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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/m^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., in press), 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., in press), neurotoxicity (O'Callaghan et al., in press), reproductive toxicity (Gray et al., in press), and developmental toxicity testing in mice and rats (Roberts et al., in press-a-b). 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., in press). 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 evaporative emissions that are encountered during refueling of vehicles, compared

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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., in press) 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 phase 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., in press; Clark et al., in press).

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

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