The Effects of MTBE (Methyl Tertiary Butyl Ether) on the Immune System of the amphibian, *Rana pipiens*.

Roxanne Suarez
Department of Biology
Hartwick College
Oneonta, NY
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Thesis Advisor Date

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Chair, Biology Department Date

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Dr. Stanley Sessions, Advisor

ABSTRACT

MTBE is a fuel additive present in gasoline. It functions as an oxygenate in fuel to increase its oxygen content to reduce carbon monoxide and ozone levels. Four groups of adult *R. pipiens* frogs where exposed to three different concentrations of MTBE in aged, dechlorinated tap water for three weeks, and then chronically injected with sheep red blood cells for five days to create an immune response. Lymphocytes were isolated from the frogs' spleens and a hemocytometer was used to count the number of viable spleen cells. The number of antibody-producing B-cell lymphocytes was determined using a hemolytic plaque assay. The results indicate a dose response in which B-cell production increases with increasing concentration of MTBE. These results suggest that MTBE induces hypersensitivity of the immune systems in frogs.

INTRODUCTION

The immune system functions to detect foreign bodies (antigens) that have entered the body, produce antibodies against the antigen and finally destroy the invader. There is an innate and adaptive immunity. The innate immune system is the first line of defense against pathogens. It provides fast and non-specific protection against antigens until the adaptive immune system can be mobilized. The adaptive immune system is highly specific for a given antigen due to the production of memory cells that respond quickly to a repeated antigen invader (Rollins-Smith et al., 1999). When a antigen is present inside the body a antigen presenting cell, usually a macrophage phagocytotes the antigen, digest it and displays pieces of it on its cell surface to be presented to T-helper cells that bind to the antigen via specific receptors. The T-helper cells then stimulates B-cells to proliferate and differentiate into plasma cells that produce antibodies. During this stage T-cells and B-cells also differentiate into memory cells. If the antigen enters the body a second time the memory cells recognize the invader and the plasma cells produce antibodies that destroys the invader (Male et al., 1998). The antibody or immunoglobulin produced is thought to be IgE although several species of adult amphibians produce two main classes of immunoglobulins, IgG and IgM (Steiner et al., 1973). The Immune system of the amphibian and human are functionally similar. The amphibian can be used as an indicator species for environmental pollutants because they have highly vascularized skin and breath air that is exposed to environmental pollutants (Louis Du Pasquier, 2000). Amphibians have also been indicator species regarding the role of trematode infestation. There has been widespread occurrences of limb abnormalities in amphibian natural populations and has become an environmental issue (Sessions et al., 2002). Reports of amphibians with abnormal limb development in natural populations have been in scientific literature for centuries and recently there have been high frequencies of multilegged amphibians (Sessions and Ruth, 1990). Research has suggested that environmental pollutants such as pesticides may be linked to the increased amphibian deformities and declines (Kiesecker, 2002).

The environmental pollutant MTBE (methyl tertiary butyl ether) is a fuel additive that is used as an oxygenate in gasoline. It functions to oxygenate gasoline to reduce carbon monoxide levels and damage to the ozone layer. It is introduced into the environment by leakage of under ground storage tanks, gasoline pumps, boats, and auto emissions (MTBE Contamination.com). The use of this additive is controversial due to limited knowledge concerning its harmful effects in water and in air. Research on mice suggests that MTBE is a potential carcinogen and human inhalation studies have shown side effects such as headaches, dizziness, and nausea (Summary of Workshop on Biodegradation of MTBE, 2000). The Environmental Protection Agency has set a drinking water advisory of 20 to 40 parts per billion. Although individual states lawfully set their own advisories. Presently in New York State the set limit is 10ppb but beginning in January 1, 2004 law legislation will ban gasoline-containing MTBE from being imported, sold, dispensed or offered for sale in New York State (MTBE in Drinking Water, www.epa.gov).

The effect of MTBE is interesting and important because it may be contributing to amphibian declines and to human diseases. An important question is how is MTBE in human drinking water supplies effecting the immune system of wild life and humans alike. One might assume that an environmental pollutant would have deleterious effects on the immune system. I tested the effects of MTBE on the immune system of the amphibian *Rana pipiens*. My results suggest that with increasing concentration of MTBE in dechlorinated water the immune system of the frog was heightened or produced a hypersensitive response.

MATERIALS AND METHODS

The species *Rana pipiens* was used as my test subject. I used a total of 12 frogs, three frogs per concentration of MTBE. The concentrations I used were 10^{-5}M, 10^{-4}M, 10^{-3}M and my control solution of dechlorinated water. The frogs were exposed to these concentrations for 3 weeks. The 10^{-3}M concentration was prepared by using a stock solution of 0.1M from a pure concentration of MTBE with a formula weight of 88.15ml. 10ml of the stock solution was added to 1000ml of dechlorinated water. 300ml of the solution was put into a glass jar and was done for the other two and one frog was placed in each jar. Cheesecloth was placed over the top and held with rubber bands. Adding 900ml dechlorinated water to the left over 100ml of the 10-3M solution made the 10-4M concentration. The solution was distributed into the glass jars with 300ml in each with 100ml left over to make the 10^{-5}M concentration. The same procedure was done with the 10^{-5}M concentration. The concentrations used for the first week were 10^{-3}M, 10^{-5}M, and
10^{-7}M concentration. It was decided to increase the concentrations because the current concentrations being used were not lethal. Every three full days the old water was discarded and fresh solutions were made. At the end of three weeks each frog was injected with 40% sheep red blood cells (SRBC) intraperitoneally for 5 consecutive days with a 26g needle to create an immune response. The 40% SRBC was prepared by adding 4ml of SRBC with 6ml of PBS. The solution was centrifuged at 2,000rpm and the supernatant discarded. The pellet was resuspended in PBS and brought up to 10ml (Fink et al., 2002). On the 6th day the frogs were not injected with SRBC and on the 7th they were killed by over anesthia with 2-phenoxyethanol. 2ml of 2-phenoxyethanol was added to their water and left there for up to 10min and then the spleens removed. The spleens were flushed with 3ml of sterile HBSS +FBS using a 26g needle. The lymphocytes were put into a 15ml sterile centrifuge tube and centrifuged for 5min at 3,000rpm. The pellet was resuspended in 3ml of HBSS+FBS and let sit on ice for 20min. the lymphocyte concentration was determined using a hemocytometer by adding 0.5ml of spleen cells to 2 drops of trypan blue in a eppendorf tube and was let to stain for 5 min. A small amount was placed into the hemocytometer and the number of viable cells, lymphocytes, and sporozoans were counted and determined by the equation $c = n \times 10^4$ cells/ml where c is number of cells/ml and n is number of cells counted. This was done for each frog at each concentration and recorded (Sessions, 2001).

The hemolytic plaque assay technique was used to determine how many plaques were produced per ml of active B-cells. Sticking 2 sterile microscope slides together with double sided tape made hemolytic plaque chambers. I mixed 0.5ml of SRBC, 0.5ml of guinea pig complement, and 1.5ml of 5% HBSS+FBS in a sterile centrifuge tube on ice. Then I mixed 100_l of SRBC mixture and 100_l of spleen cells in an eppendorf tube and kept it on ice for 5 min. 100_l of this mixture was then pipetted into each side of one chamber. The chamber was then sealed with melted vasper (1:1 mixture of vasoline and paraffin) and the chamber was incubated at room temperature for 1-5 hours with 3 hours being the optimum time. The hemolytic plaques/ml were then counted under a phase contrast microscope and the percent of active B-cells was obtained by dividing the viable cells/ml by number of plaques/ml (Sessions, 2001).

RESULTS

My viable cell count in cells/ml x 10^6 M and parasites/ml were obtained from the hemocytometer. Viable cells were easily distinguishable under phase contrast, dead cells were a dark blue color, lymphocytes were small and round with a yellow color, the frog's red blood cells were oval with a clearly visible nucleus (Fig.1). The range of viable cells/ml was 1.3ml to 5.3ml (Table. 1). Numbers of plaques/ml were obtained from the hemolytic plaque chambers. A plaque was identified as a lymphocyte in the middle of a cleared circular area surrounded by sheep red blood cells and frog red blood cells (Fig. 2). The range of plaques found was from 16.7ml to 755ml and the range of percent active B cells was 7.95 to 156.5 (Table. 1). My data suggest that with an increase in MTBE concentration there is an increase in the percent of active B cells (Table 2). The data in Table 2 comparing concentration with average percent plaque producing B lymphocytes were used to construct the graph in Fig. 6. A Kruskal-Wallis test showed that one or more of the MTBE sample concentrations are significantly different from the other concentrations.

Several of the frogs were found to be heavily infected with parasites identified assporozoans (Fig. 3). High parasitic infection was found in one control frog, one of the frogs treated with 10^{-5}M MTBE, and two of the frog that were treated with 10^{-4} M MTBE (Table 1). The sporozoans were found moving throughout the blood, attached to lymphocytes or red blood cells, and were occasionally found inside lymphocytes (Fig. 3). The parasite infection was not correlated with the percent of active B cells (Table 1).

Table 1. A comparison of MTBE concentration, cells/ml, # of plaques/ml, parasites, and % of active B cells

Concentrations	Cells/ ml (10^6)	# of Plaques/ml (per ml)	Parasites (# of parasites/ml)	% of active B cells
Control 1	2.10	16.7	$2x10^5$ml	7.95
Control 2	2.20	35	$1.7x10^5$ml	15.9
Control 3	2.90	316.7	0	109.2
5 (1)	9.5	685	0	72.1
5 (2)	1.3	55	$4x10^4$ml	42.3
4 (1)	4.5	500	0	111.1
4 (2)	5.3	755	$2.2x10^5$ml	142.5
4 (3)	1.8	85	$2.0x10^4$ml	47.2
3 (1)	2.3	310	0	156.5
3 (2)	3.2	325	0	101.6

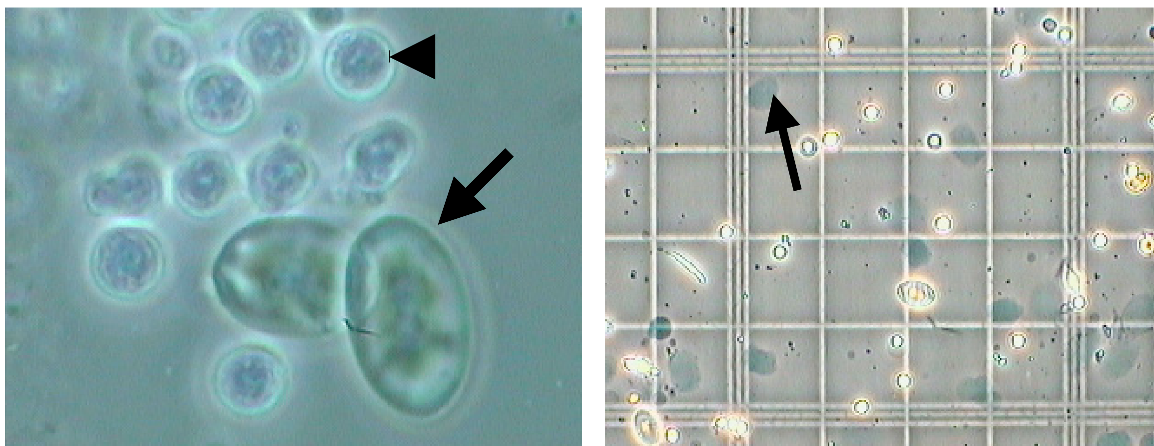

Figure 1. Left: Frog lymphocytes (arrowhead) and red blood cells (arrow) under phase contrast. Right: Field with hemocytometer showing differential staining of dead cells (arrow); viable lymphocytes and red blood cells are easily distinguishable.

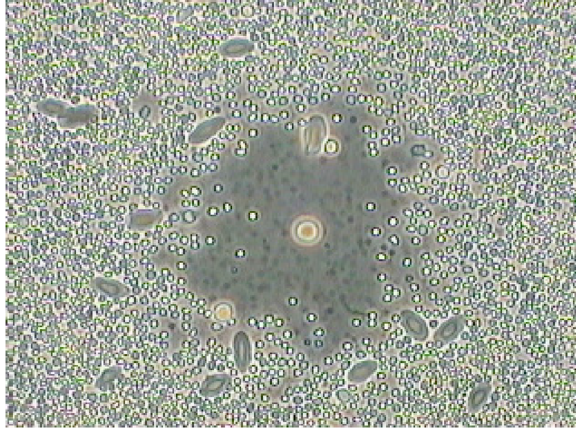

Figure 2. Example of a hemolytic plaque showing an antibody producing frog B-cell surrounded by lysed sheep red blood cells.

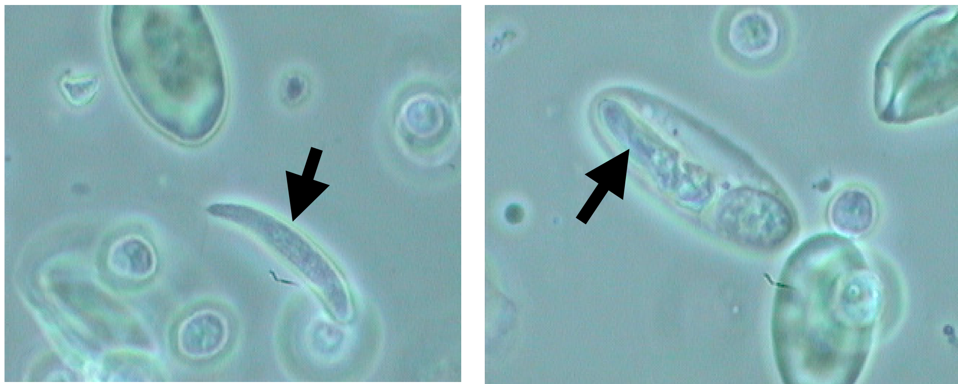

Figure 3. Left: sporozoan parasite (arrow); Right: sporozoan (arrow) that has invaded a frog leucocyte.

Table 2. MTBE concentration versus the average percent plaque-producing lymphocytes (x 10^{-4}); range in parenthesis.

Treatment	Avg. % plaque-producing lymphocytes x 10^{-4}
Control (n=3)	44.5 (7.95 - 109.2)
10^{-5} (n=2)	57.2 (42.3 - 72.1)
10^{-4} (n=3)	100.3 (47.2 – 142.5)
10^{-3} (n=2)	129.1 (101.6 – 156.5)

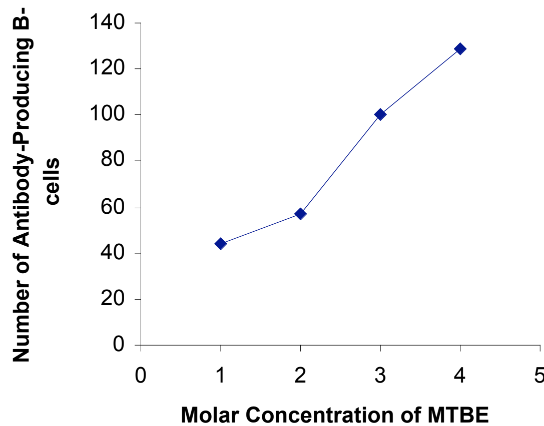

Figure 4. The immunological response of B-cells to concentrations of MTBE. 1 = control; 2 = 10^{-5} M; 3 = 10^{-4} M; 4 = 10^{-3} M).

DISCUSSION

My results suggest that with increasing concentrations of MTBE there is an increase in percent producing B-cells and a heightened immune system (Fig. 6). I reject my hypothesis that with increasing concentrations of MTBE the percent number of active B-cells producing plaques will decrease and therefore signify a weakened immune system. The parasites were identified as sporozoans and did not show any correlation with the percent of active B-cells producing plaques. I have proposed two models that may explain why my results show a heightened immune system with increasing concentration of MTBE. My first model is a hypersensitivity model that states that MTBE is causing a hypersensitivity immune response, which is over responding to an antigen. The antigen being either the SRBC or MTBE. The immune response may be type I hypersensitivity where the immunoglobulin secreted by the B-cell is IgE. The IgE binds to a mast cell on specific receptors and when the antigen is presented to the body a second time the memory cells allow an immediate reaction. The antigen binds to two IgE's and cross links causing an increase in Ca+ in the mast cells which in turn causes degranulation and the release of mediators such as histamine that causes an allergic response (Male et al., 1998). The frog that died at 10^{-5} M concentration two weeks into the experiment had red spots all over the its body especially on its hind legs indicating an inflammatory response which is a symptom of type I hypersensitivity. The frog that died at 10^{-3}M almost 3 weeks into the experiment also had inflammation on its body especially on its hind legs. Since I did not test for a hypersensitivity response I am unable to conclude that the reason for the heightened immune system to MTBE was in fact hypersensitivity.

My second model is a regenerative model that states that MTBE is cytotoxic, causing an increase in proliferation in lymphocyte stem cells as a regenerative response to B-cells being killed off by MTBE. MTBE might be causing lysis of cells and the frogs immune system is trying to combat the loss of B-cells and the presence of a cytotoxin by overproducing lymphocytes. This model may be compatible with research that has been done on mice with MTBE. In research that has been done cancerous tumors were found. In my experiment the frogs were not dissected and diagnosed for cancerous tumors but speculation of the proliferation of B-cells might be an explanation as to why there was an increase in percent number of B-cells producing plaques.

Future research includes a replication of my experiment with a larger sample size of test subjects to see if similar results are produced. If similar results are produced then further examination should be done to distinguish between the two models I have proposed.

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APPENDIX I

Protocol for Hemolytic Plaque Assay

1. Make hemolytic chambers from sterile microscope slides
   stick two slides together using two-sided tape placing the tape along each edge of the slide and one down the middle.
2. anesthetize frog with 2-phenoxyethanol
3. remove spleen
4. place in petri dish and flush with 3 ml of sterile HBSS+FBS using a 26g needle and a 3cc syringe
5. put lymphocytes in 15ml centrifuge tube
6. centrifuge at 3,000rpm for 5 min
7. resuspend lymphocyte in 3 ml of HBSS+FBS and let sit for 20min on ice
8. Determine lymphocyte concentration with a hemocytomer
9. add 0.5 ml of spleen cells and 2 drops of trypan blue into an eppendorf tube
10. let stain for 5 min
11. count with a hemocytometer wash SRBC (make 40% solution) by adding 4ml of SRBC with 6ml of PBS. The solution was centrifuged at 2,000rpm and the supernatant discarded. The pellet was resuspended in PBS and brought up to 10ml.
12. mix 0.5ml of SRBC +0.5ml complement +1.5l of 5% FBS+HBSS in sterile centrifuge tube on ice
13. mix 100_l of SRBC mixture +100_l of spleen cells in eppendorf tube, keep on ice for 5 min
14. put 100_l of SRBC and spleen cells into a chamber
15. seal chamber with melted vesper
16. incubate for 1-5 hours (3 hours optimum time)
17. count hemolytic plaques