Effect of Endocrine Disrupting Chemicals on Lipopolysaccharide-Induced Tumor Necrosis Factor- α and Nitric Oxide Production by Mouse Macrophages

Chih-Chun HONG, Mifumi SHIMOMURA-SHIMIZU, Masashi MUROI, and Ken-ichi TANAMOTO*

Division of Microbiology, National Institute of Health Sciences; 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan.

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Little is known about the development of infectious diseases during exposure to endocrine disrupting chemicals (EDCs), although several studies have reported on the effect of EDCs on the immune function of the human body. To assess the effect of EDCs on the development of infectious disease, we investigated the effect of eighteen possible EDCs on mouse macrophage production of tumor necrosis factor- α (TNF- α) and nitric oxide (NO) in response to bacterial endotoxin *in vitro* and *ex vivo*. Of chemicals we examined, simazine, nitrofen, and benzyl butyl phthalate inhibited lipopolysaccharide (LPS)-induced TNF- α production by mouse macrophage cell line RAW 264 *in vitro*. Carbaryl, alachlor, nonylphenol, octylphenol, tributyltin, and triphenyltin inhibited LPS-induced NO production *in vitro*, whereas 2,4-dichlorophenoxy acetic acid and bisphenol A enhanced its production. Zineb and alachlor, on the other hand, enhanced LPS-induced TNF- α production by mouse peritoneal macrophages *ex vivo*, while alachlor inhibited LPS/interferon- γ -induced NO production *ex vivo*. These results indicate that some EDCs exert modulatory activity on endotoxin-induced macrophage activation either positively or negatively, suggesting that these compounds may affect the development of infectious diseases. This is the first report that systematically compared the effect of EDCs on LPS action.

Key words endotoxin; hormone; environmental pollutant; RAW 264

Over the past several years there has been growing concern about endocrine disrupting chemicals (EDCs), such as dioxins, pesticides, and plasticizers for plastics, because exposure of organisms, both animals and humans, can result in disruption of the endocrine system. EDCs are ingested into the body mainly through food, and since most EDCs are highly liposoluble and minimally degradable, they tend to accumulate in the body *via* the food chain. When even very low levels of EDCs accumulate *in vivo*, they mimic, antagonize, impair, enhance, or inhibit the actions of endogenous hormones and in turn cause abnormalities of growth, reproduction, development, behavior, and immune function, or cause malignant tumors.^{1–3)}

Infectious diseases are caused by microbial invasion. In order to overcome this invasion, the human body utilizes a sophisticated immune system. Disturbance of this well-organized immune system results in the development of serious infectious diseases. It is, therefore, plausible to consider that EDCs may cause the disturbance because disruption of endocrine function seriously affects the immune system. A number of biological mediators, including tumor necrosis factor- α (TNF- α) and nitric oxide (NO) participate in the defense mechanisms,^{4–8)} and they are produced by macrophages in response to microbial toxins, such as bacterial endotoxin. Under normal conditions, the human body can tolerate a certain amount of endotoxin challenge, but toxicity is dramatically increased when the body has been exposed to sensitizing agents.⁹⁾ A number of chemicals, including estrogen¹⁰⁾ and environmental pollutants^{11–16)} have also been reported to enhance the toxicity of endotoxin. Therefore, EDCs may disturb the human immune system by enhancing the toxicity of endotoxin. Thus, we investigated the effect of chemicals suspected of having an endocrine disrupting effect on endotoxin-induced TNF- α and NO productions by mouse macrophages *in vitro* and *ex vivo*. In our knowledge, al-

though a number of immunomodulatory effects of EDCs have been reported, this is the first report examined systematically the effect of EDCs on lipopolysaccharide (LPS)-induced activation of macrophages.

MATERIALS AND METHODS

Chemicals The eighteen chemicals used as EDCs, carbaryl, zineb, β -hexachlorocyclohexane, *cis*-permethrin, simazine, 2,4-dichlorophenoxy acetic acid, alachlor, 2,4-dichlorophenol, malathion, nitrofen, benzyl *n*-butyl phthalate, bisphenol A, *p*-*n*-nonylphenol, *p*-*n*-octylphenol, styrene, tributyltin (IV) chloride, triphenyltin (IV) chloride, and benzophenone, were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Their CAS No. and abbreviations are shown in Table 1. Those chemicals were selected from the SPEED'98 list.¹⁷⁾ LPS was prepared from *Salmonella abortus equi* as described previously.¹⁸⁾ Iscove's medium and actinomycin D were obtained from SIGMA (St. Louis, MO, U.S.A.). Recombinant murine interferon (IFN)- γ was purchased from Pepro Tech EC LTD. (London, U.K.). Unless otherwise stated, all other chemicals were of reagent grade or suitable chemical purity and obtained from Wako Pure Chemical Industries, Ltd. For the *in vitro* experiments, the EDCs listed above were dissolved in dimethyl sulfoxide (DMSO) at 100 mg/ml to produce a stock solution, and the stock solution was diluted to the desired concentration with Dulbecco's modified Eagle medium (DMEM) (Invitrogen Co., N.Y., U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen Co.). DMSO had no effect at the concentration used ($\leq 0.1\%$). For the *ex vivo* experiments, the EDCs were dissolved in DMSO at 10 mg/ml and further diluted with corn oil.

Animals and Cell Preparations Female Balb/c mice,

* To whom correspondence should be addressed. e-mail: tanamoto@nihs.go.jp

6–10 weeks of age were purchased from Charles River Japan Inc. (Kanagawa, Japan) and acclimated for a minimum of one week prior to use. The mice were handled in accordance with our institutional guidelines. Murine macrophage cell line RAW 264 was purchased from the RIKEN Cell Bank (Tsukuba, Japan) and cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37 °C in a humidified incubator under a 5% CO₂ atmosphere. TNF- α sensitive murine fibroblast cell line L929 was obtained from the Human Science Research Resources Bank (Tokyo, Japan) and cultured in RPMI 1640 (Invitrogen Co.) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37 °C in a humidified incubator under 5% CO₂ atmosphere.

In Vitro Experiments To assess TNF- α production, RAW 264 cells were plated in 24-well plates (1×10^6 cells/ml/well), and 24 h later the cells were washed with PBS twice and treated with one of the EDCs and 10 ng/ml LPS in the culture medium for 4 h. To assess NO production, RAW 264 cells were plated in 96-well plates (5×10^5 cells/0.1 ml/well), and 16 h later the cells were washed with phosphate-buffered saline (PBS) twice and treated with each EDC and 10 ng/ml LPS in phenol red-free culture medium for 24 h. In one experiment, 3–16 EDCs were evaluated at once, and the experiment was repeated several times (shown as *n* in Table 2) independently on different days.

Ex Vivo Experiments Mice (3 mice for each control and EDC groups) were subcutaneously injected with each EDC (1 mg/0.1 ml/mouse). Four EDCs were evaluated at once in one experiment. The mice were sacrificed 24 h later and abdominal macrophages were harvested into Iscove's medium. The cells were washed with PBS and plated in 96-well plates (5×10^5 cells/well). After 3 h, the cells were washed with PBS twice and stimulated with 10 ng/ml LPS in the culture medium for 4 h for the TNF- α measurements or stimulated with 1 or 10 ng/ml LPS plus 1000 U/ml IFN- γ in phenol red-free culture medium for 20 h for the NO measurements. This experiment was repeated several times (shown as *n* in Tables 3 and 4) independently on different days.

Determination of TNF- α and NO Productions After

exposure to the EDCs, the culture supernatants obtained were centrifuged at 2000 rpm for 10 min, and the supernatant obtained was used to measure the TNF- α and NO concentrations. TNF- α production by RAW 264 cells and peritoneal macrophages was determined by using an ELISA kit (Techne Co., U.S.A.) and L929 cytotoxicity assay, respectively. The L929 cytotoxicity assay was performed by the method described previously.¹⁸⁾ Briefly, L929 cells were plated in 96-well plates (5×10^5 cells/0.1 ml/well) and cultured for 24 h. Actinomycin D (final concentration, 2 μ g/ml) and a test sample or TNF- α standard were then added. After culturing for 20 h, the cells that survived were stained with crystal violet (50 mg/ml), and the cells that stained were solubilized with an extraction solution (50 mM KH₂PO₄, 50% ethanol). The OD of the solution was measured at 550 nm to assess cytotoxicity. NO levels produced by RAW 264 cells and peritoneal macrophages were determined by measuring the nitrite levels of the culture supernatants with Griess reagent.¹⁹⁾

Statistical Analysis The paired Student's *t*-test was used to evaluate statistical significance. A *p*-value of less than 0.05 was considered to be significant.

RESULTS

We selected eighteen chemicals (described as EDCs in this study) that are suspected of having endocrine disrupting effects from among agrochemicals (*i.e.*, insecticides, herbicides), industrial chemicals (*i.e.*, plasticizers, resins) and other groups of chemicals (see Table 1). Their effects on LPS-induced TNF- α and NO production was first investigated *in vitro* in a mouse macrophage cell line RAW 264. RAW 264 cells were exposed to 10 μ g/ml of each EDC, except ZNB, TBT, and TPT. Crystal violet staining revealed that ZNB, TBT and TPT at 10 μ g/ml caused cytotoxicity in RAW 264 cells, therefore, ZNB was used at 1 μ g/ml, and TBT and TPT were used at 0.1 μ g/ml. Other EDCs at 10 μ g/ml did not cause cytotoxicity. The cells were then stimulated with 10 ng/ml LPS, and TNF- α and NO production was measured. Table 2 summarizes the results. The TNF- α and NO levels were expressed as percentages of the values in the absence of any EDCs. None of EDCs them-

Table 1. Endocrine Disrupting Chemicals Used in This Study

Chemicals	Use	Abbreviation	CAS No.
Carbaryl	Insecticide	NAC	63-25-2
Zineb	Bactericide	ZNB	12122-67-7
β -Hexachlorocyclohexane	Insecticide	β -BHC	319-85-7
<i>cis</i> -Permethrin	Insecticide	PMT	61949-76-6
Simazine	Herbicide	CAT	122-34-9
2,4-Dichlorophenoxyacetic acid	Herbicide	2,4-D	94-75-7
Alachlor	Herbicide	ACL	15972-60-8
2,4-Dichlorophenol	Dye intermediate	DCP	120-83-2
Malathion	Insecticide	MAT	121-75-5
Nitrofen	Herbicide	NIP	1836-75-5
Benzyl <i>n</i> -butyl phthalate	Plasticizer for plastics	BBP	85-68-7
Bisphenol A	Raw material for resins	BPA	80-05-7
<i>p</i> - <i>n</i> -Nonylphenol	Raw material for surfactants or oil-solvent phenol resins	NNP	104-40-5
<i>p</i> - <i>n</i> -Octylphenol	By-product of organic chlorine compound	OTP	1806-26-4
Styrene	By-product of organic chlorine compound	STR	100-42-5
Tributyltin (IV) chloride	Antifouling paints on ships, rot prevention for fishnets	TBT	1461-22-9
Triphenyltin (IV) chloride	Synthetic raw materials for medical products, perfume, etc.	TPT	639-58-7
Benzophenone	Synthetic raw materials for medical products, perfume, etc.	BZP	119-61-9

Table 2. *In Vitro* Effect of EDCs on LPS (10 ng/ml)-Induced TNF- α and NO Production by Mouse Macrophage Cell Line RAW 264

EDCs	TNF- α production		NO production	
	% $\pm$ S.E.M	n	% $\pm$ S.E.M	n
NAC	109.6 $\pm$ 9.2	4	59.7 $\pm$ 7.0**	6
ZNB	97.4 $\pm$ 5.5	6	86.9 $\pm$ 6.7	4
β -BHC	97.8 $\pm$ 1.0	3	107.2 $\pm$ 2.9	6
PMT	104.9 $\pm$ 10.9	4	101.4 $\pm$ 4.4	10
CAT	81.3 $\pm$ 3.3**	6	103.6 $\pm$ 5.5	9
2,4-D	96.5 $\pm$ 3.8	4	105.1 $\pm$ 1.4*	6
ACL	100.2 $\pm$ 12.2	3	19.0 $\pm$ 4.3**	7
DCP	95.1 $\pm$ 3.4	4	98.6 $\pm$ 2.0	4
MAT	91.4 $\pm$ 3.3	4	93.9 $\pm$ 2.4	4
NIP	91.6 $\pm$ 2.3**	9	94.4 $\pm$ 11.2	4
BBP	92.8 $\pm$ 1.8**	7	101.2 $\pm$ 2.4	4
BPA	86.2 $\pm$ 8.9	4	109.1 $\pm$ 2.9*	8
NNP	107.7 $\pm$ 7.7	3	83.9 $\pm$ 0.9**	4
OTP	99.1 $\pm$ 6.8	7	80.8 $\pm$ 1.7**	4
STR	98.5 $\pm$ 1.4	4	102.2 $\pm$ 1.9	4
TBT	92.9 $\pm$ 5.2	4	79.0 $\pm$ 3.8**	7
TPT	92.3 $\pm$ 4.1	4	90.8 $\pm$ 3.2*	6
BZP	98.9 $\pm$ 1.7	5	94.6 $\pm$ 3.5	4

Values are expressed as percent (mean $\pm$ S.E.M.) of respective control where no EDC treatment was performed. TNF- α and NO productions in control cells were 51.4 $\pm$ 5.8 ng/ml ($n=10$) and 37.0 $\pm$ 3.2 μ M ($n=17$), respectively. n : number of experiments. *: $p<0.05$, **: $p<0.01$, compared with the control by paired Student's t -test.

selves induced TNF- α or NO production. The LPS-induced TNF- α production was inhibited by CAT, NIP, and BBP, and no significant enhancement of production was observed in response to any of the EDCs. NO production was inhibited by NAC, ACL, NNP, OTP, TBT, and TPT, but was enhanced slightly by 2,4-D and BPA. The inhibition of NO production by NAC and ACL was especially marked. None of the other EDCs tested significantly affected their production.

The effect of the eighteen EDCs on LPS-induced TNF- α and NO production was also investigated *ex vivo* in peritoneal macrophages obtained from mice exposed to EDCs. Mice were subcutaneously injected with an EDC (1 mg/mouse), and peritoneal macrophages were obtained from the mice 1 d later. Macrophages were stimulated with LPS and IFN- γ /LPS to induce TNF- α production and NO production, respectively. Tables 3 and 4 summarize the level of TNF- α and NO production. Production is expressed as a percentage of the values in the absence of any EDCs. No TNF- α or NO production was observed in the absence of LPS or IFN- γ /LPS stimulation. ZNB and ACL enhanced TNF- α production when 1 ng/ml LPS was used, and ACL inhibited NO production at LPS concentrations of both 1 and 10 ng/ml. None of the other EDCs tested significantly affected their production.

DISCUSSION

The toxicity of bacterial endotoxin is dramatically enhanced when the human body has been exposed to sensitizing agents.⁹⁾ Since a number of chemicals, including estrogens¹⁰⁾ and environmental pollutants, such as dioxins,^{11,12,14–16)} lead,¹³⁾ and dibenzofuran¹⁴⁾ have been reported to enhance the toxicity of endotoxin, EDCs may increase the toxicity of endotoxin. We therefore investigated the effect of EDCs on LPS-induced TNF- α and NO production by macrophages,

Table 3. *Ex Vivo* Effect of EDCs on LPS-Induced TNF- α Production by Peritoneal Macrophages Obtained from EDC-Exposed Mice

EDCs	LPS concentration (ng/ml)			
	1		10	
	% $\pm$ S.E.M	n	% $\pm$ S.E.M	n
NAC	109.1 $\pm$ 11.2	10	96.0 $\pm$ 8.3	9
ZNB	143.0 $\pm$ 8.0*	5	106.3 $\pm$ 17.3	6
β -BHC	129.7 $\pm$ 15.3	16	106.7 $\pm$ 6.2	17
PMT	160.6 $\pm$ 27.2	7	125.9 $\pm$ 11.0	7
CAT	130.4 $\pm$ 23.9	9	103.1 $\pm$ 13.3	9
2,4-D	83.2 $\pm$ 7.9	7	97.7 $\pm$ 9.8	8
ACL	149.3 $\pm$ 18.0*	6	107.4 $\pm$ 12.5	6
DCP	90.6 $\pm$ 8.7	3	78.8 $\pm$ 11.3	4
MAT	104.7 $\pm$ 13.7	6	91.3 $\pm$ 11.3	6
NIP	128.4 $\pm$ 13.9	11	108.9 $\pm$ 8.8	11
BBP	133.7 $\pm$ 22.5	6	121.0 $\pm$ 14.3	6
BPA	101.6 $\pm$ 14.8	9	111.3 $\pm$ 18.0	11
NNP	104.6 $\pm$ 7.9	8	82.3 $\pm$ 10.9	8
OTP	122.8 $\pm$ 11.8	11	97.3 $\pm$ 13.8	10
STR	92.7 $\pm$ 11.8	7	79.4 $\pm$ 10.5	7
TBT	138.6 $\pm$ 21.2	7	108.8 $\pm$ 15.2	8
TPT	131.8 $\pm$ 16.9	10	113.5 $\pm$ 10.9	10
BZP	90.9 $\pm$ 11.1	7	79.0 $\pm$ 13.9	7

Values are expressed as percent (mean $\pm$ S.E.M.) of respective control where no EDC treatment was performed. TNF- α productions in control cells were 38.5 $\pm$ 5.8 ng/ml ($n=39$) and 60.5 $\pm$ 7.2 μ M ($n=39$) at 1 and 10 ng/ml LPS, respectively. n : number of experiments. *: $p<0.05$, compared with the control by paired Student's t -test.

Table 4. *Ex Vivo* Effect of EDCs on IFN- γ /LPS-Induced NO Production by Peritoneal Macrophages Obtained from EDC-Exposed Mice

EDCs	LPS concentration (ng/ml)			
	1		10	
	% $\pm$ S.E.M	n	% $\pm$ S.E.M	n
NAC	109.8 $\pm$ 12.1	14	124.0 $\pm$ 23.4	14
ZNB	84.4 $\pm$ 19.5	9	100.4 $\pm$ 20.8	8
β -BHC	100.0 $\pm$ 13.6	17	111.2 $\pm$ 16.5	17
PMT	120.7 $\pm$ 20.5	10	113.8 $\pm$ 14.9	10
CAT	114.0 $\pm$ 10.9	11	115.7 $\pm$ 12.9	11
2,4-D	110.5 $\pm$ 10.7	10	111.1 $\pm$ 7.8	10
ACL	65.0 $\pm$ 13.3*	7	57.9 $\pm$ 11.2**	7
DCP	89.3 $\pm$ 8.3	6	98.7 $\pm$ 6.6	6
MAT	104.1 $\pm$ 7.4	9	97.0 $\pm$ 8.5	9
NIP	99.8 $\pm$ 16.1	12	95.2 $\pm$ 7.4	12
BBP	85.6 $\pm$ 12.7	6	101.0 $\pm$ 14.1	5
BPA	105.5 $\pm$ 21.9	11	101.6 $\pm$ 7.7	11
NNP	101.0 $\pm$ 6.2	7	99.7 $\pm$ 5.7	6
OTP	100.2 $\pm$ 16.1	11	113.3 $\pm$ 13.8	10
STR	96.0 $\pm$ 23.3	8	97.1 $\pm$ 20.6	8
TBT	99.9 $\pm$ 11.8	9	104.0 $\pm$ 10.1	9
TPT	109.5 $\pm$ 10.3	9	110.8 $\pm$ 9.0	9
BZP	110.0 $\pm$ 22.4	8	95.4 $\pm$ 12.4	8

Values are expressed as percent (mean $\pm$ S.E.M.) of respective control where no EDC treatment was performed. NO productions in control cells were 29.5 $\pm$ 3.0 μ M ($n=45$) and 37.3 $\pm$ 3.8 μ M ($n=45$) at 1 and 10 ng/ml LPS, respectively. n : number of experiments. *: $p<0.05$, **: $p<0.01$, compared with the control by paired Student's t -test.

because both TNF- α and NO are known to be the major cytotoxic mediators.^{20,21)} We selected eighteen agrochemicals and industrial chemicals that are suspected of having endocrine disrupting effects (see Table 1). As expected, the EDCs themselves did not induce TNF- α or NO productions, but productions induced by LPS was affected by some EDCs, positively or negatively, depending on the chemical, indicat-

ing that they affected the LPS response by different mechanisms.

Among the EDCs we tested, ACL significantly inhibited LPS-induced NO production both *in vitro* and *ex vivo*, suggesting a direct action of ACL on macrophages. ZNB and ACL, on the other hand, enhanced TNF- α production *ex vivo*, but did not affect production *in vitro*. These agents, therefore, seem to exert their actions by acting at sites other than macrophages, or metabolites of these compounds may be involved in their *ex vivo* activity. These explanations may also be applicable to the opposite effect of ACL on TNF- α and NO productions *ex vivo* because ACL enhanced TNF- α production *ex vivo* but not *in vitro*. Or this opposite effect may simply reflect the difference in the mechanism between TNF- α and NO productions. On the contrary, EDCs, such as NAC, CAT *etc.*, that affected *in vitro* but not *ex vivo*, may receive rapid metabolism in the *ex vivo* situation. ZNB and ACL enhanced TNF- α production at 1 ng/ml LPS but not at 10 ng/ml LPS. Since TNF- α production reached to the maximum at 10 ng/ml LPS, these agents may shift the LPS dose response curve to the left.

Taken together, the results of this study show that the effect of EDCs on LPS-induced activation of macrophages varies with the EDC and that some EDCs exert modulatory activity on the activation. Although overproduction of TNF- α and NO is harmful to the human body because of their cytotoxic effects, inhibiting LPS-induced TNF- α and NO production could also be harmful, because these mediators play an important role in bacterial clearance. Thus, EDCs that affect the LPS response either way have the potential to exacerbate infectious diseases. The mechanisms by which EDCs affect LPS-induced macrophage activation need to be clarified.

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REFERENCES

- 1) Eskenazi B., Mocarelli P., Warner M., Samuels S., Vercellini P., Olive D., Needham L., Patterson D., Brambilla P., *Chemosphere.*, **40**, 1247—1253 (2000).
- 2) Safe S., Wang F., Porter W., Duan R., McDougal A., *Toxicol. Lett.*, **102—103**, 343—347 (1998).
- 3) Schrenk D., *Biochem. Pharmacol.*, **55**, 1155—1162 (1998).
- 4) Araya A. V., Aguirre A., Romero C., Miranda C., Molina M. C., Ferreira A., *Eur. Cytokine Netw.*, **13**, 419—424 (2002).
- 5) Goering P. L., Kuester R. K., Neale A. R., Chapekar M. S., Zaremba T. G., Gordon E. A., Hitchins V. M., *In Vitro. Mol. Toxicol.*, **13**, 125—136 (2000).
- 6) Gorelik L., Bar-Dagan Y., Mokyr M. B., *J. Immunol.*, **156**, 4298—4308 (1996).
- 7) Willeaume V., Kruys V., Mijatovic T., Huez G., *J. Inflamm.*, **46**, 1—12 (1995).
- 8) Xie Q. W., Cho H. J., Calaycay J., Mumford R. A., Swiderek K. M., Lee T. D., Ding A., Troso T., Nathan C., *Science*, **256**, 225—228 (1992).
- 9) Bradley S. G., “Handbook of Endotoxin,” Vol. 3, Chap., ed. by Berry L. J., Elsevier Science Publishers, Amsterdam, 1985, p. 340.
- 10) Ikejima K., Enomoto N., Iimuro Y., Ikejima A., Fang D., Xu J., Forman D. T., Brenner D. A., Thurman R. G., *Am. J. Physiol.*, **274**, G669—G676 (1998).
- 11) Clark G. C., Taylor M. J., Tritscher A. M., Lucier G. W., *Toxicol. Appl. Pharmacol.*, **111**, 422—431 (1991).
- 12) Clark G. C., Taylor M. J., *Exp. Clin. Immunogenet.*, **11**, 136—141 (1994).
- 13) Kuttner R. E., Schumer W., *Circ. Shock*, **13**, 233—240 (1984).
- 14) Oishi S., Hiraga K., *J. Environ. Sci. Health B*, **15**, 77—85 (1980).
- 15) Rosenthal G. J., Lebetkin E., Thigpen J. E., Wilson R., Tucker A. N., Luster M. I., *Toxicology*, **56**, 239—251 (1989).
- 16) Vos J. G., Kreeftenberg J. G., Engel H. W., Minderhoud A., Van Noorle J. L., *Toxicology*, **9**, 75—86 (1978).
- 17) Ministry of the Environment of Japan: (<http://www.env.go.jp/en/pol/speed98/sp98t3.html>), 1998.
- 18) Tanamoto K., Azumi S., *J. Immunol.*, **164**, 3149—3156 (2000).
- 19) Ding A. H., Nathan C. F., Stuehr D. J., *J. Immunol.*, **141**, 2407—2412 (1988).
- 20) Galanos C., Freudenberg M. A., *Immunobiology*, **187**, 346—356 (1993).
- 21) MacMicking J. D., Nathan C., Hom G., Chartrain N., Fletcher D. S., Trumbauer M., Stevens K., Xie Q. W., Sokol K., Hutchinson N., *Cell*, **81**, 641—650 (1995).