Arch Pharm Res Vol 28, No 1, 100-105, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

Effects of Mancozeb on the Activities of Murine Peritoneal Macrophages *In Vitro* and *Ex Vivo*

Ae-Hee Chung and Myoung-Yun Pyo[1]

Seoul Metropolitan Government Research Institute of Public and Environment, Seoul 137-734, Korea and [1]*College of Pharmacy, Sookmyung Women's University, Seoul 140-742, Korea*

(Received November 3, 2004)

Mancozeb (MCZ) is known to have detrimental effects on the reproductive system, but the toxicity of MCZ on immune responses has not been systematically investigated. We investigated the effects of MCZ exposure on the activities of murine peritoneal macrophages through evaluation of MCZ-induced alteration of nitric oxide (NO) production and tumor necrosis factor-α (TNF-α) synthesis. Macrophages were examined *ex vivo* from mice orally treated with various doses of MCZ for 5 consecutive days per week for 4 weeks (subacute exposure, 250, 1000, 1500 mg/kg/day) followed by culture for 2 (TNF-α) or 3 days (NO) in the presence of LPS plus IFN-γ. Macrophages from naive mice were also cultured with various concentrations of MCZ (0.05, 0.25, 0.5, 1 and 2 μg/mlL in the presence of LPS plus IFN-γ for 2 (TNF-α) or 3 days (NO) *in vitro*. NO production was decreased with the *in vitro* exposure to all concentrations of MCZ. However, the amount of NO production by peritoneal macrophages from MCZ-subacutely exposed mice was increased in comparision with that of control group. *In vitro*, MCZ suppressed TNF-α secretion with significant reduction at 2 μg/mL MCZ. Conversely, TNF-α release was enhanced ex vivo. This study provides the substantial evidence on MCZ-induced alternation in macrophage activity. In order to clearly understand the contrasting effect of MCZ on peritoneal macrophage activity, it is necessary to further investigate the influence of major metabolite of MCZ (ETU) exposure on the NO production and TNF-α synthesis.

Key words: Mancozeb, Macrophages, Nitric oxide, Tumor necrosis factor-α

INTRODUCTION

Mancozeb (MCZ), a polymeric complex of manganese ethylenebisdithiocarbamate with zinc salt, is widely used in agriculture as fungicide and herbicide. Their use is increasing worldwide and in Korea, because they have a low toxicity and a short half life in the environment (Vermeulen *et al.*, 2001). The metabolic pathway of MCZ are not known fully, however, a part of the absorbed compound is metabolized to ethylenethiourea (ETU) and urine concentrations of ETU must be as exposure indicators (Colosio, 2002).

MCZ caused a significant increase in thyroid/body weight ratio and reduced activity of thyroid peroxidase (Kackar *et al.*, 1997). MCZ disrupted the normal secretory response of adrenal cortical cells and was identified as the pesticide with the highest endocrine disrupting potential in the trout adrenal cells. A number of xenobiotics including TCDD (Fine *et al.*, 1988; Shepherd *et al.*, 2000), PCB (Daniel *et al.*, 2001), DDT (Lukic *et al.*, 1973; Gablicks *et al.*, 1973) possess estrogenic activity, the potential immunotoxicological consequences of which are obvious. Reported effects of MCZ on the immune system are scarce. An increase in IgG, IgE and α_2-macroglobulin serum levels was observed during occupational exposure to MCZ (Vergova *et al.*, 1988). However, Colosio *et al.* (1996) reported that no difference in serum IgG classes was found between exposed and unexposed groups and an increase in T-cell functional response was found in the group of MCZ-exposed manufacturers, suggesting a slight immunomodulating effect of MCZ in conditions of low-level, prolonged occupational exposure. Bisphenol A, endocrine disruptor, was reported to cause allergic contact dermatitis (Estlander *et al.*, 1999), in which antigen presenting cells, including macrophages could be involved

Correspondence to: Myoung-Yun Pyo, College of Pharmacy, Sookmyung Women's University, 53-12 Chungpa-dong 2 Ka, Yongsan-ku, Seoul 140-742, Korea
Tel: +82-2-710-9573
E-mail: mypyo@sdic.sookmyung.ac.kr

with occurrence of the disease (Kiekens *et al.*, 2001). Environmental estrogens such as genistein, 4-octylphenol, or estradiol have been reported to modulate the function of macrophages, resulting in suppression (Salem *et al.*, 1999), or enhancement (Ruh *et al.*, 1998) of its activities probably through estrogen receptor (Gulshan *et al.*, 1990). MCZ was also reported to cause allergic disease (Koch, 1996). However, few reports are available about the effects of MCZ on macrophage activities.

Herein, we investigated the effects of MCZ on activation of murine peritoneal macrophages through analysis of nitric oxide (NO) production, TNF-α synthesis following MCZ exposure *in vitro* and *in vivo*.

MATERIALS AND METHODS

Animals

Female ICR mice, 3 wks of age, were obtained from Yuhan Corporation in Korea where mice have been managed under the Korean Government Guideline on specific pathogen-free animal facility. Mice were acclimated for 3-4 wks prior to use in our experiments. They were housed in the College of Pharmacy, Sookmyung Women's University (Seoul, Korea) and maintained at 21-24 °C, 40-60% humidity, and 12 h cycle of light/dark. Animals were freely accessible to feed and water. Mice were weighing 25 ± 2 g at the beginning of the experiments.

Medium and reagents

MCZ (86.5%, technical grade, Dau Agro Science, France) were obtained from Kyungnong Co. (Korea), and Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Grand Island, NY, USA) was supplemented with nonessential amino acids (1%), sodium pyruvate (1 mM), sodium bicarbonate (0.044 mM) from Biowhittaker (Walkersville, MD, USA), antimycotic-antibiotics (1 mM) and heat-inactivated fetal bovine serum (10%, FBS, Gibco-BRL). LPS derived from Escherichia coli was purchased from Sigma and recombinant IFN-γ and TNF-α detection kit from BD PharMingen (San Diego, CA, USA).

Treatment with MCZ and preparation of peritoneal macrophage

MCZ was dissolved in DDW and administered through oral route at a dose of 250, 1000, or 1500 mg/kg b.w./day for 5 consecutive days per wk for 4 wks (subacute exposure). MCZ doses were based on the results of the acute toxicity (LD_{50}) evaluation demonstrating 25,550 mg/kg (19,803~32,962 mg/kg, p=0.05) in female ICR mice (po). Control mice were administered with the same volume of DDW. Five mice were used for each group per experiment and the experiment was performed three times. For evaluation of *in vitro* MCZ effects, peritoneal macrophages from naive mice untreated with MCZ were exposed to DMEM with 1% DMSO containing 0.05, 0.25, 0.5, 1 and 2 μg/mL MCZ. Mice were sacrificed and injected intraperitoneally with 8 mL cold DMEM media, thereafter, peritoneal fluid was withdrawn using a 20 mL syringe. The peritoneal fluid was centrifuged 5 min at 800 x g at 4 °C. Red blood cells (RBC) were lysed with cold 0.2% NaCl. The remaining cells were washed with cold PBS, resuspended in DMEM media containing 10% FBS, and adjusted to 2×10^6 viable cells/ml. Viable cell counts were determined with hemacytometer by trypan blue exclusion method (Mishell and Shiigi, 1980).

Determination of nitric oxide (NO)

The peritoneal exudates minus RBC (2×10^6 cells/mL per well) were placed into wells of 24-well culture plate for 2 h at 37 °C in 5% CO_2 humidified incubator for adherence isolation of macrophages. After washing out nonadherent cells, the remaining peritoneal macrophages were stimulated with 1 mL DMEM culture medium in the presence of LPS (1 μg/mL) and IFN- γ (1 ng/mL) for NO production. This assay was performed for assessment of *ex vivo* and *in vitro* analysis of MCZ. After 3 days incubation, culture supernatants were collected for NO measurement. The effect of MCZ on NO production was evaluated through measurement of nitrite (NO_2^-), a stable oxidative end product of NO. The method for measuring nitrite was performed as described previously (Green *et al.*, 1992). Briefly, 100 μL of culture supernatants was mixed with 100 μL of Griess reagent (1:1 mixture of 2% sulfanilamide and 0.2% napthylethylenediamine dissolved in 5% phosphoric acid, respectively) in the wells of a flat-bottom 96-well plate. The plate was incubated on a shaker for 10 min at room temperature and read using a microplate reader (Bio-Tek ELx800, Burlington, VT, USA) at 570 nm.

Determination of TNF-α production

Stimulation protocol for evaluation of MCZ effects on TNF-α production from peritoneal macrophages was the same as for the NO production except analysis was performed 2 days after stimulation. The amounts of TNF-α in the supernatants were assayed by a sandwich ELISA using a kit from BD PharMingen. The lower limit of detection was 20 pg/mL.

Statistical analysis

Data were expressed as arithmetical mean ± S.D. Student's *t*-test was used to establish the differences between control and experimental groups. Significance was concluded at p<0.05 and p<0.01.

RESULTS

Effect of MCZ on NO production by peritoneal macrophages

A major proportion of the cytotoxic effects of macrophages are mediated by production of NO, a reactive oxygen intermediate. NO is generated by inducible NO synthase. We evaluated whether MCZ has the capacity to modulate NO production by peritoneal macrophages stimulated with LPS plus IFN-γ. Our preliminary experiment demonstrated that production of NO was higher at day 3 than other times. Thus, evaluation of MCZ-modulated NO production was repeated using the supernatants collected at day 3. As shown in Fig. 1, the amount of NO production was significantly decreased in the presence of 0.05, 0.25, 0.5, 1 and 2 μg/mL MCZ (12.4, 11.3, 26.8, 26.5, and 25.1%, respectively, in comparison with that of the control culture with no MCZ addition). Based on the MTT assay (Mosman, 1983) for cell viability at day 3, high concentration of MCZ (2 μg/mL) was not cytotoxic (data not shown), indicating that the significant decrease of NO production was not due to loss of macrophage viability. However, the production of NO in MCZ-subacutely treated groups were increased about 13%, 17% and 42% ($p< 0.01$) as compared to that of control group at 250, 1,000 and 1,500 mg/kg/day, respectively (Table I).

TNF-α production by peritoneal macrophages

TNF-α is one of the principle mediators for an acute inflammatory response against infectious microorganisms including gram-negative bacteria, and is involved with occurrence of systemic complications following microbial infections (Xing, 2000). Activated mononuclear phagocytes are major source of TNF-α. The effect of MCZ on *in vitro* modulation of TNF-α production from peritoneal macrophages was assessed. A preliminary experiment demonstrated that production of TNF-α was higher at day 2 than other times. Thus, evaluation of MCZ-modulated TNF-α production was repeated using the supernatants collected at day 2. As shown in Fig. 2, the amount of TNF-α production was slightly decreased in the presence of 0.05, 0.25, 0.5, 1 and 2 μg/mL MCZ (6.8%, 7.6% ($p<0.05$), 8.3%, 6.2%, and 15.8% ($p<0.05$), respectively, in comparison with that of the control culture with no MCZ addition). However, the amount of TNF-α for the 1,500 mg/kg/day group (556 ± 52 pg/mL) was significantly ($p<0.05$) higher than that of control mice (353 ± 13 pg/mL) as shown in Table I.

Table I. Production of NO and TNF-α from murine peritoneal macrophages obtained from the MCZ-subacutely administered mice followed by stimulation with LPS plus IFN-γ for 3 and 2 days, respectively.

MCZ (mg/kg b.w./day)	Concentration in the supernatants	
	Nitrite (nM/mL)	TNF-α (pg/mL)
0	40.7 ± 8.4	352.6 ± 13.3
250	45.9 ± 10.4	359.7 ± 10.9
1,000	47.6 ± 11.4	379.8 ± 23.5*
1,500	57.8 ± 10.5**	555.9 ± 52.5*

NO production was evaluated through measurement of nitrite, a stable oxidation end point of NO. The results on nitrite and TNF-α production are shown as the means ± S.D. of three separate experiments for 5 mice per treatment. Statistically significant differences from the control mice unexposed to MCZ are *$p<0.05$, **$p<0.01$.

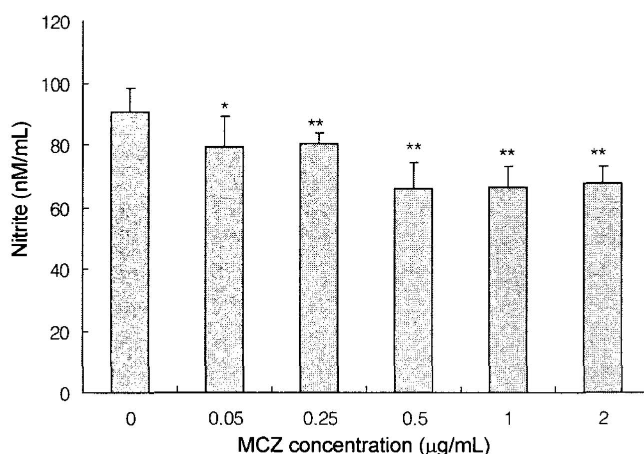

Fig. 1. Production of NO from murine peritoneal macrophages stimulated with LPS plus INF-γ in the presence of MCZ *in vitro* for 3 days. The results are expressed as the means ± S.D. of three separate experiments (duplicate per experiment). Statistically significant differences from the control culture in the absence of MCZ are *$p<0.05$, **$p<0.01$.

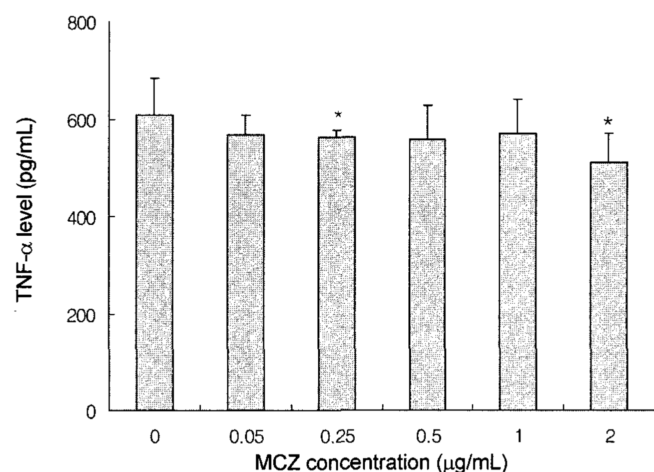

Fig. 2. Production of TNF-α from murine peritoneal macrophages stimulated with LPS plus IFN-γ in the presence of MCZ *in vitro* for 2 days. The results are expressed as the means ± S.D. of three separate experiments (duplicate per experiment). Statistically significant differences from the control culture in the absence of MCZ is *$p<0.05$.

DISCUSSION

MCZ is used to protect many fruit, vegetable and field crops against a wide spectrum of fungal disease and a practically nontoxic ethylenebisdithiocarbamate in EPA toxicity class IV. However, occupational and environmental exposure has been known to cause various pathophysiological consequences including impaired thyroid functions and altered endocrine functions ((Bisson and Hontela, 2002). With regard to immunotoxicological effects of MCZ, few systemic investigations have been undertaken on human or experimental animal immune system. To initiate delineation of the immunotoxic mechanisms of MCZ, we evaluated the effects of MCZ exposure on activities of macrophages through evaluation of MCZ-induced alteration of NO production, TNF-α synthesis by peritoneal exudates macrophages. Macrophages have been known to play an important role in the host protection and also present antigen to lymphocytes during the development of specific immunity. When activated, macrophages release various materials such as cytokines and reactive intermediates and then carry out nonspecific immune responses (Narthan, 1987).

Nitric oxide has been known to play a critical role of prevention against microbial infection, cancer occurrence (Underhill and Ozinsky, 2002). In addition, NO may be involved with regulation of apoptosis (Kim *et al.*, 1997) and progression of autoimmune disease through altering differentiation of helper T cell phenotype (Kob and Kolb-Bchofen, 1998). The biological activities of NO became more complicated since NO production was reported to suppress functional activities of T lymphocytes or APCs (MacMicking *et al.*, 1997). Regarding NOs role on pro- or anti-pathophysiologic conditions, there is no definite explanation, but the level of NO production in the microenvironment may be a decisive factor (Chung *et al.*, 2001). In this paper, we have demonstrated that MCZ induced suppression of NO production *in vitro* (Fig. 1). In contrast, the amount of NO production by peritoneal macrophages from *in vivo* MCZ-subacutely exposured mice was increased at high dose (Table I).

TNF-α is a pleiotrophic cytokine that plays an important role in inflammatory reaction in response to various pathologic conditions such as microbial infections, trauma, ischemia, and airway hypersensitivity (Thomas, 2001). TNF-α is also known to induce IL-1, IL-6, prostaglandins, reactive oxygen species and nitric oxide from macrophages. Hence, alternation in TNF-α may modulate macrophage function and overall immune defence mechanism in multiple way (Binder *et al.*,1999; Hakoda *et al.*,1999; Van Dervort *et al.*,1994).

17β-estradiol, an estrogenic compound acting through estrogen receptor, has been shown to inhibit TNF-α secretion by bone marrow-derived macrophages and central nervous system mononuclear cells from rodents (Matejuk *et al.*, 2001) as well as to suppress induction of NO synthase in murine microglial cells (Zhang *et al.*, 2001; Vegeto *et al.*, 2001). However, conflicting reports have been shown that 17β-estradiol stimulates NO release from human peripheral monocytes (Stefano *et al.*, 1999) and has no effect on NO production by a murine macrophage cell line (Woodfork *et al.*, 2001). The mechanisms associated with 17β-estradiol has not yet been resolved.

MCZ-*in vitro* induced suppression of TNF-α production from peritoneal macrophages was shown at high concentration in this study (Fig. 2). Conversely, the amount of TNF-α produced by peritoneal macrophages from the MCZ-subacutely exposed mice was increased at high dose (Table I). This result is almost consistent with the alternation of NO production, in that NO production is decreased *in vitro* and increased *ex vivo* in present study.

Colosio *et al.* (1996) also reported the contrasting results in *in vitro* and *ex vivo* experiments that in MCZ-occupationally exposed subjects the proliferation of peripheral blood mononuclear cells to phytohamagglutinin (PHA) and PHA-induced IL-2 were higher than in MCZ-unexposed subjects and *in vitro* studies, MCZ strongly inhibited lymphocyte proliferation to PHA at high doses. However, occupational exposure to MCZ didn't affect IL-2 production. In addition, Siddiqui *et al.* (1993) reported that multiple treatment of MCZ resulted in a dose dependent increase in the activity of hepatic glutathione S-transferase (GST) whereas MCZ inhibited GST activity under *in vitro* conditions. These contrasting findings are consistent with our current results and it is suggested that the production of a reactive metabolite or the induction of the synthesis of an unknown inducer, or both.

MCZ was identified as the pesticide with the highest endocrine disrupting potential and is rapidly absorbed into the body from GI tract, and almost completely excreted in 96 h. ETU is the major MCZ metabolite, and it has been reported that the impairment of thyroid function induced by MCZ-chronic exposure may be due to the metabolite ETU (Colosio *et al.*, 2002). It is, therefore, possible that an increase of NO and TNF-α production by peritoneal macrophages from *in vivo* MCZ-subacutely exposured mice may be due to ETU.

In conclusion, on the basis of our data, we propose that MCZ induces an modulation of macrophage activity, in that NO production and TNF-α release are suppressed *in vitro* and enhanced *ex vivo*. In order to clearly understand the contrasting effect of MCZ on peritoneal macrophage activity, it is necessary to determine other immunological parameters and to further investigate the influence of major metabolite of MCZ (ETU) exposure on the NO production and TNF-α synthesis.

ACKNOWLEDGEMENT

The authors would like to thank Dr. J.A. Byun for helpful discussion during the study. This research was supported by the Sookmyung Womens University Research Grants 2003.

REFERENCES

Binder, C., Schulz, M., Hiddemann, W., and Oellerich, M., Induction of inducible nitric oxide synthase is an essential part of tumor necrosis factor-α-induced apoptosis in MCF-7 and other epithelial tumor cells. *Lab Invest.*, 79, 1703-1712 (1999)

Bisson, M. and Hontela, A., Cytotoxic and endocrine-disrupting potential of atrazine, diazinon, endosulfan, and mancozeb in adrenocortical steroidogenic cells of rainbow Trout exposed *in vitro*. *Toxicol. Appl. Pharmacol.*, 180, 110-117 (2002).

Chung, H-T., Pae, H-C., Choi, B-M., Billiar, T. R., and Kim, Y-M., Nitric oxide as a bioregulator of apoptosis. *Biochem. Biophys. Res. Commun.*, 282, 1075-1079 (2001).

Colosio, C., Barcellini, W., Maroni, M., Alcini, D., Bersani, M., Cavallo, D., Galli, A., Meroni, P., Pastorelli, R., Rozzardi, G. P., Soleo, L., and Foa V., Immunomodulatory effects of occupational exposure to mancozeb. *Arch. Environ. Health,* 51, 445-451 (1996).

Colosio, C., Fustinoni, S., Birindelli, S., Bonomi, I., Paschale, G. D., Mammone, T., Tiramani, M., Vercelli, F., Visentin, S., and Maroni, M., Ethylenethiourea in urine as an indicator of exposure to mancozeb in vineyard workers. *Toxicol. Lett.*, 134, 133-140 (2002).

Daniel, V., Huber, W., Baue, K., Suesal, C., Conradt, C., and Opelz, G., Associations of blood levels of PCB, HCHS, and HCB with numbers of lymphocytes subpopulations, in vitro lymphocyte reponse, plasma cytokine levels, and immunoglobulin autoantibodies. *Environ. Health Perspect.*, 109, 173-178 (2001).

Estlander, T., Jolanki, R., Henriks-Eckerman, M., and Kanerva, L., Occupational contact allergy to bisphenol A. *Contact Dermititis,* 40, 52-53 (1999).

Fine, J. S., Gasiewicz, T. A., and Silverstone, A. E., Lymphocyte stem cell alteration following perinatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Mol. Pharmacol.*, 35, 18-25 (1988).

Gablicks, J., Askari, E. M., and Yolen, N., DDT and immunological responses. 1. Serum antibodies and anaphylactic shock in guinea-pigs. *Arch. Environ. Health,* 26, 305 (1973).

Green, L. C., Wagner, D. A., Glogowski, J. S., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R., Analysis of nitrate, nitrite and [15N] nitrite in biological fluids. *Analyt. Biochem.*, 126, 131-138 (1992).

Gulshan, S., Mccrrden, A. B., and Stimson, W. H., Oestrogen receptors in macrophages. Scand. *J. Immunol.*, 31, 691-697 (1990).

Hakoda, S., Ishikura, H., Takeyama, N., and Tanaka, T., Tumor necrosis factor-α-plus actinomycin D-induced apoptosis of L929 cells is prevented by nitric oxide. *Surg Today,* 29, 1059-1067 (1999)

Kackar, R., Srivastava, M. K., and Raizada, R. B., Studies on rat thyroid after oral administration of mancozeb: Morphological and biochemical evaluations. *J. Appl. Toxicol.*, 17, 369-375 (1997).

Kiekens, R. C. M., Thepen, T., Ossting, A. J., Bihari, I. C., van de Winkel, J. G. J., Bruijnzeel-Koomen, C. A. F. M., and Knol, E. F., Heterogeneity within tissue-specific macrophage and dendritic cell populations during cutaneous inflammation in atopic dermatitis. *Br. J. Dermatol.*, 145, 957-965 (2001).

Kim, Y. M., Talanian, R. V., Li, J., and Billiar, T. R., Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J. Bio. Chem.,* 272, 31138-31148 (1997).

Koch, P., Occupational allergic contact pdermatitis and airborne contact dermatitis from 5 fungicides in a vineyard worker. Cross-reactions between fungicides of the dithiocarbamate group. *Contact Dermatitis*, 34, 324-329 (1996).

Kolb, H. and Kolb-Bachofen, V., Nitric oxide in autoimmune disease: Cytotoxic or regulatory mediator. *Immunol., Today,* 19, 556-561 (1998).

Lukic, M. L., Popeskovic, L., and Jan, B. D., Potentiation of immune responsiveness in rats treated with DDT. *Fed. Proceed.*, 32, 1037 (1973).

MacMicking, J., Xie, Q., and Nathan, C., Nitric oxide and macrophage function. *Annu. Rev. Immunol.*, 15, 323-350 (1997).

Matejuk, A., Adlard, K., Zamora, A., Silverman, M., Vandenbark, A. A., and Offner, H., 17β-estradiol inhibits cytokine, chemokine, and chemokine receptor mRNA expression in the central nerveous system of female mice with experimental autoimmune encephalomyelitis. *J. Neurosci.*, 65, 529-542 (2001).

Mishell, B. B. and Shiigi, S. M., Selected methods in cellular immunology. W. H. Freeman and Company, p.14, (1980).

Mosmann, T., Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, 65, 55-63 (1983).

Narthan, C. F., Secretory products of macrophages. *J. Clin. Invest.*, 79, 319-326 (1987).

Ruh, M. F., Bi, Y., Cox, D., Berk, D., Howlett, A. C., and Bellone, C. J., Effect of environmental estrogens on IL-1beta promoter activity in a macrophage cell line. *Endocrine,* 9, 207-211 (1998).

Salem, M. L., Matsuzaki, G., Madkour, G. A., and Nomoto, K., Beta-estradiol-induced decrease in IL-12 and TNF-α expression suppresses macrophage function in the course of Listeria monocytogenes function in mice. *Int. J.*

Immunopharmacol., 21, 481-497 (1999).

Shepherd, D. M., Dearstyne, E. A., and Kerkvliet, N. I., The effects of TCDD on the activation of ovalbumin(OVA)-specific DO11.10 transgenic $CD4^{+}$ T cells in adoptively transferred mice. *Toxicol. Science,* 56, 340-359 (2000).

Siddiqui, A., Ali, B., and Srivastava, S. P., Effect of mancozeb on hepatic glutathione S-transferase in rat. *Toxicology Letters.*, 68, 301-305 (1993)

Stefano, G. B., Prevot, V., Beauvillain, J. C., Fimiani, C., Welters, I., Cadet, P., Breton, C., Pestel, J., Salzet, M., and Bilfinger, T. V., Estradiol coupling to human monocytes nitric oxide release is dependent on intracellular calcium transients: Evidence for an estrogen surface receptor. *J. Immunol.*, 163, 3758-5763 (1999).

Thomas, P. S., Tumor necrosis factor-α: The role of this multifunctional cytokine in asthma. *Immunol. Cell. Biol.*, 79, 132-140 (2001).

Underhill, D. M. and Ozinsky, A., Phagocytosis of microbes: Complexity in action. *Annu. Rev. Immunol.*, 20, 825-852 (2002).

USA Environmental Protection Agency (EPA), Mancozeb Pesticide Fact Sheet (1995).

Van Dervort, A. L., Yan, L., Madara, P. J., Cobb, J. P., Wesley, R. A., Corriveau, C. C., *et al.*, Nitric oxide regulates endotoxin induced TNF-α production by human neutrophils. *J. Immunol.*, 152, 4102-4109 (1994)

Vegeto, E., Bonincontro, C., Pollio, G., Sala, A., Viappiani, S., Nardi, F., Brusadelli, A., Viviani, B., Ciana, P., and Maggi, A., Estrogen prevents the lipopolysaccharide-induced inflammatory response in microglia. *J. Neurosci.*, 21, 1809-1818 (2001).

Vergova, M., Jablonica, A., and Janota, S., Occupational exposure to mancozeb in employees in the production of Novozir Mn 80. *Prakov Lak,* 40, 425-430 (1988).

Vermeulen, L. A., Reinecke, A. J., and Reinecke, S. A., Evaluation of the fungicide manganese-zinc ethylene bis(dithiocarbamate) (mancozeb) for sublethal and acute toxicity to Eisenia fetida(Oligochaeta). *Ecotoxico. Environ. Saf.*, 48, 183-189 (2001).

Woodfork, K. A., Schuller, K. C., and Huffman, L. J., Cytokine and nitric oxide release by J774A.1 macrophages is not regulated by estradiol. *Life Sci.*, 69, 2287-2294 (2001).

Xing, Z., Current understanding of macrophage type 1 cytokine responses during intracellular infections. *Histol. Histopathol.*, 15, 199-205 (2000).

Zhang, X., Wang, L., Zhang, H., Guo, D., Qiao, Z., and Qiao, J., Estrogen inhibits lipopolysaccharide-induced tumor necrosis factor-alpha release from murine macrophages. *Methods Fund. Exp. Clin. Pharmacol.*, 23, 169-173 (2001).