Environment Health and Safety Division, Environmental Health Department, Environmental Agency Government of Japan (1999) *Chemicals in the Environment*, Tokyo, p. 9 (in Japanese).
3) Salvaggio, J. E. (1996) Understanding Clinical Immunological Testing in Alleged Chemically Induced Environmental Illnesses. *Regul. Toxicol. Pharmacol.*, **24**, S16–S27.
4) Munson, A. E., McCay, J. A. and Cao, W. (1991) Approaches to Immuno-toxicologic Studies with Emphasis on Chemical-induced Immunomodulation. *Ann. Allergy*, **66**, 505–518.
5) Gleichmann, E., Kimber, I. and Purchase, I. F. H. (1989) Immunotoxicology: Suppressive and Stimulatory Effects of Drugs and Environmental Chemicals on the Immune System. *Arch. Toxicol.*, **63**, 257–273.
6) Stack, A. S., Altman-Hamamdzic, S., Morris, P. J., London, S. D. and London, L. (1999) Polychlorinated Biphenyl Mixtures (Aroclors) Inhibit LPS-induced Murine Splenocyte Proliferation *in vitro*. *Toxicology*, **139**, 137–154.
7) Prell, R. A., Oughton, J. A. and Kerkvliet, N. I. (1995) Effect of 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin on Anti-CD3-induced Changes in T-cell Subsets and Cytokine Production. *Int. J. Immunopharmacol.*, **17**, 951–961.
8) White, K. L., Jr., Sanders, V. M., Barnes, D. W., Shopp, G. M., Jr. and Munson, A. E. (1985) Immunotoxicological Investigations in the Mouse: General Approach and Methods. *Drug. Chem. Toxicol.*, **8**, 299–331.
9) Pallardy, M., Kerdine, S. and Lebrec, H. (1998) Testing Strategies in Immunotoxicology. *Toxicol. Lett.*, **102–103**, 257–260.
10) Luster, M. I., Portier, C., Pait, D. G., White, K. L., Gennings, C., Munson, A. E. and Rosenthal, G. J. (1992) Risk Assessment in Immunotoxicology I. Sensitivity and Predictability of Immune Tests. *Fundam. Appl. Toxicol.*, **18**, 200–210.
11) Luster, M. I., Portier, C., Pait, D. G., Rosenthal, G. J., Germolec, D. R., Corsini, E., Blaylock, B. L., Pollock, P., Kouchi, Y., Craig, W., White, K. L., Munson, A. E. and Comment, C. E. (1993) Risk Assessment in Immunotoxicology II. Relationships Between Immune and Host Resistance Tests. *Fundam. Appl. Toxicol.*, **21**, 71–82.
12) Luster, M. I., Portier, C., Rosenthal, G. J., Germolec, D. R., Comment, C. E., Munson, A. E., White, K. and Pollock, P. (1992) Qualitative and Quantitative Experimental Models to Aid in Risk Assessment for Immunotoxicology. *Toxicol. Lett.*, **64–65**, 71–78.
13) Robert, F. Ashman (1984) In *Fundamental Immunology* (Paul, W. E., Ed.), Raven Press Books, Ltd., New York.
14) Inoue, T., Horii, I. and Udaka, K. (1989) Immunotoxicological Alterations Induced by Fluoropyrimidine Derivatives, Cyclosporine A and Uberimax in Rats. *Int. Cong. of Toxicology*, **5**, 131.
15) Vos, J. G. (1981) Screening and Function Studies in Immunotoxicity Testing. *Vet. Q.*, **3**, 190–195.
16) Nyfeler, B. and Pichler, W. J. (1997) The Lymphocyte Transformation Test for the Diagnosis of Drug Allergy: Sensitivity and Specificity. *Clin. Exp. Allergy*, **27**, 175–181.
17) The ICICIs Group Investigators (1998) Report of Validation Study of Assessment of Direct Immunotoxicity in the Rat, *Toxicology*, **125**, 183–201.
18) Gogal, R. M., Jr., Ahmed, S. A. and Larsen, C. T. (1997) Analysis of Avian Lymphocyte Proliferation by a New, Simple, Nonradoactive Assay (Lymphopro). *Avian Dis.*, **41**, 714–725.
19) Betel, I., Martijnse, J. and van der Westen, G. (1979) Mitogenic Activation and Proliferation of Mouse Thymocytes. Comparison Between Isotope Incorporation and Flow-microfluorometry. *Exp. Cell. Res.*, **124**, 329–337.
20) Itoh, Y., Fukamachi, I., Nakajima, K., Kawai, T. and Nakao, K. (1983) A New Method for the Evaluation of Lymphocyte Activation by Ethidium Bromide Fluorescence Assays. *Journal of Clinical and Experimental Medicine*, **126**, 21–22 (in Japanese).
21) Sayato, Y. and Nakamuro, K. (1987) Studies of Preconcentration Methods for Detecting the Mutagenicity of Organics in Drinking Water. *Eisei Kagaku*, **3**, 328–336.
22) Vukajlovich, A. W. and Morrison, D. C. (1983) Conversion of Lipopolysaccharides to Molecular Aggregates with Reduced Subunit Heterogeneity: Demonstration of LPS-responsiveness in "Endotoxin-unresponsive" C3H/HeJ Splenocytes. *J. Immunol.*, **130**, 6.
23) National Center for Biotechnology Information, National Library of Medicine, U.S.A. *PubMed*. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi
24) Jensen, K. G., Onfelt, A., Wallin, M., Lidums, V. and Andersen, O. (1991) Effects of Organotin Compounds on Mitosis, Spindle Structure, Toxicity and *in vitro* Microtubule Assembly. *Mutagenesis.*, **6**, 409–416.
25) Low, K. W. and Sin, Y. M. (1998) Effects of Mercuric Chloride and Sodium Selenite on Some Immune Responses of Blue Gourami, Trichogaster

Trichopterus (Pallus). *Sci. Total. Environ.*, **214**, 153–164.
26) Payette, Y., Lachapelle, M., Daniel, C., Bernier, J., Fournier, M. and Krzystyniak, K. (1995) Decreased Interleukin-2 Receptor and Cell Cycle Changes in Murine Lymphocytes Exposed *in vitro* to Low Doses of Cadmium Chloride. *Int. J. Immunopharmacol.*, **17**, 235–246.
27) Nordlind, K. and Henze, A. (1984) Investigation of Ability of the Metal Allergens Cobalt Chloride, Nickel Sulfate and Potassium Dichromate to Give a Mitogenic Response in Human Thymocytes. *Int. Arch. Allergy Appl. Immunol.*, **75**, 330–332.
28) Luebke, R. W., Rogers, R. R., Riddle, M. M., Rowe, D. G. and Smialowicz, R. J. (1987) Alteration of Immune Function in Mice Following Carcinogen Exposure. *Immunopharmacology*, **13**, 1–9.
29) Suthanthiran, M., Rubin, A. L., Novogrodsky, A. and Stenzel, K. H. (1982) Immunosuppressive Properties of Polar Organic Compounds that Induce Cellular Differentiation in Friend Erythroleukemia Cells. *Transplantation*, **33**, 534–540.
30) Hsieh, G. C., Parker, R. D. R., Sharman, R. P. and Hughes, B. J. (1990) Subclinical Effects of Groundwater Contaminants. *Arch. Toxicol.*, **64**, 320–328.