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Health assessment of gasoline and fuel oxygenate vapors: Immunotoxicity evaluation

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ABSTRACT

Female Sprague Dawley rats were exposed via inhalation to vapor condensates of either gasoline or gasoline combined with various fuel oxygenates to assess potential immunotoxicity of evaporative emissions. Test articles included vapor condensates prepared from "baseline gasoline" (BGVC), or gasoline combined with methyl tertiary butyl ether (G/MTBE), ethyl t-butyl ether (G/ETBE), t-amyl methyl ether (G/TAME), diisopropyl ether (G/DIPE), ethanol (G/EtOH), or t-butyl alcohol (G/TBA). Target concentrations were 0, 2000, 10,000 or 20,000 mg/mg^3 administered for 6 h/day, 5 days/week for 4 weeks. The antibody-forming cell (AFC) response to the T-dependent antigen, sheep erythrocyte (sRBC), was used to determine the effects of the gasoline vapor condensates on the humoral components of the immune system. Exposure to BGVC, G/MTBE, G/TAME, and G/TBA did not result in significant changes in the IgM AFC response to sRBC, when evaluated as either specific activity (AFC/10^6 spleen cells) or as total spleen activity (AFC/spleen). Exposure to G/EtOH and G/DIPE resulted in a dose-dependent decrease in the AFC response, reaching the level of statistical significance only at the high 20,000 mg/m^3 level. Exposure to G/ETBE resulted in a statistically significant decrease in the AFC response at the middle (10,000 mg/m^3) and high (20,000 mg/m^3) exposure concentrations.

1. Introduction

The 1990 amendments to the Clean Air Act (CAA) mandated the use of oxygenates in motor gasoline. In 1994, the U.S. Environmental Protection Agency (EPA) issued a final rule under the CAA which added new health effects information and testing requirements to the Agency's existing registration requirements. As described in more detail in a companion paper (Henley et al., 2014), requirements include inhalation exposures to evaporative emissions of the gasoline or additive in question. The health endpoints include assessments for standard subchronic toxicity, neurotoxicity, genotoxicity, immunotoxicity, developmental and reproductive toxicity, and chronic toxicity/carcinogenicity. The results of chronic toxicity testing of gasoline and gasoline combined with MTBE have already been reported (Benson et al., 2011) and reported elsewhere in this issue are the findings for genotoxicity (Schreiner et al., 2014), neurotoxicity (O'Callaghan et al., 2014), reproductive toxicity (Gray et al., 2014), and developmental toxicity testing in mice and rats (Roberts et al., 2014a; Roberts et al., 2014b). This paper describes the results of immunotoxicity testing submitted to EPA.

Seven test materials were evaluated in 13 week toxicity studies. They were vapor condensates prepared from an EPA described "baseline gasoline" (BGVC), as well as gasoline combined with methyl tertiary butyl ether (G/MTBE), ethyl t-butyl ether (G/ETBE), t-amyl methyl ether (G/TAME), diisopropyl ether (G/DIPE), ethanol (G/EtOH), or t-butyl alcohol (G/TBA) (Henley et al., 2014). The immunotoxicity studies described here were conducted with satellite groups of the 13 week subchronic studies. The goal of the studies was to provide information on the extent to which the use of oxygenates in gasoline might alter the hazard of

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evaporative emissions that are encountered during refueling of vehicles, compared to those from gasoline alone. The studies were conducted in female rats because female rats elicit a more robust immune response than male rats and have a greater sensitivity for detecting an adverse effect of a compound should one occur. Routine immunotoxicology evaluations conducted by the National Toxicology Program (NTP) evaluate compounds only in female animals (Luster et al., 1988).

The IgM antibody-forming cell (AFC) response to the T-dependent antigen sheep erythrocytes, also referred to as the plaque assay, was the immunological assay conducted to evaluate the effect of baseline gasoline and baseline gasoline/ oxygenate blend vapor condensates on the immune response. For a single test, the plaque assay has been shown to be the most comprehensive and predictive assay for determining the immunotoxicological potential of a compound (Luster et al., 1992).

As background, sheep erythrocytes (sRBC) are a T-dependent antigen, T cells, B cells, and macrophages (dendritic cells) are required to function properly in order to obtain an antibody-forming cell (AFC) response. If the test article affects any of these cell types to a significant degree, an altered response will be observed which could be capable of modifying the humoral immune response and, thus, has the potential for causing whole animal immunotoxicity. As a result, the T-dependent IgM response to sRBC is one of the most sensitive immunotoxicological assays accepted by regulatory agencies currently in use.

The plaque assay is regarded as the "gold standard" for evaluating effects of compounds on humoral immunity. Although the plaque assay is not considered to be an assay for other mechanisms of immune response such as innate, T-cell independent, or cell-mediated immunity, by utilizing a T-dependent antigen, it provides valuable information on T-helper cells, macrophages, and B-/ plasma cells. As indicated above, if these cells are adversely affected, then an effect on humoral immunity can be detected with this assay. This assay is one of the tier I assays used by the NTP (Luster et al., 1988).

2. Methods and materials

Seven separate inhalation studies involving exposures to vapor condensates were conducted. Test materials included vapors of baseline gasoline (BGVC) and BGVC combined with 10–20% (see Henley et al., 2014) methyl t-butyl ether (G/MTBE), ethanol (G/EtOH), t-amyl ethyl ether (G/TAME), ethyl t-butyl ether (G/ETBE), diisopropyl ether (G/DIPE), or t-butyl alcohol (G/TBA). The in- life phases of these studies (animal exposures) were conducted by Huntingdon Life Sciences Princeton Research Center (PRC), East Millstone, NJ, and the immunological evaluations were conducted by ImmunoTox, Inc., Richmond, VA. The animals were satellite groups of a larger subchronic toxicity study; the generation and composition of the vapor concentrations and additional details on the exposure methods are reported in companion articles (Henley et al., 2014; Clark et al., 2014).

2.1. Experimental design

Each of the seven immunotoxicological satellite studies consisted of five groups of animals: a vehicle control group, three test material exposure groups (one of the gasoline vapor condensates), and a positive control group. There were 10 female Sprague Dawley rats in each of the groups. Animals were exposed by PRC personnel to either vehicle (air only) or test material at exposure levels of 2000, 10,000 or 20,000 mg/m^3 via inhalation for 4 weeks (5 days per week). Cyclophosphamide (CPS, CAS #6055-19-2, purity 99.2%; from Sigma Chemical Company) was used as the positive control. CPS was dissolved and diluted in phosphate buffered saline to stock concentrations of 5.0 mg/mL. The positive control animals received 50 mg/kg CPS, a known immunosuppressive agent, administered intraperitoneally (i.p.) on the last 4 days of exposure. These animals were not chamber exposed because the purpose of the positive control group is merely to verify that the assay can detect immunological effects. If the positive control group fails to cause a significant reduction in AFC, the assay is considered invalid and repeated.

Rats were immunized by ImmunoTox, Inc. personnel by intravenous injection of 2×10^8 sheep red blood cells (sRBC), four days prior to sacrifice. One day after the last test material exposure and four days after immunization with sRBC, rats were sacrificed and PRC personnel aseptically removed the spleen from each animal, weighed it, placed it in a collecting tube containing Earle's Balanced Salt Solution (EBSS) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and gentamicin solution and shipped the spleens on ice in individual shipping containers at 2–8 °C by overnight delivery to ImmunoTox laboratories for immunological evaluation. Upon receipt, spleens were further processed for determination of IgM antibody response.

2.2. Terminal body and organ weights

The terminal body weights were obtained by PRC personnel, who also collected blood (serum) samples (orbital collection anesthetized via carbon dioxide/oxygen inhalation) and then sacrificed (carbon dioxide inhalation) the animals on the day after the final exposure. The serum samples were frozen (−70 °C). The thymuses were removed, weighed and preserved in formalin for possible histopathology.

2.3. Splenocyte preparation

Upon arrival at the ImmunoTox testing facility, single-cell suspensions were prepared from each spleen using a Stomacher® 80 Lab Blender. Cell suspensions were then centrifuged and resuspended in EBSS with HEPES buffer in a 6-ml volume, from which 1:50 and 1:150 dilutions were prepared. Viability of splenocytes was determined using propidium iodide (PI) and the Coulter EPICS XL-MCL Flow Cytometer.

2.4. Spleen lgM antibody response to the T-dependent antigen, sRBC-day 4 response

The primary IgM response to sheep erythrocytes was measured using a modified hemolytic plaque assay of Jerne (Jerne et al., 1974), as detailed by White et al., 2010. A 0.1-ml aliquot of spleen cells from each suspension was added to separate test tubes, each containing 25 µl guinea pig complement, 25 µl sRBC, and 0.5 ml of warm agar (0.5%). After thoroughly mixing, each test tube mixture was plated onto a separate petri dish, covered with a microscope cover slip, and incubated at approximately 36–38 °C for 3 h.

Spleen cell number, following lysis of RBC, was performed on the 6-ml samples using a Model Z1 Coulter Counter. The spleen weight, cells/spleen, antibody forming cells (AFC)/10^6 spleen cells, and AFC/spleen were determined. The plaques that developed were counted using a Bellco plaque viewer. For each spleen, 2 dilutions (1:50 and1:150) were prepared. At the time of counting, each plate was examined. Routinely, the plate that had between 100 and 300 plaques was counted. When the number of plaques is in excess of 350 plaques per plate, it becomes difficult to obtain an accurate count using the Bellco viewer. A plaque, occurring from the lysis of sRBC, is elicited as a result of the interaction of complement and antibodies (produced in response to the i.v. immunization) directed against sRBC. Each plaque is generated from a single IgM antibody-producing B cell, permitting the number of AFC

present in the whole spleen to be calculated. The data are expressed as specific activity (AFC/10^6 spleen cells) and total spleen activity (AFC/spleen).

In accordance with practices of NTP immunotoxicology assays, these plaque forming assay results were not adjusted for spleen cell viability. In mixed cell populations, lymphocytes, particularly those antibody forming cells (plasma cells) making antibody to sheep erythrocytes die off more slowly than more fragile polymorphonuclear cells White et al., 2010). Thus, correcting for individual cell viability in a mixed population can artificially inflate plaque forming cell values.

2.5. Data handling and statistical analysis

The data obtained in this study were first tested for homogeneity of variances using the Bartlett's Chi Square Test (Bartlett, 1937). Homogeneous data were evaluated by a parametric one-way analysis of variance (Kruskal and Wallis, 1952). When significant differences occurred, exposed groups were compared to the vehicle control group using the Dunnett's *t* Test (Dunnett, 1955). Non-homogeneous data were evaluated using a non-parametric analysis of variance (Kruskal and Wallis, 1952). When significant differences occurred, exposed groups were compared to vehicle control group using the Gehan-Wilcoxon Test (Gross and Clark, 1975) when appropriate. The Jonckheere's Test (Hollander and Wolfe, 1973) was used to test for exposure level-related trends across the vehicle and exposed groups. The positive control was compared to the vehicle control group using the Student's *t* Test (Sokal and Rohlf, 1981). The criteria for accepting the results of the positive control in the assay were a statistically significant ($p < 0.05$) decrease in the response as compared to the vehicle control group.

2.6. Compliance

These studies were conducted in accordance with the United States Environmental Protection Agency's (EPA) Good Laboratory Practice Standards (US EPA, 1994), and complied with all appropriate parts of the Animal Welfare Act Regulations (USDA, 1989, 1991). The study also met the requirements of EPA's guidelines for inhalation toxicity and immunotoxicity screening (US EPA, 1998a,b).

3. Results

3.1. Terminal body and organ weights

No statistically significant effect was observed on terminal body weights in any of the vapor condensate exposure groups, as compared to the corresponding vehicle controls. In addition, exposure to the test materials in the seven studies did not significantly affect spleen or thymus weights when evaluated either as absolute or relative weight, with the exception of the G/DIPE exposed animals. When evaluated as relative weight, there was a statistically significant increase in the spleen of 21% at the low dose G/DIPE exposure level when compared to the vehicle control. However, the effect on relative weight was not dose dependent and is not considered to be biologically significant. Furthermore, there was no effect on the relative weight of the thymus in G/DIPE exposed animals.

Treatment with the positive control, cyclophosphamide, significantly decreased both absolute and relative spleen and thymus weights in all of the studies, compared to the vehicle controls. Values for absolute spleen weight were depressed between 50–57% and between 45–53% for relative spleen weight. Absolute thymus weights were depressed 73–80% and between 71% and 79% for relative thymus weights.

3.2. Spleen IgM antibody forming cell response

The spleen IgM antibody-forming cell response, i.e. plaque assay, was evaluated on spleens removed 1 day after the last exposure, which was Day 4 after antigen sensitization. Day 4 after antigen sensitization is the peak day for the sRBC IgM AFC response in rats. Female rats were sensitized in the morning and also sacrificed in the morning. Viabilities were conducted on all cell suspensions and ranged from 70% to greater than 95% for BGVC, 87% (G/MTBE), 84% (G/TAME, G/EtOH, G/ETBE), 71% (G/TBA), and 70% (G/DIPE).

There were no statistically significant differences in spleen cell numbers resulting from exposure to any of the test materials (Tables 1 and 2), consistent with the lack of effect on spleen weights noted above. (There was a slight, albeit not statistically significant increase in spleen cell number in the low dose G/DIPE group, which is not consistent with its lower spleen weights).

Table 1
Spleen antibody-forming cell response to T-dependent antigen sheep erythrocytes in female rats exposed to BGVC, G/MTBE, G/TAME, or G/TBA via inhalation for 4 weeks.

Test material	Exposure group	Body weight (g)	Spleen weight (mg)	Spleen cells (×107)	IgM AFC/106 spleen cells	IgM AFC/spleen (×103)
BGVC	Vehicle	247.8 ± 3.6	615 ± 38	53.18 ± 2.15	1639 ± 408	880 ± 209
	2000 mg/m^3	258.0 ± 5.2	647 ± 26	62.26 ± 2.35	1540 ± 194	980 ± 143
	10,000 mg/m^3	250.0 ± 5.7	600 ± 23	55.82 ± 4.02	1687 ± 235	903 ± 120
	20,000 mg/m^3	245.7 ± 2.7	675 ± 102	57.96 ± 3.33	1175 ± 111[a]	685 ± 77[a]
	Positive control	224.4 ± 5.5**	265 ± 10**	9.69 ± 0.41**	3 ± 3**	0 ± 0**
G/MTBE	Vehicle	248.3 ± 6.1	646 ± 35	72.09 ± 3.82	1646 ± 218	1162 ± 137
	2000 mg/m^3	256.9 ± 3.3	637 ± 34	76.52 ± 4.07	1128 ± 190	887 ± 171
	10,000 mg/m^3	242.5 ± 6.5	574 ± 25	65.13 ± 2.81	1490 ± 282	966 ± 185
	20,000 mg/m^3	254.8 ± 7.2	651 ± 43	76.28 ± 5.97	1680 ± 199	1245 ± 122
	Positive control	231.3 ± 9.2	283 ± 18**	10.65 ± 0.74**	0 ± 0**	0 ± 0**
G/TAME	Vehicle	255.7 ± 5.1	587 ± 24	60.71 ± 3.45	1560 ± 342	928 ± 184
	2000 mg/m^3	252.0 ± 5.8	623 ± 39	64.78 ± 5.15	1250 ± 194	785 ± 114
	10,000 mg/m^3	261.9 ± 8.2	676 ± 27	68.09 ± 5.01	1384 ± 208	908 ± 132
	20,000 mg/m^3	255.8 ± 6.3	618 ± 25	55.34 ± 3.00	1514 ± 289	874 ± 177
	Positive control	238.5 ± 8.9	291 ± 16**	10.41 ± 0.52**	0 ± 0**	0 ± 0**
G/TBA	Vehicle	262.1 ± 5.1	676 ± 31	85.01 ± 5.36	1863 ± 308	928 ± 184
	2000 mg/m^3	255.4 ± 6.7	630 ± 27	83.78 ± 3.20	1734 ± 350	785 ± 114
	10,000 mg/m^3	252.6 ± 6.8	614 ± 27	78.71 ± 5.28	1960 ± 408	908 ± 132
	20,000 mg/m^3	254.2 ± 5.2	611 ± 24	74.30 ± 4.03	1759 ± 368	874 ± 177
	Positive control	243.4 ± 5.0*	304 ± 12**	14.46 ± 0.64**	27 ± 9**	4 ± 1**

* Statistically significant from vehicle control at $p \leq 0.05$.
** Statistically significant from vehicle control at $p \leq 0.01$.
[a] Excludes outlier in BGVC high dose group with IgM AFC/106 value of 216 and AFC/spleen value of 123.

Table 2
Spleen antibody-forming cell response to T-dependent antigen sheep erythrocytes in female rats exposed to G/ETOH, G/ETBE or G/DIPE via inhalation for 4 weeks.

Test material	Exposure group	Body weight (g)	Spleen weight (mg)	Spleen cells ($\times 10^7$)	IgM AFC/10^6 spleen cells	IgM AFC/spleen ($\times 10^3$)
G/EtOH	Vehicle	242.1 ± 4.4	625 ± 39	74.34 ± 5.29	974 ± 259	705 ± 161
	2000 mg/m^3	243.8 ± 6.7	645 ± 38	70.36 ± 2.86	692 ± 137	477 ± 93
	10,000 mg/m^3	243.7 ± 4.9	586 ± 28	65.44 ± 3.32	505 ± 148	347 ± 111
	20,000 mg/m^3	240.1 ± 7.2	591 ± 34	67.97 ± 6.29	143 ± 29**	98 ± 18**
	Positive control	223.2 ± 7.6*	271 ± 14**	11.60 ± 0.86**	0 ± 0**	0 ± 0**
G/ETBE	Vehicle	259.0 ± 5.0	597 ± 37	71.48 ± 4.48	556 ± 76	391 ± 48
	2000 mg/m^3	263.0 ± 3.2	608 ± 41	74.21 ± 3.69	576 ± 94	427 ± 72
	10,000 mg/m^3	259.4 ± 4.0	608 ± 25	72.95 ± 1.27	136 ± 23**	100 ± 17**
	20,000 mg/m^3	250.3 ± 3.5	603 ± 17	72.34 ± 2.78	153 ± 39**	117 ± 35
	Positive control	247.0 ± 4.0	299 ± 17**	15.79 ± 1.21**	0 ± 0**	0 ± 0**
G/DIPE	Vehicle	251.6 ± 4.3	544 ± 24	62.65 ± 2.50	688 ± 128	440 ± 95
	2000 mg/m^3	254.5 ± 4.9	667 ± 30	75.17 ± 4.34	657 ± 166	503 ± 137
	10,000 mg/m^3	249.8 ± 3.5	579 ± 33	65.87 ± 3.29	553 ± 143	377 ± 106
	20,000 mg/m^3	247.9 ± 5.4	604 ± 39	64.82 ± 3.92	252 ± 44**	159 ± 26**
	Positive control	228.7 ± 4.6**	267 ± 14**	12.52 ± 0.73	0 ± 0**	0 ± 0**

* Statistically significant from vehicle control at $p \leqslant 0.05$.
** Statistically significant from vehicle control at $p \leqslant 0.01$.

The positive control, cyclophosphamide, consistently decreased spleen cell numbers in all of the studies compared to the vehicle control group, ranging from 78% depression in the G/ETBE positive control group to 85% in G/MTBE positive control animals.

The functional results from the AFC assay for the BGVC, G/MTBE, G/TAME and G/TBA groups are shown in Table 1. There were no statistically significant differences in the IgM antibody-forming cell response between animals exposed to these vapor condensates and their corresponding vehicle control group when evaluated either as specific activity (AFC/10^6 spleen cells) or as total spleen activity (AFC/spleen). (In the BGVC high dose group there was one animal which had an IgM AFC/10^6 value of 216 and an AFC/spleen value of 123, which was considered a significant enough outlier that it was not included in the analysis. Furthermore, there was no significant difference in the trend analysis when evaluated by the Jonckheere's Test.

The results from the AFC assay for the G/EtOH, G/ETBE, and G/DIPE groups are summarized in Table 2. Dose related decreases in IgM antibody forming cell response to the T-dependent antigen sRBC, were observed in the G/EtOH, G/ETBE, and G/DIPE exposed groups when the data were evaluated as either specific activity or as total spleen activity. For both parameters, exposure produced a reduced response at all exposure levels, but only the high dose reached a level of statistical significance. The response of the high dose group was suppressed 85% and 63% for the G/EtOH and G/DIPE groups, respectively, and 86% and 64% (G/EtOH v G/DIPE) when evaluated as total spleen activity. While the overall response of the DIPE animals was somewhat lower than had been observed in the past, the positive and negative control animal responses are consistent with the range of responses observed with the Sprague Dawley outbred strain of rats.

Statistically significant decreases in IgM antibody forming cell response were observed at the mid and high doses in the G/ETBE exposed group (Table 2). When evaluated as specific activity, the responses of the mid and high dose groups were suppressed 76% and 72%, respectively, and 64% and 70% (mid v high) when evaluated as total spleen activity. There was no significant difference in the trend analysis when evaluated by the Jonckheere's Test. As anticipated, the positive control, CPS, produced a significant decrease in both specific activity and total spleen cell activity when compared to the vehicle control animals (Tables 1 and 2). The results of both the positive and negative (vehicle) controls were consistent with the historical controls for the laboratory.

4. Conclusions and discussion

Exposure of female Sprague Dawley rats to the gasoline vapor condensates BGVC, G/MTBE, G/TAME and G/TBA 6 h/day, 5 days/week for 4 weeks did not result in alterations of the humoral immune response as evaluated in the IgM antibody-forming cell response to the T-dependent antigen sheep erythrocytes. There were no statistically significant effects on spleen weight, spleen cell number, or IgM antibody production when evaluated as either specific activity or as total spleen activity. Based on the immunological parameters evaluated, under the experimental conditions of these studies, the four vapor condensates did not adversely affect the humoral immune response of the test animals. Furthermore, the addition of MTBE, TAME or TBA did not affect the immune AFC response of gasoline vapor condensate alone.

There was statistically significant depression of the humoral immune response for three of the vapor condensates: G/EtOH and G/DIPE at the 20,000 mg/m^3 dose and G/ETBE at the 10,000 mg/m^3 and 20,000 mg/m^3 doses. Plaque forming cell assays conducted in rats following ethanol administered alone by the oral route have shown decreased antibody responses in sheep red blood cells (Loose et al., 1975; Razani-Boroujerdi et al., 1994). However the responses to ethanol have been somewhat contradictory and may depend on the age of the animal, amount of ethanol consumed, and nutritional composition of the administered diet (Loose et al., 1975; Razani-Boroujerdi et al., 1994).

There are no peer-reviewed published studies suggesting either ETBE or DIPE are capable of causing immunotoxicity, including hypersensitivity responses. An unpublished evaluation of ETBE in the guinea pig minimization assay with induction via intradermal injection of a 10% solution, followed by challenge using an occluded patch with 100% ETBE, did not provoke any signs of cutaneous sensitization (no positive controls were used in the study; European Chemicals Agency website). DIPE did not elicit a positive response in an unpublished laboratory study using the mouse local lymph node assay (European Chemicals Agency website).

In conclusion, gasoline vapor condensates and condensates of gasoline mixed with MTBE, TAME and TBA were negative in the plaque assay indicating that, similar to gasoline vapor condensate alone, the addition of these additives do not affect rat humoral immunity. Gasoline mixed with EtOH, ETBE and DIPE elicited positive responses thus suggesting that the addition of EtOH, ETBE, or DIPE does depress the rat humoral response as compared to the response from gasoline vapor condensate alone. The no observed

effect levels in female Sprague Dawley rats were 10,000 mg/m^3 for G/EtOH and G/DIPE and 2000 mg/m^3 for G/ETBE under the described test conditions.

Conflict of interest statement

K.L. White, V.L. Peachee and L.E. Twerdok are hired for work by American Petroleum Institute, and Armstrong's employer, Cambridge Environmental Inc., was a consulting company; Renewable Fuels Association was a client, during the conduct of the study. The others declare no conflicts of interest.

References

Bartlett, M.S., 1937. Sub-sampling for attributes. J. R. Stat. Soc. (Suppl. 4: 13), 1–35.

Benson, J.M., Gigliotti, A.P., March, T.H., Barr, E.B., Tibbetts, B.M., Skipper, B.J., Clark, C.R., Twerdok, L., 2011. Chronic carcinogenicity study of gasoline vapor condensate (GVC) and GVC containing methyl tertiary-butyl ether in F344 rats. J. Toxicol. Environ. Health 74, 638–657.

Clark, C.R., Schreiner, C.A., Parker, C.M., Gray, T.M, Hoffman, G.M., 2014. Health assessment of gasoline and fuel oxygenate vapors: subchronic inhalation toxicity. Regul. Toxicol. Pharmacol. 70 (2S), S18–S28.

Dunnett, C.W., 1955. A multiple comparison procedure for comparing several treatments with a control. J. Am. Stat. Assoc. 50, 1096–1121.

European Chemicals Agency Registration Data for 2-ethoxy-2-methylpropane. <http://apps.echa.europa.eu/registered/data/dossiers/DISS-9ebd98b4-66aa-4922-e044-00144f67d031/AGGR-af433b38-7d96-4710-90da-1cf4c1ccbc46_DISS-9ebd98b4-66aa-4922-e044-00144f67d031.html#AGGR-af433b38-7d96-4710-90da-1cf4c1ccbc4 2-ethoxy-2-methylpropane 6>.

European Chemicals Agency Registration Data for diisopropyl ether. <http://apps.echa.europa.eu/registered/data/dossiers/DISS-9d9a4cf8-17d0-2fad-e044-00144f67d249/AGGR-5273ae27-76e0-426a-b891-a000ebb01cdb_DISS-9d9a4cf8-17d0-2fad-e044-00144f67d249.html#AGGR-5273ae27-76e0-426a-b891-a000ebb01cdb>.

Gray, T.M., Steup, D., O'Callaghan, J.P., Hoffman, G.M., Roberts, L.G., Schreiner, C.A., Clark, C.R., 2014. Health assessment of gasoline and fuel oxygenate vapors: reproductive toxicity. Regul.Toxicol. Pharmacol. 70 (2S), S48–S57.

Gross, A.J., Clark, V.A., 1975. Gehan-Wilcoxon Test. In: Gross, A.J., Clark, V.A. (Eds.), Survival Distributions: Reliability Applications in the Biomedical Sciences. John Wiley and Sons, New York, pp. 225–256.

Henley, M., Daniel, J., Letinsky, D.J., Carr, J., Caro, M., Daughtrey, W., White, R., 2014. Health assessment of gasoline and fuel oxygenate vapors: generation and characterization of test materials. Regul. Toxicol. Pharmacol. 70 (2S), S13–S17. http://dx.doi.org/10.1016/j.yrtph.2014.05.012.

Hollander, M., Wolfe, D.A., 1973. In: Hollander, M., Wolfe, D.A. (Eds.), Jonckheere's Test: Non-parametric Statistical Methods. John Wiley and Sons, New York, pp. 120–123.

Jerne, N.K., Henry, C., Nordin, A.A., Fun, H., Koros, M.C., Lefkovits, I., 1974. Plaque-forming cells: methodology and theory. Transplant Rev. 18, 130–191.

Kruskal, W.H., Wallis, W.A., 1952. Use of ranks in one-criterion variance analysis. J. Am. Stat. Assoc. 47, 583–621.

Loose, L.D., Stege, T., Diluzio, N.R., 1975. The influence of acute and chronic ethanol or bourbon administration on phagocytic and immune responses in rats. Exp. Mol. Pathol. 23, 159–172.

Luster, M.I., Munson, A.E., Thomas, P., Holsapple, M.P., Fenters, J., White Jr., K.L., Lauer, L.D., Dean, J.D., 1988. Development of a testing battery to assess chemical-induced immunotoxicity. Fundam. Appl. Toxicol. 10, 2–19.

Luster, M.I., Portier, C., Pait, D.G., White Jr., K.L., Gennings, C., Munson, A.E., Rosenthal, G.J., 1992. Risk assessment in immunotoxicology. I. Sensitivity and predictability of immune tests. Fundam. Appl. Toxicol. 18, 200–210.

O'Callaghan, J.P., Felton, C.M., Billig, B.K., Daughtrey, W.C., Clark, C.R., Schreiner, C.A., White, R., 2014. Health assessment of gasoline and fuel oxygenate vapors: neurotoxicity evaluation. Regul. Toxicol. Pharmacol. 70 (2S), S35–S42. http://dx.doi.org/10.1016/j.yrtph.2014.05.002.

Razani-Boroujerdi, S., Savage, S.M., Sopori, M.L., 1994. Alcohol-induced changes in the immune response: immunological effects of chronic ethanol intake are genetically regulated. Toxicol. Appl. Pharmacol. 127 (1), 37–43.

Roberts, L.G., Gray, T.M., Marr, M.C., Tyl, R.W., Trimmer, G.W., Hoffman, G.M., Murray, F.J., Clark, C.R., Schreiner, C.A., 2014a. Health assessment of gasoline and fuel oxygenate vapors: developmental toxicity in mice. Regul. Toxicol. Pharmacol. 70 (2S), S58–S68.

Roberts, L.G., Gray, T.M., Trimmer, G.W., Murray, F.J., Parker, R., Schreiner, C.A., Clark, C.R., 2014b. Health assessment of gasoline and fuel oxygenate vapors: developmental toxicity in rats. Regul. Toxicol. Pharmacol. 70 (2S), S69–S79. http://dx.doi.org/10.1016/j.yrtph.2014.05.009.

Schreiner, C., Hoffman, G.,Gudi, R., Clark, C.R., 2014. Health assessment of gasoline and fuel oxygenate vapors: micronucleus and sister chromatid exchange tests by inhalation exposure. Regul. Toxicol. Pharmacol. 70 (2S), S29–S34.

Sokal, R.R., Rohlf, F.J., 1981. Biometry. Freeman, San Francisco, pp. 222–229.

USDA, 1989. Animal Welfare Act Regulations: 9 CFR Parts 1 and 2 Final Rules, Federal Register, vol. 54, No. 168, August 31, 1989, pp. 36112–36163, effective October 30, 1989.

USDA, 1991. Animal Welfare Standards, 9 CFR Part 3; Final Rule, Federal Register, vol. 56, No. 32, February 15, 1991, pp. 6426–6505, effective March 18, 1991.

US EPA, 1994. Good Laboratory Practice Standards 79.60, CFR vol. 59, No. 122, 27 June 1994.

US EPA, 1998. OPPTS Health Effects Test Guidelines 870.3465, 90-Day Inhalation Toxicity, August 1998.

US EPA, 1998. OPPTS Health Effects Test Guidelines 870.7800, Immunotoxicity, August 1998.

White, K.W., Musgrove, D.L., Brown, R.D., 2010. The sheep erythrocyte T-dependent antibody response (TDAR). Methods Mol. Biol. 598, 173–184.