Rapid detection of cytotoxicity of food additives and contaminants by a novel cytotoxicity test, menadione-catalyzed H_2O_2 production assay

Shiro Yamashoji¹ and Kenji Isshiki^{2,*}

¹Kobe Gakuin Women's College, 27-1 Hayashiyama-cho, Nagata-ku, Kobe 653-0861, Japan; ²Current address: National Food Research Institute, 2-1-12 Kannondai, Tsukuba 305-8642, Japan; *Author for correspondence (e-mail: issiki@nfri.affrc.go.jp; phone: +81-298-38-8067; fax: +81-298-38-7996)

Received 10 January 2001; accepted in revised form 13 August 2001

Key words: Cytotoxicity, Food safety evaluation, Menadione-catalyzed H_2O_2 production

Abstract

Menadione-catalyzed H_2O_2 production by viable animal cells was proportional to the viable cell number, and H_2O_2 production decreased with increasing cytotoxic effects after the incubation of cells with cytotoxic compounds. The cytotoxic effects of food additives, pesticides, antibiotics, heavy metals, phytotoxins, mycotoxins, and marine toxins were estimated using the above test employing NIH/3T3 and Neuro-2a cells. Synergistic effects of the toxin mixture were observed and acute cytotoxicity detected 1 h after the incubation of cells with toxins. This menadione-catalyzed H_2O_2 production assay is rapid and simple compared to other popular cytotoxicity tests such as the MTT reduction assay and Neutral red inclusion test, requiring 4 h. The menadione-catalyzed H_2O_2 production assay is expected to be a useful food safety test for rapidly detecting toxic compounds having a basic cytotoxic effect on common animal cells.

Introduction

A variety of cytotoxicity tests has been proposed as alternatives to animal tests, and applied to the detection of cytotoxicity of drugs and chemicals. However, the use of cytotoxicity tests to evaluate food safety has not been widely proposed due to the complexity and variation of foods. Additive, synergistic, and antagonistic actions could be caused by various factors, including toxic compounds. These actions would make evaluation of the safety of whole foods difficult (Konemann and Pieters 1996; Van Zorge 1996; Yamashoji and Isshiki 1998).

Cytotoxicity tests are useful in detecting basal cytotoxic events common to various cells, but not in detecting organ-specific toxins (Ekwall 1983). We showed that 50% inhibition concentration of toxins was not proportional to the 50% lethal dose (LD_{50}) when a cytotoxicity test was conducted involving 17 substances used for international validation of fixed-

dose procedures as an alternative to the conventional LD_{50} test (Yamashoji and Isshiki 1999). Cytotoxicity tests modified to allow rapid and automated analysis of large numbers of samples will be able to detect additive, synergistic, and antagonistic actions. Organ-specific toxins may also be detected using more differentiated cells. The application of cytotoxicity tests to food safety evaluation is expected to be useful for determining total cytotoxicity potential resulting from additive, synergistic, and antagonistic actions in the presence of different toxins.

We propose a method of food safety evaluation involving the use of a rapid cytotoxicity test based on menadione-catalyzed H_2O_2 production by viable animal cells. This test is simpler, more rapid and sensitive than the widely used neutral red inclusion test and MTT reduction test (Yamashoji et al. 1992). We discuss assay conditions using food additives, pesticides, antibiotics, heavy metals, phytotoxins, and marine toxins.

Materials and methods

Organism and growth conditions

NIH/3T3 and Neuro-2a cells were cultured in DMEM and RPMI1640 supplemented with 10% fetal bovine serum. Cells were grown in tissue dishes in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. Exponentially growing cells were trypsinized, and single cells were collected by centrifugation and resuspended in a 96-well tissue culture plate. Cells at 40,000 cells/well were cultured overnight under the above conditions. Cell lines, media, and culture plates were purchased from the following; NIH/3T3 and Neuro-2a: Dainippon Pharmaceutical Co., Ltd.; fetal bovine serum: Life Technologies Oriental, Inc.; DMEM and RPMI1640: Sigma Chemical Co.; and culture plate: Falcon Labware.

Cytotoxicity test

One to 10 μ l of the test solution was added to about 80% confluent cells at 100 μ l per well on a 96-well tissue culture plate, and the mixture was incubated in a humidified atmosphere of 95% and 5% CO₂ at 37 °C for 4–24 h. Each well was washed twice with 100 μ l of phenol red-free MEM, and 100 μ l of phenol red-free MEM containing 0.2 mM menadione was added to each well. After 10 min incubation in a humidified atmosphere of 95% air and 5% CO₂, 150 μ l of the colorimetric reagent solution was added to each well and the absorbance at 725 nm in each well was measured using a microplate reader 1 min later. The colorimetric reagent solution contained 0.1% bovine albumin, 10 U ml⁻¹ horseradish peroxidase, and 200 μ M (N-(carboxymethylaminocarbonyl)-4-4'-bis(dimethylthiazol-2-yl)-diphenylamine (DA-64) in 1 M acetate buffer (pH 5.0). The viability of cells exposed to test samples was determined as a percentage based on the absorbance of wells containing intact cells. The growth of cells exposed to tetrodotoxin or brevetoxin was expressed as the increase in the absorbance at 725 nm.

Cytotoxicity test of tetrodotoxin and brevetoxin

To 90% confluent Neuro-2a cells in 100 μ l of culture medium containing 0.5 mM ouabain and 0.05 mM veratridine per well in 96-well tissue culture plates was added 2–10 μ l of tetrodotoxin or brevetoxin, and this mixture was incubated for 2–20 h.

Cell growth was determined by following the increase in the absorbance at 725 nm according to the above colorimetric assay. Marine toxin was assayed according to the method described by Manger et al. (1993).

Preparation of the test solution

Water-soluble compounds such as antibiotics, heavy metal salts other than tributyltin (IV) chloride, color additives, and toxins were diluted with distilled water, and diluted to the appointed concentration with culture medium before been added to cell culture. Ethanol-soluble compounds such as alkaloids, antioxidants, and antimicrobial agents were dissolved in ethanol, or more hydrophobic compounds such as pesticides, tetrodotoxin, and brevetoxin were dissolved in dimethylsulfoxide, and then diluted with culture medium. The maximum concentration of the above solvent in the culture media was 5%.

Chemicals

Antibiotics, pesticide, heavy metals, food additives, and DA-64 were purchased from Wako Pure Chemical Industries Ltd. Plant and marine toxins were purchased from Sigma Chemical Co. Other chemicals were of analytical grade.

Results and discussion

Cytotoxicity of antibiotics

Many types of antibiotics have been used to protect domestic animals and poultry from disease germs and to maintain the animal's rate of growth. Such antibiotics are found in meat and poultry, but these effects on human health are not well known. Some antibiotics in meat, for example, may produce side effects in patients undergoing chemotherapy.

Though it is difficult to directly compare IC₅₀ (g l⁻¹ medium) with LD₅₀ (g kg⁻¹ body weight), IC₅₀ may be estimated as the 50% inhibition concentration of toxins necessary to inhibit cell activity in the body. Table 1 shows the cytotoxicity of antibiotics after 4 or 24 h incubation with animal cells. IC₅₀ level determined after 4 h incubation was lower than that of LD₅₀, and 24 h incubation decreased IC₅₀ more than 4 h incubation. However,

the IC_{50} of colistin sodium methansulfonate was higher than that of LD_{50} . Few differences in IC_{50} were observed between NIH/3T3 and Neuro-2a cells. These facts indicate that the cytotoxicity test is more sensitive to antibiotics than is the animal test. In general, the toxic effects of antibiotics on animals have been considered much lower than those on bacteria (Russel and Chopra 1990), but the above results suggest that antibiotics may produce stress in animal cells (Ikemoto and Ueda 1994). Combining cytotoxicity and antimicrobial susceptibility tests could contribute greatly to the detection of antibiotics and their overall cytotoxicity in whole foods.

Cytotoxicity of pesticides

Foods are suspected the contamination by pesticides used in attempts to good harvest, and pesticides extracted from food have been chemically analyzed to evaluate food safety. The toxicity of pesticides interfering with neurotransmission has been detected using animal tests but rarely using cytotoxicity tests employing animal cells, while LD_{50} in animal test and IC_{50} in cytotoxicity test correlate poorly, presumably because many pesticides that specifically disturb neurotransmission in the central or peripheral nervous system produce little damage in animal cell cultures or organs that do not contain nervous systems, unless the pesticides are basically cytotoxic to common animal cells (Ekwall 1983; Ecobichon 1996).

We tested the cytotoxic effects of pesticides after 4 h culture with Neuro-2a and NIH/3T3 cells (Table

2). The IC_{50} of pesticides other than BHC was lower than LD_{50} and the difference in IC_{50} between NIH/3T3 and Neuro-2a cells was negligible. TPN showed a high toxic effect. These facts suggest that the cytotoxicity test is more useful in detecting the basic cytotoxicity of some pesticides than is the animal test.

The detection of basic cytotoxic effects of pesticides is thus as important as that of the inhibitory effects on the neurotransmission system. In the future, pesticides disturbing neurotransmission are expected to be detected by cells whose menadione-catalyzed H_2O_2 production is related to cholin esterase activity.

Cytotoxicity of heavy metals

Heavy metals are known to bind to food components and to have chronic toxicity. The cytotoxicity of inorganic salts of mercury, silver, and chrome was detected at 0.01 g l^{-1} (10 ppm) in the culture medium after 5 h culture (Table 3). The sensitivity of menadione-catalyzed H_2O_2 production assay to metal cytotoxicity increased with increasing culture period, and Neuro-2a cells were more sensitive to this cytotoxicity than were NIH/3T3 cells. The cytotoxicity test of most metals tested may not be practical for food safety evaluation because most metals are toxic at the level of $\mu\text{g kg}^{-1}$ body weight and are specifically detected by chemical analysis (Table 3). The cytotoxicity test of organic tin compounds, however, is expected to be useful because tin or its inorganic compounds require rela-

Table 1. IC_{50} and LD_{50} of antibiotics

	IC ₅₀ (g l ⁻¹)				LD ₅₀ (g kg ⁻¹)
	NIH/3T3		Neuro-2a		
	4 h	24 h	4 h	24 h	
Chlortetracycline Hydrochloride	1.2	0.9	1.6	0.08	
Cefazolin Sodium	> 2.5	0.7	> 2.5	0.75	> 11 (M,R)
Colistin Sodium Methansulfonate	> 2.5	0.4	> 2.5	0.48	> 0.767(M)
Tylosin Tartrate	> 2.5	0.8	> 2.5	0.45	
Oleandomycin Phosphate	> 2.5	1.7	> 2.5	1.48	
Ampicillin	> 2.5	> 2.5	> 2.5	> 2.5	
Tetracycline Hydrochloride	0.16	0.9	1.9	0.13	7.2(M)
Gentamicin Hydrochloride	> 2.5	> 2.5	> 2.5	> 2.5	> 9.05(M)
Oxytetracycline Hydrochloride	> 2.5	1.5	> 2.5	0.09	7.2(M)
Morantel Citrate Momohydrate	> 2.5	0.7	0.9	0.27	
Erythromycin	1.2	0.8	1.5	0.53	2.927(M)
Josamycin	0.8	0.7	1.4	0.57	> 7(M,R)

(R) represents LD_{50} for rats and (M) for mice. Number of assays (N) was 4.

Table 2. IC₅₀ and LD₅₀ of pesticide

	IC ₅₀ (g l ⁻¹)		LD ₅₀ (g kg ⁻¹)
	NIH/3T3	Neuro-2a	
TPN	0.008	0.01	> 10 (R)
Thiocyclam Hydrogen Oxalate	0.03	0.33	0.31 (R)
NAC	0.37	0.5	0.8 (R)
pp'-DDD	0.15	0.05	3.4 (R)
PCP	0.19	0.19	0.21 (R)
Oxadiazon	0.14	> 0.5	> 8 (M)
DCPA	0.13	0.35	1.38 (R)
Isoprothiolane	0.03	0.38	1.19–1.34 (R)
Malathion	0.32	> 0.5	2.8 (R)
BHC	0.23	0.15	0.076–0.128 (R)

(R) represents LD₅₀ for rats and (M) for mice. Number of assays (N) was 4. Abbreviations are as follows: TPN, tetrachlorophthalonitrile; NAC, 1-naphthylmethylcarbamate; pp'-DDD, 1,1-dichloro-2,2-bis (p-chlorophenyl) ethane; PCP, pentachlorophenol; DCPA, 1,4-dichloropropionanilide; BHC, isomers of 1,2,3,4,5,6-hexachlorocyclohexane.

tively large doses (500 mg kg⁻¹ for 14 months) to produce toxicity when ingested orally (Goyer 1996), and the use of tin in food processing seems to show little hazard. The average U.S. daily intake, mostly as a result of processed food, is estimated at 17 mg (Goyer 1996). Some organic tin compounds are highly toxic, however, and trialkyl compounds cause encephalopathy and cerebral edema (Goyer 1996). Tributyltin (IV) chloride is much more cytotoxic than tin (II) chloride (Table 3).

Cytotoxicity of food additives

LD₅₀ and the acceptable daily intake (ADI) of antioxidants, antimicrobial agents, and color additives are listed in Table 4. IC₅₀ of food additives (Table 5) was lower than the LD₅₀ (Table 4), and the difference between NIH/3T3 cells and Neuro-2a cells in regard to their sensitivity to cytotoxicity was negligible. The sensitivity of cells to the cytotoxicity of food additives increased with increasing culture

Table 3. IC₅₀ of heavy metals

	IC ₅₀ (g l ⁻¹)			
	NIH/3T3		Neuro-2a	
	5 h	24 h	5 h	24 h
Mercury(II) Acetate	0.04	0.06	0.07	0.04
Silver Nitrate	0.06	0.06	0.02	0.01
Potassium Ferricyanide	> 1	0.5	> 1	> 1
Manganese (IV) Oxide	> 1	1	> 1	0.08
Aluminum Nitrate	> 1	1	> 1	> 1
Cadmium Nitrate 4H ₂ O	0.1	0.1	0.5	0.02
Barium Chloride	> 1	> 1	> 1	> 1
Ammonium Molybdate 4H ₂ O	> 1	> 1	> 1	> 1
Ammonium Vanadate (V)	0.9	0.3	0.4	0.06
Sodium Pentacyano-nitrosylferrate	0.8	0.1	0.4	0.1
Tin (II) Chloride	> 1	> 1	> 1	> 1
Tributyltin (IV) Chloride	0.0008	0.00025	0.0008	0.00035
Chromium (IV) Oxide	0.5	0.07	0.09	0.006
Nickel (II) Nitrate	> 1	0.7	> 1	0.2
Cobalt (II) Acetate 4H ₂ O	> 1	0.4	1	0.1
Zinc Chloride	0.3	0.2	0.8	0.4
Potassium Dichromate	0.7	0.04	0.1	0.006
Copper (II) Acetate	0.6	0.2	0.8	0.06

Number of assays (N) was 4.

Figure 1. Synergistically cytotoxic effect of sorbic acid and *n*-butyl *p*-hydroxybenzoate to NIH/3T3 cells. The incubation period of NIH/3T3 cells with additives was 4 h and viability (%) represents the mean of 4 different determinations. $\square$ sorbic acid; $\diamond$ sorbic acid and 0.004% *n*-butyl hydroxybenzoate; $\circ$ sorbic acid and 0.006% *n*-butyl hydroxybenzoate.

period but color additives had little cytotoxic effect at 1 g l^{-1} . Color additives have high molecular weight and polarity, so they are not considered to be adsorbed by the gastrointestinal tract or to enter cells (Kotsonis et al. 1996). Even weak cytotoxic food additives, however, may become stronger in the presence of other food additives and composition.

Sorbic acid, for example, showed a synergistic effect with *p*-oxybenzoic acid ester, acting as a preservative, because the dose-dependent cytotoxic effect of sorbic acid in the presence of 0.006% *p*-oxybenzoic acid was greater than that in its absence (Figure 1). These facts suggest that the cytotoxicity test is more rapid and simple than is the animal test in detecting synergistic effects in the different combinations among food composition and food additives. The present study, a 4-h culture period was enough to estimate the cytotoxic effects of food additives.

Cytotoxicity of plant toxins

The most toxic amanitin, mushroom toxin, has a strong affinity for hepatocytes and to RNA polymerase II, and inhibits protein synthesis (Jaeger et al. 1993). Serious clinical signs of liver develop slowly, beginning on about the third day following ingestion (Norton 1996a). Colchicine has an antimitotic effect by blocking the formation of microtubules from tubulin and death occurs if contamination is heavy (Norton 1996b). The IC_{50} of these toxins was about 100 times the LD_{50} of those after 4 h culture (Table 6), suggesting that menadione-catalyzed H_2O_2 production is affected little by the inhibition of DNA, RNA, and protein syntheses within 4 h incubation.

The LD_{50} of tomatine, and solanine contained in tomato, potato, and other plants are about 10 times their IC_{50} after 4 h culture. Solanine showed synergistic effect with chaconine, but not in the presence

Table 4. LD_{50} and acceptable daily intake of food additives

	LD ₅₀ (g kg ⁻¹)		Acceptable daily intake (g kg ⁻¹ day ⁻¹)
Antioxidants			
BHA	2.9 (R)	1.25 (M)	0.0005
BHT	2.45 (R)	2.0 (M)	0.0005
Propyl gallate	3.8 (R)	3.5 (M)	0.0002
Ethyl gallate			
Gallic acid			
Antimicrobial agents			
n-propyl p-hydroxybenzoate		3.7 (M)	
n-butyl p-hydroxybenzoate		16 (M)	
Dehydroacetic acid	1.0 (R)		0.50
Sorbic acid	10.5 (R)		0.025
Color additives			
Food red No2			0.0005
Food green No3	> 2 (R)		0.0125
Food yellow No4		12.75 (M)	0.0075
Food yellow No5	> 2 (R)		0.0025

(R) represents LD_{50} for rats and (M) for mice.

Table 5. IC₅₀ of food additives

	IC ₅₀ (g l ⁻¹)			
	NIH/3T3		Neuro-2a	
	4 h	24 h	4 h	24 h
Antioxidants				
BHA	0.12	0.014	0.1	0.003
BHT	0.1	0.03	0.14	0.1
Propyl gallate	0.22	0.046	0.14	0.09
Ethyl gallate	0.25	0.058	0.15	0.03
Gallic acid	0.24	0.036	0.1	0.17
Antimicrobial agents				
n-propyl p-hydroxybenzoate	0.25	0.1	0.19	0.12
n-butyl p-hydroxybenzoate	0.13	0.026	0.14	0.05
Dehydroacetic acid	0.44	0.17	0.55	0.75
Sorbic acid	0.57	0.32	0.58	0.15
Color additives				
Food red No2	> 1	> 1	> 1	> 1
Food green No3	> 1	> 1	> 1	> 1
Food yellow No4	> 1	> 1	> 1	> 1
Food yellow No5	> 1	> 1	> 1	> 1

Number of assays (N) was 4.

of tomatine (Figure 2). These alkaloids are well known to disrupt the biomembrane by binding to cholesterol and sterol, and the synergism between solanine and chaconine has been observed using a model membrane (Kenkens et al. 1995). Menadione-catalyzed H₂O₂ production by viable cells is considered sensitive to cell lysis rather than inhibition of macromolecule syntheses (RNA and protein) by amanitin and colchicine. These toxins are difficult to analyze chemically due to their complex structure and high molecular weight, so a rapid cytotoxicity test should be useful in food safety evaluation.

Gossypol exists at 0.1–0.75% in cotton oil, causing heart failure if contamination is heavy (Yamazaki et al. 1995). The IC₅₀ estimated after 4 h incubation was lower than the concentration in cotton oil, and the LD₅₀ concentration was about 10 to 100 times

that of IC₅₀. Amygdalin is a compound of cyanogens in plants. It is found in the highest amounts in the seeds of the bitter almond. In the stomach, amygdalin releases hydrocyanic acid, which combines with ferric ions in cytochrome oxidase or methemoglobin (Norton 1996c). The cytotoxic effect of amygdalin was detected at 0.5 g l⁻¹.

Cytotoxicity of marine toxins

Some marine toxins are active in voltage-sensitive sodium channels and prevent the increase of early transient ionic permeability of the nerve normally associated with the inward movement of sodium during excitation. With tetrodotoxin, or puffer (fugu) poison, the intraperitoneal minimal lethal dose in mice is 8 µg kg⁻¹ body weight (Kao and Fuhrman

Table 6. IC₅₀ of plant toxins

	IC ₅₀ (g l ⁻¹)				LD ₅₀ (g kg ⁻¹)
	NIH/3T3		Neuro-2a		
	4 h	24 h	4 h	24 h	
Amanitin	0.036	0.068	0.0195	0.0192	0.0003 (M)
Gossypol	0.152	0.032	0.076	0.011	2.5 (R)
Colchicine	0.292	0.58	0.56	0.04	0.00353 (R)
Amygdalin	0.364	0.526	0.518	0.5	
Solanine	0.049		0.058		0.45 (Rabbit)
Tomatine	0.027		0.008		0.5 (M)
Chaconine	0.014		0.022		0.03 (M)

(R) represents LD₅₀ for rats and (M) for mice. Number of assays (N) was 4.

Figure 2. Synergistically cytotoxic effect of solanine and chaconine to Neuro-2a cells. The incubation period of Neuro-2a cells with plant toxins was 4 h and viability (%) represents the mean of 4 different determinations. $\square$ solanine; $\diamond$ solanine and 10 μ M tomatine; $\circ$ solanine and 10 μ M chaconine.

1963). The cytotoxicity assay for tetrodotoxin is based on the following principle described by Manger et al. (1993): When a neuroblastoma cell line is

Figure 3. Dose-dependent cytotoxicity of tetrodotoxin to Neuro-2a cells. Incubation conditions of tetrodotoxin and Neuro-2a cells are described in Materials and Methods. The incubation period was 20 h and the cell growth was determined by following the increase in the absorbance at 725 nm. The absorbance represents the mean of four different determinations.

Figure 4. Dose-dependent cytotoxicity of brevetoxin to Neuro-2a cells. Incubation conditions of brevetoxin and Neuro-2a cells and the cell growth are described in Materials and Methods. The incubation period was as follows: $\diamond$ 2 h and $\square$ 4 h. Absorbance represents the mean of 4 different determinations.

treated with a fixed concentration of the sodium channel activator, veratridine, in the presence of ouabain, an inhibitor of Na^+/K^+ ATPase, to block sodium efflux, the combined effect results in elevated intracellular sodium leading to a decrease in cell viability. Tetrodotoxin, saxitoxin, and related toxins that block the sodium channel antagonize this effect, essentially rescuing cells dose-dependently. As shown in Figure 3, the menadione-catalyzed H_2O_2 production (the absorbance at 725 nm) of Neuro-2a cells was proportional to the dose of tetrodotoxin at the range of 0.1–1 ng. On the other hand, brevetoxins and ciguatoxins enhancing the sodium channel, induce cell death. Figure 4 shows that a menadione-catalyzed H_2O_2 production decreases with increasing concentrations of brevetoxin at the range of 0.2–10 ng. These results indicate that the menadione-catalyzed H_2O_2 production assay of the above marine toxins is more sensitive than is the animal test.

References

- Ecobichon D.J. 1996. Toxic effects of pesticides. In: Klaassen C.D., Amdur M.O. and Doull J. (eds), Casarett & Doull's Toxicology. McGraw-Hill, New York, pp. 643–689.

- Ekwall B. 1983. Correlation between cytotoxicity in vitro and LD₅₀ values. *Acta. Pharmacol. et. Toxicol.* 52: 80–99.
- Goyer R.A. 1996. Toxic effects of metals. In: Klaassen C.D., Amdur M.O. and Doull J. (eds), *Casarett & Doull's Toxicology*. McGraw-Hill, New York, pp. 738.
- Ikemoto H. and Ueda S. 1994. *Manual of Antibiotics and Infectious Disease*. Hirokawa Publishing Co., Tokyo.
- Jaeger A., Jehl F. and Flesch F. 1993. Kinetics of amatoxins in human poisoning: Therapeutic implication. *Clin. Toxicol.* 31: 68–80.
- Kao C.Y. and Fuhrman F.A. 1963. Pharmacological studies on tarichatoxin, a potent neurotoxin. *J. Pharmacol. Exp. Ther.* 140: 31.
- Kenkens E.A.J., de Vrije T., van den Boom C., de Waard P., Plasman H.H., Thiel F. et al. 1995. Molecular basis of glycoalkaloid induced membrane disruption. *Biochim. Biophys. Acta.* 1240: 216–228.
- Konemann W.H. and Pieters M.N. 1996. Confusion of concepts in mixture toxicology. *Food and Chem. Toxicol.* 34: 1025–1031.
- Kotsonis F.N., Burdock G.A. and Flamm W.G. 1996. Food toxicology. In: Klaassen C.D., Amdur M.O. and Doull J. (eds), *Casarett & Doull's Toxicology*. McGraw-Hill, New York, pp. 922.
- Manger R.L., Leja L.S., Lee S.Y., Hungerford J.M. and Wekell M.M. 1993. Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. *Anal. Biochem.* 214: 190–194.
- Norton S. 1996a. Toxic effects of plants. In: Klaassen C.D., Amdur M.O. and Doull J., *Casarett & Doull's Toxicology*. McGraw-Hill, New York, pp. 848.
- Norton S. 1996b. Toxic effects of plants. In: Klaassen C.D., Amdur M.O. and Doull J., *Casarett & Doull's Toxicology*. McGraw-Hill, New York, pp. 842.
- Norton S. 1996c. Toxic effects of plants. In: Klaassen C.D., Amdur M.O. and Doull J., *Casarett & Doull's Toxicology*. McGraw-Hill, New York, pp. 849.
- Russel A.D. and Chopra I. 1990. *Understanding Antibacterial Action and Resistance*. Ellis Horwood Ltd., Publisher, Chichester, England.
- Van Zorge J.A. 1996. Exposure to mixtures of chemical substances: is there a need for regulation? *Food and Chem. Toxicol.* 34: 1033–1036.
- Yamashoji S., Nishimoto F., Usuda F., Kubota H. and Isshiki K. 1992. Application of the chemiluminescent assay to cytotoxicity test: detection of menadione-catalyzed H₂O₂ production by viable cells. *Anal. Biochem.* 207: 255–260.
- Yamashoji S. and Isshiki K. 1998. Cytotoxicity testing for evaluating food safety. *Altern. Anim. Test Exp.* 5: 146–161.
- Yamashoji S. and Isshiki K. 1999. Novel cytotoxicity test based on menadione-catalyzed H₂O₂ productivity for food safety evaluation. *Cytotechnology* 31: 45–51.
- Yamazaki M., Nakajima T. and Fusetani N. 1995. *Natural Toxins*. Kodansha, Tokyo.