Suppression of immunity by some pesticides, xenobiotics, and industrial chemicals. *In vitro* model

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ABSTRACT

In recent years, attention has focused on suppression of immunity in immunocompromised patients. The definition of immunocompromise is impairment of the immune system caused by a disease or treatment. In addition to disease and treatment, other factors such as exposure to pesticides or toxic chemicals can damage the immune system. An *in vitro* test was used to assess the ability of monochloroacetic acid (MCA) (CAS number 79-11-8), dichloroacetic acid (DCA) (CAS number 79-43-6), trichloroacetic acid (TCA) (CAS number 76-03-9), and 2,2-dichloropropionic acid sodium salt (2,2-DCPANa, Dalapon) (CAS number 127-20-8) to suppress bacteriolysis by hen egg white lysozyme (HEWL). The system for bacteriolysis of Gram-negative bacteria *Actinobacillus*, *Haemophilus*, and *Pasteurella* in Tris-maleate buffer supplemented with EDTA and HEWL developed by Brondz *et al.* [1-4] was used to monitor bacteriolysis of Gram-negative bacteria in the *Actinobacillus-Haemophilus-Pasteurella* group. The halogenated acetic acid DCA is produced as a toxic artifact of the degradation of TCA and by disinfection of drinking and pool water and industrial waste. Nearly all humans consume DCA in drinking water during their lifetime; the concentration of DCA in drinking water can be higher than that associated with the upper-bound excess lifetime cancer risk of 10^{-4} (40 μg/L) [5]. Lysozyme found in tears, saliva, nasal secretions, and excretions from all mucous membranes can break down the cell walls of bacteria and destabilize bacterial membranes. Lysozyme is involved in innate (nonspecific) immunity; the innate immune system is the first line of defense against invading organisms. *Actinobacillus actinomycetemcomitans* (ATCC29522), a Gram-negative bacterium whose primary ecological niche is the respiratory tract and oral cavity, provided the peptidoglycan substrate for HEWL. The optical density (OD) of a standardized suspension of *A. actinomycetemcomitans* decreased from 100% (OD 0.6 at 540 nm) to 23.5% after exposure to EDTA/HEWL for 50 min. After 50 min of exposure to 10.0 mg/mL of MCA, DCA, TCA, or 2,2-DCPANa as a supplement, EDTA/HEWL-induced lysis of *A. actinomycetemcomitans* decreased to 66.3%, 66.8%, 65.7%, and 73.6%, respectively. The aim of the presented study was the development of a model for measuring possible immunotoxic effects of chemicals, degradation products, xenobiotics and metabolites by these chemicals on the immune system. The method used is an *in vitro* bacteriolysis of Gram-negative bacterium induced by HEWL, described previously [1-4]. In the present study, several halogenated acids, MCA, DCA, and TCA, and the Na salt of 2,2-DCPA, exhibited immunosuppressive activity. The ability of MCA, DCA, TCA, and 2,2-DCPANa (Dalapon) to suppress HEWL induced bacteriolysis was demonstrated *in vitro* document.

Keywords: Immunosuppression; Immunotoxicity; Chlorinated Aliphatic Acids; Dalapon

1. INTRODUCTION

Lysozyme belongs to the innate (nonspecific) immune system; it is a bacteriolytic enzyme that is distributed widely in nature [6] and is found across the entire range of vertebrates and in certain invertebrates, plants, and bacteriophages. In humans, lysozyme found in tears, saliva, nasal secretions, and excretions from all mucous membranes can break down the cell walls of bacteria and destabilize bacterial membranes. Bird egg white is

particularly rich in lysozyme [7]. Lysozyme is also present in many types of cells including phagocytes, in tissues and organs [8], and in body fluids such as blood and urine.

The natural substrate for lysozyme is peptidoglycan of the bacterial cell wall. Peptidoglycan is a polymer comprising two amino sugars, N-acetylglucosamine and N-acetylmuramic acid, and amino acids. Lysozyme causes hydrolysis of the β(1-4) bond between N-acetylmuramic acid and N-acetylglucosamine, resulting in the dissolution of the bacterial cell wall. Lysozyme easily breaks down the cell walls of Gram-positive bacteria *in vitro*, although some microorganisms, particularly Gram-negative microorganisms, are resistant to lysozyme *in vitro*. It is likely that lysozyme acts synergistically *in vivo* with other immune factors such as IgM, IgA (sIgA secetory IgA1 and IgA2), complement, lactoferrin, or lactoperoxidase, thereby increasing the lytic sensitivity of such organisms [9].

Lysozyme belongs to the innate (nonspecific) immune system, which provides the first line of defense against invading organisms. Lysozyme is also an important part of the adaptive or specific immune system, which works together with the production by phagocytes of toxic oxygen compounds such as the superoxide anion (O_2^-), H_2O_2, the singlet oxygen ($^1O_2$), hypochlorite (OCl^-), and the hydroxyl radical ($OH\cdot$) [10]. Lysozyme also stimulates immunoglobulin production [11].

During the last century, many chemicals have been introduced in the biosphere as food additives, herbicides, insecticides, industrial waste and medicines. The array of potential hazards which these chemicals can have to humans is not always clear. The growing number of immunocompromised people together with the adaptation of some microorganisms toward antibiotics has changed the balance between the host organism and a possible pathogen. In the immunocompromised patient, local infections (which are non-threatening for a normal person) can be generalized and cause death.

The method for judging the influence of chemicals on the human immune system in particular and all living creatures in general is needed.

A. actinomycetemcomitans, a Gram-negative bacterium whose primary ecological niche is the respiratory tract and oral cavity, was chosen as the target for lysozyme in this study.

Halogenated acids such as monochloroacetic acid (MCA) (CAS number 79-11-8), dichloroacetic acid (DCA) (CAS number 79-43-6), and trichloroacetic acid (TCA) (CAS number 76-03-9) occur widely in the biosphere as xenobiotics derived from herbicides and pesticides, as artifacts from the disinfection by chlorine of drinking or pool water, and as industrial pollutants

[5,12,13]. The broad spectrum of toxicity for humans and animals of MCA, DCA, and TCA is described in [5,12,13]. Nearly all humans consume DCA in drinking water throughout the entire lifespan, and the concentration of DCA may exceed that associated with the upper-bound excess lifetime cancer risk of 10^{-4} (40 µg/L) [5]. According to the World Health organization (WHO), the tolerable daily intake (TDI) of MCA in drinking water is 20 µg/L [12], and the TDI of TCA is 200 µg/L [13].

Increased hepatocellular carcinomas were observed in male mice given DCA in drinking water [14-16]. The hepato- and immunotoxicity of DCA are described in [17]. Immunotoxicity was characterized as a pronounced response in this investigation. 2,2-Dichloropropionic acid (2,2-DCPA) is a standard herbicide also known as Dowpon, Basinex P, Devipon, Gramevin, Kenapon, Liropon, Dalapon, and Unipon. Because of its selectivity, it kills only certain plants; the major use of Dalapon is on sugarcane and sugar beets [18]. The oral LD_{50} values range from 3860 mg/kg for female rabbits to 9330 mg/kg for male rats [19]. Dalapon may cause irritation to the mucous membrane linings of the mouth, nose, throat, lungs, and eyes [20].

2. MATERIALS AND METHODS

2.1. Biological Material and Chemicals

A. actinomycetemcomitans (strain ATCC 29522) was obtained from (American Type Culture Collection, Rockville MD, USA). Stock cultures were kept in liquid nitrogen after reconstitution from the lyophilized state. The bacteria were maintained anaerobically (80% N_2, 10% CO_2, and 10% H_2) on blood agar plates at 35°C and were transferred weekly. To measure cell lysis, inoculated blood agar plates were incubated anaerobically for 2 days at 35°C, and the colonies were transferred on agar slants to preheated (37°C) Brain Heart Infusion ® (Difco™) broth (Voigt Global Distribution Inc., USA) and were cultured anaerobically at 35°C for 15.5 hours.

2.2. Lysozyme

The mucopeptide N-acetylmuramoylhydrolase (muramidase, EC 3.2.1.17; BioChemica; powder (crystalline), activity: 70,000 units/mg, M_r~14.600) was obtained from Sigma-Aldrich (Sigma-Aldrich Norway AS, Oslo, Norway).

2.3. Halogenated Acids

MCA, DCA, and TCA were obtained from Sigma-Aldrich Norway AS, and 2,2-DCPA sodium salt (2,2-DCPANa) was obtained from Dow Chemical Co. (Midland, MI, USA).

2.4. Ultraviolet-Visible (UV-Vis) Spectrometry

UV-vis spectrometry was recorded using a Lambda 35 UV-vis spectrometer (PerkinElmer Inc., San Jose, CA, USA) using a quartz colorimetric cell under heated conditions. Spectrometry was recorded in six parallel controls and in six parallel samples for each pesticide added. The measurements were repeated on four separate days. The results for days 1, 2, 3, and 4 are presented in **Tables 1**, **2**, **3**, and **4**, respectively. The day-to-day variations are presented in **Table 5**.

2.5. Assessment of Bacteriolysis

The sensitivity of *A. actinomycetemcomitans* to EDTA (0.01 M) and hen egg white lysozyme (HEWL) was examined in Tris-maleate buffer (0.005 M, pH 7.2) as described previously [1-3]. Briefly, washed bacterial cells were suspended in the buffer, and the optical density (OD) was adjusted to 1.0 at 540 nm before preincubation for 5 min in the buffer at 37˚C. The OD was then adjusted to 0.6, and 20 μL of buffer or lysozyme solution was added to the spectrophotometric cells, each containing bacteria suspended in 1.980 μL of 0.005 M Trismaleate buffer supplemented with EDTA and one of the halogenated substances at a concentration of 10 mg/mL,

Table 1. Induced lyses of *A. actinomycetemcomitans.*

Amount of Chemical in mg/mL added to EDTA in Tris-maleate buffer + HEWL 0.1 mg/mL 1 mg/mL 10 mg/mL or Amount of Chemical in mM/mL added to EDTA in Tris-maleate buffer + HEWL		
MCA	1 mM	10 mM	100 mM
EDTA in Tris-maleate buffer + HEWL + MCA		
	24.0*	28.5*	66.0*
DCA	800 μM	8 mM	80 mM
EDTA in Tris-maleate buffer + HEWL + DCA		
	24.9*	29.4*	67.1*
TCA	600 μM	6 mM	60 mM
EDTA in Tris-maleate buffer + HEWL + TCA		
	23.9*	27.8*	65.7*
	600 μM	6 mM	60 mM
EDTA in Tris-maleate buffer + HEWL + 2,2-DCPANa		
	27.7*	38.9*	73.5*

Induced lyses of *A. actinomycetemcomitans* on day 1 were in EDTA in Tris-maleate buffer 61.7, and EDTA in Tris-maleate buffer + HEWL 23.8, *n* = 6. Optical density measured at 540 nm in percentage of initial OD after 50 min. *Mean value, *n* = 6, the RSD = 5%.

Table 2. Induced lyses of *A. actinomycetemcomitans.*

Amount of Chemical in mg/mL added to EDTA in Tris-maleate buffer + HEWL 0.1 mg/mL 1 mg/mL 10 mg/mL or Amount of Chemical in Amount of Chemical in mM/mL added to EDTA in Tris-maleate buffer + HEWL		
MCA	1 mM	10 mM	100 mM
EDTA in Tris-maleate buffer + HEWL + MCA		
	24.2*	28.8*	66.1*
DCA	800 μM	8 mM	80 mM
EDTA in Tris-maleate buffer + HEWL + DCA		
	24.3*	28.9*	66.8*
TCA	600 μM	6 mM	60 mM
EDTA in Tris-maleate buffer + HEWL + TCA		
	23.7*	27.5*	65.9*
2,2-DCPANa	600 μM	6 mM	60 mM
EDTA in Tris-maleate buffer + HEWL + 2,2-DCPANa		
	27.9*	38.7*	73.9*

Induced lyses of *A. actinomycetemcomitans* on day 2 were in EDTA in Tris-maleate buffer 62.0, and EDTA in Tris-maleate buffer + HEWL 23.5, *n* = 6. Optical density measured at 540 nm in percentage of initial OD after 50 min. *Mean value, *n* = 6, the RSD = 5%.

Table 3. Induced lyses of *A. actinomycetemcomitans.*

Amount of Chemical in mg/mL added to EDTA in Tris-maleate buffer + HEWL 0.1 mg/mL 1 mg/mL 10 mg/mL or Amount of Chemical in mM/mL added to EDTA in Tris-maleate buffer + HEWL		
MCA	1 mM	10 mM	100 mM
EDTA in Tris-maleate buffer + HEWL + MCA		
	24.5*	28.9*	66.7*
DCA	800 μM	8 mM	80 mM
EDTA in Tris-maleate buffer + HEWL + DCA		
	24.1*	28.7*	67.1*
TCA	600 μM	6 mM	60 mM
EDTA in Tris-maleate buffer + HEWL + TCA		
	23.7*	27.5*	65.9*
	600 μM	6 mM	60 mM
EDTA in Tris-maleate buffer + HEWL + 2,2-DCPANa		
	28.0*	38.5*	73.3*

Induced lyses of *A. actinomycetemcomitans* on day 1 were in EDTA in Tris-maleate buffer 61.9, and EDTA in Tris-maleate buffer + HEWL 23.2, *n* = 6. Optical density measured at 540 nm in percentage of initial OD after 50 min. *Mean value, *n* = 6, the RSD = 5%.

Table 4. Induced lyses of *A. actinomycetemcomitans.*

Amount of Chemical in mg/mL added to EDTA in Tris-maleate buffer + HEWL 0.1 mg/mL 1 mg/mL 10 mg/mL or Amount of Chemical in mM/mL added to EDTA in Tris-maleate buffer + HEWL			
MCA	1 mM	10 mM	100 mM
EDTA in Tris-maleate buffer + HEWL + MCA			
	24.2*	28.7*	66.3*
DCA	800 µM	8 mM	80 mM
EDTA in Tris-maleate buffer + HEWL + DCA			
	24.4*	29.0*	66.8*
TCA	600 µM	6 mM	60 mM
EDTA in Tris-maleate buffer + HEWL + TCA			
	23.8*	27.5*	65.6*
	600 µM	6 mM	60 mM
EDTA in Tris-maleate buffer + HEWL + 2,2-DCPANa			
	27.9*	38.7*	73.5*

Induced lyses of *A. actinomycetemcomitans* on day 1 were in EDTA in Tris-maleate buffer 61.8, and EDTA in Tris-maleate buffer + HEWL 23.5, *n* = 6. Optical density measured at 540 nm in percentage of initial OD after 50 min. *Mean value, *n* = 6, the RSD = 5%.

1.0 mg/mL, or 0.1 mg/mL.

After 50 min of exposure to MCA, DCA, TCA, or 2,2-DCPANa as a supplement, EDTA/HEWL-induced lyses of *A. actinomycetemcomitans* were recorded.

3. RESULTS AND DISCUSSION

The amounts of chemicals added to EDTA in Tris-maleate buffer + HEWL are expressed in mg/mL, as often expressed in toxicological studies; for comparison, these values are also expressed in their closest approximations to mM or µM. The OD data are presented in **Tables 1-4** respectively. The data presented in **Tables 1-4** are the data from measurements on four separate days. The data presented in **Table 5** summarize the day-to-day variations.

After exposure of *A. actinomycetemcomitans* to EDTA for 50 min, the measured OD decreased from 100% to 61.7%, 62.0%, 61.9%, and 61.8%, respectively (**Tables 1-4**).

After exposure of *A. actinomycetemcomitans* to EDTA/HEWL for 50 min, the measured OD declined from 100% to 23.8%, 23.5%, 23.2%, and 23.5%, respectively (**Tables 1-4**).

After exposure of *A. actinomycetemcomitans* to EDTA/HEWL + 10.0 mg/mL MCA the OD decreased from 100% to 66.0%, 66.1%, 66.7%, and 66.3%, respectively.

After exposure of *A. actinomycetemcomitans* to EDTA/

Table 5. Induced lyses of *A. actinomycetemcomitans* day-to-day performance.

EDTA in Tris-maleate buffer + HEWL+ 10 mg/mL MCA	
Total days 4	
Mean	66.275
Standard deviation	0.30957
EDTA in Tris-maleate buffer + HEWL + 1 mg/mL MCA	
Total days 4	
Mean	28.725
Standard deviation	0.17078
EDTA in Tris-maleate buffer + HEWL + 0.1 mg/mL MCA	
Total days 4	
Mean	24.225
Standard deviation	0.20616
EDTA in Tris-maleate buffer + HEWL + 10 mg/mL DCA	
Total days 4	
Mean	66.8
Standard deviation	0.24495
EDTA in Tris-maleate buffer + HEWL + 1 mg/mL DCA	
Total days 4	
Mean	29.0
Standard deviation	0.29439
EDTA in Tris-maleate buffer + HEWL + 0.1 mg/mL DCA	
Total days 4	
Mean	24.425
Standard deviation	0.34034
EDTA in Tris-maleate buffer + HEWL + 10 mg/mL TCA	
Total days 4	
Mean	65.65
Standard deviation	0.20817
EDTA in Tris-maleate buffer + HEWL + 1 mg/mL TCA	
Total days 4	
Mean	27.525
Standard deviation	0.20616
EDTA in Tris-maleate buffer + HEWL + 0.1 mg/mL TCA	
Total days 4	
Mean	23.825
Standard deviation	0.9574
EDTA in Tris-maleate buffer + HEWL + 10 mg/mL 2,2-DCPNa	
Total days 4	
Mean	73.575
Standard deviation	0.25
EDTA in Tris-maleate buffer + HEWL + 1 mg/mL 2,2-DCPNa	
Total days 4	
Mean	38.7
Standard deviation	0.1633
EDTA in Tris-maleate buffer + HEWL + 0.1 mg/mL 2,2-DCPNa	
Total days 4	
Mean	27.875
Standard deviation	0.12583
Induced lyses of *A. actinomycetemcomitans* EDTA in Tris-maleate buffer + HEWL	
Total days 4	
Mean	23.5
Standard deviation	0.24495

HEWL + 1.0 mg/mL MCA the OD declined from 100% to 28.5%, 28.8%, 28.9%, and 28.7%, respectively.

After exposure of *A. actinomycetemcomitans* to EDTA/ HEWL + 0.1 mg/mL MCA the OD declined from 100% to 24.0%, 24.2%, 24.5%, and 24.2%, respectively.

After exposure of *A. actinomycetemcomitans* to EDTA/ HEWL + 10.0 mg/mL DCA the OD decreased from 100% to 67.1%, 66.8%, 66.5%, and 66.8%, respectively.

After exposure of *A. actinomycetemcomitans* to EDTA/ HEWL + 1.0 mg/mL DCA the OD decreased from 100% to 29.4%, 28.9%, 28.7%, and 29.0%, respectively.

After exposure of *A. actinomycetemcomitans* to EDTA/ HEWL + 0.1 mg/mL DCA the OD declined from 100% to 24.9%, 24.3%, 24.1%, and 24.4%, respectively.

After exposure of *A. actinomycetemcomitans* to EDTA/ HEWL + 10.0 mg/mL TCA the OD decreased from 100% to 65.7%, 65.9%, 65.4%, and 65.6%, respectively.

After exposure of *A. actinomycetemcomitans* to EDTA/ HEWL + 1.0 mg/mL TCA the OD declined from 100% to 27.8%, 27.5%, 27.3%, and 27.5%, respectively.

After exposure of *A. actinomycetemcomitans* to EDTA/ HEWL + 0.1 mg/mL TCA the OD decreased from 100% to 23.9%, 23.7%, 23.9%, and 23.8%, respectively.

After exposure of *A. actinomycetemcomitans* to EDTA/ HEWL + 10.0 mg/mL 2,2-DCPANa the OD decreased from 100% to 73.5%, 73.9%, 73.3%, and 73.6%, respectively.

After exposure of *A. actinomycetemcomitans* to EDTA/ HEWL + 1.0 mg/mL 2,2-DCPANa the OD declined from 100% to 38.9%, 38.7%, 38.5%, and 38.7%, respectively.

After exposure of *A. actinomycetemcomitans* to EDTA/ HEWL + 0.1 mg/mL 2,2-DCPANa the OD decreased from 100% to 27.7%, 27.9%, 28.0%, and 27.9%, respectively.

In the present study, several halogenated acids, MCA, DCA, and TCA, and the Na salt of 2,2-DCPA, exhibited immunosuppressive activity. MCA, DCA, and TCA are usually found in treated drinking or swimming pool water or as a result of the degradation of pesticides and xenobiotics; 2,2-DCPA is a pesticide. These substances exerted marked suppression of the bacteriolytic effect of HEWL on bacterial peptidoglycan *in vitro*. The mechanisms responsible for this suppression are not known in detail, but enzyme deactivation was not related to acid strength because 2,2-DCPANa had the most potent effects in the experiments, and 2,2-DCPANa was in a salt form, in contrast to the acidic form of MCA, DCA, and TCA. It is possible that chlorinated ions were responsible for the deactivation of HEWL because 2,2-DCPANa exerted the most potent suppressive action.

Most chemical noxae are capable of binding proteins in organisms. TCA has a high capacity for protein bind-ing and probably interacts readily with the basic protein, lysozyme. Iodoacetic acid modifies methionine and lysine situated in the primary structure of lysozyme [21]. It has been suggested that EDTA permits HEWL access to peptidoglycan to initiate autolysis activity in *A. actinomycetemcomitans* [1-4].

There has been a recent increase in the isolation of facultative anaerobic, Gram-negative rods in clinical samples [22]. This increase cannot be related to improved diagnostic methods alone. In addition to the increased isolation of such organisms, people are being exposed increasingly to halogenated substances in the biosphere [5,12-17]. Farmers and chemical workers, who tend to be exposed most heavily [18-20], have higher rates of chronic bronchitis than do physicians and firemen [23]. Such exposure may predispose to secondary infection by opportunists residing on the nasal, bronchial, and oral mucosa, particularly when important aspects of nonspecific immunity are inhibited. In addition to its industrial and agricultural uses, MCA is often found as a contaminant of TCA and the herbicide Dalapon, in which 2,2-DCPANa is the active ingredient, and in other chemicals such as EDTA. Such contamination may increase the toxicity of the main substance. MCA is also produced as a metabolite of chemicals such as vinylidene chloride, vinyl chloride, 1,1,2-trichloroethane, trichloroethylene, and 1,2-dichloroethane [24-27]. MCA is known to cause severe damage to energy-rich tissues and to interfere with the tricarboxylic acid cycle and gluconeogenesis [28,29]. The kinetics of MCA metabolism in adult male rats is described in [30].

DCA is generated as a metabolite after exposure to trichloroethene and as a byproduct of the chlorination of drinking water. The *in vivo* immunotoxicity of DCA is reported in [17].

TCA tends to accumulate in the organism, where it may counteract the effect of lysozyme. TCA is a metabolite of trichloroethylene (TCE) and perchloroethylene (PCE) and is detectable in the urine of humans exposed to these solvents. TCA has been shown to act as a liver tumor promoter in several studies in which rats or mice were treated with an initiating dose of a carcinogen followed by chronic exposure to TCA in the drinking water.

Dalapon or 2,2-DCPANa is an herbicide and plant growth regulator used to control specific annual and perennial grasses. The major food crop use of Dalapon is on sugarcane and sugar beets [31]. Repeated and/or prolonged exposure to Dalapon may cause irritation of the mucous membrane linings of the mouth, nose, throat, lungs, and eyes [32], and the possible involvement of the kidneys in the death of animals has been described [33]. Its interference with the body's ability to synthesize pro-

tein and to use sugar from food is described in [34].

Lysozyme deactivation by the halogenated substances did not parallel their acute toxicity. Even when tested at the lowest molar concentration, Dalapon exhibited the highest deactivation potential toward lysozyme in the present study.

It is possible that the substances examined may affect lysozyme *in vivo*, *i.e.*, those present in organs, tissues, cells, and body fluids such as blood and urine.

Immunotoxicology is a subject of recent interest because the physiology and biochemistry of the immune system and the basic processes involved in host defense mechanisms are now understood better. Successful interfacing between immunology testing and toxicology evaluation of chemicals is still in the early stages. Immunotoxicology assays should be included routinely in the evaluation of chemicals, especially herbicides and pesticides. The present study provides the basis for judging whether chemicals present a potential risk to nonspecific host defense.

4. CONCLUSIONS

1) An *in vitro* model for recording immunosuppressive effect of some chlorinated aliphatic acids on the bacteriolytic effect of HEWL was developed. This effect is general for lysozyme of any origin. Some of the used chemicals are artifacts from chlorination of water.

2) Dalapon exhibited the highest immunosuppressive effect. Enzyme deactivation was not related to acid strength, but instead the chlorinated ions were responsible for the deactivation of HEWL.

3) The immunosuppressive effect by the halogenated substances did not parallel their acute toxicity. Therefore, the acute toxicity and LD_{50} examination of chemicals should be supplemented with a test for immunosuppressive effectiveness for every chemical halogenated as for non-halogenated.

5. ACKNOWLEDGEMENTS

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